Type One Protein Phosphatase Regulates Fixed-Carbon Starvation-Induced Autophagy by Dephosphorylating ATG13a to Facilitate ATG1a-ATG13a Formation in Arabidopsis

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Running title: TOPP regulates autophagy through ATG1a-ATG13a complex

One sentence summary: Dephosphorylation of ATG13a by TOPP facilitates the formation of the ATG1a-ATG13a complex, activating fixed-carbon starvation-induced autophagy in Arabidopsis.

ABSTRACT

Autophagy, a conserved pathway which carries out the bulk degradation of cytoplasmic material in eukaryotic cells, is critical in plant physiology and development. It is tightly regulated by ATG13, a core component of ATG1 kinase complex which initiates autophagy. Although it has been reported that ATG13 is dephosphorylated immediately after nutrient starvation, the phosphatase regulating this process is poorly understood. Here, we demonstrated that the septuple mutant (topp-7m) and octuple mutant (topp-8m) of type one protein phosphatase (TOPP) exhibited significantly reduced tolerance to fixed-carbon (C) starvation due to compromised autophagy activity. Genetic analysis placed TOPP upstream of autophagy. Interestingly, ATG13a was found to be an interactor of TOPP. And TOPP directly dephosphorylated ATG13a in vitro and in vivo. Meanwhile, eighteen phosphorylation sites of ATG13a were identified by LC-MS. Mimic
dephosphorylation of ATG13a at these 18 sites significantly promoted autophagy and increased the \textit{atg13ab} mutant tolerance to fixed-C starvation. Further study showed that the dephosphorylation of ATG13a facilitated ATG1a-ATG13a complex formation. Consistently, the recruitment of ATG13a for ATG1a was markedly inhibited in \textit{topp-7m-1}. In addition, TOPP-controlled dephosphorylation of ATG13a boosted ATG1a phosphorylation. Taken together, our study reveals the crucial role of TOPP in regulating autophagy by stimulating the formation of ATG1a-ATG13a complex through dephosphorylating ATG13a in \textit{Arabidopsis}.

\textbf{Key Words:} Type one protein phosphatase (TOPP); ATG13a; dephosphorylation; ATG1a; autophagy

\section*{INTRODUCTION}

Autophagy is a conserved biological process in which eukaryotic cells recycle damaged or unwanted cell components in double-membrane vesicles termed autophagosomes to maintain cell homeostasis (Michaeli et al., 2016; Marshall and Vierstra, 2018). In plants, autophagy is implicated in almost every aspect of life, including development, reproduction, metabolism, and stress tolerance (Ren et al., 2014; Li et al., 2019; Sedaghatmehr et al., 2019; Rodriguez et al., 2020; Zhao et al., 2020).

Autophagy is mainly driven by autophagy-related (ATG) proteins. Numerous ATGs have been discovered in plants over the past few decades (Liu and Bassham, 2012). Proteins that are essential for the autophagic process predominately assemble into several functional complexes: (a) the ATG1 kinase or ATG1-ATG13 complex, which initiates autophagy by responding to changes in nutritional status; (b) the ATG9 cycling system complex, which helps supply the membrane during phagophore expansion; (c) the class III phosphatidylinositol (PI) 3-kinase (PI3K-III) complex, which mediates vesicle nucleation; (d) two ubiquitin-like conjugation systems (ATG8-PE and ATG5-ATG12), which regulate autophagosome expansion and maturation; and (e) the soluble NSF (N-ethylmaleimide sensitive factor) attachment
protein receptor (SNARE) complex, which mediates the fusion of autophagosomes with the vacuole, thus degrading nonfunctional cytoplasmic components (Liu and Bassham, 2012; Marshall and Vierstra, 2018). Among these, the activation of ATG1-ATG13 complex is the most upstream step of autophagosome formation (Mizushima, 2010). Therefore, a complete understanding of the function of this complex is essential for the development of autophagy regulation mechanism.

Studies in yeast and vertebrates have demonstrated that phosphorylation events play vital roles in the initiation phase of autophagy by regulating the assembly and activity of the Atg1/ULK (Unc-51-like kinase) kinase complex (Xie et al., 2015). The yeast Atg1 kinase complex is composed of the Ser/Thr kinase Atg1, the adaptor protein Atg13, and the ternary complex of Atg17-Atg31-Atg29 (Suzuki et al., 2007; Suzuki and Ohsumi, 2010). Target of rapamycin complex 1 (TORC1) and cAMP-dependent protein kinase (PKA) phosphorylate Atg13 under nutrient-sufficient conditions, preventing autophagy by inhibiting the Atg1-Atg13 interaction (Kamada et al., 2000; Stephan et al., 2009). Inactivation of TORC1 results in Atg13 dephosphorylation, leading to the formation of the Atg1-Atg13-Atg17 complex, which functions in the induction of autophagy (Kamada et al., 2000; Kabeya et al., 2005). It has previously been shown that protein phosphatase 2A (PP2A) antagonizes Atg13 phosphorylation and promotes autophagy after the inactivation of TORC1 in yeast (Yeasmin et al., 2016). In addition, the PP2C protein phosphatases Ptc2 and Ptc3 in yeast promote DNA damage and rapamycin-induced autophagy by dephosphorylating Atg1 and Atg13 (Memisoglu and Haber, 2019; Memisoglu et al., 2019). In mammals, the ULK complex consists of ULK1/2, ATG13, focal adhesion kinase family interacting protein of 200 kDa (FIP200), and ATG101. Under nutrient-rich conditions, mammalian TORC1 (mTORC1) represses autophagy by directly phosphorylating ULK1 and ATG13 (Chang and Neufeld, 2009; Mizushima, 2010). Inhibition of mTORC1 results in increased PP2A activity towards ULK1, thus activating autophagy (Alers et al., 2011; Pui-Mun Wong, 2015). Meanwhile, the expression of unphosphorylated ATG13 bypasses the TORC1 pathway to directly...
induce autophagy in vegetative cells (Puente et al., 2016).

Although the regulation mechanism of phosphorylation of Atg1-Atg13 complex in yeast and mammals has been largely elucidated, it is not well-defined in plants until now. In *Arabidopsis*, the ATG1 kinase complex includes the Ser/Thr kinase ATG1 and its accessory proteins ATG13, ATG11, and ATG101. Plants lacking ATG1, ATG13, or ATG11 are hypersensitive to nutrient limitation and senesce prematurely due to repressed autophagy, suggesting these components are indispensable regulators of plant autophagy (Suttangkakul et al., 2011; Li et al., 2014; Huang et al., 2019; Qi et al., 2019). Nutrient starvation could change the phosphorylation state of ATG1-ATG13 complex (Suttangkakul et al., 2011; Li et al., 2014). Recently, some upstream kinases affecting the activity of the complex have been identified. Overexpression of *TOR* significantly inhibits autophagy in *Arabidopsis* (Pu et al., 2017); while the inactivation of TOR triggers dephosphorylation of ATG13, activating the origination of autophagosome (Liao and Bassham, 2020). In addition, the regulatory associated protein of mTOR (RAPTOR) interacts with ATG13a via its TOS motif to regulate autophagy (Son et al., 2018). Thus, TOR kinase may be involved in the phosphorylation of ATG1-ATG13 complex (Van Leene et al., 2019). SNF1 (sucrose nonfermenting1) kinase homolog 10 (KIN10, also known as SnRK1.1) is another kinase implicated in the regulation of the ATG1-ATG13 complex (Chen et al., 2017). Additionally, the mitogen-activated protein kinases (MPK3) and MPK6 may be also served as possible kinase targets of ATG13a (Hoehenwarter et al., 2013). However, the phosphatases responsible for the reversal of ATG13 phosphorylation in plants are almost unknown. Besides, the phosphorylation sites of ATG13 and the downstream events regulated by its phosphorylation are poorly understood.

Plants express a variety of protein phosphatases (PP), including PP1, PP2A, PP2C, PP4, and PP6. Among these, PP1 plays an important role in regulating plant growth, development, and stress response (Bheri et al., 2021). There are nine isoforms of PP1 in *Arabidopsis*, named type one protein phosphatase (TOPP). It has been demonstrated that TOPP enzymes regulate the DELLA-mediated GA signaling...
pathway (Qin et al., 2014), phyB-mediated hypocotyl elongation (Yue et al., 2016),
pin-formed (PIN1) polarity and trafficking (Guo et al., 2015), plant immunity (Liu et
al., 2019; Yan et al., 2019), ABA signaling (Hou et al., 2016; Zhang et al., 2020), and
cell wall integrity in pollen tip-growing cells (Franck et al., 2018). In addition,
OsPP1a and TdPP1a play potential roles in the salt stress response in rice and wheat,
respectively (Liao et al., 2016; Bradai et al., 2018). The latest study shows TdPP1
participates in the brassinosteroid control of root growth via activation of BRII-EMS
suppressor1 (BES1) (Bradai et al., 2021). In this research, we found that TOPP, as a
novel regulator of the ATG1-ATG13 complex in Arabidopsis, promotes autophagy by
dephosphorylating ATG13a and modulating the formation of the ATG1a-ATG13a
complex under fixed-carbon (C) starvation.

