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Shedding Light on the Role of Phosphorylation in Plant Autophagy

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Autophagy is a highly conserved quality control process that maintains cellular health by eliminating deleterious cargoes. Compared with the extensive studies in yeast and mammalian models, the molecular details and significance of post-translational modifications (PTMs) in the autophagy process in plants remain less well defined. In this review, we discuss recent progress in our understanding of phosphorylation, one of the most extensively studied PTMs, in the regulation of autophagosome biogenesis and autophagic degradation in plants. Based on the plant mass spectrometric database, we summarize the experimentally verified phosphorylation sites of the core autophagy machinery in plants. Furthermore, we put forward several approaches to test the roles of phosphorylation in the regulation of plant autophagy.

Keywords: autophagy; dephosphorylation; phosphorylation; post-translational modifications; SnRK1; TOR kinase

Introduction

In eukaryotes, autophagy is a highly conserved catabolic process that delivers cargos into lysosome (in mammals) or vacuole (in yeast and plant) for degradation. As one of the survival pathways and quality control mechanisms, autophagy plays a wide range of physiological roles in plant growth and development [1–3], leaf senescence [4–6], plant immunity [7–9] and response to environmental stresses [10–15]. Based on the morphological and mechanistic characteristics, autophagy can be categorized into three distinct types: macroautophagy, microautophagy and chaperone-mediated autophagy. Among them, the macroautophagy (hereafter autophagy) is the most widely studied. The typical feature of this type autophagy is the formation of a double-membrane structure, termed autophagosome, which finally fuses with lysosome or vacuole to release the inner membrane and inclusions. In the past decades, more than 40 conserved autophagy-related (ATG) genes have been identified [16]. There are about 20 core ATG proteins involved in the process of autophagosome formation, which can be subdivided into several units: ATG1 complex, phosphatidylinositol 3-kinase complex (PI3K complex), ATG9 vesicles, ATG2-ATG18 complex and the ATG conjugation systems [17]. These core ATG proteins act either sequentially or synergistically to initiate and organize the autophagosome formation.

Abbreviations

AMPK, AMP-regulated kinase; ATG, autophagy-related; FLZs, FCS-like zinc fingers; FREE1, FYVE domain protein required for endosomal sorting 1; LST8, lethal with Sec13 protein 8; O-GlcNAc, O-linked β-N-acetylglucosamine; PI3K, phosphatidylinositol 3-kinase; PP2A, phosphatase 2A; PP2C, type 2C protein phosphatases; PTMs, post-translational modifications; RAPTOR, regulatory-associated protein of TOR; SnRK1, sucrose non-fermenting-related kinases 1; TOR, target of rapamycin.

BOX 1. A brief summary of several typical PTMs in the regulation of autophagy.

Phosphorylation: Phosphorylation, a critical signal initiating autophagy, is highly dynamic and fine-tuned by various Ser/Thr kinases and phosphatases.

Ubiquitination: Ubiquitination not only affects the stability of ATG proteins but also functions as an important signal that recognized by autophagic receptors/adaptors such as p62/SQSTM1 and NBR1 (neighbour of BRCA1) [23].

Acetylation: Acetylation works at multiple levels in the autophagy process, including direct acetylation of the core ATG proteins such as ATG5, ATG7 and ATG12 [26], as well as acetylation of some transcription factors affecting the *ATG* gene transcription [10].

Lipidation: Lipidation involves the covalent conjugation of the lipid phosphatidylethanolamine (PE) to ATG8 via the ATG5-ATG12 conjugate [27]. The ATG8-PE imbeds in the membranes of phagophore, facilitating its assembly.

SUMOylation: The covalent attachment of SUMO (small ubiquitin-like modifier) at the Lys840 of VPS34 increases its activity bound to Beclin 1 and autophagosome formation [28].

Methylation: Methylation acts at multiple levels in the autophagy process, including affecting the *ATG5* and *ATG7* transcripts [30], cargo selection [29] and the binding of ATG16L1 to the ATG5-ATG12 conjugate [31].

Glycosylation: Glycosylation influences multiple steps of autophagy, including the induction of autophagy and several key steps in the autophagy process [32].

Persulfidation: Persulfidation acts as a signalling mechanism or directly targets the active-site cysteine of ATG18a, ATG4, ATG3, ATG5 and ATG7 to be involved in autophagy regulation [34,35].

Oxidation: The regulation of oxidation in autophagy occurs in all steps of autophagy. It can not only function as a signal to initiate autophagy but also regulate autophagy by directly oxidizing ATG3, ATG4, ATG7 and other key autophagic factors [36–38].

Palmitoylation: Palmitoylation affects cargo selection, autophagosome formation and fusion [40].

S-nitrosylation: S-nitrosylation is a process entailing the covalent addition of nitric oxide to a cysteine residue, which impacts on autophagy by affecting the upstream regulators of AMPK, mTOR and the PI3K complex [41,42].

