

Does the Ubiquitination Degradation Pathway Really Reach inside of the Chloroplast? A Re-Evaluation of Mass Spectrometry-Based Assignments of Ubiquitination

Klaas J. van Wijk,* Tami Leppert, Zhi Sun, and Eric W. Deutsch*

Cite This: *J. Proteome Res.* 2023, 22, 2079–2091

Read Online

ACCESS |



Metrics & More



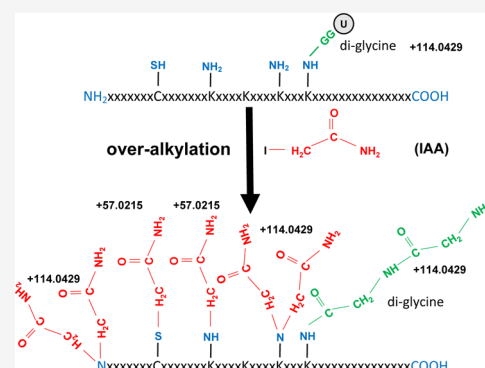
Article Recommendations



Supporting Information

ABSTRACT: A recent paper in *Science Advances* by Sun et al. claims that intra-chloroplast proteins in the model plant *Arabidopsis* can be polyubiquitinated and then extracted into the cytosol for subsequent degradation by the proteasome. Most of this conclusion hinges on several sets of mass spectrometry (MS) data. If the proposed results and conclusion are true, this would be a major change in the proteolysis/proteostasis field, breaking the long-standing dogma that there are no polyubiquitination mechanisms within chloroplast organelles (nor in mitochondria). Given its importance, we reanalyzed their raw MS data using both open and closed sequence database searches and encountered many issues not only with the results but also discrepancies between stated methods (e.g., use of alkylating agent iodoacetamide (IAA)) and observed mass modifications. Although there is likely enrichment of ubiquitination signatures in a subset of the data (probably from ubiquitination in the cytosol), we show that runaway alkylation with IAA caused extensive artifactual modifications of N termini and lysines to the point that a large fraction of the desired ubiquitination signatures is indistinguishable from artifactual acetamide signatures, and thus, no intra-chloroplast polyubiquitination conclusions can be drawn from these data. We provide recommendations on how to avoid such perils in future work.

KEYWORDS: ubiquitination, chloroplasts, mass spectrometry, iodoacetamide, alkylation



INTRODUCTION

A recent paper by Sun et al.¹ studying *Arabidopsis thaliana* suggested that proteins in the chloroplast stroma and thylakoid membrane, including chloroplast-encoded proteins, can be polyubiquitinated and subsequently transferred into the cytosol for proteasomal degradation. This closely followed a prior publication which suggested that the cytosolic CDC48 complexes mediate ubiquitin-dependent degradation of intra-chloroplast proteins in *Arabidopsis*.² However, mechanisms and enzymatic machinery to polyubiquitinate within the chloroplast stroma or thylakoids have not been determined, nor has a translocation pathway to export proteins from the intra-chloroplast space (the stroma) been identified. Polyubiquitination (especially with lysine-48 (K48) linkages between the ubiquitin molecules) is recognized by the 26S proteasome in the cytosol and nucleus and results in degradation of the polyubiquitinated substrates.^{3–6} So far, extensive research has shown that proteins within the chloroplast (i.e., the space confined by the double envelope membranes) are degraded by intra-chloroplast proteases or degraded in the vacuole after export through autophagy-independent transport vesicles or through autophagosomes.^{7–13} Different speculative and confirmed degradation pathways of chloroplast proteins and possible involvement on (poly)ubiquitination are summarized and listed in Figure 1A.