RESULTS

Plants Lacking at Least Seven Members of TOPP are Hypersensitive to Fixed-C
Starvation

Autophagy-defective mutants are characterized by hypersensitivity to fixed-C
deprivation (Yoshimoto et al., 2004; Chung et al., 2010; Suttangkakul et al., 2011;
Huang et al., 2019; Qi et al., 2019; Liu et al., 2020). We previously identified that a
dominant-negative mutant, topp4-1 (Qin et al., 2014), displayed early senescence after
fixed-C starvation, similar to autophagy-defective mutants (Supplementary Figure 1A,
B). Overexpression of TOPP4 in topp4-1 partially inhibited premature senescence.
However, no obvious phenotype was observed in the TOPP4 T-DNA insertion mutant
topp4-3 (Supplementary Figure 1A, B). To gain further insight into the role of TOPP
in carbon deprivation, we first analyzed the transcript levels of TOPPs after fixed-C
starvation. Our results showed that the expression of TOPPs was induced after
treatment (Supplementary Figure 2), suggesting that there may be functional
redundancy among TOPPs in response to fixed-C starvation. However, all of the
homozygous TOPP single mutants appeared similar to the wild type (WT) in response
to fixed-C starvation (Supplementary Figure 3). We subsequently generated topp-2m
(topp1/4, topp6/7, topp8/9), topp-3m (topp1/2/4, topp3/6/7, topp1/4/5), topp-5m (topp1/4/6/7/9), and topp-6m (topp1/2/4/6/7/9) mutants (Supplementary Figure 3 and Supplementary Table 4). Unfortunately, these mutants did not display obvious phenotypes under fixed-C starvation conditions (Supplementary Figure 3). However, when the septuple mutant topp-7m-1 (topp1/4/5/6/7/8/9) which was reported in our previous studies (Liu et al., 2019; Zhang et al., 2020), underwent fixed-C starvation, an early senescence phenotype, similar to that observed for topp4-1, was identified (Figure 1A, D). This phenotype was rescued by expression of TOPP1, TOPP4, or TOPP5 driven by their native promoters in topp-7m-1 #79 (all topp-7m-1 in the following text refers to line #79) (Figure 1B, E and Supplementary Figure 4A, B). We further generated topp-7m-2 (topp1/2/4/6/7/8/9), topp-7m-3 (topp1/2/4/5/6/7/9), and octuple mutant topp-8m (topp1/2/4/5/6/7/8/9) (Supplementary Table 4). Similar to topp-7m-1, these mutants were extremely sensitive to fixed-C starvation (Supplementary Figure 1C, D and Figure 1A, D). Together, these results suggest that TOPPs function redundantly in fixed-C starvation-induced senescence.

In contrast, we found that the TOPP1, TOPP4, and TOPP5 overexpression lines displayed higher tolerance to fixed-C starvation than the WT (Figure 1C, F and Supplementary Figure 4C, D). To understand the genetic relationship between TOPP and autophagy-dependent genes, TOPP4 was overexpressed in atg7-3 and atg13ab mutants. Interestingly, atg7-3 and atg13ab contained 35S:TOPP4 lines displayed similar sensitivity as the atg7-3 and atg13ab mutants under carbon starvation (Figure 1C). This was further supported by the decreased chlorophyll content (Figure 1F). These results indicate that the enhanced tolerance to fixed-C starvation in the TOPP4-OE lines is dependent on the autophagy pathway.

**Fixed-C Starvation Induced Autophagy is Repressed in the Absence of TOPP**

To further address the role of TOPP in fixed-C starvation-induced autophagy, calyculin A (CL-A), a pharmacological inhibitor of the protein phosphatases PP2A and PP1, was used (Stubbs et al., 2001; Wright et al., 2002; Takahashi et al., 2012). Upon CL-A treatment, the fixed-C starvation-induced translocation of GFP-ATG8e to
the vacuoles and the accumulation of YFP-ATG1a and YFP-ATG13a labeled vesicles were strongly reduced (Supplementary Figure 5A and B). These findings suggest that phosphatase activity is required for fixed-C starvation-induced autophagy.

To investigate whether inhibition of TOPP activity reduces the formation of autophagosomes, we expressed YFP-ATG8e, YFP-ATG1a, and YFP-ATG13a driven by the 35S promoter in WT and topp-7m-1 lines. The formation of YFP-labeled puncta structures was more significantly reduced in topp-7m-1 than in WT after fixed-C starvation (Figure 2A, B). In addition, the release of free YFP from YFP-ATG8e was used to monitor the autophagic transport (Wang et al., 2016; 2019).

Consistent with the microscopy results, free YFP levels were substantially reduced in the topp-7m-1 upon fixed-C starvation (Figure 2C). Moreover, immunoblotting revealed a higher accumulation of ATG1a and ATG13a in topp-7m-1 than in WT (Figure 2D). We further checked the autophagy flux in topp4-1, and similar results were observed (Supplementary Figure 5C-F). These data demonstrate that TOPP plays an important role in autophagy.

**TOPP Directly Interacts with ATG13a and Associates with Autophagosomes**

To understand the mechanism of TOPP in regulating autophagy, we first analyzed the transcript levels of ATGs in WT and topp-7m-1 plants following fixed-C starvation. qRT-PCR analyses indicated that the transcription of ATG1a, ATG13a, ATG8a, ATG8e, ATG5, and ATG7 was the same in the mutant and WT (Supplementary Figure 6), suggesting that TOPP does not regulate autophagy at the transcriptional level.

Then liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed to analyze the co-immunoprecipitated proteins with TOPP4-GFP. One candidate protein identified with three specific peptides was ATG13a (Supplementary Table 1). Through yeast two-hybrid (Y2H) and pull-down analysis, we found that TOPP4 physically interacts with ATG13a. Bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation (Co-IP) assays further confirmed their interaction in vivo (Figure 3A-D). We next examined the interactions between TOPP family proteins and ATG13a through Y2H and BiFC. The results showed that besides TOPP4, TOPP1 and...
TOPP5 also directly interact with ATG13a (Figure 3A). All of the nine TOPP proteins could interact with ATG13a in BiFC experiments (Supplementary Figure 7A). However, no interaction was detected between TOPPs and ATG13b both in vitro and in vivo (Figure 3A-D, Supplementary Figure 7A). Therefore, all the evidence demonstrates that ATG13a is a potential target of TOPP in Arabidopsis.

To explain how TOPP is involved in fixed-C starvation-induced autophagy, we first examined the localization of TOPP by using the transgenic lines expressing TOPP-GFP driven by 35S promoter. Under normal growth conditions, TOPP1, TOPP4, and TOPP5 were localized in the cytoplasm and nucleus and were not altered by fixed-C starvation (Supplementary Figures 8A and B). However, TOPP translocated to the vacuole with small vesicles upon concanavalin A (ConcA) treatment, and much more puncta structures were observed after fixed-C deprivation (Supplementary Figure 8A and B). The same results were observed in the pTOPP4:TOPP4-GFP/WT lines (Supplementary Figure 8C and D), indicating that TOPP trafficking into vesicle is not the result of its overexpression. To further confirm these TOPP-labeled puncta structures were autophagic vesicle-related, p35S:TOPP4-RFP/WT was crossed with pATG8e:GFP-ATG8e/WT, a well-characterized autophagosome marker line (Xiao et al., 2010). Confocal microscopy analyses of root cells showed that TOPP4-RFP partially (approximately 70%) colocalized with GFP-ATG8e-decorated autophagosomes (Figure 3E). Additionally, we found that the TOPP4-GFP marked vesicles were almost undetectable in atg7-3 and only little could be observed in atg13ab (Supplementary Figure 8E and F). The reason for this phenomenon might be that plants lacking core component ATG7 completely block autophagy, while residual autophagy still exists in atg13ab (Suttangkakul et al., 2011; Li et al., 2014; Huang et al., 2019). These results suggest that TOPP4 puncta are autophagy-dependent. Given that ATG8 is unstable under acidic conditions and degrades in vacuoles (Yoshimoto et al., 2004; Thompson et al., 2005; Chung et al., 2010), and that TOPP4 enters vacuoles like ATG8e, we examined the stability of TOPP4 fusion protein under fixed-C starvation (Figure 3F).
The degradation of TOPP4 was observed, although it was not decreased as rapidly as that of ATG8e (Figure 3F). In contrast, the accumulation of free GFP from TOPP4-GFP was lower in atg13ab and atg7-3 mutants than in WT (Supplementary Figure 8G). These findings indicate that TOPP4 is associated with autophagosomes induced by fixed-C starvation.

**TOPP Dephosphorylates ATG13a in Vitro and in Vivo**

ATG13a is a reversibly modified phosphoprotein in *Arabidopsis* (Suttangkakul et al., 2011). We further confirmed this by using p35:SYFP-ATG13a/WT transgenic lines (Supplementary Figure 9A and B) and found that ATG13a shifted to a faster band due to rapid dephosphorylation upon fixed-C starvation by using phosphoserine (pSer) antibody (Figure 4A). We next examined whether TOPP dephosphorylates ATG13a directly. The recombinant protein TOPP4-GST purified from *E. coli* was confirmed to display a phosphatase activity as it could catalyze the hydrolysis of the general substrate p-nitrophenyl phosphate (pNPP) (Figure 4B). After TOPP4-GST treatment, faster migration of ATG13a bands was observed, and this was immediately suppressed by a protein phosphatase inhibitor (PhosSTOP), suggesting that TOPP4, similar to λPP (Supplementary Figure 9C), directly dephosphorylates ATG13a in vitro (Figure 4C). Other TOPP family proteins like TOPP1, TOPP3, and TOPP9, which exhibit phosphatase activity in vitro in our previous study (Zhang et al., 2020), could also directly dephosphorylate ATG13a like TOPP4 (Figure 4D), suggesting TOPP redundantly regulates the dephosphorylation of ATG13a.