Increasing evidence demonstrates that the activity of autophagy is strictly regulated by post-translational modifications (PTMs) (Box 1). So far, PTMs including phosphorylation [18–20], ubiquitination [21–23], acetylation [24–26], lipidation [27], SUMOylation [28], methylation [29–31], glycosylation [32,33], persulfidation [34,35], oxidation [36–38], palmitoylation [39,40] and S-nitrosylation [41–43] have been shown to regulate autophagy (Box 1 and Figure 1). These PTMs may influence the structural conformation, stability, enzymatic activities, and subcellular localization of ATG proteins and other accessory autophagy regulators. For example, autophosphorylation of Thr226 and Ser230 induces a conformational change in the catalytic loop and disrupts the active site of yeast ATG1 [44]. In human ULK1 (Unc-51-like kinase 1), the phosphorylation of Ser1042 and Thr1046 sites increases its interaction with Cul3 ubiquitin ligase, promoting ubiquitination and degradation of ULK1 [45]. Although the detailed modification residues remain undetermined, the stability and activity of ATG6 in plants are regulated by ubiquitination and phosphorylation, respectively [46,47].

In some cases, the regulation of autophagy by PTMs is highly dynamic and can compete at the same residue site or cooperate at different residue sites. Phosphorylation is a critical signal for triggering autophagy and is antagonistically regulated by two conserved Ser/Thr kinase complexes, TOR (target of rapamycin) and AMPK (AMP-regulated kinase) [18]. In response to nutrient levels, the phosphorylated site can be competitively modified with O-GlcNAc (O-linked β-N-acetylglucosamine), which influences autophagy activity at multiple steps including autophagosome maturation and the fusion between autophagosome and lysosome [32,33]. In the process of ATG8 lipidation, ATG8 and ATG12 are conjugated to the active-site cysteine of ATG7, ATG3 and ATG10 [48]. The high-energy thioester bond is possibly blocked by persulfidation, S-nitrosylation, palmitoylation and oxidation. Several recent reports clearly demonstrated that persulfidation of ATG4a at Cys170 or ATG18a at Cys103 negatively regulates autophagy in plants [34,35]. Besides cysteine, lysine residue can also undergo various covalent PTMs, such as ubiquitination [21–23], acetylation [24–26], SUMOylation [28] and methylation [29–31], which act as important regulatory signals involved in the regulation of autophagy. The complicated interplay among PTMs is critical to precisely modulate autophagy activity under different environmental stimuli.