Polyubiquitination requires recognition of substrate by E3 ubiquitin ligases, as well as E1 and E2 enzymes for activation of ubiquitin and transfer to the E3 ligase (Figure 1B). There are two E1 and eight E2 ligases and a large number (~1400) of E3 UBI ligases in *A. thaliana*⁶ that can be found in the cytosol and nucleus or associated with the cytosolic surface of the endoplasmic reticulum or outer membranes of plastids/chloroplasts, mitochondria, and peroxisomes.^{6,14} Each E3 ligase is generally believed to target a specific set of substrates, and it is therefore the E3 ligases that provide selectivity to proteasomal degradation (in case of polyubiquitinated substrates). Two E3 ligases, SP1 and SP2, are associated with the cytosolic side of chloroplasts.^{15–17} Furthermore, nuclear-encoded chloroplast precursor proteins synthesized in the cytosol can be polyubiquitinated for proteasomal degradation en route to the chloroplast by cytosolic E3 ligases such as CHIP,¹⁸ and the cytosolic E3 ligase PUB4 appears also

Received: March 27, 2023

Published: April 24, 2023



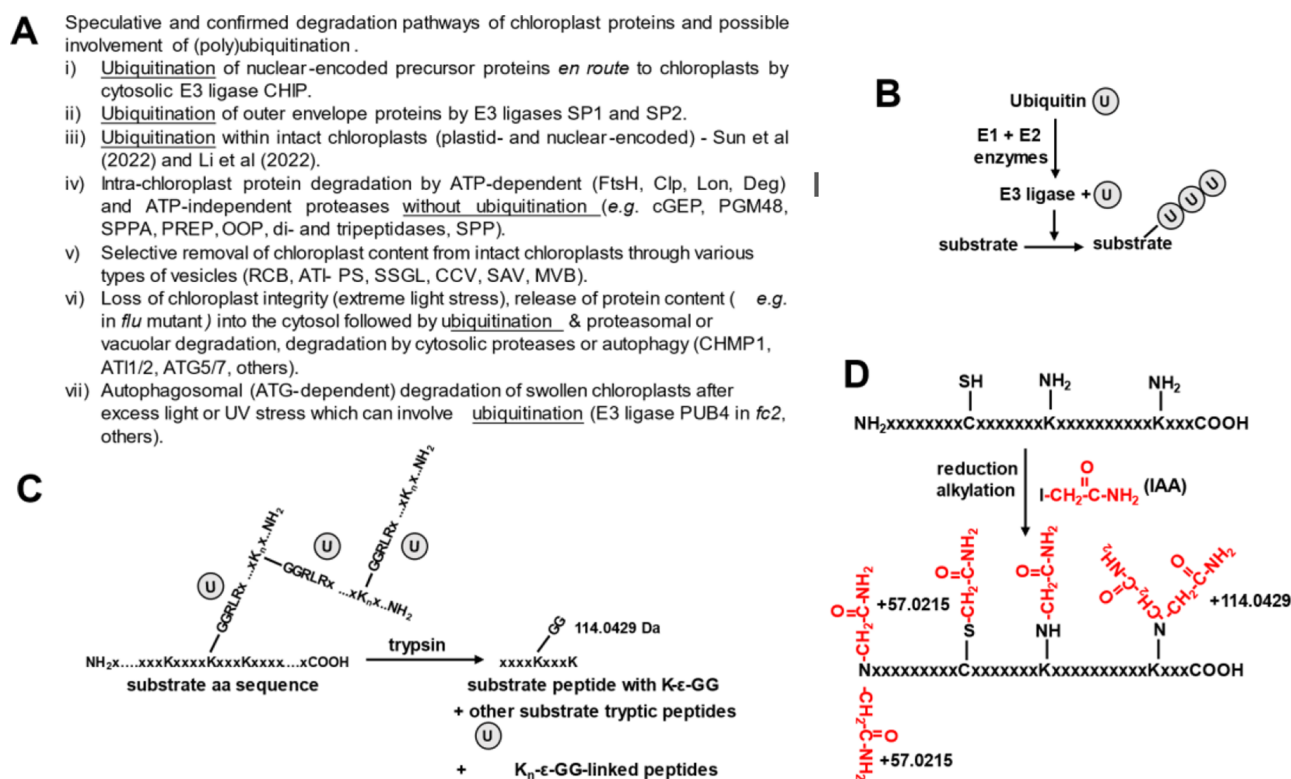


Figure 1. Points for consideration and background information when evaluating ubiquitination of chloroplast proteins. (A) Speculative and confirmed degradation pathways of chloroplast proteins and possible involvement of (poly)ubiquitination. Scenario (iii) is proposed in refs 1, 2. (B) UBI-protease pathway (precursor UBI processing and activation, conjugation to E3 ligases, and selective ubiquitination of substrates by E3 ligases). Ubiquitin is shown as circled U. (C) UBI-protein isopeptide linkage (K- ϵ -GG) and consequence of digestion with trypsin protease. The products are tryptic peptides from the substrate and from the (poly)ubiquitination chain. For proteasomal degradation, the internal isopeptide linkages in the polyubiquitination chain are typically from K₄₂. (D) Desired and unwanted off-target alkylation can result in +57.0215 and 114.0429 modifications on cysteine, lysine, peptide N-termini, as well as several other amino acid residues (S, T, D, E, Y, H, and M). The di-GG footprint of ubiquitination also results in a +114.0429 Da mass modification on lysine, thus preventing unequivocal identification of this modification.

involved in chloroplast proteostasis.