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Authors

Xu, Shou-Ling
Chalkley, Robert J
Maynard, Jason C
et al.

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Proteomic analysis reveals O-GlcNAc modification on proteins with key regulatory functions in *Arabidopsis*

Shou-Ling Xu[a,b], Robert J. Chalkley[b], Jason C. Maynard[b], Wenfei Wang[c], Weimin Ni[d,e], Xiaoyue Jiang[f], Kihye Shin[c], Ling Cheng[c], Dasha Savage[a], Andreas F. R. Hühmer[f], Alma L. Burlingame[b,1], and Zhi-Yong Wang[a,1]

[a]Department of Plant Biology, Carnegie Institution for Science, Stanford, CA 94305; [b]Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94158; [c]Basic Forestry and Proteomics Research Center, Fujian Agriculture and Forestry University, Fuzhou 350002, China; [d]Department of Plant and Microbial Biology, University of California, Berkeley, CA, 94720; [e]Plant Gene Expression Center, United States Department of Agriculture/Agriculture Research Service, Albany, CA94710; and [f]Thermo Fisher Scientific, San Jose, CA 95134

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Genetic studies have shown essential functions of O-linked *N*-acetylglucosamine (O-GlcNAc) modification in plants. However, the proteins and sites subject to this posttranslational modification are largely unknown. Here, we report a large-scale proteomic identification of O-GlcNAc–modified proteins and sites in the model plant *Arabidopsis thaliana*. Using lectin weak affinity chromatography to enrich modified peptides, followed by mass spectrometry, we identified 971 O-GlcNAc–modified peptides belonging to 262 proteins. The modified proteins are involved in cellular regulatory processes, including transcription, translation, epigenetic gene regulation, and signal transduction. Many proteins have functions in developmental and physiological processes specific to plants, such as hormone responses and flower development. Mass spectrometric analysis of phosphopeptides from the same samples showed that a large number of peptides could be modified by either O-GlcNAcylation or phosphorylation, but cooccurrence of the two modifications in the same peptide molecule was rare. Our study generates a snapshot of the O-GlcNAc modification landscape in plants, indicating functions in many cellular regulation pathways and providing a powerful resource for further dissecting these functions at the molecular level.

O-GlcNAcylation | proteomics | plant | *Arabidopsis* | phosphorylation

O-GlcNAcylation is a posttranslational modification (PTM) of proteins consisting of a single O-linked *N*-acetylglucosamine attached to serine and threonine residues. It has been extensively studied in animals, where it regulates a wide range of developmental and metabolic processes. O-GlcNAcylation is dynamically controlled by two enzymes: an O-GlcNAc transferase (OGT) and an O-GlcNAcase (OGA), which add and remove O-GlcNAc, respectively. O-GlcNAcylation occurs in the cytoplasm, nucleus, and mitochondria and has been implicated in cellular processes, including transcription, translation, signal transduction, nuclear pore function, epigenetic regulation and proteasomal degradation (1). Altered levels of protein O-GlcNAcylation in animals have been associated with neurodegeneration, diabetes, cardiovascular diseases, and cancer (2) whereas knock out of OGT is embryonically lethal (3).

The model plant *Arabidopsis* has two putative OGTs: SPINDLY (SPY) and SECRET AGENT (SEC). The *spy* mutant was identified based on its phenotypes that mimic gibberellin-treated plants, with elongated stems (4). The *spy* plants also show defects in light and cytokinin responses, leaf morphology and phyllotaxy, root growth, meristem activity, and circadian rhythms (5). The *sec* mutant displays defects in flower development (6). Although OGT enzymatic activity has been demonstrated in SEC, similar activity in SPY has not been confirmed (7). However, the *spy;sec* double mutants show severe defects in the development of gametes and are embryonically lethal (7), similar to the OGT knockout mutant in animals. Thus, genetic evidence indicates that O-GlcNAc modification is as important in plants as in animals. But little is known about its specific functions because few O-GlcNAc–modified proteins have been identified in plants (8).

Progress in deciphering possible biological functions of O-GlcNAcylation has been historically hampered due to the lack of a sensitive and rigorous methodology required to establish the sites of O-GlcNAcylation on protein substrates. However, the recent development of lectin weak affinity chromatography (LWAC) and chemical/enzymatic tagging strategies has facilitated enrichment of these modified peptides from complex proteolytic digest mixtures (9, 10), and the advent of electron transfer dissociation (ETD) mass spectrometry has facilitated the robust assignment of modification sites (11). These advances have been used effectively for studies in mammalian systems, and over 1,000 O-GlcNAc–modified proteins have been identified (9–14). However, no similar study has been reported in plants, and thus it's unclear whether O-GlcNAc modification controls similar cellular process in plants and animals.

Yeast two-hybrid screens have identified several putative SPY-interacting proteins in *Arabidopsis*, including GIGANTEA (GI) (15), two transcriptional regulators from the MYB- and NAC-like families (16), and two class I TCP proteins (TCP14 and TCP15) (17). These studies suggest that SPY-mediated O-GlcNAcylation

Significance

Studies in mammalian systems have shown important functions of O-linked *N*-acetylglucosamine (O-GlcNAc) modification of proteins (O-GlcNAcylation) in a wide range of cellular, physiological, and disease processes. Genetic evidence indicates that O-GlcNAcylation is essential for plant growth and development. However, very few O-GlcNAc–modified proteins have been identified in plants. Here, we report identification of 262 O-GlcNAc–modified proteins in *Arabidopsis*, revealing both conserved and distinct functions of O-GlcNAc modification in plants. This study uncovers potentially important functions of O-GlcNAcylation in many cellular and developmental pathways and also provides a large number of modification sites for further genetic and molecular dissection of these specific functions. Our study provides the framework of an O-GlcNAc modification network underlying plant growth and development.

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The authors declare no conflict of interest.

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Data deposition: The O-GlcNAc ETD and HCD results from these searches have been uploaded to MS-viewer (prospector2.ucsf.edu), which allows viewing of annotated spectra of all results with the searchkeys 3hpyufjcel and 94xlgafvxf. The phosphopeptide HCD results from these searches have been uploaded to MS-viewer with the searchkey vivvtc8reo.

[1]To whom correspondence may be addressed. Email: zywang24@stanford.edu or alb@cgl.ucsf.edu.

