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The mRNA decapping machinery targets \textit{LBD3/ASL9} to mediate apical hook and lateral root development

Zhangli Zuo\textsuperscript{1}, Milena E Roux\textsuperscript{1}, Jonathan R Chevalier\textsuperscript{1}, Yasin F Dagdas\textsuperscript{2}, Takafuli Yamashino\textsuperscript{3}, Søren D Højgaard\textsuperscript{1}, Emilie Knight\textsuperscript{4}, Lars Østergaard\textsuperscript{4}, Eleazar Rodriguez\textsuperscript{1}, Morten Petersen\textsuperscript{1}

Multicellular organisms perceive and transduce multiple cues to optimize development. Key transcription factors drive developmental changes, but RNA processing also contributes to tissue development. Here, we report that multiple decapping deficient mutants share developmental defects in apical hook, primary and lateral root growth. More specifically, \textit{LATERAL ORGAN BOUNDARIES DOMAIN 3 (LBD3)/ASYMMETRIC LEAVES 2-LIKE 9 (ASL9)} transcripts accumulate in decapping deficient plants and can be found in complexes with decapping components. Accumulation of ASL9 inhibits apical hook and lateral root formation. Interestingly, exogenous auxin application restores lateral roots formation in both ASL9 over-expressors and mRNA decay–deficient mutants. Likewise, mutations in the cytokinin transcription factors type-B \textit{ARABIDOPSIS RESPONSE REGULATORS} \textit{(B-ARR}s) \textit{ARR10} and \textit{ARR12} restore the developmental defects caused by over-accumulation of capped ASL9 transcript upon ASL9 overexpression. Most importantly, loss-of-function of \textit{asl9} partially restores apical hook and lateral root formation in both \textit{dcp5-1} and \textit{pat} triple decapping deficient mutants. Thus, the mRNA decay machinery directly targets ASL9 transcripts for decay, possibly to interfere with cytokinin/auxin responses, during development.

**Introduction**

Understanding proper tissue development requires information about diverse cellular mechanisms controlling gene expression. Much work has focused on the transcriptional networks that govern stem cell differentiation. For example, ectopic expression of \textit{LATERAL ORGAN BOUNDARIES DOMAIN (LBD)/ASYMMETRIC LEAVES 2-LIKE (ASL)} genes is sufficient to induce spontaneous proliferation of pluripotent cell masses in plants, a reprogramming process triggered in vitro by complementary/Yin-Yang phytohormones auxin and cytokinin (Fan et al, 2012; Schaller et al, 2015). Auxin and cytokinin responses are essential for a vast number of developmental processes in plants including postembryonic reprogramming and formation of the apical hook to protect the meristem during germination in darkness (Chaudhury et al, 1993; Hu et al, 2017) and lateral root (LR) formation (Jing & Strader, 2019). Loss-of-function mutants in genes that regulate auxin-dependent transcription such as \textit{auxin-resistant1 (axr1)} exhibit defective hooking and LR formation (Estelle & Somerville, 1987; Lehman et al, 1996). In addition, type-B \textit{ARABIDOPSIS RESPONSE REGULATORS} \textit{(B-ARR}s) \textit{ARR1}, \textit{ARR10}, and \textit{ARR12} work redundantly as transcriptional activators to regulate cytokinin targets including type-A \textit{ARR}s, which are negative regulators of cytokinin signaling in shoot development and LR formation (Riefler et al, 2006; Ishida et al, 2008; Xie et al, 2018). Exogenous cytokinin application disrupts LR initiation by blocking pericycle founder cell transition from G2 to M phase (Li et al, 2006; Laplaze et al, 2007). Thus, reshaping the levels of certain transcription factors leads to changes in cellular identity. As developmental programming must be tightly regulated to prevent spurious development, the expression of these transcription factors may be controlled at multiple levels (Tatapudy et al, 2017). However, most developmental studies focus on their transcription rates and overlook the contribution of mRNA stability or decay to these events (Crisp et al, 2016).