To further verify the dephosphorylation of ATG13a by TOPP, we co-expressed YFP-ATG13a and TOPP4-RFP in *N. benthamiana* leaves. The result showed that YFP-ATG13a exerted a weaker phosphorylation signal than that co-expressed with GUS-RFP (Figure 4E), indicating ATG13a undergoes TOPP4-mediated dephosphorylation in planta. Similar results were obtained by TOPP1, TOPP3, and TOPP9 (Figure 4F). We next investigated the phosphorylation of ATG13a in topp-7m-1. After exposure to fixed-C starvation, the migration of ATG13a bands became faster in WT plants. However, this trend was impeded in topp-7m-1 and larger
ATG13a proteins appeared in the mutant (Figure 4G, H). In addition, λPP could remove these larger bands of ATG13a (Figure 4H), suggesting that the slow migration of ATG13a in topp-7m-1 was caused by phosphorylation rather than other protein modification. To solidify these results, immunoprecipitated proteins were detected with pSer antibody. The results showed that the dephosphorylation of YFP-ATG13a was significantly prevented in topp-7m-1 (Figure 4I). Taken together, all these experiments support the notion that ATG13a is the substrate of TOPP and the autophagy defects in topp-7m-1 might be due to the repression of ATG13a dephosphorylation.

**ATG13a Undergoes Multi-Site Phosphorylation**

After establishing ATG13a as a substrate of TOPP, we tried to identify the potential phosphorylation sites of ATG13a. YFP-ATG13a protein from the non-starved transgenic plant p35S:YFP-ATG13a/WT was enriched. And in vivo phosphorylation sites were analyzed by LC-MS/MS. At least eleven phosphorylation peptides and eighteen unique phosphorylation sites were identified in ATG13a (Figure 5A and Supplementary Data Sheet1). Among these, five serine residues (S248, S397, S404, S406, and S407) have been previously reported (Van Leene et al., 2019). Sequence analysis showed that S248, S343, S397, and S473 are highly conserved in different plants, whereas others are specific to *Arabidopsis* (Supplementary Figure 10). Additionally, our previous quantitative phosphorylation proteomics analysis showed that three specific peptides of ATG13a which contain the conserved residues S248, S397, and S473 were found both in WT and topp4-1, and the phosphorylation levels of these peptides were all changed in topp4-1 compared to WT (Supplemental Table 2). These results suggest that S248, S343, S397, and S473 may affect the function of ATG13a.

To evaluate the contribution of these four conserved phosphorylation sites to fixed-C starvation tolerance, we substituted these residues with alanine (A) or aspartate (D) as a dephosphorylation-mimic or phosphorylation-mimic form of ATG13a, respectively. However, overexpression of *ATG13a*, *ATG13a*4A, and
ATG13a<sup>4D</sup> in <i>atg13ab</i> completely restored the sensitivity of the mutant to fixed-C starvation (Supplementary Figure 11). Consistently, similar to ATG13a, both ATG13a<sup>4A</sup> and ATG13a<sup>4D</sup> were still sensitive to λPP and TOPP4-GST treatment in <i>vitro</i> (Figure 6A). Besides, we found that when YFP-ATG13a<sup>4A</sup> or YFP-ATG13a<sup>4D</sup> co-expressed with TOPP4-RFP in <i>N. benthamiana</i> leaves, the mutated proteins could be still dephosphorylated like the wild ATG13a (Figure 6C). In addition, the migration caused by dephosphorylation of the ATG13a<sup>4A</sup> and ATG13a<sup>4D</sup> after fixed-C starvation was not significantly changed compared with ATG13a (Figure 6E). Collectively, these results indicate that the four conserved residues are not enough to activate ATG13a and TOPP dephosphorylates ATG13a at multiple sites in autophagy.

ATG13a Dephosphorylation Enhances Fixed-C Starvation Induced Autophagy

Given that the substitutions of S248, S343, S397, and S473 have no significant effect on the rescue of <i>atg13ab</i>, we speculate that the activity of ATG13a might be influenced by the numbers rather than the location of available phosphorylation sites. To test this, we substituted all the identified 18 phosphorylation site residues with alanine (ATG13a<sup>18A</sup>) or aspartate (ATG13a<sup>18D</sup>) and overexpressed them in <i>atg13ab</i> mutant (Figure 5B). We found that ATG13a<sup>18A</sup> significantly improved the tolerance of the mutant to fixed-C starvation, since the transgenic line <i>p35S:YFP-ATG13a<sup>18A</sup>/atg13ab</i> showed obviously delayed senescence compared with <i>p35S:YFP-ATG13a/atg13ab</i> (Figure 5B, C). In contrast, ATG13a<sup>18D</sup> only partially recovered the sensitivity of <i>atg13ab</i> upon fixed-C starvation (Figure 5B, C). These results suggest that dephosphorylation of ATG13a helps plants to tolerate carbon deficiency.

The ATG1-ATG13 protein kinase complex is both a regulator and a target of autophagic recycling in <i>Arabidopsis</i> (Suttangkakul et al., 2011). To assess whether the delayed starvation-induced senescence by ATG13a<sup>18A</sup> does indeed occur due to autophagy induction, we examined the deposition of YFP-ATG13a-labeled autophagic bodies inside the central vacuole. The results showed that the translocation of YFP-ATG13a<sup>18A</sup> was much stronger than that of ATG13a and ATG13a<sup>18D</sup> (Figure 5D
and E). YFP-release assays further confirmed the confocal microscopy observations (Figure 5F). When autophagy is activated, ATG8 proteins associate with phosphatidylethanolamine (PE) to form ATG8-PE, and the ATG8-PE level has been used to show the strength of autophagy activity (Chung et al., 2010; Bassham, 2015; Li et al., 2015). We found that ATG13a^{18A} strongly enhanced the protein level of ATG8a (Figure 5G), although we were unable to detect lipidated-ATG8a using our ATG8a antibody. These results indicate that dephosphorylation of up to 18 sites of ATG13a contributes to the regulation of its activity in autophagy.

We next investigated the effect of these eighteen residues on the dephosphorylation of ATG13a. *In vitro* assay showed that both ATG13a^{18A} and ATG13a^{18D} were significantly less sensitive to λPP and TOPP4-GST than ATG13a (Figure 6B). Meanwhile, phospho-immunoblotting assay confirmed that TOPP4-RFP-mediated dephosphorylation of YFP-ATG13a^{18A} and YFP-ATG13a^{18D} was significantly inhibited (Figure 6D). When exposed to fixed-C starvation, the migration caused by dephosphorylation of the ATG13a^{18A} and ATG13a^{18D} was difficult to observe (Figure 6F), suggesting these sites are important for the maintenance of ATG13a phosphorylation state. However, the dephosphorylation of ATG13a^{18A} and ATG13a^{18D} was not completely blocked through pSer antibody test (Figure 6F), implying that other phosphorylation sites exist. Taken together, these results suggest that dephosphorylation of ATG13a at multiple sites enhances its function in fixed-C starvation-induced autophagy.

**TOPP-Controlled ATG13a Dephosphorylation Promotes the Formation of ATG1a-ATG13a Complex**

*Arabidopsis* ATG13a contains a classical ATG13 domain at its N-terminal and an intrinsically disordered region (IDR) at the C-terminal. All of the 18 phosphorylation sites we identified were located in this IDR (Figure 7A). Tobacco transient expression experiments showed ATG13a was localized in the cytoplasm and formed a puncta structure. When IDR was missing, ATG13a accumulated in the nucleus, suggesting that the IDR was required for ATG13a correct subcellular localization (Figure 7A, B...
and Supplementary Figure 12A). Moreover, repeated experiments showed that ATG13a<sup>18D</sup> significantly reduced the puncta formation in the cytoplasm compared with ATG13a and ATG13a<sup>18A</sup> (Figure 7B and Supplementary Figure 12A), implying that the phosphorylation state affects ATG13a localization.

We then examined the interaction of ATG1a and ATG13a. Y2H assay showed that IDR, excluding the N-terminal, mediated the interaction between ATG1a and ATG13a (Figure 7C). BiFC and Co-IP experiments further confirmed this result (Figure 7D and Supplementary Figure 12B). Given that IDR contains multiple phosphorylation sites, we analyzed whether the phosphorylation state of ATG13a affects the interaction of ATG1a and ATG13a. Y2H showed both ATG13a<sup>18A</sup> and ATG13a<sup>18D</sup> could bind to ATG1a physically (Figure 7C). However, in vivo BiFC and Co-IP assays revealed that ATG13a<sup>18D</sup> significantly reduced their interaction (Figure 7D, E).