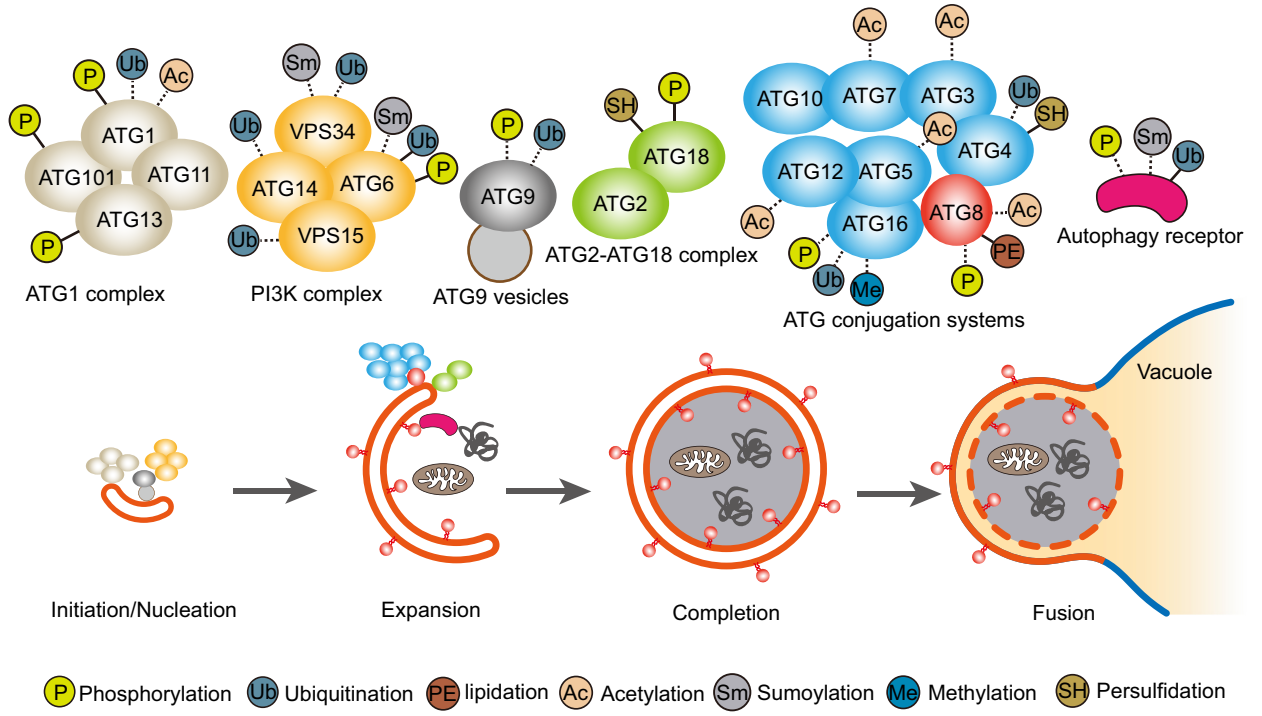


Fig. 1. Overview of post-translational modifications in the core ATG proteins. In the process of autophagy, the core ATG proteins can be divided into several categories: (1) ATG1 complex, (2) PI3K complex, (3) ATG9 vesicles, (4) ATG2-ATG18 complex and (5) ATG conjugation systems. These core ATG proteins are tightly regulated by various PTMs, including, but are not limited to, phosphorylation, ubiquitination, lipidation, acetylation, SUMOylation, methylation, glycosylation, persulfidation, oxidation, palmitoylation and S-nitrosylation. The PTMs occur in all steps of autophagy, including cargo selection, phagophore initiation/nucleation, expansion, autophagosome completion, and fusion with the lysosome or vacuole. Solid lines mark PTMs validated in plants, whereas dotted lines represent PTMs that are not unproven in plants, but experimentally verified in other organisms.

At present, our understanding of the regulation of PTMs in plant autophagy has not yet reached a level of precision comparable to the studies in mammals and yeast systems. In this review, we focus mainly on the regulation of autophagy by phosphorylation and dephosphorylation, and discuss several potential phosphorylation sites of the core autophagy machinery in plants.

Regulation of plant autophagy by phosphorylation

Phosphorylation is one of the most intensively studied PTMs in the process of autophagy. Similar to yeast and animals, autophagy in plants is ultimately regulated by the conserved TOR and SnRK1 (sucrose non-fermenting-related kinases 1, one of the animal AMPK orthologs) signalling pathways [49–52]. In mammals, TOR kinase is a large protein (~280 kDa) that exists as two functionally and biochemically distinct complexes, mTORC1 (mammalian TOR complex 1) and mTORC2, which are formed by different associated elements [19], whereas, in plants, there is only one form of TOR complex (i.e. TORC1) consisting of TOR kinase, RAPTOR (regulatory-associated protein of TOR) and LST8 (lethal with Sec13 protein 8) [53–55]. There are approximately 40 SnRKs encoded by Arabidopsis genome, of which SnRK1 typically represents the ortholog of mammalian AMPK [56,57]. Similar to the architecture of AMPK, SnRK1 exhibits a highly conserved heterotrimeric structure comprising a catalytic α subunit (two isoforms in Arabidopsis, KIN10 and KIN11) and regulatory β and γ subunits [57,58]. FCS-like zinc finger proteins (FLZs) are identified as the plant-specific adaptors of SnRK1, which might be involved in the regulation of TOR signalling through affecting the interaction between SnRK1 and RAPTOR in plants [59–61].

Under nutrient-rich growth conditions, the TOR signalling pathway promotes cell proliferation and metabolism and negatively regulates autophagy. Autophagy directly affects cell metabolism through degradation of cellular constituents in the vacuole. Using etiolated Arabidopsis seedlings as a model, Avin-Wittenberg

et al. have revealed that the levels of free amino acids were reduced in *atg* mutants [62]. Thus, there should be an amino acid-sensing mechanism by TOR to maintain cellular homeostasis. Actually, in yeast and mammals, the levels of amino acids affect the guanine nucleotide-binding status of RAG/Gtr GTPases and eventually the activity of TORC1 [63,64]. The perception of amino acid levels or energy status helps to determine whether activation of TOR inhibits autophagy or inactivation of TOR enhances autophagy. Nonetheless, how the upstream amino acid sufficiency is sensed to integrate TOR signalling and autophagy in plants remains to be uncovered.

The antagonistic phosphorylation of ATG1 complex by TOR kinase and the AMPK/SnRK1 is likely a decisive step in initiating autophagy [18,49,52]. Generally, under nutrient-replete conditions, TOR negatively regulates autophagy through hyperphosphorylating ATG1 and ATG13, thereby reducing the activity of ATG1 kinase. By contrast, nutrient or energy starvation increases the AMP/ATP ratio and activates AMPK/SnRK1 that positively phosphorylates ATG1 to activate autophagy [52,65,66]. In yeast, TOR-mediated phosphorylation of ATG13 reduces the interaction of ATG1 with its associated proteins [67]. Conversely, the affinity between ATG13 and ULK1 in mammals seems to be unaffected by TOR kinase [68]. The complicated relation might be due to the different accessory compartments in the ATG1 complex of mammalians and yeast. It is worth to note that most plants, such as Arabidopsis, are relatively rapamycin-insensitive, due to mutations in the FKBP12 that prevent the formation of the FKBP12–rapamycin–TOR complex [69]. However, recent studies have revealed that several TOR-specific inhibitors, such as TORINs and AZD8055, efficiently restrain plant root growth [70,71]. Inhibition of TOR activity by AZD8055 leads to constitutive autophagy in Arabidopsis seedlings [49,51]. At present, the direct biochemical evidences linking TOR to ATG1 complex in plants are still lacking. Whether the affinity between ATG1 and ATG13 is affected by plant TOR kinase remains to be examined.