Eukaryotic mRNAs contain stability determinants including the 5′-7-methylguanosine triphosphate cap (m7G) and the 3′ poly-(A) tail. mRNA decay is initiated by deadenylation, followed by degradation via either 3′–5′ exosomal exonucleases and SUPPRESSOR OF VCS (SOV)/DIS3L2 or via the 5′–3′ exoribonuclease (XRN) activity of the decapping complex (Garneau et al, 2007; Sorensen et al, 2018). This complex includes the decapping holoenzyme composed of the catalytic subunit Decapping 2 (DCP2) and its cofactor DCP1 along with other factors (DCPS, DHH1, VCS, LSM-7 complex, and PAT1), and the XRN that degrades monophosphorylated mRNA. As a central platform, PAT1 (Protein Associated with Topoisomerase II, PAT1b in mammals) forms a heterooctameric complex with LSM (Like-sm)–7 at 3′ end of a
mRNA to engage transcripts containing deadenylated tails thereafter recruits other decapping factors and interacts with them using different regions; these decapping complex and mRNAs can aggregate into distinct cytoplasmic foci called processing bodies (PBs) (Brengues et al, 2005; Balagopal & Parker, 2009; Ozgur et al, 2010; Chowdhury et al, 2014; Charpenton et al, 2017; Lobel et al, 2019). Beyond DCP genes, deletion of PAT1 gene in yeast exhibits the strongest temperature sensitive phenotype compared with other decapping factors genes (Bonnerot et al, 2000).

mRNA decay regulates mRNA levels and thereby impacts cellular reprogramming (Newman et al, 2017; Essig et al, 2018). We and others have shown that the decapping machinery is involved in stress and immune responses (Xu & Chua, 2012; Merret et al, 2013; Roux et al, 2015; Perea-Resa et al, 2012; Crisp et al, 2017; Yu et al, 2019), and that RNA-binding proteins can target selected mRNAs for decay (Gerstberger et al, 2014; Perea-Resa et al, 2016; Yu et al, 2019). Postembryonic lethality (Xu et al, 2006) and stunted growth phenotypes (Xu & Chua, 2009; Perea-Resa et al, 2012) associated with disturbance of the decay machinery indicate the importance of mRNA decapping and decay machinery during plant development. However, although much has been learned about how mRNA decapping regulates plant stress responses (Perea-Resa et al, 2016; Yu et al, 2019; Zuo et al, 2021), far less is known about how decapping contributes to plant development.

*Arabidopsis* dcp1, dcp2, and vcs mutants display postembryonic lethality, whereas ism1alsm1b, pat triple mutant, and dcp5 knock-down mutants only exhibit abnormal development (Xu et al, 2006; Xu & Chua, 2009; Perea-Resa et al, 2012; Zuo et al, 2022a, 2022b Preprint). All these differences suggest that mutations in mRNA decay components may cause pleiotropic phenotypes not directly linked to mRNA decapping and decay deficiencies (Riehs-Kearnam et al, 2012; Gloggnitzer et al, 2014; Roux et al, 2015). For example, it has been proposed that lethality in some mRNA decay loss-of-function mutants is not due to decay deficiencies per se but to the activation of immune receptors which evolved to surveil microbial manipulation of the decay machinery (Roux et al, 2015). In line with this, loss-of-function of AtPAT1 inappropriately triggers the immune receptor SUMM2, and Atpot1 mutants consequently exhibit dwarfism and autoimmunity (Peterson et al, 2000; Zhang et al, 2012; Roux et al, 2015; Rodriguez et al, 2020). Thus, PAT1 is under immune surveillance and PAT proteins are best studied in SUMM2 loss-of-function backgrounds.

Here, we studied the impact of mRNA decapping during development. For this, we have analyzed three sequential mRNA decapping mutants dcp2-1, dcp5-1, and pat triple mutant (pat1path1-4path2-1summ2-8), revealing that the mRNA decay machinery targets the important developmental regulator ASL9. Specifically, disruption of the mRNA decay machinery promotes ASL9 accumulation, and this in turn contributes to inhibit apical hook and lateral root formation. Interestingly, these developmental defects, which are observed in mRNA decapping deficient mutants and ASL9 over-expressors, can be salvaged through disruption of cytokinin signaling or exogenous application of auxin. Importantly, mutations in asl9 also partially restores the developmental defects including apical hook and lateral root formation in decapping mutants. These observations indicate that the mRNA decay machinery is fundamental to developmental decision-making.