To further understand ATG1a and ATG13a interaction during fixed-C starvation, we performed Co-IP experiments. Under normal growth conditions, the basal interaction between ATG1a and ATG13a could be detected. After treatment with fixed-C starvation, this affinity significantly increased (Figure 7F, G). Interestingly, the dephosphorylation-mimic form ATG13a<sup>18A</sup> strongly enhanced the recruitment of ATG1a, while the phosphorylation state form ATG13a<sup>18D</sup> reduced their interaction (Figure 7H). Since ATG13a was hyperphosphorylated in topp-7m-1 (Figure 4G-I), it is reasonable to assume that the ATG1a-ATG13a association was impaired in the mutant. To test this hypothesis, the same Co-IP was performed (Figure 7I, J). The results showed that the interaction of ATG1a and ATG13a was significantly reduced in topp-7m-1 under fixed-C starvation (Figure 7I, J). Based on these results, we conclude that the formation of the ATG1a-ATG13a complex is regulated by the ATG13a phosphorylation state and this process is tightly controlled by TOPP.

**TOPP-Controlled Dephosphorylation of ATG13a Boosts ATG1a Phosphorylation**

As ATG1a-ATG13a interaction was impaired in topp-7m-1, we set out to find whether TOPP directly regulates ATG1a. Through Y2H analysis, we found that TOPP4 physically interacted with ATG1a (Figure 8A). BiFC and Co-IP assays were
performed to confirm this interaction in vivo (Figure 8B and C). Besides TOPP4, TOPP1 could bind to ATG1a (Figure 8A and B). We also found that TOPP4 could interact with the ATG1a homologous proteins ATG1b and ATG1c both in vitro and in vivo, but not ATG1t (Supplementary Figure 7B-D).

To investigate whether TOPP dephosphorylates ATG1a in vitro, we used atg7-3, a mutant in which ATG1a is hyperphosphorylated following fixed-C/N starvation (Li et al., 2014), as material (Figure 8D). The result showed that the phosphorylated state of ATG1a could be removed by protein phosphatase λ.PP. However, the hyperphosphorylated ATG1a could not be directly dephosphorylated by TOPP4 (Figure 8E). When YFP-ATG1a co-expressed with TOPP4-RFP in tobacco, there were no significant changes in phosphorylation signal compared with GUS-RFP (Figure 8F). To further test the effect of TOPP4 on ATG1a in Arabidopsis, we overexpressed TOPP4 in the atg7-3 background. Interestingly, it showed TOPP4 significantly promoted the phosphorylation of ATG1a during autophagy (Figure 8G). However, this phenomenon was not observed in the atg13ab mutant (Figure 8H). Therefore, TOPP-mediated promotion of ATG1a phosphorylation depends on the dephosphorylation of ATG13a.

**DISCUSSION**

PP1 is a major class of the PPP family of Ser/Thr protein phosphatases and is ubiquitously distributed throughout higher eukaryotes (Farkas et al., 2007; Uhrig et al., 2013). In mammals, PP1 determines the fate of cardiomyocytes during hypoxia/reoxygenation by regulating the dephosphorylation of ATG16L1 during autophagy (Song et al., 2015). This implies that TOPP may play a role in plant autophagy. In this study, we first found that the TOPP dominant-negative mutant topp4-1 displayed an autophagy-deficient phenotype, consistent with the well-known autophagy mutant atg7-3 under fixed-C starvation. However, the homozygous single mutants of TOPP did not show similar phenotypes, nor did the mutants of topp-2m, topp-3m, topp-5m, and topp-6m. The septuple mutant (topp-7m) and octuple mutant (topp-8m) were sensitive to fixed-C starvation like atg13ab, suggesting that highly
functional redundancy exists among TOPPs in responding to fixed-C starvation.

Although we recovered only three (TOPP1, TOPP4, and TOPP5) alleles of the topp1/4/5/6/7/8/9 (topp-7m-1) mutant, and additional TOPP genes, such as TOPP3 was not mutated in the multiple knockouts, we believe that the above phenotypes observed in topp-7m and topp-8m mutants were caused by TOPP mutation, and that higher-order mutant topp-9m must also have the same phenotype as topp4-1.

Autophagosomes could be observed upon ConcA treatment (Suttangkakul et al., 2011; Qi et al., 2017; Huang et al., 2019; Liu et al., 2020). We found that TOPP entered the vacuoles in the form of puncta in the presence of ConcA. However, these TOPP-labeled dots did not completely overlap with GFP-ATG8e, and TOPP4 did not degrade as quickly as GFP-ATG8e under fixed-C starvation conditions. In a previous study, we demonstrated that TOPP4 is required for the endocytic trafficking of PIN1 in pavement cells (Guo et al., 2015). Therefore, these TOPP-puncta in vacuoles may also contain other non-autophagosome acidic vesicles as ConcA is a vacuolar ATPase inhibitor that prevents acidic vesicle (either endosome or autophagosome) trafficking and inhibits vacuolar degradation activity (Dröse et al., 1993; Huss et al., 2002; Dettmer et al., 2006). On the other hand, ATG8 also plays a non-autophagic function through the endosomal pathway in plant senescence (Jia et al., 2019). Anyway, their main colocalization indicates that TOPP is involved in autophagy.

To detect whether TOPP performs a function in plant autophagy through a mechanism similar to that observed in mammals, we first examined the relationship between TOPP and ATG16. Unfortunately, we did not detect any interaction between TOPPs and ATG16 (Supplemental Figure 7E and F). Therefore, TOPP may regulate plant autophagy through a different mechanism in plants. The ATG1-ATG13 complex plays an essential role in initiating autophagy, sensing nutritional status signals, recruiting downstream ATG proteins, and governing autophagosome formation (Huang et al., 2019; Li et al., 2014; Qi et al., 2019; Suttangkakul et al., 2011). However, the functional phosphorylation sites of ATG13a have not been verified and its associated protein phosphatase has not been uncovered so far. In this work, we
found that TOPP is the phosphatase that directly targets ATG13a. Moreover, eighteen crucial phosphorylation sites of ATG13a were identified. Even though S248 was previously considered as a potential carbon/nitrogen-responsive phosphorylation site of ATG13a (Li et al., 2020), the substitution of the four conserved serine residues including S248 among the 18 phosphorylation sites did not significantly affect the function of ATG13a, suggesting that these four residues are not the key phosphorylation sites of ATG13a. However, when all the 18 phosphorylation sites were mutated to A simultaneously, the function of ATG13a was markedly enhanced and autophagy was activated. This multi-site phospho-regulation of ATG13a suggests that the number of sites available for phosphorylation is a more important determinant of ATG13a activity than the exact location of those sites. This multi-site phosphorylation regulation mechanism has been reported in plants (Smertenko et al., 2006; Hardwick and Philpott, 2015). Similarly, in yeast, Atg13 is a hyperphosphorylated protein that contains at least 51 phosphorylation sites, and dephosphorylation of Atg13 at eight serine residues is sufficient for induction of autophagy (Fujioka et al., 2014; Kamada et al., 2010; Yasmin et al., 2016; Memisoglu and Haber, 2019). It is also worth noting that three potential phosphorylation sites, T251, S268, and S558 of ATG13a, were not identified in our experiment (Van Leene et al., 2019), suggesting that there are additional phosphorylation sites in ATG13a that require further investigation. Indeed, there may exist at least 37 phosphorylation sites in Arabidopsis ATG13a according to bioinformatics analysis (Supplemental Figure 12C). Additionally, the presence of Y351 indicates that other types of protein phosphatases, such as PTP (protein tyrosine phosphatase), might be involved in the dephosphorylation of ATG13a. Thus, compared to yeast, the phosphorylation regulation of ATG13a in plant autophagy is more complicated. How these phosphorylation sites of ATG13a respond to different environmental conditions and nutrient deficiency requires further research.

As one of the core components of the ATG1-ATG13 complex, Arabidopsis ATG13a was structurally divided into an N-terminal ATG13 domain and a C-terminal IDR
region which contains a large number of phosphorylation sites. We found that the IDR was necessary for the localization of ATG13a in the puncta structures of the cytoplasm. Furthermore, ATG13a-puncta formation was dependent on the dephosphorylation of ATG13a. Interestingly, these puncta-like signals were specifically generated by ATG13a and ATG13b, but not other ATG proteins like ATG8e, ATG1a, ATG1b, or ATG1c (Supplemental Figure 12D), although these ATGs also included the classic IDR (Supplemental Figure 12E). The yeast Atg13 also contains an IDR having 51 phosphorylation sites at the C-terminal, which is essential for PAS (pre-autophagosomal structure) localization of Atg13 itself (Jao et al., 2013; Suzuki et al., 2015; Yamamoto et al., 2016), suggesting the IDR of ATG13 is conserved in different organisms. It was found that the yeast PAS is a liquid-like condensate of Atgs, in which the Atg1 complex undergoes phase separation to form liquid droplets, and this process is dynamically controlled by Atg13 phosphorylation (Fujioka et al., 2020). However, the exact nature of these ATG13a-puncta in Arabidopsis remains unknown, since it is not clear whether the PAS exists in plants. Future work should determine whether these ATG13a-puncta in plants are the result of a similar phase separation like yeast.