As an initial core machinery for autophagosome formation, the ATG1 complex in plants is extensively phosphorylated in the experimentally verified phosphorylation sites (Table 1, Figure 2A), which are retrieved from the available mass spectrometric phosphoproteomic database [72]. The phosphorylation site in protein potentially affects its conformation, the ability of protein binding and the catalytic activity. Using tandem mass spectrometry, a large number of phosphorylation sites of ULK1/ATG1 and ATG13 have been identified in yeast and mammals [73]. In yeast ATG13, multiple residues such as Ser348, Ser496, Ser535 and Ser541 have a conserved motif (S-X-S*-P) that shares structurally homology to the sequence S-T-T*-P of eIF4E-binding protein 1 (4E-BP1), a well-known conserved motif recognized and phosphorylated by mTORC1 [74,75]. Although the motif enrichment analysis showed that there is no clear linear consensus motif, the presence of proline at position +1 and glycine at position −1 seems to be conserved features targeted by TOR kinase in plants [76]. Consistent with this criterion, Ser182 of ATG1b (S174 in ATG1c), lies in the kinase domain (Figure 2B), is detected to be phosphorylated with high confidence under nutrient-rich growth condition [77]. Similarly, Ser248 in ATG13a (Ser282 in ATG13b) also has a conserved TOR substrate motif (V-G-S*-P) (Figure 2C), which has been shown to be phosphorylated in up to 16 individual experiments [76–92]. However, whether these sites are directly phosphorylated by TOR kinase and their roles in plant autophagy regulation in plants await further investigation.

The cellular concentrations of ATP, ADP and AMP are usually maintained close to equilibrium via the reversible reaction 2ADP ↔ ATP + AMP. As a crucial cellular energy sensor, AMPK/SnRK1 is activated by increases in the ratios of AMP/ATP and ADP/ATP under energy deprivation [20,57,58]. Generally, AMPK acts as a positive regulator of autophagy, especially under energy-depriving conditions such as glucose starvation [18]. One important pathway of AMPK that activates autophagy is inactivation of mTORC1. The mTOR binding partner RAPTOR is directly phosphorylated by AMPK in two conserved serine residues (Ser722 and Ser792) [93]. The phosphorylation of RAPTOR by AMPK is required for the inhibition of mTORC1 induced by energy stress. Noteworthily, the critical residues flanking RAPTOR Ser792 are also highly conserved in Arabidopsis [93]. In future, it will be interesting to test whether the conserved residue (Ser786) in Arabidopsis RAPTOR can be phosphorylated by SnRK1 and whether such phosphorylation plays a crucial role in plant autophagy regulation.

Studies from a number of groups have revealed that the ULK1 complex is activated through direct phosphorylation by AMPK, even though there is a discrepancy in identified phosphorylation sites [18,94,95]. However, AMPK can also negatively regulate autophagy by phosphorylating ULK1 at Ser638 [96], as well as ATG13 at Ser224 [97], thus reducing the autophagy activity. In this context, the effect of AMPK in autophagy seems to be more complicated than that of mTORC1. The specific phosphorylation sites may

Table 1. Summary of the phosphorylation sites of the core ATG proteins in *Arabidopsis thaliana*. The phosphorylation sites of indicated ATG proteins were obtained from the Plant PTM Viewer database [72]. The symbol '/' indicates that no phosphorylation sites are currently found in the Plant PTM viewer.