## Results

mRNA decapping deficiency causes deregulation of apical hooking

We and others have reported that mutants of mRNA decay components exhibit abnormal developmental phenotypes including postembryonic death and stunted growth (Xu et al, 2006; Xu & Chua, 2009; Perea-Resa et al, 2012; Roux et al, 2015; Zuo et al, 2022b Preprint), indicating mRNA decay may be needed for proper development. To assess this, we explored readily scorable phenotypic evidence of defective development. Because apical hooking can be exaggeratedly induced by exogenous application of ethylene or its precursor ACC, we germinated seedlings in darkness in the presence or absence of ACC (Bleecker et al, 1988; Guzman & Ecker, 1990). Interestingly, all three sequential mRNA decapping mutants tested dcp2-1, dcp5-1, and pat triple mutant were hookless and unable to make the exaggerated apical hook under ACC treatment (Figs 1A and B and S1A and B), being that dcp2-1 exhibit the strongest hookless phenotype. Because dcp2-1 is postembryonic lethal, we used seeds from a parental heterozygote to score for hook formation, and subsequentially confirmed by genotyping that all hookless seedlings were dcp2-1 homozygotes. This, and the fact that ACC treatment leads to massive increase of DCP5–GFP (Chicois et al, 2018) and Venus–PAT1(Zuo et al, 2022b Preprint) foci in hook regions (Fig 1C), all suggest that mRNA decapping is required for apical hooking.

mRNA decay machinery targets ASL9 for decay

To search for transcripts responsible for the hookless phenotype, we revisited our previous RNA-seq data for pat triple mutant (Zuo et al, 2022b Preprint) and verified that transcripts of ASL9 (ASYMMETRIC LEAVES 2-LIKE 9, also named LBD3, LOB DOMAIN–CONTAINING PROTEIN 3) accumulated specifically in pat triple mutants (Zuo et al, 2022b Preprint). ASL9 belongs to the large AS2/LOB (ASYMMETRIC LEAVES 2/LATERAL ORGAN BOUNDARIES) family (Matsumura et al, 2009) which includes key regulators of organ development (Xu et al, 2016). Interestingly, the ASL9 homologue ASL4 negatively regulates brassinosteroids accumulation to limit growth in organ boundaries, and overexpression of ASL4 impairs apical hook formation and leads to dwarfed growth (Bell et al, 2012). Although ASL4 mRNA did not accumulate in pat triple mutants (Zuo et al, 2022b Preprint), we hypothesized that ASL9 could also interfere with apical hook formation. We therefore analyzed mRNA levels of ASL9 in ACC-treated seedlings and verified that all three sequential mRNA decay mutants accumulated up to 30-fold higher levels of ASL9 transcript compared with ACC-treated Col-0 seedlings (Fig 2A). Concordantly, two over-expressor lines of ASL9 Col-0/oxASL9 and Col-0/oxASL9–VP16 (Naito et al, 2007) also exhibited hookless phenotypes (Fig 2B and C). However, we did not observe any changes including tighter apical hooks in asl9-1 mutants (Fig S1C and D), suggesting other members of the AS2/LOB family act redundantly in this process. Nevertheless, these results indicate that apical hook formation in mRNA decapping deficient mutants is compromised, in part, might be due to misregulation of ASL9.
To determine whether ASL9 is a target of the decapping complex, we performed 5′-RACE assays and found significantly higher levels of capped ASL9 in mRNA decapping mutant seedlings than in Col-0 (Fig 2D). We also assayed for capped ASL9 transcripts in ACC and mock-treated mRNA decapping mutants. By calculating the ratio between capped and total ASL9 transcripts, we verified that with ACC treatment, mRNA decapping mutants accumulated significantly higher levels of capped ASL9 transcripts than Col-0 (Fig 2E). Moreover, RNA immunoprecipitation (RIP) revealed enrichment of ASL9 in DCP5-GFP and Venus–PAT1 plants compared with a MYC–YFP control line (Fig 2F), indicating mRNA-decapping components directly bind ASL9 transcripts. These data confirm that ASL9 mRNA can be found in mRNA-decapping complexes, and that mRNA decapping regulates ASL9 mRNA levels and contributes to ACC-induced apical hook formation.

**Accumulation of ASL9 suppresses LR formation**

LR formation is another example of postembryonic development. In Arabidopsis, the first stage of LR formation requires that xylem pericycle pole cells change fate to become LR founder cells, a process positively regulated by auxin and negatively regulated by cytokinin and ethylene (Jung & McCouch, 2013; Weijers et al, 2018). We therefore examined LR formation in mRNA decapping deficient mutants dcp5-1 and pat triple mutants and in both ASL9 over-expressors and verified that LR formation was dramatically impaired in all genotypes tested (Figs 3A and B and S2A and B). However, like seen for apical hooking, asl9-1 also appeared to display normal LR formation (Fig S2C and D). Nevertheless, LR formation defects in dcp5-1 and pat triple mutants indicate that mRNA decapping is required for the commitment to LR formation.