^{19–21} However, so far, none of the E1–E2–E3 proteins are known to reside within the plastid/chloroplast stroma and thylakoids. To the best of our knowledge, there is also no demonstration for ubiquitin (a 76 amino acid peptide encoded by UBQ10—AT4G05320 and homologs) within the stroma/thylakoids of plastids/chloroplast or the mitochondrial matrix. The conclusion that a ubiquitin-based pathway acts inside chloroplasts to regulate photosynthesis¹ is thus truly surprising, and it is therefore prudent to take a critical look at the various data presented in this recent study. The conclusion mostly hinges on mass spectrometry (MS) of affinity-enriched peptides using either an antibody directed to a lysine- ϵ -diglycine (K- ϵ -GG) peptide or using anti-Myc affinity materials that recognize an overexpressed 6Myc-tagged version of ubiquitin.

Canonical ubiquitination occurs on the sidechain of lysine residues through a covalent isopeptide bond of the ϵ -amine to the C-terminal glycine residue of ubiquitin (Figure 1C). The C-terminus of the activated ubiquitin ends with the amino acid residues-RLRGG. Upon digestion of a ubiquitinated protein with trypsin, which cleaves the peptidyl bond C-terminal of lysine and arginine, a di-G “footprint” is left on the modified lysine (Figure 1C). This K- ϵ -GG footprint is therefore considered evidence for mono- or polyubiquitination. Using MS, this GG is identified as an extra mass of 114.0429 associated with the modified lysine residue (Figure 1C). It should be noted that the K- ϵ -GG footprint can also result from

other UB-like modifications (NEDDylation and some SUMOylation) and cannot distinguish between monoUBI and different polyUBI linkages.²² Finally, there are reports of non-lysine (non-canonical) ubiquitination at the N-terminus of proteins, as well as of cysteine, serine, or threonine, but this possibility has not really been investigated in plants.²³

There are several pitfalls of relying on the additional +114.0429 Da mass for assigning ubiquitination. Unfortunately, covalent attachment of two acetamide molecules to lysine results in an identical mass modification (Figure 1D). The main confounder for the GG footprint therefore stems from covalent attachment of two acetamide molecules to lysine, or if a peptide has two lysines, an extra mass of 114.0429 can be obtained by single alkylation of each lysine (2×57.0215)^{24,25} (Figure 1D). Such lysine modifications can occur during the alkylation reaction to block the sulfhydryl of cysteine residues, in particular using iodoacetamide (IAA) (a α -halocarbonyl electrophile). The frequency of off-target alkylation can be reduced by using lower IAA concentrations at lower temperatures or by using chloroacetamide (CAA).^{26,27} However, it should be noted that the delta mass of acetylation with IAA or CAA is identical and potential (di)-acetylation of lysine by even CAA cannot be ignored.²⁸ Furthermore, CAA has shown to produce other artifacts not seen with IAA, in particular very high levels of oxidation of M and W.²⁶ Different abbreviations for iodoacetamide and chloroacetamide are used