ATG machinery	Protein name	AGI accession	Protein accession	Modified position	Search algorithm(s)	Refs
ATG1 complex	ATG1a	AT3G61960	Q94C95	S306, S352, S400, S419	MaxQuant	[77]
	ATG1b	AT3G53930	F4JBP3	S182, S303, S304, S345, S349, S358, S392, S397, S398, S448, S450, T473	MASCOT, MaxQuant, SEQUEST	[76,77,79,81–84,86,87]
	ATG1c	AT2G37840	F4IRW0	S174, S293, S295, S296, S298, S366, S367, S448, S566	MaxQuant, SEQUEST, MASCOT	[76,77,81,82,84,86,91]
	ATG1t	AT1G49180	F4I1N8	/	/	/
	ATG11	AT4G30790	Q9SUG7	S4, S686, S785, S1082, S1084, S1086	MaxQuant, MASCOT	[77, 83]
	ATG13a	AT3G49590	Q9SCK0	S48, S248, T251, S259, S263, S268, T270, S337, S341, S353, S357, T366, S374, T376, S382, S383, S386, S397, S404, S406, S407, S410, S422, S425, S446, S450, S454, S455, S456, S458, S464, S466, S509, S511, S513, S521, S543, S544, S551, S558, S560, S564, S571, S572, S573	MaxQuant, SEQUEST, MASCOT	[76–92]
	ATG13b	AT3G18770	F4J8V5	S42, S47, S54, S123, S133, S300, S311, S320, S333, S335, S395, S415, S421, S425, S437, S507, S523	MaxQuant, SEQUEST, MASCOT	[76,77,81,84,91]
	ATG101	AT5G66930	F4K265	S88, Y90, S98, S141, S212	MaxQuant	[76,77]
ATG9 vesicles	ATG9	AT2G31260	Q8RUS5	/	/	/
ATG2-ATG18 complex	ATG2	AT3G19190	F8S296	S1321, S1383, S1390, S1744	MaxQuant, MASCOT	[77,83]
	ATG18a	AT3G62770	Q93VB2	S343, S344	MaxQuant, SEQUEST, MASCOT	[77,86,87]
	ATG18b	AT4G30510	Q8H1Q8	S316, S317	MaxQuant, SEQUEST, MASCOT	[77,81,84,86]
	ATG18c	AT2G40810	Q8GYD7	/	/	/
	ATG18d	AT3G56440	Q0WPK3	/	/	/
	ATG18e	AT5G05150	Q9FHK8	/	/	/
	ATG18f	AT5G54730	Q9FH32	S9, S12, S543, S693, S696, S706, S754	MaxQuant, SEQUEST, MASCOT	[77,83,84]
	ATG18g	AT1G03380	Q8GUL1	S16, S36, S37, S336, S598, S725, S762, S772	MaxQuant, MASCOT	[77,83]
	ATG18h	AT1g54710	Q8H1Q5	S152, S338, S340, S341, S342, S352, S357, S606, T656, S657, S659, S759, S790, S796, S837, S851	MaxQuant, SEQUEST, MASCOT	[76,77,83,84,86–88,90]
PI3K complex	ATG6	AT3G61710	Q9M367	S10, S72, S96	MaxQuant, MASCOT	[80,83]
	ATG14a	AT1G77890	Q147F2	/	/	/
	ATG14b	AT4G08540	Q8H1E1	S175, S296, S299, S301, S306, S308, S420	MaxQuant, SEQUEST, MASCOT	[77,82–84]

Table 1. (Continued).

ATG machinery	Protein name	AGI accession	Protein accession	Modified position	Search algorithm(s)	Refs
	VPS15	AT4G29380	Q9M0E5	S350, S373, S374, S978, S994, S1037, S1039, S1042, S1047, S1065	MaxQuant, SEQUEST, MASCOT	[76,77,83,84]
	VPS34	AT1G60490	P42339	S37, S181	MaxQuant	[77]
	VPS38	AT2G32760	O48841	/	/	/
ATG conjugation system	ATG3	AT5G61500	Q0WWQ1	S86, S127	MaxQuant	[77]
	ATG4a	AT2G44140	Q8S929	/	/	/
	ATG4b	AT3G59950	Q9M1Y0	S26	MaxQuant	[77]
	ATG5	AT5G17290	Q9FFI2	S188, S280	MaxQuant	[77]
	ATG7	AT5G45900	Q94CD5	S320, T688	MaxQuant, SEQUEST,	[77,86]
	ATG10	AT3G07525	Q8VZ52	/	/	/
	ATG12a	AT1G54210	Q8S924	S7	MaxQuant, MASCOT	[77,83]
	ATG12b	AT3G13970	Q9LVK3	/	/	/
	ATG16	AT5G50230	Q6NNP0	/	/	/
ATG8s	ATG8a	AT4G21980	Q8LEM4	/	/	/
	ATG8b	AT4G04620	Q9XEB5	/	/	/
	ATG8c	AT1G62040	Q8S927	S5	MaxQuant	[77]
	ATG8d	AT2G05630	Q9SL04	/	/	/
	ATG8e	AT2G45170	Q8S926	/	/	/
	ATG8f	AT4G16520	Q8VYK7	/	/	/
	ATG8g	AT3G60640	Q9LZZ9	/	/	/
	ATG8h	AT3G06420	Q8S925	/	/	/
	ATG8i	AT3G15580	Q9LRP7	/	/	/
Autophagy receptor	NBR1	AT4G24690	Q9SB64	S174, S178, S224, T274	MaxQuant, SEQUEST, MASCOT	[77,86,88]

determine whether autophagy activity is activated or inhibited. In Arabidopsis, the overexpression of the SnRK1 catalytic subunit KIN10 can enhance autophagy, which is crucial for plant tolerance against a wide range of abiotic stresses [49,52]. Of note, although the detailed information about the phosphorylation sites on ATG1 is still lacking, KIN10 overexpression greatly increases the phosphorylation status of ATG1 upon carbon starvation [52]. Interestingly, another more recent study reported that autophagy initiation in plants could bypass ATG1, but use an alternative route through direct phosphorylation of the PI3K component ATG6 by SnRK1 during prolonged fixed-carbon starvation but not nitrogen starvation or short-term fixed-carbon starvation conditions [47]. At present, it is still unclear why prolonged nitrogen stress does not trigger a similar backup route. The stress stimulus condition is therefore of supreme importance to determine how the interplay between TOR kinase and SnRK1 regulates autophagy in plants.