This is further substantiated by the fact that auxin application leads to a massive increase of DCP5-GFP and Venus–PAT1 foci in root regions (Fig 3C). Collectively, these data indicate mRNA decapping machinery, targeting ASL9, also contributes to LR formation.

**ASL9 contributes to apical hooking and LR formation**

The overexpression of ASL9 is sufficient to suppress apical hook and lateral root development. To examine more directly if ASL9 accumulation contributes to the developmental defects in decapping mutants, we crossed asl9-1 to both dcp5-1 and pat triple mutant to generate dcp5-1asl9-1 and pat asl9-1 (pat1-1pat1-4pat2-1summ2-8asl9-1) mutants. We then germinated dcp5-1asl9-1 and/or pat asl9-1 seedlings in darkness in the presence or absence of ACC, and under both conditions, dcp5-1asl9-1 and/or pat asl9-1 made more stringent hooks than dcp5-1 and/or pat triple but not as tight as Col-0 or asl9-1 did, indicating that the loss-of-function of asl9 can partially suppress decapping deficient mutants hookless phenotype (Figs 4A and B and S3A and B). Moreover, the LR phenotype of dcp5-1 and pat triple was also partially restored by mutating ASL9 (Figs 4C and D and S3C and D). Thus, our data indicate that ASL9 contributes to both apical hooking and LR development in mutant with decapping deficiencies.

**Interference with cytokinin signaling and/or exogenous auxin restores developmental defects of ASL9 over-expressor and mRNA decay–deficient mutants**

ASL9 has been implicated in cytokinin signaling (Naito et al, 2007; Ye et al, 2021) in which ARR1, ARR10, and ARR12 are responsible for activation of cytokinin transcriptional responses (Ishida et al, 2008; Xie et al, 2018), and cytokinin acts antagonistically with auxin. Apical
Figure 2. mRNA decay machinery targets ASL9 for decay.
(A) ASL9 mRNA levels in cotyledons and hook regions of dark-grown Col-0, dcp2-1, dcp5-1, and pat triple seedlings under control or ACC treatment. Error bars indicate SE of bio-triplicates. (B, C) Hook phenotypes (B) and apical hook angles (C) of triple response to ACC treatment of etiolated seedlings of Col-0, Col-0/oxASL9, and Col-0/oxASL9-VP16. The experiment was repeated three times, in each repeat sample size (n)>15 for each genotype and treatment, and representative pictures are shown. The scale bar indicates 1 mm. (D) Accumulation of capped transcripts of ASL9 analyzed in 4-d-old MS grown etiolated seedlings of Col-0, pat triple, dcp5-1, and dcp2-1 by 5' RACE-PCR. RACE-PCR products obtained using low (upper panel) and high (bottom panel) number of cycles are shown. EIF4A1 RACE-PCR products were used as loading control. (E) Capped ASL9 transcript levels using XRN1 susceptibility assay in cotyledons and hook regions of dark-grown Col-0, dcp2-1, dcp5-1, and pat triple seedlings. Error bars indicate SE (n = 3). (F) DCP5 and PAT1 bind ASL9 transcripts. 4-d dark-grown plate seedlings with DCP5–GFP or Venus–PAT1 were taken for RIP assay. ASL9 transcript levels were normalized to those in RIP of YFP-WAVE as a non-binding control. EIF4A1 was used as a negative control. Error bars indicate SE (n = 3). Bars marked with the same letter are not significantly different from each other (P-value > 0.05).