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may affect the activity of the circadian clock and both gibberellin and cytokinin responses. Two recent studies showed evidence for O-GlcNAc modification of a wheat RNA-binding protein involved in flowering induction by vernalization (18) and of the master growth repressor DELLA (19). However, O-GlcNAc modification of plant proteins has only been inferred from Western blotting with an O-GlcNAc antibody (18) or mass spectrometric analysis of immunoprecipitated targeted protein DELLA (19).

Here, we report a large-scale identification of O-GlcNAcylated peptides in plants. We identified 971 O-GlcNAc–modified peptides representing 262 proteins. Our study reveals potential roles of O-GlcNAc modification in diverse cellular and developmental processes, including transcription and translation, chromatin remodeling, signal transduction, meiosis, and flower development. The dataset lays the foundation for future investigations of O-GlcNAcylation and possible PTM cross-talk in this model plant system.

Results

Enrichment and Identification of GlcNAcylated and Phosphorylated Peptides in *Arabidopsis*. The phenotype of *spy* and *sec* mutants suggests that O-GlcNAc modification plays important roles in reproductive development. Therefore, to maximize the identification of O-GlcNAc–modified proteins, the *Arabidopsis* inflorescence tissues containing open flowers and young floral buds were harvested for protein extraction and modified peptide enrichment. A workflow previously developed in our laboratory (12) was modified to sequentially enrich phosphorylated and O-GlcNAcylated tryptic peptides (Fig. 1*A*). Phosphorylated peptides were isolated using immobilized metal affinity chromatography (IMAC) enrichment as described previously (20), but using Ga^{3+} as the metal cation. Subsequently O-GlcNAcylated peptides were isolated using three rounds of lectin weak affinity chromatography (LWAC) (12). The peak after each round of enrichment clearly eluted later, indicating efficient enrichment of O-GlcNAcylated peptides (Fig. 1*B*).

The LWAC-enriched fractions were analyzed on an LTQ-Orbitrap Velos mass spectrometer equipped with a nano-Acquity Ultra Performance Liquid Chromatography (UPLC), using ETD, sequential higher energy collisional dissociation (HCD) and ETD, or HCD-triggered ETD. Analysis of HCD data from these LWAC-enriched fractionations using MS-Filter (21) determined that 51% of precursors fragmented produced a HexNAc oxonium ion at *m/z* 204.087, confirming a high level of enrichment of glycosylated peptides.

ETD MS analysis of enriched peptides in many cases allowed assignment of the mass spectrum to a particular peptide sequence and unambiguous site localization of the modification. In HCD, the O-GlcNAc moiety usually dissociates during the internal vibronic energy randomization, preventing the use of mass shifts in the peptide sequence ion series to establish the site(s) of this labile modification. However, in many cases, HCD data provided confident assignment of a particular peptide sequence, and, in the rare instances when there was only one potential site of the modification in the peptide, it could also provide modification site localization.

Our MS analysis of LWAC-enriched fractions identified 971 distinct O-GlcNAcylated peptides, mapped to 262 proteins (Datasets S1 and S2). These assignments correspond to at least 533 unique sites of O-GlcNAcylation, of which 365 could be determined with greater than 95% site localization confidence (22). A list of these modified peptides and sites of modification is provided in Dataset S1, and annotated spectra for all identifications can be visualized using MS-Viewer (23) (see *Materials and Methods* for details). Among the proteins that have been previously implicated to be O-GlcNAc–modified, we identified SPY and TCP 14 (17), but not any DELLA proteins (19). The 262 O-GlcNAc–modified proteins included several homologs from the same family or multiple proteins involved in the same biological process or pathway, suggesting overrepresentation of specific functions.

The Functions of Many O-GlcNAc–Modified Proteins Are Similar in Plants and Animals. Analysis of the peptide sequences with unambiguous O-GlcNAcylation site assignment indicated that there is a minor preference for proline at −2 and −3 residues N-terminal to the modification site and serine in positions C-terminal to the modification site (Fig. 2*A*). Interestingly, similar sequence preference has been observed from O-GlcNAcylation sites identified in animals (11, 12, 24, 25), suggesting that the plant and animal OGTs have consistent enzymatic preferences.

The subcellular localization and biochemical functions of many O-GlcNAc–modified proteins are similar in plants and animals. About 80% of the identified O-GlcNAcylated proteins are predicted to be nuclear localized, in contrast to the N-linked glycosylation found mostly on proteins predicted to be localized in the apoplast, membrane, endoplasmic reticulum, or Golgi (26). Gene Ontology (GO) analysis showed that the majority of O-GlcNAc–modified proteins have either DNA-binding or RNA-binding properties (Fig. 2*B*). The modified proteins also overrepresent functions in transcription, RNA binding/processing, translation, and chromatin remodeling (Fig. 3). In addition, several nuclear pore proteins (At1g55540/LNO1; At5g20200) were found to be O-GlcNAc–modified, consistent with the observation of O-GlcNAc modification of the nuclear pore complex in animals (27).

Interestingly, several highly conserved proteins are O-GlcNAc–modified in both *Arabidopsis* and animals. For example, the transcription repressor TOPLESS (TPL) and its homologs TPR2

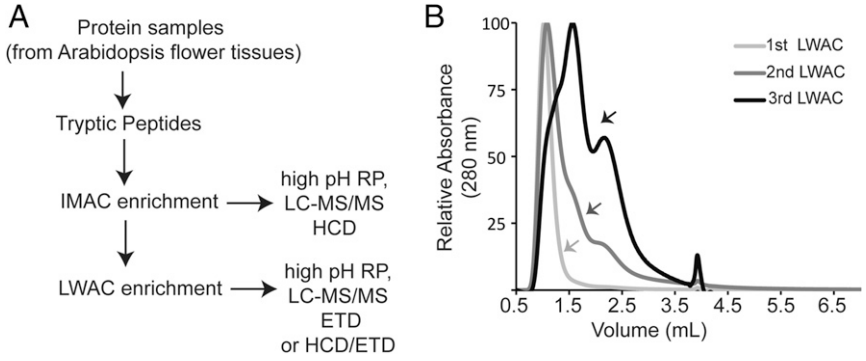

Fig. 1. Combined analysis for O-GlcNAcylation and phosphorylation from *Arabidopsis* inflorescence tissues. (*A*). Flowchart for the serial enrichment and analysis of in vivo phosphorylated and O-GlcNAc–modified peptides. (*B*) The UV trace of absorbance at 280 nm of three sequential lectin weak affinity chromatography (LWAC) separations of tryptic digest of proteins extracted from *Arabidopsis* inflorescence tissues, showing enrichment of O-GlcNAc–modified peptides through their retardation on the column (arrows). In each case, an aliquot of GlcNAc (eluting at 4 mL) was injected to elute any complex glycans. Peptides were collected as a single fraction starting at 1.3 mL, desalted, and rerun for a total of three rounds.