In addition to being a substrate, ATG1 also acts as an important kinase that phosphorylates other core ATG modules to fine-tune the autophagy activity. ATG9A, Beclin1, ATG14, ATG4B and ATG16L1 have been shown as the direct substrates of ATG1 kinase in mammals and yeast [98–101]. The phosphorylation of these proteins is required for redirecting these regulators to the phagophore and recruiting other cooperators to initiate autophagy. In contrast to ATG1 complex, the level of phosphorylation in the downstream core ATG proteins is relatively weak (Table 1). Of note, the number of phosphorylation sites varies greatly among ATG18 isoforms (Table 1). The Ser344 site on ATG18a has recently been demonstrated to be continuously phosphorylated [102]. Upon necrotrophic pathogen *Botrytis cinerea* infection, Thr241, Ser328, Ser361 and Thr387 in the ATG18a were identified to be phosphorylated in a BAK1-dependent manner [102]. Although the phosphorylation of Ser344 site is independent on BAK1, dephosphorylation of all five sites (Ser344, Ser361, Thr241, Ser328 and Thr387) on ATG18a is required for inducing strong autophagy, which enhances plant resistance against *Botrytis cinerea* [102]. Nonetheless, the kinases responsible for the phosphorylation of Ser344 on ATG18a remain to be uncovered.

Growing evidence has revealed that phosphoregulation of some ATG-interacting proteins is closely related to the progression of autophagy. DSK2 (dominant suppressor of KAR 2) is a ubiquitin-binding receptor that

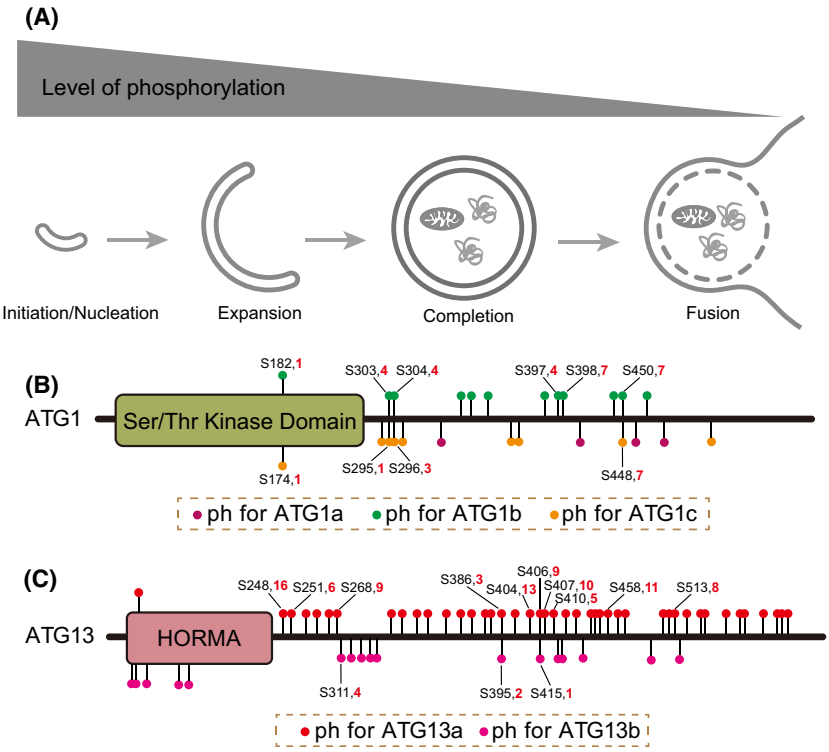


Fig. 2. Level of phosphorylation in the core ATG proteins. (A) Changes of phosphorylation level in the core ATG proteins at different autophagy stages. As a crucial regulator of autophagy initiation, the ATG1 complex has the highest levels of phosphorylation. (B and C) Schematic summary of the phosphorylation sites in *Arabidopsis* ATG1 (B) and ATG13 (C) annotated in the mass spectrometric phosphoproteomics database (the Plant PTM Viewer [72]). The phosphorylated identical residues in each isoform and the phosphorylation sites that were identified by more than 3 independent experiments are specially displayed. The red number after each residue indicates the number of independent experiments.