We found that ATG13a was quickly dephosphorylated, while ATG1a was phosphorylated upon fixed-C starvation. And fixed-C starvation or dephosphorylation-mimic ATG13a (ATG13a 18A) enhanced the ATG1a-ATG13a interaction. Therefore, the regulation mode of the ATG1-ATG13 complex in Arabidopsis is similar to that of yeast (Cebollero and Reggiori, 2009; Kamada et al., 2010; Fujioka et al., 2014). In contrast, in mammalian cells, both ULK1 and ATG13 are dephosphorylated when starvation triggers autophagy. The binding of ULK1 and ATG13 is not regulated by nutritional conditions (Mizushima, 2010; Yang and Klionsky, 2010). On the contrary, in Drosophila, Atg13 is highly phosphorylated, whereas Atg1 is dephosphorylated when autophagy is induced by starvation. The formation of ATG1-ATG13 complex occurs constitutionally in fed and starved conditions (Chang and Neufeld, 2010). Hence, although the Atg1-Atg13 complex is
conserved in eukaryotes, it has complicated regulatory mechanisms in different species with regard to the switching of its phosphorylation state and formation in autophagy. Besides, in yeast, the phosphorylation of Atg13 not only affects its binding to Atg1 but also alters its interaction with Atg17 (Fujioka et al., 2014; Memisoglu et al., 2019). While *Arabidopsis* appears to be missing a separate canonical ATG17 protein, instead, an ATG11/17 hybrid is detected (Li et al., 2014). Therefore, it could be speculated that the phosphorylation status of ATG13 may affect its interaction with ATG11 in *Arabidopsis*.

Interestingly, our result showed that TOPP4 could not dephosphorylate ATG1a, though they interacted with each other directly. And the phosphorylation of ATG1a was increased when TOPP4 overexpressed in *atg7-3*. A reasonable explanation for this is that the accumulated ATG13a is first dephosphorylated by TOPP4, resulting in the formation of hypo-phosphorylated ATG13a binding to ATG1a. Then ATG1a is auto-phosphorylated or phosphorylated by other kinases through an unclear mechanism.

Collectively, our observations present strong evidence that TOPP is a positive regulator of fixed-C starvation induced autophagy in *Arabidopsis*. In WT, under nutrient-rich conditions, ATG13a is highly phosphorylated by TOR, reducing its affinity for the ATG1a kinase. After treatment with fixed-C starvation, TOR is inactivated. An unknown component then activates TOPP to dephosphorylate ATG13a, promoting ATG1a-ATG13a complex formation. Thus, autophagy is induced. However, the deletion of TOPP phosphatase (*Δ*topp) leads to the continued presence of ATG13a which is phosphorylated by TOR. These hyperphosphorylated ATG13a weakens its ability to recruit ATG1a, resulting in impaired autophagy (Figure 9). In summary, our work illustrates how ATG13a is dephosphorylated after induction of autophagy by fixed-C starvation and discovers the key phosphatase that regulates this process in plants.

**METHODS**
Plant Materials and Growth Conditions

All materials used in this work were Arabidopsis accession Columbia (Col-0). Mutants of topp1-1 (SALK_057537), topp2 (GK_187C10_014623/CS305848), topp6 (SALK_093747), topp7 (SALK_023073), topp8-1/aun2-1 (SALK_137888), topp9/aun1-1 (SALK_045433C), and atg7-3 (SAIL_11_H07) were obtained from the Arabidopsis Biological Resource Center. topp4-3 (SALK_098324) was from Prof. Yong Ding. The dominant-negative mutant topp4-1 has been described (Qin et al., 2014). Mutants of topp3, topp5, topp-2m, topp-3m, topp-5m, topp-6m, topp-7m, and topp-8m were generated as Supplementary Table 4. atg13ab (SALK_044831) was from Prof. Fa Qiang Li (Suttangkakul et al., 2011). The transgenic line pATG8e:GFP-ATG8e/Col-0 was from Prof. Shi Xiao (Huang et al., 2018). Other stable expression transgenic plants were generated as listed in Supplemental Table 5. All the plants obtained by CRISPR-cas9 were screened with hygromycin. After separation of the Cas9 gene from the mutants, non-hygromycin-resistant seeds were used for experiments.

Arabidopsis seedlings were surface-sterilized and vernalized at 4°C for 2 to 3 d, then germinated on 1/2 MS medium at 22°C under an LD photoperiod (16 h light/8 h darkness) with illumination at ~100 μmol m⁻² s⁻¹. After 1 week, the seedlings were transferred to soil for further growth. Nicotiana benthamiana was grown under LD conditions. One-month-old tobacco plants were used for transient expression assays.

Fixed-Carbon Starvation Treatment

Effects of fixed-carbon starvation on plant growth and survival were determined as described (Suttangkakul et al., 2011). Briefly, for +C, seedlings were grown on 1/2 MS solid medium with sucrose under LD for 20 d. For –C, seedlings were grown on sucrose-free 1/2 MS solid medium under LD for one week, transferred to darkness for 9-10 d, then allowed to recover for 3-4 d in LD. For detecting protein expression, seedlings grown under LD on 1/2 MS solid medium for 1 week were transferred to –C liquid medium for different times.
Measurement of Chlorophyll Contents

Chlorophyll was extracted by adding 450 μL 95% ethanol to 50 μL tissue resuspension and incubating at 4°C in the dark. Supernatants were separated from tissue debris by centrifugation at 14,000 rpm at 4°C for 10 min. Absorbances at 652 nm of the supernatant were measured. The chlorophyll content was calculated as described previously (Qi et al., 2017; Yang et al., 2020).

Plasmid Construction

Most plasmids used in this study were generated using Gateway Cloning Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Briefly, full-length coding sequence fragments of TOPPs or ATGs were amplified and inserted into pDONR-Zeo via the Gateway BP clonase I reaction. Sequence-confirmed coding sequences were then subcloned into destination vectors. To generated vectors harboring mutated versions of ATG13a, primers carrying mutated bases were designed, pDONR-Zeo carrying sequence-confirmed ATG13a was used as the template. The PCR products were digested at 37°C for 5 h with DpnI, then transformed to E. coli. To generate CRISPR-cas9 vectors, the golden gate method to construct a vector expressing one or two gRNAs specific targets was used as described (Xing et al., 2014).

Yeast Two-Hybrid (Y2H) Assays

For direct protein-protein interaction by Y2H, pairwise AD and BD were co-transformed into yeast strain Y2H gold (Clontech). Cells transformed with both plasmids were selected after growth 2 d at 30°C on synthetic dropout medium lacking leucine and tryptophan. Protein-protein interactions were then identified by growing for 2 d at 30°C on synthetic dropout medium lacking adenine, leucine, tryptophan, histidine, and containing 20 mg/ml X-α-gal. To confirm interactions, single colony was diluted in sterile H2O to an OD600 of 0.1, and 10 μL was spotted onto both types of selective medium and again grown for 2 d at 30°C.
Bimolecular Fluorescence Complementation (BiFC)

In planta protein-protein interactions were assayed using bimolecular fluorescence complementation in *N. benthamiana* leaves. The resulting plasmids were introduced into *A. tumefaciens* strain GV3101. Overnight cultures were resuspended in 5 mL infiltration buffer (0.15 M acetosyringone dilute in DMSO; 0.01 M MES, pH 7.5; 0.01 M MgCl₂), incubated at room temperature for 4 h in the darkness, and then used for direct infiltration of 4 to 6-week-old *N. benthamiana* leaves. Leaf sections of approximately 2 mm × 2 mm excised 36-48 h after infiltration were visualized by confocal fluorescence microscopy.

Drug Treatment and Confocal Laser Scanning Microscopy

For concanavalin A (ConcA) treatment, *Arabidopsis* seedlings expressing GFP or YFP reporters were grown on 1/2 MS medium for 5 d, then transferred to sucrose-free liquid medium with or without 0.5 μM ConcA for 12 h in the darkness. For calyculin A (CL-A) treatment, five-day-old seedlings grown on 1/2 MS agar medium were first preincubated with or without 0.5 μM CL-A in 1/2 MS liquid medium for 60 min. Then transferred to sucrose-deficient liquid medium with 1 μM ConcA for an additional 12 h in the darkness. Following incubation, the root elongation zone was visualized with a Nikon A1+ confocal laser scanning microscope, using 40 × water objectives. Excitation was performed at 488 nm and 543 nm, and emission was collected at 500-530 nm or 565-615 nm, for GFP and RFP signals, respectively. Identical settings were used for imaging of *N. benthamiana* leaf sections subjected to *A. tumefaciens*-mediated infiltration as described above.

Protein Isolation and Immunoblot Analysis

For protein extraction, *Arabidopsis* samples were ground and homogenized in ice-cold extraction buffer (10 mM HEPES, pH 7.5; 100 mM NaCl; 1 mM EDTA pH 8.0; 10% Glycerol; 0.5% Triton X-100; 1 × cocktail). Samples were incubated on ice for 10-15 min and centrifuged at 4°C for 10 min at 12,000 g. The supernatant was used for electrophoresis. For immunoblot analysis, total proteins were subjected to
SDS-PAGE and electrophoretically transferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore). Antibodies used in the protein blotting analysis were ATG1α (Agrisera, AS194274, 1:3000), ATG13α (Agrisera, AS194279, 1:2000), PBA1 (Abcam, ab98861, 1:10,000), ATG8α (Abcam, ab77003, 1:1000), GFP (Abmart, M20004, 1:5000), FLAG (Proteintech, 80010-1-RR, 1:5000), GST (Abmart, M20007, 1:5000), MBP (Proteintech, 15089-1-AP, 1:5000), RFP (MBL, M155-3, 1:5000), HIS (Abmart, M2000S, 1:5000), pSer (Abcam, 9332, 1:750), pThr (Cell signaling technology, 9381S) and α-Tubulin (Sigma, T6199, 1:10,000). For dephosphorylation experiments, samples were subjected to 8% SDS-PAGE, running at 80 V for 3 h, electrophoretically transferred to PVDF membrane for 1 h at 100 V.