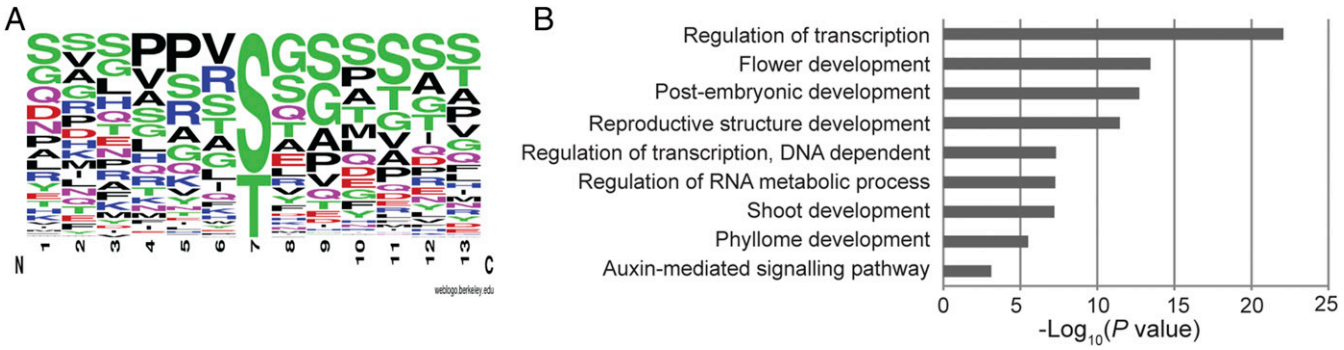

Fig. 2. Summary of identified O-GlcNAcylated proteins and peptides. (*A*) The weblogo motif for an alignment of O-GlcNAc modification sites identified in this study. (*B*) Gene ontology (GO) analysis of detected O-GlcNAcylated proteins in vivo. GO terms for biological processes are shown on the *y* axis.

and TPR4 were O-GlcNAc–modified in *Arabidopsis*, and their human homolog Transducin-like Enhancer of Split (TLE) was also O-GlcNAc–modified (28). O-GlcNAc modification was shown to play an important role in TLE-mediated transcriptional repression in the Wnt signaling pathway (28). TPL, on the other hand, plays a key role in transcriptional repression in the brassinosteroid (BR) signaling pathway (29), which shares many similar features with the metazoan Wnt pathway (30). TPL is recruited to promoters by the BZR1 family transcription factors (29), which act downstream of both BR and gibberellin pathways (31). Therefore, O-GlcNAc modification of TPL may be related to the gibberellin-related cell elongation phenotypes of the *spy* mutant (4).

O-GlcNAc Modification of Proteins Involved in Transcription, Translation, and Chromatin Remodeling. Many transcription factors and RNA polymerase II and associated general transcription factor proteins are O-GlcNAcylated in humans and mice (32–34). O-GlcNAc cycling also regulates epigenetic mechanisms (35). Similarly, many O-GlcNAc–modified proteins identified in this study are involved in transcription, ranging from transcription initiation, elongation, and termination to RNA splicing (Fig. 3). These proteins include general transcription factors TFIIE and TFIIS, mediator 8 and 25 (which are Polymerase II coactivators), and transcription repressors TOPLESS and its related proteins (TPR2 and TPR4), which are modified by OGT on multiple sites. Members of the CCR4-NOT complexes are O-GlcNAc–modified in plants and mammals: five members were identified in this study (Fig. 3) whereas three subunits were previously shown to be O-GlcNAc–modified in mouse synapse (12). CCR4-NOT has been shown to be associated with Polymerase II and to regulate transcription elongation, mRNA decay, and translational repression (36). Furthermore, we found eight proteins involved in RNA 3′ end processing that are O-GlcNAc–modified, including FY.

We detected O-GlcNAcylation on many components that are involved in mRNA processing and translation. These components include proteins involved in mRNA splicing (SUS2 and ACINUS), decapping (DCP5-L), degradation (ECT/YTH domain), and nonsense mRNA decay (SMG7) (Fig. 3). In plants, YTH domain proteins are particularly abundant, with 11 family members (37, 38). The YTH domain is called an evolutionarily conserved C-terminal region (ECT) domain in plants. Intriguingly, we found that 7 out of 11 members of the ECT/YTH family (ECT2, -4, -5, -6, -7, -8, and -10) are modified by O-GlcNAcylation, often on multiple sites. YTH proteins bind to m(6)A-containing mRNA and regulate messenger RNA stability (39). They have been shown to be modified by O-GlcNAcylation in murine synapse and human tissues (25). We generated transgenic *Arabidopsis* overexpressing a YFP-ECT7 fusion protein and performed two replicates of immunoprecipitation, followed by mass spectrometry. In both repeat experiments, we detected O-GlcNAc modification of ECT7 (Fig. S1).