mediates BES1 (BRI1-EMS-Suppressor 1) degradation and enhances plant adaptation to drought and fixed-carbon starvation conditions [3]. DSK2 is phosphorylated by the GSK3-like kinase BIN2, which promotes DSK2-ATG8 interaction and facilitates BES1 degradation [3]. As a plant unique ESCRT protein, FREE1 (FYVE domain protein required for endosomal sorting 1) is associated with the autophagic regulator SH3P2 and the PI3K regulatory subunit ATG6 to regulate autophagy activity [103,104]. Recently, multiple phosphorylation sites have been identified in FREE1 by mass spectrometry [76,105]. SnRK2 could directly interact with and phosphorylate FREE1, which promotes the nuclear shuttling of FREE1 [105]. In the nucleus, FREE1 interacts with ABI5 and ABF4 to inhibit their transcriptional activation activities, thereby attenuating ABA signalling [105]. At present, it is unclear whether the nuclear-localized FREE1 is involved in regulating the expression of autophagy-related genes.

Regulation of plant autophagy by dephosphorylation

Protein phosphorylation is a reversible post-translational modification catalysed by protein kinases and reversed by phosphatases. It is well established that, following the inactivation of TOR under nutrient depletion conditions, ATG13 undergoes dramatic dephosphorylation, which is crucial for ATG1 complex assembly and initiation of autophagy in yeast [67]. Similarly, starvation or rapamycin treatment results in a rapid dephosphorylation of ULK1, particularly at Ser638 and Ser758 [96]. In these processes, the Ser/Thr phosphatases play specific roles in resetting the levels of protein phosphorylation. Based on the distinct structures, the Ser/Thr phosphatases are generally classified into three families: (1) the FCP family, (2) the PPM family, such as PP2C (type 2C protein phosphatases), and (3) the PPP family, including PP2A (phosphatase 2A), PP2B (calcineurin) and many others [106]. Among them, PP2A and PP2C are two well-characterized phosphatases in the regulation of autophagy in yeast and mammals [107–111].

Yeast Tap42 (type 2A phosphatase-associated protein 42) is an important regulatory subunit of PP2A, which is phosphorylated by TOR under nutrient-rich conditions, resulting in the inhibition of PP2A activity [109]. Double deletion of Pph21 (protein phosphatase 21) and Pph22, the catalytic subunit of PP2A, impairs dephosphorylating of ATG13 and limits autophagy activity [97]. In mouse embryonic fibroblasts, PP2A promotes autophagy activity by specifically dephosphorylating Ser637 (corresponding to Ser638 in humans) of ULK1, the same target site by mTORC1 and AMPK kinase [108]. In plants, Tap46, the homologue of Tap42, has been shown to be directly phosphorylated by TOR kinase [110]. Mutation of Tap46

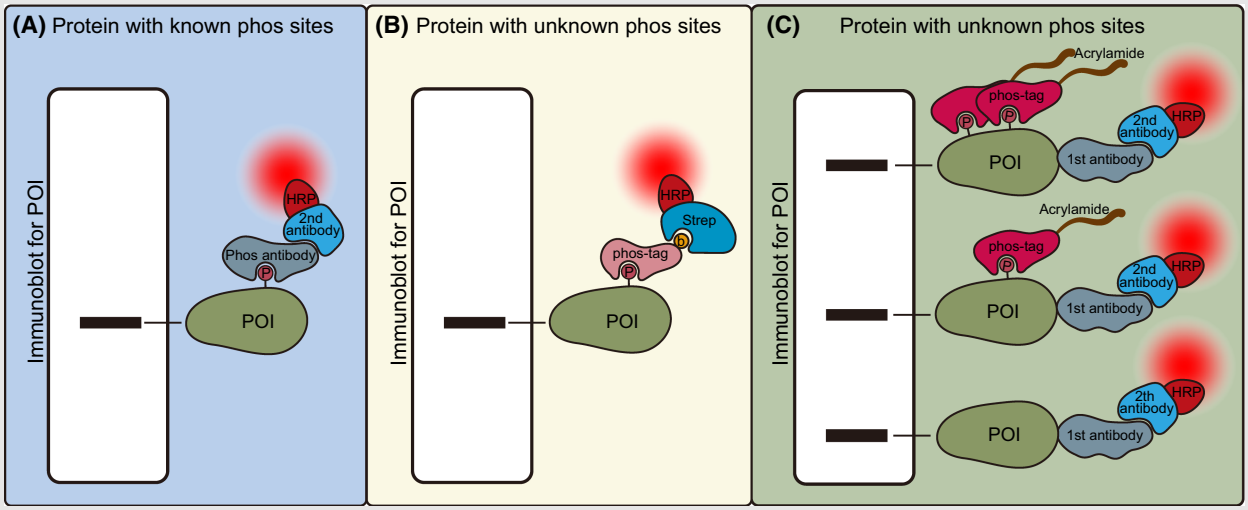
reproduces the characteristic features of TOR inactivation, such as activation of autophagy and suppression of protein translation, indicating that Tap46 functions as a positive effector of TOR signalling in plants [110]. How the PP2A contributes to autophagy in plants and what the target substrates are in this process remain to be examined.