Figure 3. Accumulation of ASL9 suppresses LR formation.
(A, B) Phenotypes (A) and emerged LR density (B) of 10-d old seedlings of Col-0, dcp5-1, pat triple, Col-0/oxASL9, and Col-0/oxASL9-VP16. The experiment was repeated four times, in each repeat sample size (n)>10 for each genotype, and representative pictures are shown. The scale bar indicates 1 cm. Bars marked with the same letter are not significantly different from each other (P-value > 0.05). (C) Representative confocal microscopy pictures of root regions from 7-d old seedlings with either Venus–PAT1 or DCP5–GFP treated with MS or MS + 0.2 μM IAA for 15 min. Scale bars indicate 10 μm.
hooking and lateral root formation represent classic examples of auxin-dependent development (Peer et al, 2011). In support of this, axr1 mutants showed defective apical hook formation and reduced LR numbers (Estelle & Somerville, 1987; Lehman et al, 1996). We therefore examined cytokinin- and auxin-related gene expression in both mRNA decay–deficient mutants and ASL9 over-expressor (Figs S4 and S5). The cytokinin responsive and signaling repressors type-A ARR genes ARR8 and ARR15, the auxin-induced gene SAUR23 and the auxin biosynthesis gene TAR2 are all repressed in these genotypes tested, which suggest a misregulation of cytokinin signaling and abrogated auxin homeostasis. To test if the developmental defects of mRNA decay mutants and Col-0/oxASL9 are due to misregulation of cytokinin signaling, we interfered with cytokinin-signaling activators ARR10 and ARR12 (Ishida et al, 2008). Interestingly, both apical hooking and LR formation phenotypes of ASL9 over-expressors were largely restored in arr10-5arr12-1 background (Fig 5), indicating that the developmental defects in ASL9 over-expressors are most likely caused by misregulation of cytokinin signaling. As for dcp5-1, the apical hooking and LR phenotype were partially restored by mutating arr10 and arr12 (Fig 6), which despite not reaching the same extent as seen in ASL9 over-expressors, was still similar to our observations in dcp5-1as19-1 double mutants (Figs 4 and 5). Furthermore, the expression of ARR8, ARR15, SAUR23, and TAR2 in dcp5-1 was also partially restored in arr10-5arr12-1 background (Fig S5). Therefore, our data suggest that apical hooking and LR developmental defects in ASL9 over-expressors and to some degree in mRNA-decapping mutants depend on functional cytokinin signaling.

To test if repressed auxin signaling is also responsible for the developmental defects in mRNA-decapping mutants and ASL9 over-expressors, we first confirmed the repressed auxin signaling in mRNA decay mutants by introducing the indirect auxin-responsive reporter DR5::GFP. We found increased GFP signals in the concave side of Col-0 apical hook region when dark-grown on MS with/without ACC but not in dcp5-1 or dcp2-1 under either growth condition, and the overall GFP signals in dcp2-1 were markedly lower than Col-0 (Fig S6). We also examined DR5::GFP signal in the root area of 7-d old seedlings of Col-0 and dcp5-1, and overall GFP signal in dcp5-1 were strikingly lower than Col-0 (Fig S7). Collectively, these data confirmed our supposition that repressed auxin responses in the mRNA decapping mutants affect apical hook and root developmental processes. Consistent with this notion, exogenous auxin supplementation (0.2 μM IAA) lead to partial restoration of LR formation in dcp5-1, pat triple, and Col-0/oxASL9 (Fig S8). Collectively, our findings indicate that misregulation of cytokinin/auxin responses is partially responsible for the developmental defects in the mRNA decay mutants and ASL9 over-expressors.

**Discussion**

Developmental changes require massive overhauls of gene expression (Miyamoto et al, 2015). Apart from unlocking, effectors needed to install a new program, previous states or programs also need to be terminated (Tatapudy et al, 2017; Rodriguez et al, 2020).
We report here that mRNA decay is required for certain auxin-dependent developmental processes. The stunted growth phenotype and down-regulation of developmental and auxin-responsive mRNAs in the mRNA decapping mutant (Zuo et al, 2022b Preprint) supports a model in which defective clearance of mRNAs hampers decision-making upon hormonal perception. Apical hooking and LR formation are classic examples of auxin-dependent developmental processes (Peer et al, 2011). In line with this, we and others observed that mRNA decay-deficient mutants are impaired in apical hooking (Fig 1) and LR formation (Fig 3)( Perea-Resa et al, 2012; Jang et al, 2019). Interestingly, among the transcripts up-regulated in these decay-deficient mutants was that of capped ASL9/LBD3 (Fig 2), which is involved in cytokinin signaling (Naito et al, 2007). Cytokinin and auxin can act antagonistically (Su et al, 2011), and cytokinin can both attenuate apical hooking (Tantikanjana et al, 2001) and cytokinin can directly affect LR founder cells to prevent initiation of lateral root primordia (Laplaze et al, 2007). Our findings were that defective processing during those developmental events in mRNA decay-deficient mutants involves ASL9 was supported by our observation that ASL9 mRNA is directly regulated by the decapping machinery and that Col-0/oxASL9 transgenic lines cannot reprogram to attain an apical hook or to form LRs (Figs 2 and 3), whereas loss-of-function of asl9 partially restores the developmental defects in the decapping deficient mutants (Figs 4 and S3). In line with this, we argue that the misregulation of cytokinin-dependent and auxin-dependent signaling is partially responsible for the developmental defects in mRNA decay-deficient mutants. This is supported by the observation that auxin responses in the dcp5-1 and dcp2-1 mutants are repressed (Figs S6 and S7) and treating dcp5-1, pat triple, and Col-0/oxASL9 with exogenous auxin partially restores LR formation (Fig S8). Besides misregulation of cytokinin signaling pathway in plants overexpressing ASL9, short-term accumulation of ASL9 also led to down-regulation of cytokinin-responsive genes (Ye et al, 2021), indicating a negative role of ASL9 in regulating cytokinin responses. However, the fact that the developmental defects of ASL9 overexpressors are largely restored by knocking out two cytokinin signaling activator genes ARR10 and ARR12 suggest the function of ASL9 during apical hooking, and LR formation largely depends on ARR10 and ARR12. In line with this, the developmental defects of dcp5-1 are also partially restored in asl9-1 and ar10-5arr12-1 backgrounds (Figs 4 and 6), but in addition to ASL9 and ARRs, other unidentified factors also contribute to the defects in apical hook and LR formation in decapping mutants.