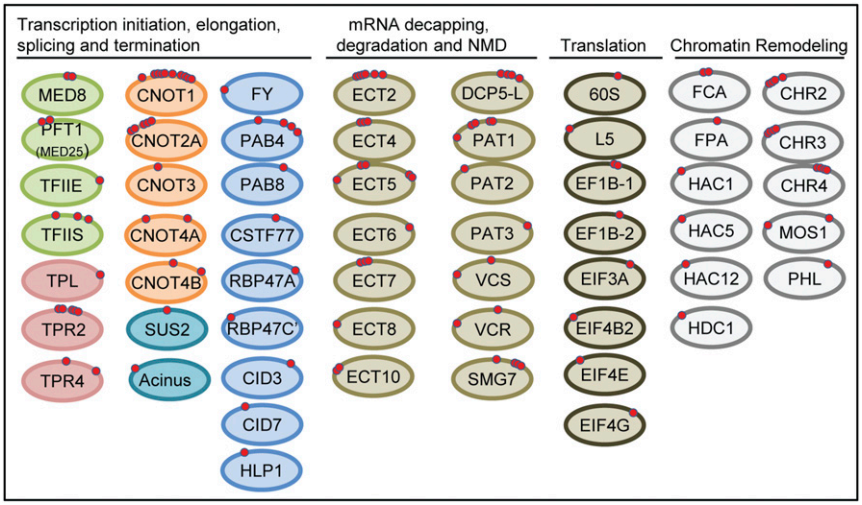

Fig. 3. O-GlcNAcylated proteins are involved in transcription, translation, and chromatin remodeling. O-GlcNAcylated proteins were grouped into several categories based on their known functions or predicted functions. Each red dot represents an O-GlcNAcylation site.

SMG7, a component of the nonsense-mediated mRNA decay pathway, is O-GlcNAcylated on multiple sites near its C terminus (Dataset S1). SMG7 has been shown to be regulated by phosphorylation and is required to exit meiosis; disruption of SMG7 results in embryo lethality (40). We also found O-GlcNAc-modification on several proteins in translation, such as ribosomal proteins 60S, L5, EF1B, EIF3A, EIF4B2, EIF4G, EIF4E, and EIF4G.

Several chromatin modifiers were found to be O-GlcNAcylated, including histone acetyltransferases HAC1, -5, and -12, histone deacetylation complex 1 (HDC1), and chromatin remodeling 2, 3, and 4 (CHR2, -3, and -4) (Fig. 3 and Dataset S1). CHR2, -3, and -4 encode a SWI/SNF chromatin remodeling ATPase. In addition, FCA and FPA, both of which contain an RNA-recognition motif (RRM), are O-GlcNAcylated. These two proteins are required for RNA-mediated chromatin silencing of many genes (41) whereas FCA interacts with SWI/SNF chromatin remodeler SWI3B (42).

These findings suggest conserved functions and common themes of O-GlcNAcylation between animals and plants in regulating transcription, translation, and chromatin remodeling.

O-GlcNAc Modification of Proteins Involved in Plant Hormone Signaling Pathways. Previous genetic studies have shown functions of SPY and SEC in processes regulated by several hormones, including gibberellin, cytokinin, auxin, and abscisic acid, but the molecular mechanisms are not fully understood. The *spy* mutant was categorized based on its phenotypes that mimic gibberellin-treated plants, with elongated stems (4), indicating that SPY is a negative regulator of the GA pathway. Interestingly, a recent study showed that SEC functions as a positive regulator of the GA pathway, by O-GlcNAcylating the GA-signaling protein DELLA (19). O-GlcNAc modification of the TCP14 transcription factor has been indicated in cytokinin hormone responses (17). Our data provide direct evidence that TCP3, -8, and -14 are O-GlcNAc–modified in vivo, with multiple sites detected on TCP8 (Fig. 4 *A* and *B*). ABA-binding factor 3 protein is also detected as an O-GlcNAc–modified protein (Fig. 4*A*). A previous study showed that ABA-regulated dehydrin gene expression is induced when a barley SPY (HvSPY) is expressed (43).

Two key signal transduction proteins in the ethylene pathway, EIN2 and EIN5, are O-GlcNAcylated (Fig. 4 *A*, *C*, and *D*). The modification site on EIN2 S906 is near the cytosolic terminus, surrounded by phosphorylation sites (Dataset S3). In addition to previously reported phosphorylation sites (S645, S757, S924, S1283) (44), we detected additional 20 phosphorylation sites on the C-terminal cytosolic region (S598, S650, S655, S657, S659, S719, S731, S769, S801 or S802, S808 or S809, S819, T848 or S849, S889, S945, S960, S972, S988/S990, S1037, S1199, S1292) (Dataset S3). Phosphorylations on S645 and S924 are regulated by ethylene and have been previously shown to play roles in cleavage and nuclear localization of EIN2 (45, 46). It will be interesting to determine whether S906 O-GlcNAc modification