**Recombinant Protein Expression and Pull-Down**

For the expression of recombinant protein in prokaryotic cells, the CDSs of TOPPs were cloned into the pGEX 4T-3 (GST tag) or pET28a (HIS tag) vector and transformed to *E. coli* strain Rosetta. GST tagged proteins were purified with Glutathione Sepharose 4B beads (10250335, GE Healthcare) following the manufacturer’s instructions. HIS tagged proteins were purified with BeaverBeads™ IDA-Nickel (17B026101, Beaver) following the instructions. Protein concentration was determined using BCA protein assay kit (Solarbio, PC0021).

*In vitro* pull-down assay was performed as described (Guo et al., 2015). ATG13a-MBP and ATG13b-MBP were purified using PurKine™ MBP-Tag Dextrin Resin 6FF (BMR20206, Abbkine). MBP beads of ATG13a-MBP and ATG13b-MBP were incubated with purified GST or TOPP4-GST protein at 4°C for 2 h with gentle shaking. After wash for several times with wash buffer, the proteins were detected by immunoblotting with GST antibody.

**p-Nitrophenyl Phosphate (pNPP) Phosphatase Activity Assay**

Phosphatase activity assays were performed as described previously (Templeton et al., 2011; Hou et al., 2016). General substrate p-nitrophenyl phosphate (pNPP, Sigma, N3002) was used to measure the phosphatase activity of TOPP4. Reactions were...
performed in assay buffer (50 mM Tris-HCl, pH 7.5, 2 mM MnCl$_2$, 1 mM EDTA, 0.5% 
β-mercaptoethanol, 2 mg/ml BSA and 50 mM pNPP) with an increasing gradient of 
recombinant protein TOPP4-GST at 37°C for 1 h. After incubation, the reactions were 
quenched with 5 volumes of 0.5 M EDTA. The hydrolysis of pNPP was measured by 
following the absorbance at 405 nm (A405).

Coimmunoprecipitation (Co-IP)

For Co-IP in Arabidopsis, one-week-old seedlings of WT or transgenic plants 
overexpressing TOPP4-GFP were ground in liquid nitrogen and homogenized in 
ice-cold IP buffer (10 mM HEPES, pH 7.5; 100 mM NaCl; 1 mM EDTA pH 8.0; 10%
Glycerol; 0.5% Triton X-100; 1 × cocktail). Samples were incubated on ice for 10-15 
min and centrifuged at 4°C for 10 min at 12,000 g. The supernatant was then incubated 
with GFP magnetic beads (D153-11, MBL) for 2 h at 4°C to immunoprecipitate the 
target protein. The beads were collected and washed with cold IP buffer 3-5 times.

Co-IP in tobacco was performed as described with minor modification (Li et al., 
2017). Firstly, constructs of p35S:TOPP4-FLAG and p35S:YFP-ATGs were 
infiltrated into N.benthamiana via the Agrobacterium-mediated method. After 48 h, 
total proteins were extracted with IP buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 
5 mM dithiothreitol; 1% Triton X-100; 2% NP40 and 1 × cocktail) followed by 
incubating with anti-FLAG magnetic beads (M185-11R, MBL) for 2 h. Beads were 
washed 6-8 times with washing buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1%
Triton X-100; 2% NP40). The immunoprecipitated proteins were analyzed by 
SDS-PAGE and immunoblotted with GFP antibody.

λ. Protein Phosphatase Treatment and Dephosphorylation Assay in Vivo

Transgenic plants p35S:YFP-ATG13a/atg13ab grown on 1/2 MS medium for 7 d were 
immunoprecipitated at 4°C for 2 h. The GFP-beads were washed three times with cold 
IP buffer and another three times with reaction buffer (50 mM HEPES, pH 7.5; 100 
mM NaCl; 5 mM DTT; 0.01% Brij35; 1 mM MnCl$_2$; 10 mM Na$_2$EDTA and 0.1%
Triton X-100; supplemented with 1 mM phenylmethane-sulfonfyl fluoride and 1 × cocktail). The beads were then suspended in fresh 50 μL reaction buffer, incubated with λ protein phosphatase (40 units/μL) (P0753S, New England Biolabs) or recombinant TOPP4-GST/HIS for 1 h at 30°C. The reactions were quenched by adding an equal volume of 2 × SDS-PAGE sample buffer and heating to 95°C for 5 min. For dephosphorylation of ATG1a in atg7-3, total proteins were directly extracted with the 1.25:1 reaction buffer (volume to fresh weight). Clarified extracts were then incubated with λ protein phosphatase or TOPP4-GST as above.

**Mass Spectrometry Analysis**

To prepare samples for mass spectrometry analysis, at least 2 g seedlings of p35S::YFP-ATG13a/atg13ab grown on 1/2 MS medium for one week were collected. YFP-ATG13a was immunoprecipitated as described above, 50 μL of concentrated immunoprecipitated was separated by SDS-PAGE. Gels were stained with G-250, and then the YFP-ATG13a band was excised. In-gel tryptic digestion and mass spectrometry analysis were performed as described (Sun et al., 2021). The samples were analyzed by an Orbitrap fusion lumos mass spectrometer (Thermo Fisher Scientific) connected to an EASY-nLC 1200 system. The software Proteome Discoverer Daemon 2.2 (Thermo Fisher Scientific) was used for data analysis.

**qRT-PCR Analysis**

RNA extraction was performed as the instructions of total RNA kit (OMEGA). 5 × PrimeScript™ RT Master Mix (TAKARA) was used to synthesize cDNA. qRT-PCR analysis was performed using SYBR Premix Ex Taq II (TaKaRa) and Stepone PCR instrument. Ubiquitin10 (UBQ10) was used as a reference gene. The gene-specific primers used for qRT-PCR were listed in Supplemental Table 3.

**Sequence Alignments**

Predicted full-length protein sequences were obtained from Uniprot (https://www.uniprot.org/). Sequences were aligned with Clustal Omega
Sequence data for the genes used in this study could be found in The Arabidopsis Information Resource (www.arabidopsis.org) under the following accession numbers:

- **TOPP1** (AT2G29400), **TOPP2** (AT5G59160), **TOPP3** (AT1G64040), **TOPP4** (AT2G39840), **TOPP5** (AT3G46820), **TOPP6** (AT4G11240), **TOPP7** (AT5G43380), **TOPP8** (AT5G27840), **TOPP9** (AT3G05580), **ATG1a** (AT3G61960), **ATG1b** (AT3G53930), **ATG1c** (AT2G37840), **ATG1t** (AT1G49180), **ATG8a** (AT4G21980), **ATG8e** (AT2G45170), **ATG13a** (AT3G49590), **ATG13b** (AT3G18770), **ATG16** (AT5G50230), **ATG7** (AT5G45900).

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**AUTHOR CONTRIBUTIONS**

S.W.H and Q.L.W designed the research, Q.L.W, Q.Q.Q, M.F.S, N.L, J.Z, Y.L carried out the experiments, S.W.H and Q.L.W wrote the manuscript.

**COMPETING INTERESTING**

The authors declare no competing interests.

**SUPPLEMENTAL DATA**

- **Supplementary Figure 1.** Dominant-negative mutant *topp4-1* and loss function mutants *topp-7m-2* and *topp-7m-3* are hypersensitive to fixed-C starvation.
- **Supplementary Figure 2.** Transcription of **TOPPs** upon fixed-C starvation.
- **Supplementary Figure 3.** TOPP members function redundantly in regulating plant
response to fixed-C starvation.

**Supplementary Figure 4.** The sensitivity of *topp-7m-1* to fixed-C starvation is recovered by *TOPP*, and overexpression of *TOPP1* and *TOPP5* enhances plant tolerance to fixed-C starvation.

**Supplementary Figure 5.** Autophagic flux is affected by TOPP phosphatase activity.

**Supplementary Figure 6.** The expression of ATGs in WT and *topp-7m-1* upon fixed-C starvation.

**Supplementary Figure 7.** Interaction between TOPP and ATG13a, ATG13b, ATG1a, ATG1b, ATG1c, ATG1t, or ATG16.

**Supplementary Figure 8.** Subcellular localization of TOPP depends on autophagy upon fixed-C starvation.

**Supplementary Figure 9.** ATG13a is dephosphorylated upon fixed-C starvation.

**Supplementary Figure 10.** Conservation analysis of 18 phosphorylation sites of ATG13a in different species.

**Supplementary Figure 11.** Substitution of S248, S343, S397, and S473 did not affect the function of ATG13a in response to fixed-C starvation.