Arabidopsis contains 42 LBD/ASL genes (Matsumura et al, 2009), among these genes, LBD16, LBD17, LBD18, and LBD29 control lateral roots formation and regulate plant regeneration (Fan et al, 2012),

Figure 5. ARR10 and ARR12 loss-of-function restores apical hook and LR formation in ASL9 overexpressor.

(A, B) Hook phenotypes (A) and apical hook angles (B) in triple response to ACC treatment of etiolated Col-0, arr10-Sarr12-1, Col-0/oxASL9, and arr10-Sarr12-1/oxASL9 seedlings. The treatment was repeated three times, in each repeat sample size (n)>20 for each genotype and treatment, and representative pictures are shown. The scale bar indicates 1 mm.

(C, D) Phenotypes (C) and emerged LR density (D) of 10-d old seedlings of Col-0, arr10-Sarr12-1, Col-0/oxASL9, and arr10-Sarr12-1/oxASL9. Treatment was repeated three times, in each repeat sample size (n) >10 for each genotype, and representative pictures are shown. The scale bar indicates 1 cm. Bars marked with the same letter are not significantly different from each other (P-value > 0.05).
and overexpression of another member ASL4 also impairs apical hook (Bell et al, 2012). The partial restoration of apical hooking and LR formation caused by asl9 mutation in dcp5-1 and pat triple mutant (Figs 4 and S3) suggest that other ASLs and/or non-ASL genes also contribute to the developmental defects in decapping mutants. Besides lateral root formation, it was recently reported that Arabidopsis LBD3, together with LBD4, functions as rate-limiting components in activating and promoting root secondary growth, which is also tightly regulated by auxin and cytokinin, indicating that LBDs balance primary and secondary root growth (Smetana et al, 2019; Smith et al, 2020; Xiao et al, 2020; Ye et al, 2021). Together with auxin, cytokinin plays crucial roles in vascular development through the two-component signaling system, and plants with mutations in cytokinin receptor or type B-ARRs exhibit vasculature defects (Mahonen et al, 2006; Yokoyama 2007; Kondo et al, 2011). Hence, we cannot exclude the possibility that ASL9 might be also regulated at the translational level by the decapping machinery. Nevertheless, our finding of direct interaction of ASL9 transcripts with DCP5 and PAT1, together with the accumulation of capped ASL9 in mRNA decay mutants, indicates that ASL9 misregulation in dcp2-1, dcp5-1, and pat triple mutants is due to mRNA decapping deficiency (Fig 2).

Deadenylated mRNA can be degraded via either 3′–5′ exosomal exonucleases and SOV/DIS3L2 or via the 5′–3′ XRN activity of the decapping complex (Garneau et al, 2007; Sorenson et al, 2018). Sorenson et al (2018) found that ASL9 expression is dependent on both VCS and SOV based on their transcriptome analysis, so that ASL9 might be a target of both pathways (Sorenson et al, 2018). Although more direct data are needed to conclude whether SOV can directly regulate ASL9 mRNA levels, we have shown that ASL9 is a target of the mRNA decapping machinery. However, because the Col-0 accession is a sov mutant and has no developmental defects, the SOV decay pathway probably only plays an accessory role in regulation of ASL9. The function of PBs in mRNA regulation has been controversial because mRNAs in PBs may be sequestered for degradation or re-enter polysomal translation complexes (Franks & Lykke-Andersen, 2008). Yeast PAT1 has also been found to repress translation (Coller & Parker, 2005), and a recent study has confirmed that PBs function as mRNA reservoirs in dark-grown Arabidopsis seedlings (Jang et al, 2019). These data open the possibility that ASL9 might be also regulated at the translational level by the decapping machinery. Nevertheless, our finding of direct interaction of ASL9 transcripts with DCP5 and PAT1, together with the accumulation of capped ASL9 in mRNA decay mutants, indicates that ASL9 misregulation in dcp2-1, dcp5-1, and pat triple mutants is due to mRNA decapping deficiency (Fig 2).