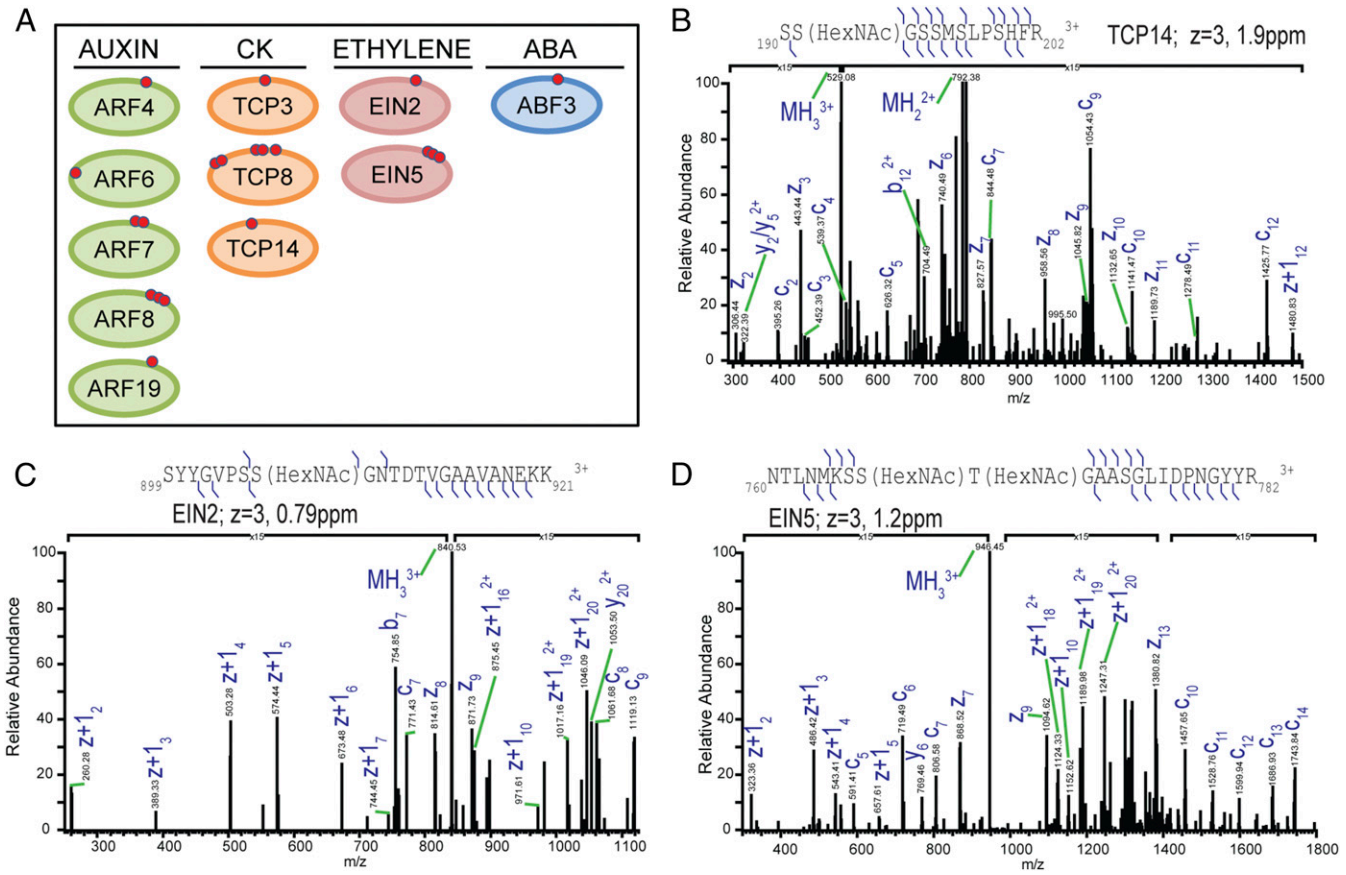

Fig. 4. O-GlcNAcylation regulates hormone responses. (*A*) O-GlcNAcylation was found on auxin signaling pathway ARF transcription factors, cytokinin signaling pathway TCP transcription factors, the abscisic acid (ABA) signaling pathway (ABF3), and the ethylene signaling pathway (EIN2 and EIN5). The proteins with the same colored background are proteins that are involved in the same hormone pathway. (*B–D*). ETD mass spectra of peptide from TCP14, EIN2, and EIN5. (*B*) ETD spectrum of an *m/z* 528.2437 3+ precursor identifies a peptide from TCP14 spanning from amino acid 190–202 with modification on Ser-191. (*C*) ETD spectrum of *m/z* = 840.0703 precursor identifies a peptide from EIN2 with O-GlcNAc modification on Ser-906. (*D*) ETD spectrum of *m/z*= 946.1162 precursor identifies a peptide from EIN5 with O-GlcNAc modification on Ser-767 and Thr-768.

affects EIN2 phosphorylation and cleavage. An ethylene-related phenotype has not been reported in either *spy* or *sec* single mutants, and the early lethality of *spy;sec* homozygous mutant prevented genetic dissection of the functions in later developmental stages.

Auxin response proteins are particularly enriched in our O-GlcNAc proteome dataset (e value = 3.66e−4). These auxin response proteins include five of the 23 members of the auxin response factor (ARF) family transcription factors (ARF4, -6, -7, -8, -19), which are O-GlcNAc–modified (Fig. 4*A*). Although the high resolution mass spectrometry data provide convincing evidence for in vivo O-GlcNAc modification, we further verified the modification of ARF8 using the anti–O-GlcNAc antibody. Several anti–O-GlcNAc antibodies commercially available can detect the GlcNAc moiety on proteins, but each of them only recognizes a subset of O-GlcNAc–modified proteins (47). To validate the O-GlcNAcylation modification, ARF8-MYC was affinity purified from lysates from transgenic *Arabidopsis* plants (48) using an anti-MYC antibody and was immunoblotted using anti-O-GlcNAc antibody RL2. A band with expected size was detected in the ARF8-MYC sample but was absent in the WT control (Fig. S2), supporting that ARF8 is O-GlcNAc–modified. Although the effect of O-GlcNAc modification on ARF function awaits further study, similar phenotypes between the *sec-1*/*sec-1* SPY/*spy-4 ga1*/*ga1* mutant and auxin transport mutants suggested a role of O-GlcNAc modification in auxin hormone function (6).

Potential Roles of O-GlcNAcylation in the Circadian Clock, Flowering Time, and Floral Organ Development. In mammals, O-GlcNAc modification has been implicated in circadian clock function through regulating circadian clock genes, including PERIOD (49), BMAL1 (50), and CLOCK (51, 52). Although these clock components are not conserved in plants, we found O-GlcNAc modification of proteins involved in plant circadian clock function, including TIME FOR COFFEE (TIC) (53). Previous reports have shown SPY and GIGANTEA in *Arabidopsis* work together to regulate circadian rhythms in transpiration and cotyledon movement (15, 54). GIGANTEA was shown to interact with SPY by yeast two-hybrid and by genetic interaction, but the functional relation between these two proteins is still unclear (15). TIC plays a role in the free-running circadian rhythms (53, 55) and was found to be O-GlcNAc–modified on multiple sites in our study (Fig. 5).

We found O-GlcNAcylation of key regulators of floral organ development, including HEN4, HUA1, NGA1, NGA3, NGA4, and SEU and its homologs, LUG and its homolog. It has been shown that SEC and SPY have overlapping roles in floral meristem and carpel development (6). HEN4 and HUA1 promote reproductive floral organ development by specifically promoting the processing of AGAMOUS pre-mRNA (56). SEU interacts with LUG to repress AGAMOUS expression in the outer floral whorls (57–60), and SEUSS has been shown to interact with ETT (ARF3) in auxin responses to promote floral organ patterning and growth (58).

We found O-GlcNAcylation of several key components that regulate flowering time, including FY, LUMINIDEPENDENS (LD), FCA, FPA, PFT1, and SBP-box gene SPL2, -8, -11, and -13 (Fig. 5). FCA, FY, and FPA are all negative regulators of FLC in the autonomous flowering. FY is an RNA 3′ processing/polyadenylation factor, which interacts directly with FCA to regulate FLC transcriptional silencing (61). LD is a nuclear homeodomain protein that regulates transcription (62). SPL transcription factors are involved in floral transition by regulating the expression of floral meristem identity gene AP1 (63). These results suggest that O-GlcNAc modification might be involved in the regulation of reproductive transition by nutrient and energy status.