**Supplementary Figure 12.** Interaction between ATG1a and ATG13a different domains; illustration of ATG13 possible phosphorylation sites; subcellular localization of ATG1a/1b/1c, ATG8e, ATG13a/13b; and IDR prediction of ATG1a/1b/1c, ATG8e, ATG13a/13b.

**Supplementary Table 1.** Identification of ATG13a peptides in GFP-tagged TOPP4 immunoprecipitation product.

**Supplementary Table 2.** Analysis of phosphorylation of ATG13a in WT and *topp4-1* by iTRAQ quantitative phosphoproteomics.

**Supplementary Table 3.** Oligonucleotide primers for qRT-PCR.

**Supplementary Table 4.** Mutants generated in this study.

**Supplementary Table 5.** Transgenic plants generated in this study.

**Supplementary Table 6.** List of ATG13a homolog proteins.

**Supplemental Dataset 1.** Detailed ATG13a phosphorylation peptides identified by
LC-MS/MS.

Reference


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Figure 1. Plants Lacking at Least Seven Members of TOPP are Hypersensitive to Fixed-C Starvation.

(A) Phenotypes of topp-7m-1 (topp1/4/5/6/7/8/9) and topp-8m (topp1/2/4/5/6/7/8/9) in response to fixed-C starvation. Seedlings of WT, topp-7m-1, topp-8m, and atg13ab grown on 1/2 MS solid medium with sucrose under long day (LD) (16 h light/8 h dark)
constantly (+C), or grown on sucrose-free 1/2 MS solid medium under LD for 7 d, placed in darkness for 9-10 d, and then allowed to recover for 3-4 d in LD (−C). #79, #85, and #88 refer to different lines of topp-7m-1, and all topp-7m-1 in the following text indicates line #79.

(B) TOPP4 recovers the sensitivity of topp-7m-1 to fixed-C starvation. One-week-old WT, pTOPP4:TOPP4-GFP/topp-7m-1, and atg7-3 seedlings were treated the same as (A).

(C) Enhanced tolerance of the TOPP4-OE lines to fixed-C starvation is dependent on autophagy pathway. One-week-old WT, p35S:TOPP4-GFP/WT, p35S:TOPP4-GFP/atg7-3, and p35S:TOPP4-GFP/atg13ab seedlings were treated the same as (A).

(D)-(F) Relative chlorophyll contents of the seedlings with or without fixed-C starvation shown in (A)-(C). The relative chlorophyll contents were calculated by comparing the values in −C seedlings versus +C ones. Values are means ± SD of three biological replicates. Statistical differences were calculated by one-way ANOVA. Different letters above each bar indicate statistically significant differences as determined by Tukey’s multiple testing methods (P < 0.05).
Figure 2. TOPP are Required for Autophagy Turnover.

(A) Confocal images of YFP-(ATG8e, ATG1a, ATG13a)-labeled autophagic bodies inside the central vacuoles. Seedlings of WT or topp-7m-1 expressing YFP-ATG8e, YFP-ATG1a, and YFP-ATG13a grown for 5 d on 1/2 MS solid medium were transferred to fresh 1/2 MS liquid medium (+C), or sucrose-free 1/2 MS liquid medium and incubated in darkness for 12 h (−C) with or without the addition of 0.5 μM ConcA. Scale bars, 10 μm.

(B) Statistical data of the autophagic bodies shown in (A). Values are means ± SD from 4 different optical sections from 10 individual roots. Statistical differences were calculated by one-way ANOVA. Different letters above each bar indicate statistically significant differences as determined by Tukey’s multiple testing methods ($P < 0.05$).

(C) Immunoblotting analysis showing the processing of YFP-ATG8e fusion proteins in WT and topp-7m-1 after fixed-C starvation. Seedlings were treated upon fixed-C
starvation for the indicated time. Total protein was extracted and analyzed with GFP antibody. PBA1 was used as a protein loading control. Values below each lane represent the ratio of free YFP to YFP-ATG8e as quantified by ImageJ.

(D) ATG1a and ATG13a proteins in WT and topp-7m-1. The relative intensities of each band against the PBA1 loading control were quantified using ImageJ, and the first lane in each experiment was set to 1. The gray value of two bands of ATG13a or ATG1a was calculated as a whole.

hpt, hours post treatment. Protein experiments were repeated at least three times with similar results.

**Figure 3. TOPP Interacts with ATG13a and Associates with Autophagosomes.**
(A) Interaction of TOPPs with ATG13a and ATG13b in Y2H assays. −LW, SD medium lacking Trp and Leu; −LWHA+X, SD medium lacking Trp, Leu, His, and Ade; X, X-α-gal.

(B) Pull-down assays showing the interaction of TOPP4 with ATG13a or ATG13b. ATG13a-MBP or ATG13b-MBP was immobilized on MBP sepharose beads, incubated with GST or TOPP4-GST protein, and subjected to immunoblot analysis with GST antibody. Asterisks represent non-specific bands. Experiments were repeated two times with similar results.

(C) BiFC assays of interaction between TOPP4 and ATG13a or ATG13b. Scale bar, 50 μm. β-glucuronidase (GUS) was used as a noninteracting control.

(D) Coimmunoprecipitation of TOPP4 with ATG13a and ATG13b. For ATG13a, total protein was extracted from WT and 35S:TOPP4-GFP/WT, immunoprecipitated with GFP magnetic beads, and then analyzed by ATG13a antibody. For ATG13b, TOPP4-FLAG was co-expressed with YFP-ATG13b in N. benthamiana. Total protein was immunoprecipitated with GFP magnetic beads and analyzed by FLAG antibody. Asterisks represent non-specific bands. Experiments were repeated three times with similar results.

(E) Intracellular colocalization of RFP signal from TOPP4-RFP with the GFP signal from GFP-ATG8e. Five-day-old co-expressing TOPP4-RFP and GFP-ATG8e seedlings grown on 1/2 MS medium were transferred to +C or −C conditions with or without 0.5 μM ConcA for 12 h. Arrows indicate the co-localized puncta. The percentages indicate the contributions of autophagic routes to the vacuolar deposition of TOPP4-RFP. Scale bars, 10 μm.

(F) Degradation of GFP-ATG8e and TOPP4-RFP in WT upon fixed-C starvation. One-week-old co-expressing TOPP4-RFP and GFP-ATG8e seedlings were treated upon fixed-C starvation for indicated time. Total protein was analyzed with GFP or RFP antibody. Values below each lane represent the ratio of free GFP to GFP-ATG8e or free RFP to TOPP4-RFP as quantified by ImageJ. hpt, hours post-treatment. Experiments were repeated three times with similar results.
Figure 4. TOPP Dephosphorylates ATG13a in Vitro and in Vivo.

(A) Fixed-C starvation induces the rapid dephosphorylation of ATG13a. Seedlings of p35S::YFP-ATG13aWT grown on 1/2 MS medium for 7 d were treated upon fixed-C starvation for the indicated time. Phosphorylation of ATG13a was conducted by pSer antibody immunoblotting. Numbers below each lane represent the relative abundance of phosphorylated ATG13a. hpt, hours post-treatment.

(B) Detection of purified TOPP4-GST enzyme activity in vitro. The phosphatase activity of TOPP4-GST was determined by a colorimetric assay using the substrate...
p-nitrophenyl phosphate (pNPP). GST was used as negative control.

(C) Dephosphorylation of ATG13a by TOPP4-GST. One-week-old unstarved seedlings of p35S:YFP-ATG13a/WT were grown on 1/2 MS medium, total protein extracts were immunoprecipitated with GFP magnetic beads, then treated with TOPP4-GST with or without the phosphatase inhibitor PhosSTOP, and immunoblotted with GFP, GST, or ATG13a antibody. UN, untreated extracts.

(D) Dephosphorylation of ATG13a by TOPP1, TOPP3, and TOPP9 in vitro. Seedlings were treated the same as (C). Total protein extracts were treated with TOPP1-HIS, TOPP3-HIS, or TOPP9-HIS, then subjected to immunoblot analysis with GFP or HIS antibody. Asterisks represent non-specific bands.

(E), (F) In vivo dephosphorylation of ATG13a by TOPP4, TOPP1, TOPP3, and TOPP9. TOPPs-RFP or GUS-RFP together with YFP-ATG13a was transiently co-expressed in N. benthamiana leaves. Phosphorylation of ATG13a was conducted by pSer antibody immunoblotting. Numbers below the blots represent the relative abundance of phosphorylated ATG13a. Asterisks represent non-specific bands.

(G)-(I) Dephosphorylation of ATG13a in WT and topp-7m-1 after fixed-C starvation confirmed by different antibodies. Seedlings were treated the same as (A). Numbers below the blots represent the relative abundance of phosphorylated ATG13a. Long exp. and short exp. indicate long time and short time exposure. All the protein experiments were repeated at least three times with similar results.
Figure 5. Dephosphorylation of ATG13a Enhances Fixed-C Starvation Induced Autophagy.

(A) Summary of ATG13a phosphorylation sites identified by LC-MS/MS.

(B) Phenotypes of the transgenic plants p35S:YFP-ATG13a/atg13ab, p35S:YFP-ATG13a18A/atg13ab, and p35S:YFP-ATG13a18D/atg13ab in response to...
fixed-C starvation. Seedlings grown on 1/2 MS solid medium with sucrose under LD constantly (+C), or grown on sucrose-free 1/2 MS solid medium under LD for 7 d, placed in darkness for 11 d, and then allowed to recover for 4 d in LD (−C).