**Materials and Methods**

**Plant materials and growth conditions**

*Arabidopsis thaliana* ecotype Columbia (Col-0) was used as a control. All mutants used in this study are listed in Table S1. T-DNA insertion lines for AT5G13570 (DCP2) dcp2-1 (Salk_000519), At3g26110 (DCP5) dcp5-1 (Salk_008881), and double mutant arr10-Sarr12-1 have been described (Xu et al, 2006; Ishida et al, 2008; Xu & Chua, 2009). The T-DNA line for AT1g16530 (ASL9) is SAIL_659_D08 with...
insertion in the first exon. Primers for newly described T-DNA lines are provided in Table S2. pat triple mutant, Venus–PAT1, and DCP5–GFP transgenic lines have also been described (Chicois et al., 2018; Zuo et al., 2022) Preprint). The YFP–WAVE line was from NASC (Geldner et al., 2009). Col-0/oxASL9 line has been described before (Naito et al., 2007).

Plants were grown in 9 × 9 cm or 4 × 5 cm pots at 21°C with 8/16 h light/dark regime, or on plates containing Murashige–Skoog (MS) salts medium (Duchefa), 1% sucrose, and 1% agar with 16/8 h light/dark.

Plant treatments

For ethylene triple response assays, seeds were plated on normal MS and MS + 50 µM ACC, vernalized 96 h, and placed in the dark at 21°C for 4 d before pictures were taken. Apical hook angle is defined as 180° minus the angle between the tangential of the apical part with the axis of the lower part of the hypocotyl, in the case of hook exaggeration, 180° plus that angle is defined as the angle of hook curvature (Vandenbussche et al., 2010). Cotyledon and hook regions of etiolated seedlings were collected after placing in the dark at 21°C for 4 d for gene expression and XRN1 assay. For LR formation assays, seeds on MS plates were vernalized 96 h and grown with 16/8 h light/dark at 21°C vertically for 10 d. For external IAA application for LR formation experiments, seeds on MS plates were vernalized 96 h and grown with 16/8 h light/dark at 21°C for 7 d and the seedlings were moved to MS or MS+IAA plates and grown vertically for 7 d.

Cloning and transgenic lines

pGreenIIIM DR5v2-ntdTomato/DR5-n3GFP has been published previously (Liao et al., 2015). Arabidopsis transformation was performed by floral dipping (Clough & Bent, 1998) for Col-0/DR5:GFP and thereafter Col-0/DR5:GFP was crossed to dcp5-1 and dcp2-1het to achieve dcp5-1/DR5:GFP and dcp2-1/DR5:GFP. arr10-5arr12-1/oxASL9 was generated by vacuum infiltrating arr10-5arr12-1 with A. tumefaciens strain EHA101 harbouring pSK1-ASL9 (Naito et al., 2007). Transformants were selected on hygromycin (30 mg/l) or methotrexate (0.1 mg/l) MS agar, and survivors were tested for transcript expression by qRT–PCR and protein expression by immuno-blotting and at least two independent lines were used for further analysis.

Protein extraction, SDS–PAGE, and immunoblotting

Tissue was ground in liquid nitrogen and 4 × SDS buffer (Novex) was added and heated at 95°C for 5 min, cooled to room temperature for 10 min, samples were centrifuged 5 min at 15,682 g. Supernatants were separated on 10% SDS–PAGE gels, electroblotted to PVDF membrane (GE Healthcare), blocked in 5% (wt/vol) milk in TBS-Tween 20 (0.1%, vol/vol), and incubated 1 h overnight with primary antibodies (anti-GFP [1:5,000; AMS Biotechnology]). Membranes were washed 3 × 10 min in TBS–T (0.1%) before 1 h incubation in secondary antibodies (anti-rabbit HRP or AP conjugate [1:5,000; Promega]). Chemiluminescent substrate (ECL Plus, Pierce) was applied before camera detection. For AP-conjugated primary antibodies, membranes were incubated in NBT/BCIP (Roche) until bands were visible.