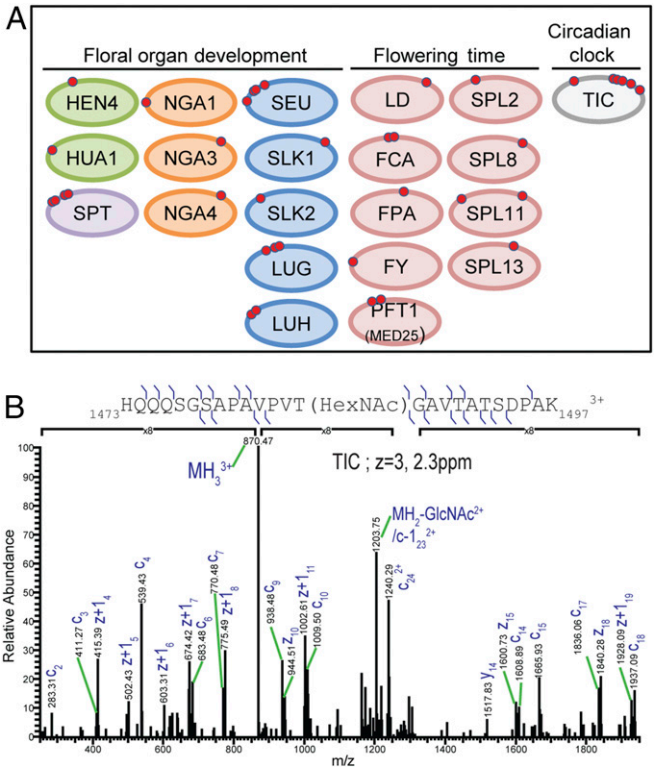

Fig. 5. O-GlcNAcylation regulates proteins involved in flora organ development, flowering time, and circadian clock. (*A*) O-GlcNAc–modified proteins are involved in floral organ development: e.g., HEN4 and HUA1 act in the specification of floral organ identity in the third whorl; NGA1, -3, and -4 act in style specification; SEU and its homolog SLK1 and SLK2 act with LUG and its homolog LUH in carpel development; LD, FCA, FPA, FY, PFT1, and SPL2, -8, -11, and -13 are involved in flowering time. TIC is involved in circadian clock. The proteins with the same colored background are proteins that are involved in the same processes. (*B*) ETD spectrum of TIC showing that Thr-1486 is modified by O-GlcNAcylation.

O-GlcNAcylation and Phosphorylation. We identified 8,041 phosphorylated proteins, corresponding to 34,114 unique sites of phosphorylation (of which 26,099 sites were unambiguous sites localized with greater than 95% confidence) from IMAC-enriched samples (Dataset S3). These results are also available for viewing using MS-Viewer. Phosphopeptides were identified from 87% of the identified O-GlcNAcylated proteins. Among the peptides we found to be O-GlcNAcylated, 35% of them were observed alternatively phosphorylated. We also identified in the IMAC-enriched fractions 109 peptides simultaneously modified by both O-GlcNAcylation and phosphorylation (Dataset S4).

Conclusion and Discussion

Genetic evidence indicates that protein O-GlcNAcylation plays essential roles in both animals and plants. Although studies in animals have uncovered functions for O-GlcNAcylation in many key cellular processes, analogous functions in plants have been largely unknown or speculative due to lack of knowledge of the identity of most modified proteins. This work describes the identification of a large set of O-GlcNAc–modified plant proteins and implicates regulatory functions for O-GlcNAcylation in many key cellular and developmental processes. These results lay a broad foundation for future studies of site-specific functions of particular proteins and for further molecular characterization of O-GlcNAc modification in other plants.

Although LWAC has been successfully used for enrichment of O-GlcNAcylated peptides from animal samples (9, 11, 12), the

use of the original protocol on plant samples was not straightforward. Our initial attempt using plant tissue provided information on only about a dozen O-GlcNAcylated peptides (8). The difficulty is likely due to low binding affinity and competition by endogenous carbohydrates, mostly N-linked complex glycans. However, the improvements reported in the enrichment protocol (repeating LWAC three times using a POROS support) have allowed us to achieve much higher enrichment of O-GlcNAcylated peptides: About 51% of the components present in this enriched fraction were glycosylated. Subsequent mass spectrometric analyses using a combination of ETD and HCD allowed the identification of O-GlcNAcylated peptides with high confidence and revealed an increase in the number of O-GlcNAcylation sites for *Arabidopsis* by two orders of magnitude. Therefore, our improved LWAC method is very effective in enriching O-GlcNAc peptides.

Several lines of evidence indicate that our O-GlcNAc dataset is of high confidence and functional relevance. First, we identified some of the few proteins previously known or expected to be O-GlcNAc–modified, such as TCP14 and SPY. Second, the identified O-GlcNAcylated proteins are mostly predicted to be nuclear and cytoplasmic, as expected. In contrast, the N-GlcNAc–modified peptides identified by LWAC enrichment were mostly from proteins localized in the secretory pathway or apoplast [these results were described in a previous publication (26)].

Third, the enrichment of several groups of evolutionarily or functionally related proteins in our O-GlcNAc dataset provides strong evidence for specific O-GlcNAc functions. For example, the 262 O-GlcNAcylated proteins strikingly include three of the four TPL/TPR family repressors, five of the twenty-two ARFs, and seven of the eleven ECT/YTH proteins. Modifications of homologous proteins suggest conservation of the modifications due to their important functions. Our O-GlcNAc dataset also includes many groups of diverse proteins involved in common functions. For example, SEUSS interacts with LEUNIG to repress *AGMOUS* gene expression (64), and both of these repressors are modified by O-GlcNAcylation. FY interacts with FCA and negatively regulates FLC in the autonomous promotion pathway for flowering time (61), and these proteins are O-GlcNAyclated. A similar phenomenon has also been observed in animals, where three proteins (Pdx1, MafA, and NeuroD1) activating insulin gene expression upon glucose induction in pancreatic beta cells are all O-GlcNAcylated (65).