(C) Relative chlorophyll contents of seedlings shown in (B). Values are means ± SD of three biological replicates. Statistical differences were calculated by one-way ANOVA. Different letters above each bar indicate statistically significant differences as determined by Tukey’s multiple testing methods ($P < 0.05$).

(D) Confocal images of YFP-ATG13a, YFP-ATG13a$^{18A}$, and YFP-ATG13a$^{18D}$ labeled autophagic bodies inside the central vacuoles. Seedlings of $p35S$:YFP-ATG13a/atg13ab, $p35S$:YFP-ATG13a$^{18A}$/atg13ab, and $p35S$:YFP-ATG13a$^{18D}$/atg13ab grown on 1/2 MS medium for 5 d were transferred to +C or −C conditions with or without 0.5 μM ConcA for 12 h. Scale bars, 10 μm.

(E) Numbers of puncta per section in the root cells of seedlings in (D). Values are means ± SD from 4 different optical sections from 10 individual roots. Statistical differences were calculated by one-way ANOVA. Different letters above each bar indicate statistically significant differences as determined by Tukey’s multiple testing methods ($P < 0.05$).

(F) Free YFP release from YFP-ATG13a, YFP-ATG13a$^{18A}$, and YFP-ATG13a$^{18D}$ after fixed-C starvation. Seedlings of $p35S$:YFP-ATG13a/atg13ab, $p35S$:YFP-ATG13a$^{18A}$/atg13ab, and $p35S$:YFP-ATG13a$^{18D}$/atg13ab grown on 1/2 MS medium for 7 d were starved for the indicated time, total protein extracts were immunoblotted with GFP antibody. Values below each lane represent the ratio of free YFP to YFP fusions as quantified by ImageJ. hpt, hours post-treatment.

(G) Immunodetection of ATG8a protein of seedlings shown in (F). Plants were treated the same as (F). Total protein was analyzed with ATG8a antibody. The relative intensities of each band against the α-Tubulin loading control were quantified using ImageJ.

Protein experiments were repeated three times with similar results.
Figure 6. Residue Substitution Effects of the Identified Phosphorylation Sites of ATG13a on Their Phosphorylation State upon Fixed-C Starvation.

(A), (B) Dephosphorylation of ATG13a^{4A}(18A) and ATG13a^{4D}(18D) by λ phosphatase and TOPP4-GST. Seedlings of p35S:YFP-ATG13a/atg13ab, p35S:YFP-ATG13a^{4A}(18A)/atg13ab, and p35S:YFP-ATG13a^{4D}(18D)/atg13ab were collected from 1/2 MS medium, total protein was immunoprecipitated with GFP magnetic beads, the purified beads were treated with λPP or TOPP4-GST, then subjected to immunoblot analysis with GFP antibody.

(C), (D) Dephosphorylation of ATG13a, ATG13a^{4A}(18A), and ATG13a^{4D}(18D) by TOPP4. TOPP4-RFP or GUS-RFP together with YFP-ATG13a, YFP-ATG13a^{4A}(18A), or YFP-ATG13a^{4D}(18D) was transiently co-expressed in N. benthamiana leaves. α-GFP
IP was used to remove the background. Phosphorylation of ATG13a was conducted by α-pSer immunoblotting. Numbers below the blots represent the relative abundance of phosphorylated ATG13a. Asterisks represent non-specific bands.

(E), (F) Effects of the mimic 4A, 4D, 18A, and 18D of ATG13a on their dephosphorylation under fixed-C starvation. Seedlings of atg13ab expressing YFP-tagged ATG13a, ATG13a4A, ATG13a4D, ATG13a18A, and ATG13a18D grown on 1/2 MS medium for 7 d were treated with fixed-C starvation for the indicated time. hpt, hours post-treatment.

All the protein experiments were repeated at least three times with similar results.
Figure 7. TOPP-Controlled Dephosphorylation of ATG13a Promotes the Formation of ATG1a-ATG13a Complex.

(A) Illustration of ATG13a domain. The numbers indicate the positions of the amino acids in the constructs.

(B) Subcellular localization of different domains of ATG13a, ATG13a<sup>18A</sup> and ATG13a<sup>18D</sup> in tobacco epidermal cells. Arrows represent ATG13a puncta in the cytoplasm. Scale bar, 50 μm.

(C), (D) Y2H and BiFC analysis of the interaction between ATG1a and the full-length,
truncated, or mutated ATG13a. Scale bar, 50 µm.

(E) Coimmunoprecipitation of ATG1a with the wild or mutated ATG13a. ATG1a together with YFP-ATG13a, YFP-ATG13a18A, and YFP-ATG13a18D was transiently co-expressed in N. benthamiana leaves for 2 d. Total protein was immunoprecipitated with GFP magnetic beads and analyzed by ATG1a antibody. Numbers below the blots represent the relative abundance of ATG1a.

(F), (G) ATG1a and ATG13a interaction upon fixed-C starvation. Seedlings of p35S:YFP-ATG13a/WT or p35S:YFP-ATG1a/WT grown on 1/2 MS medium for 7 d were treated by fixed-C starvation for the indicated time. Total protein was immunoprecipitated with α-GFP beads, and the bound protein was detected by ATG1a or ATG13a antibody. Values below each lane represent the ratio of ATG1a to ATG13a or ATG13a to ATG1a as quantified by ImageJ. The gray value of two bands of ATG1a was calculated as a whole in (G).

(H) Coimmunoprecipitation of ATG13a, ATG13a18A, and ATG13a18D with ATG1a upon fixed-C starvation. Seedlings of p35S:YFP-ATG13a/atg13ab, p35S:YFP-ATG13a18A/atg13ab, and p35S:YFP-ATG13a18D/atg13ab were treated same as (F) and (G). Values represent the ratio of ATG1a to ATG13a or mutated ATG13a.

(I), (J) The interaction of ATG1a and ATG13a in topp-7m-1 upon fixed-C starvation. Seedlings of p35S:YFP-ATG13a/WT, p35S:YFP-ATG13a/topp-7m-1, p35S:YFP-ATG1a/WT, and p35S:YFP-ATG1a/topp-7m-1 were treated same as (F) and (G). Values below each lane represent the ratio of ATG1a to ATG13a or ATG13a to ATG1a as quantified by ImageJ. The gray value of two bands of ATG1a was calculated as a whole in (J).

hpt, hours post-treatment. All the protein experiments were repeated three times with similar results.
Figure 8. TOPP-Controlled Dephosphorylation of ATG13a Boosts ATG1a Phosphorylation.

(A) Interaction of TOPPs with ATG1a in Y2H assays.

(B) BiFC assays of interaction between TOPPs and ATG1a. β-glucuronidase (GUS) was used as a noninteracting control. Scale bar, 100 μm.

(C) Coimmunoprecipitation of TOPP4 with ATG1a. Total protein was extracted from 1-week-old WT and p35S:TOPP4-GFP/WT seedlings, immunoprecipitated with GFP magnetic beads, and then analyzed by ATG1a antibody.

(D) Phosphorylation of ATG1a in atg7-3 under fixed-C starvation. Seedlings of atg7-3 grown on 1/2 MS solid medium for 7 d were transferred to +C or −C conditions for the indicated time. Total protein was extracted and then immunoblotted with ATG1a antibody.

(E), (F) TOPP4 could not dephosphorylate ATG1a. One-week-old atg7-3 seedlings were treated for 72 h under fixed-C starvation. Total protein was extracted and then
treated with λPP or TOPP4-GST, followed by immunoblot analysis with ATG1a antibody (E). TOPP4-RFP or GUS-RFP together with YFP-ATG1a was transiently co-expressed in *N. benthamiana* leaves. Phosphorylation of ATG1a was conducted by pSer and pThr antibodies immunoblotting. Numbers below the blots represent the relative abundance of phosphorylated ATG1a. Asterisks represent non-specific bands (F).

**G** TOPP4 promotes phosphorylation of ATG1a in *atg7-3*. Seedlings of *atg7-3* and *p35S:TOPP4-GFP/atg7-3* grown on 1/2 MS medium for 7 d, then treated with fixed-C starvation for different time. Total protein was extracted and then immunoblotted with ATG1a antibody.

**H** Promoting phosphorylation of ATG1a by TOPP4 is ATG13a-dependent. Seedlings of *atg13ab* and *p35S:TOPP4-GFP/atg13ab* were treated same as (G). Total protein was extracted and then immunoblotted with ATG1a antibody.

hpt, hours post-treatment. All the protein experiments were repeated three times with similar results.

Figure 9. A Proposed Model for the Regulation of Autophagy by TOPP in *Arabidopsis*. In WT, ATG13a is kept in a hyperphosphorylated state by the active
TOR under nutrient-rich conditions, leading to a weak ATG1a-ATG13a interaction. Upon carbon starvation, TOR is inactivated, and TOPP contributes to the dephosphorylation of ATG13a, forming tight ATG1a-ATG13a complex. Then ATG1a is phosphorylated and autophagy is induced. Disruption of the TOPP results in the accumulation of hyperphosphorylated ATG13a and weakened ATG1a-ATG13a interaction, impairing ATG1a phosphorylation. Thus, autophagy is inhibited.