Two of the yeast PP2C-type phosphatases, Ptc2 and Ptc3, play a key role in initiating autophagy by dephosphorylating ATG13 at Ser429 [111]. In particular, the autophagy activity is restrained in the *ptc2*Δ *ptc3*Δ double mutant and is accompanied by hyperphosphorylation of ATG13 [111]. Additionally, Ptc2 has an ability to directly dephosphorylate ATG1 at Thr226 *in vitro*, which is crucial for organizing phagophore formation [112]. The PP2C-type phosphatase responsible for autophagy regulation in plants is still unknown. Rodrigues et al. have reported that PP2Cs, including ABI1 and PP2CA, interact with the SnRK1 catalytic subunit and negatively regulate abscisic acid (ABA) signalling in plants [113]. PP2C inhibition by ABA results in SnRK1 activation, promoting SnRK1 signalling during stress response [113]. At present, it is unclear whether and how PP2C, as a negative regulator of SnRK1, is involved in plant autophagy. Of course, it cannot exclude PP2C as a positive regulatory factor, similar to yeast, participated in autophagy initiation; after all, different stimulus factors lead to discrepancy effects.

A brief guideline for studying phosphorylation in plant autophagy

Extensive studies have highlighted the regulatory significance of phosphorylation for the autophagy process. The role of phosphorylation in plant autophagy has

BOX 2. Approaches to study the phosphorylation status of a POI (protein of interest) in plant autophagy.



Three simple methods can be adopted to quickly examine the presence of phosphorylation modifications on a given POI. (A) One approach involves immunoblotting with phosphorylation of site-specific antibodies against phosphoserine, phosphothreonine or other phosphoresidues. Unfortunately, the low affinity and specificity of these antibodies can be a problem in some cases. (B) The dinuclear metal complex 1,3-bis[bis(pyridin-2-ylmethyl)-amino]propan-2-olato has been found as selectively binding phosphate [114]. Based on this feature, various phosphate-binding tag derivatives including Phos-tag biotin and Phos-tag acrylamide have been developed for phosphoproteome research [115–117]. The Phos-tag biotin method is suitable for the detection of phosphorylation status of purified proteins obtained using, for example, GFP-trap and other methods. (C) Phos-tag acrylamide is another method based on the mobility shift difference between the phosphorylated and nonphosphorylated counterparts. The Phos-tag acrylamide is copolymerized with other polymers in the separation gel, and the phosphorylated forms are trapped by the Phos-tag while electrophoresis proceeds. The phosphorylation status of POI can be indicated by the gel-shifted bands, which has an advantage in the analysis of multisite phosphorylation on a single protein. These methods provide a relatively simple way to quickly determine the phosphorylation status of ATG proteins.

been only marginally investigated in comparison with that of the mammals and yeast. Here, several primary steps were proposed to analyse the phosphorylation of the core autophagy proteins. The first step is to determine whether the protein of interest is phosphorylated. Three methods can be used to quickly and preliminarily determine the presence of phosphorylation modifications (Box 2). Next, mapping and analysis of phosphorylation sites can be done by tandem mass spectrometry (MS/MS). The interest ATG protein tagged with GFP, haemagglutinin or Flag can be purified using antibody beads, which is digested with a protease to produce short peptides. The peptide masses and fragment ion masses are measured by quantitative mass spectrometry. Phosphorylation peptide can be identified by matching the theoretical spectra. Generally, MS analysis will yield a plenty of phosphorylation sites that need to be further verified to test whether they are physiologically relevant. There may be some functional redundancy among these residues. An important last step is to mutate the sites and complement related mutants so as to ascertain their biological significance.

Conclusions and perspectives

The process of autophagy strongly depends on the coordination of different post-translational modifications. Among them, phosphorylation and dephosphorylation have been shown to act in concert to initiate autophagy in response to nutritional or environmental stimuli. However, our understanding of the regulation of phosphorylation in plant autophagy has not yet made a such great progress comparable to that of the yeast and mammals. As TOR and SnRK1 are two important regulators in autophagy, what exactly are their phosphorylation sites in plant ATG1 complex still unknown? Recently, FLZs have shown to function as plant-specific adaptors for SnRK1, which might be involved in the regulation of SnRK1-TOR dynamics [60,61]. Further studies are required to fully understand these adaptors and their roles in plant autophagy regulation. Moreover, besides TOR and SnRK1, it is likely that more kinases and phosphatases involved in autophagy regulation in plants remain to be identified. To fully understand the regulation of phosphorylation in plant autophagy, a systematic and comprehensive analysis needs to be performed to map the phosphoproteomics of the core ATG machinery in plants.

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Author contributions

H.L., Y.L., X. Zheng, C.G. and J.Z. prepared the figures and drafted the manuscript. X. Zhuang, C.G. and J.Z. edited the manuscript.

Disclosure statement

No potential conflicts of interest were disclosed.

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