Fourth, our O-GlcNAc dataset reveals similarities to results reported for animal studies on the mechanism and functions of O-GlcNAcylation. The similarity in sequence preference for O-GlcNAc modification sites supports a conserved enzymatic preference of OGTs in plants and animals. In addition, the functions of many O-GlcNAc–modified proteins are similar between plants and animals, including transcription, translation, chromatin remodeling, and the nuclear pore complex. Furthermore, many evolutionarily conserved proteins are O-GlcNAc–modified in both plants and animals. For example, the *Arabidopsis* TPL/TPRs and human TLE are members of the Groucho family of transcription repressors. O-GlcNAcylation of TLE has been shown to modulate its transcriptional activity (28), and similar modulation of TPL activity by O-GlcNAylation would be consistent with the known function of SPY and TPL in regulating cell elongation. Interestingly, some evolutionarily distinct proteins with similar functions in plants and animals, such as the circadian clock components (52), seem to be targets of O-GlcNAc regulation in both kingdoms. In this case, the biological function of O-GlcNAcylation seems to be maintained despite the divergence of the target proteins involved.

Our dataset of 262 O-GlcNAc–modified proteins is small compared with the thousands of proteins identified in the largest study of an animal tissue (12). However, the identification of multiple members of several protein families suggests that our dataset provides a reasonable coverage of the O-GlcNAc proteome. On the other hand, the failure to detect the known DELLA proteins suggests that our dataset is still incomplete. Apparently, further analysis, in more tissue types and plant species using sensitive analytical methods, will be required to determine whether there is any overall difference in the scope and selectivity of O-GlcNAc modification between plants and animals. Nevertheless, our dataset provides many interesting targets for future functional studies.

A major area of evolutionary divergence is the phytohormones, which act through signaling mechanisms distinct from animal hormones. Unsurprisingly, key components of hormone pathways, including growth hormones auxin and cytokinin as well as stress hormones ABA and ethylene, are modified by O-GlcNAcylation. Modifications of multiple components of each pathway further support their likely functional importance for modulation of hormone responses by nutrient and energy status, which is essential for optimal growth and homeostasis.

In summary, our results provide evidence for evolutionary conservation of the biological functions of O-GlcNAc modification, supporting its importance as a basic mechanism of cellular protein regulation. Our study also uncovered potentially plant-specific functions of O-GlcNAcylation, prominently in modulating key components of several plant hormone pathways. Mutagenesis studies of the identified O-GlcNAcylation sites in these key components of hormone pathways will be needed to establish the functional links between O-GlcNAcylation and hormone signaling and plant growth regulation. Similarly, the large number of O-GlcNAcylated sites identified in important regulatory proteins will allow functional dissection in the contexts of gene expression, signal transduction, and development. Quantitative studies of O-GlcNAcylation will be needed to provide insight into the functional importance of this posttranslational modification in plant responses to the environmental and endogenous cues. This ground-breaking study of O-GlcNAcylation targets opens an area of exploration that will advance our understanding of both O-GlcNAcylation and the biology of plants.

Materials and Methods

Sample Preparation. Proteins from 3 g of *Arabidopsis thaliana* (Columbia) inflorescence tissues (5 to 6 wk old, growing in greenhouse) were extracted as previously described in ref. 8, with a slight modification in extraction buffer [0.1 M Tris·HCl, pH 8.0, 2% (wt/vol) SDS, 20 mM EGTA, 20 mM EDTA, 1.2% (vol/vol) Triton X-100, PhosStop, protease inhibitor, and 20 μM PUGNAc inhibitor (Sigma)], followed by reduction using DTT and alkylation using iodoacetamide, tryptic digestion (Thermo), and then reverse-phase desalting using Sep-Pak C18 cartridge (Millipore).

Enrichment of GlcNAcylated Peptides Using a Wheat Germ Agglutinin Column. The wheat germ agglutinin (WGA)-poros column was packed as previously described in ref. 12. The enrichment of GlcNAcylated peptides was slightly modified from that described in ref. 12. Briefly, peptides were resuspended in 100 μL of LWAC buffer [100 mM Tris, pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 5% (vol/vol) acetonitrile in water]. Chromatography was performed at a flow rate of 100 μL/min. After 3.0 mL of elution, 100 μL of 40 mM GlcNAc in LWAC buffer was injected to elute out any bound glycopeptides. A GlcNAc-enriched fraction was collected between 1.3 and 6.7 mL. To decrease the chance of overloading the column, 20 mg of starting peptides was split into 12 aliquots, each portion was run separately, and the GlcNAc-enriched fractions were then pooled. For subsequent rounds of LWAC enrichment, the pooled fractions were run as before, each time collecting the same glycopeptide-enriched tail.

Enrichment of Phosphorylated Peptides Using an IMAC Column. Briefly, the Ni-NTA agarose beads (GE Healthcare) were first washed three times with water and then treated with 100 mM EDTA for 30 min with end-over-end rotation. After removal of EDTA, the beads were washed with water three times before treatment with 100 mM $GaCl_3$ for 30 min with end-over-end rotation. After removal of excess $GaCl_3$, beads were washed with water three times and then washed once with resuspension buffer [80% (vol/vol) acetonitrile in water, 0.1% TFA buffer]. Peptides were dissolved in resuspension buffer and added to the beads and incubated for 30 min with end-over-end rotation. The supernatant was removed, and beads were washed with resuspension

buffer three times. Phosphopeptides were eluted using elution buffer (1:1 acetonitrile/1:20 ammonia/water), and the eluate was acidified to pH 3.5–4.0 with TFA and dried down using a SpeedVac concentrator.

High pH Reverse-Phase Chromatography of Phosphopeptides or O-GlcNAcylated Peptides. High pH reverse-phase chromatography was performed using an AKTA purifier (GE Healthcare) equipped with a 4.6 × 150-mm Gemini 5μ C18 column for phosphopeptide and 1 × 100-mm Gemini 3μ C18 column for O-GlcNAcylated peptides (Phenomenex). Phospho-enriched fractions were loaded onto the column in 240 μL of buffer A (20 mM ammonium formate, pH 10). Buffer B consisted of buffer A with 90% (vol/vol) acetonitrile in water. Sample separation was accomplished using the following linear gradient: from 1% B to 9% B over 4 mL, from 9% to 49% B over 20 mL, from 49% to 70% B over 1.5 mL The flow rate was 550 μL/min. Fractions between 6 mL and 24 mL were collected and dried down using a SpeedVac concentrator.