Confocal microscopy

Imaging was performed using a Zeiss LSM 700 confocal microscope. The confocal images were analyzed with Zen2012 (Zeiss) and ImageJ software. Representative maximum intensity projection images of 10 Z-stacks per image have been shown in Figs 1, 3, S5, and S6.

RNA extraction and qRT–PCR

Total RNA from tissues was extracted with TRizol Reagent (Thermo Fisher Scientific), 2 µg total RNA were treated with DNase I (Thermo Fisher Scientific), and reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit according to the manufacturer’s instructions (Thermo Fisher Scientific). The ACT2 gene was used as an internal control. qRT–PCR analysis was performed on a Bio-Rad CFX96 system with SYBR Green Master Mix (Thermo Fisher Scientific). Primers are listed in Table S2. All experiments were repeated at least three times each in technical triplicates.

In vitro XRN1 susceptibility assay

Transcripts XRN1 susceptibility was determined as described (Mukherjee et al., 2012; Kiss et al., 2016) with some modification. Total RNA was extracted from tissues using the NucleoSpin RNA Plant kit (Machery–Nagel). 1 µg RNA was incubated with either 1 unit of XRN1 (New England Biolabs) or water for 2 h at 37°C. Loss of ribosomal RNA bands on gel electrophoresis was used to ensure XRN1 efficiency, after heating inactivation under 70°C for 10 min, half of the digest was then reverse transcribed into random primed cDNA with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Capped target transcript accumulation was measured by comparing transcript levels from XRN1-treated versus mock-treated samples using qRT–PCR (EJF4A1 serves as inner control) for the individual genotypes (Mukherjee et al., 2012; Roux et al., 2015; Kiss et al., 2016).

RIP assay

RIP was performed as previously described (Streitner et al., 2012). 1.5 g tissues were fixated by vacuum infiltration with 1% formaldehyde for 20 min followed by 125 mM glycine for 5 min. Tissues were ground in liquid nitrogen and RIP lysis buffer (50 mM Tris–HCl, pH 7.5; 150 mM NaCl; 4 mM MgCl2; 0.1% Igepal; 5 mM DTT; 100 U/ml Ribolock [Thermo Fisher Scientific]; 1 mM PMSF; protease inhibitor cocktail [Roche]) was added at 1.5 ml/g tissue powder. Following 15 min centrifugation at 4°C and 15,682 g, supernatants were incubated with GFP-Trap A beads (ChromoTek) for 4 h at 4°C. Beads were washed three times with buffer (50 mM Tris–HCl, pH 7.5; 500 mM NaCl; 4 mM MgCl2; 0.5% Igepal; 0.5% sodium deoxycholate; 0.1% SDS; 2 M urea; 2 mM DTT before RNA extraction with TRizol reagent [Thermo Fisher Scientific]). Transcript levels in input and IP samples were quantified by qRT–PCR, and levels in IP samples were corrected with their own input values and then normalized to YFP–WAVE lines for enrichment.
5’-RACE assay

5’-RACE assay was performed using the FirstChoice RLM-RACE kit (Thermo Fisher Scientific) following manufacturer’s instruction. RNAs were extracted from 4-d-old etiolated seedlings with the NucleoSpin RNA Plant kit (Macherey-Nagel), and PCRs were performed using a low (26–28) or high (30–32) number of cycles. Specific primers for the 5’ RACE adapter and for the genes tested are listed in Table S2.

Statistical analysis

Statistical details of experiments are reported in the figures and legends. Systat software was used for data analysis. Statistical significance between groups was determined by one-way ANOVA (analysis of variance) followed by Holm–Sidak test.

Supplementary Information

Supplementary Information is available at https://doi.org/10.26508/lsa.202302090

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Author Contributions

Z Zuo: conceptualization, data curation, software, formal analysis, validation, investigation, visualization, methodology, and writing—original draft, review, and editing.
ME Roux: conceptualization, data curation, and visualization.
JR Chevalier and SD Højgaard: data curation and investigation.
YF Dagdas: data curation, software, and investigation.
T Yamashino: resources, investigation, and visualization.
E Knight: investigation and methodology.
L Østergaard: resources, investigation, and methodology.
E Rodriguez: investigation and writing—review and editing.
M Petersen: conceptualization, resources, data curation, supervision, funding acquisition, validation, investigation, project administration, and writing—review and editing.

Conflict of Interest Statement

Correspondence and requests for materials should be addressed to M Petersen. The authors declare that they have no conflict of interest.

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