O-GlcNAcylated–enriched fractions were loaded onto the column in 240 μL of buffer A (20 mM ammonium formate, pH 10). Buffer B consisted of buffer A with 50% acetonitrile. The gradient was from 1% B to 21% B over 1.1 mL, to 62% B over 5.4 mL, then directly to 100% B. The flow rate was 80 μL/min. Fractions from 1.4 mL to 7.3 mL were collected and dried down using a SpeedVac concentrator.

Mass Spectrometry and Data Analysis. All phosphopeptide fractions were analyzed on an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher) equipped with a nano-Acquity UPLC (Waters) or on a Q-Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher) equipped with an EASY-nLC 1000 UPLC (Thermo Fisher) system using higher energy collisional dissociation (HCD) (see Dataset S5 for details of individual runs and acquisition parameters). All GlcNAcylated peptide fractions were analyzed on an LTQ-Orbitrap Velos mass spectrometer equipped with a nano-Acquity UPLC using electron transfer dissociation (ETD), sequential HCD and ETD, or HCD-triggered ETD (see Dataset S5 for details of individual runs and acquisition parameters). Peptides were analyzed using either a 1- or 2-h reverse-phase gradient. Tandem mass spectrometry (MS/MS) peaklists were extracted using in-house script PAVA or Proteome Discoverer. Data were searched against The Arabidopsis Information Resource (TAIR) database, to which randomized sequence versions were concatenated (a total of 35,386 entries) to allow estimation of a false discovery rate. Data were searched with a 10-ppm tolerance of the precursor ion, a 0.6-Da tolerance of MS/MS measured in the ion-trap (ETD), and a 20-ppm tolerance for HCD MS/MS. Carbamidomethylcysteine was searched as a constant modification. Variable modifications included protein N-terminal acetylation, peptide N-terminal Gln conversion to pyroglutamate, and Met oxidation. For the Phospho search for the HCD data, phosphorylation modification of serine, threonine, and tyrosine was set as variable modifications. For the GlcNAc search of ETD data, HexNAc modification of serine, threonine, or asparagine was set as variable modifications. For the GlcNAc search of HCD data acquired on Q-Exactive, peaklists were first filtered for the presence of the HexNAc oxonium ion at *m/z* 204.087 ± 20 ppm using MS-Filter. Variable modifications considered were HexNAc modification of serine, threonine, or asparagine, HexNAc neutral loss (i.e., precursor mass is modified, but all fragments are unmodified masses), and phosphorylation of serine, threonine, or tyrosine. The cleavage specificity was set to trypsin, allowing two missed cleavages. False discovery rate was less than 1% at the unique peptide level according to target:decoy database searching. The confidence of modification site assignment was determined by site localization in peptide (SLIP) score (22), with a score of six indicating greater than 95% site localization confidence. All SLIP scores are reported in columns J and K in Datasets S1, S3, and S4 and MS-Viewer. The O-GlcNAc ETD and HCD results from these searches have been uploaded to MS viewer (prospector2.ucsf.edu) (23), which allows viewing annotated spectra of all results with the searchkeys 3hpyufjcel and 94xlgafvxf. The phosphopeptide HCD results from these searches have been uploaded to MS-viewer with a searchkey vivvtc8reo.

The same sets of data were also searched using Protein Prospector for longer O-linked glycans and N-linked glycans, by allowing for unspecified mass modifications within the mass range of 100 to 2,500 on serine, threonine, or asparagine residues. Results from this search showed no evidence of glycosylation on serines or threonines other than a single HexNAc (26). The related N-linked profiling is described in ref. 26. The same sets of data were also searched considering modification of hydroxyproline residues because these residues have been reported as O-glycosylation sites in plants, but no additional O-glycopeptides were found.

ARF8 and ECT7 Immunoprecipitation, Immunoblotting, and Mass Spectrometry Analysis. WT (Col) and ARF8-MYC transgenic seedlings were grown for 4 d under continuous white light at 21 °C. Seedlings were ground to powder in liquid nitrogen. Proteins were extracted in the following buffer: 100 mM Mops [3-(*N*-morpholino)propanesulfonic acid], pH7.6, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 μM PUGNAc, and 2× Complete protease inhibitor Mixture and PhosStop mixture (Roche). Extracted proteins were centrifuged and filtered through two layers of miracloth and then incubated with goat anti-MYC antibodies (9132; Abcam) for 1 h at 4 °C. ARF8-MYC fusion proteins were captured with protein G agarose beads for another hour at 4 °C, washed five times with immunoprecipitation (IP) buffer, transferred into a new tube before elution with boiling SDS sample buffer. IP products were detected by standard Western blot using mouse monoclonal antibody against MYC tag (9B11; Cell Signaling) or O-GlcNAc (RL2; Santa Cruz).

Full-length ECT7 cDNA was amplified by PCR and then cloned into pEarleyGate104 binary vector downstream of the CaMV 35S promoter and yellow fluorescence protein (YFP) coding sequence. The 35S::YFP-ECT7 construct was transformed into *A. thaliana* via an Agrobacterium floral dip method. The 35S::YFP-ECT7 transgenic plants were grown for 14 d under continuous white light. Proteins were extracted by IP buffer [50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM PUGNAc, the Complete protease inhibitor mixture and PhosStop mixture (Roche)]. Immunoprecipitation was performed using a homemade rabbit anti-YFP antibody. The YFP-ECT7 proteins were captured with protein A/G agarose beads for another hour at 4 °C, washed three times with wash buffer, and transferred into a new tube before elution with boiling SDS sample buffer. The eluted sample was separated in a precast gradient SDS PAGE gel (NuPAGE Novex 4–12% [(wt/vol) acylamide in buffer] Bis-Tris Protein Gels; Invitrogen).

The YFP-ECT7 gel band was then in-gel digested with trypsin, and the resulting peptides were analyzed by HCD LC-MS/MS using an Orbitrap Fusion mass spectrometer (Thermo Fisher). Survey scans were acquired in the Orbitrap MS using a mass resolution of 140,000. Six MS/MS scans were acquired in the Orbitrap for each survey scan. Peptide identification using Protein Prospector was as described in *Mass Spectrometry and Data Analysis*.

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