Table 1. Summary of the MS-Based Experiments in Sun et al.¹ and Our Re-Analysis

proteome Xchange PXDs ^{a,b}	experiments within PXDs	# raw files	instrument (info in RAW files)	# MSMS spectra acquired (info in raw files)	reduction, alkylation, and digestion conditions (Methods section of the paper) ^{c,d}	claims in paper and our comments. UBI is inferred from +114 Da on lysine residues	summary of our open ^e and closed ^f searches
PXD0031388 ^a	exp. 1. 6xMyC-UBQ10 affinity-chloroplasts. +bortezomib	2 (beads & eluate)	QE	39,372 all; 18,220 eluate	10 mM TCEP 30 min RT, in 8 M urea in AB, 0.2% protease inhibitor cocktail (Sigma P8340) ^c 50 mM CAA (30 min, RT). Filter-aided. LysC in 6 M urea for 4 h at 37 °C; then trypsin in <1 M urea o/n at 37 °C	12 UBI proteins out of 359 proteins; only one UBI protein (P8B3) is in the plastid-spectrum shown in Figure S1C	387 proteins. 41 ATCGs & 273 TargetP predicted n-encoded (81%). No support for chloroplast protein ubiquitination
	exp. 2. UBI-diGly affinity wt of isolated chloroplasts (PTMscan)	4 (eluate, flow through, 2x input)	QE	109,959 all; 8715 eluate	10 mM TCEP 30 min RT, in 8 M urea in AB & 1% plant protease inhibitor cocktail (PPIC; Sigma-Aldrich, P9599) ^c 50 mM CAA (30 min, RT). LysC in 6 M urea for 4 h at 37 °C; then trypsin in <1 M urea o/n at 37 °C. 1% TFA afterward.	57 UBI sites in 40 proteins. Our reannotation shows that only 8–12 are plastid localized. Table S2 suggests that most +114 Da peptide modifications are from 2 lysines with each +57 Da	At 0.73% protein FDR, there are 1268 proteins. There are 15 PSMs with a potential K+114. 6 PSMs are for ubiquitin (5 at K48 and one at K63), but upon manual inspection of the spectra, none of the other nine have credible K+114 modifications.
	exp. 4. comparative proteomics isolated chloroplasts (CDC48-WT and CDC48-DN)	6 (3 replicates per genotype)	QE	277,125	listed as similar as in exp. 1 & 5	results are similar as for the comparative leaf proteomics	1385 proteins. 52 ATCGs & 1086 TargetP predicted n-encoded (82%)
PXD0031468 ^b	exp. 3. UBI-diGly affinity CDC48-DN of isolated chloroplasts (IPX0004051001)	3 (replicates of eluates)	timsTOE ^g	630,278	10 mM DTT, 1.5 h, 37 °C in 8 M urea, pH 8.5 & 600 rpm; no alkylation mentioned ; trypsin but no conditions mentioned	768 UBI sites in 316 chloroplast proteins (searching only 15% of the predicted Arabidopsis proteome). Our reannotation shows that only ~40% are plastid localized. Our closed search corroborates ~80% of the K + 114 in the ATCG proteins reported in Figure 2 of Sun et al. ¹	5546 proteins (FDR < 1%), 56 ATCGs & 1345 TargetP predicted n-encoded (25% of total). Ubiquitin comprises ~0.25% of all PSMs and RBCL comprises ~1.45% of all PSMs. High levels of alkylation and off-target alkylation, indicative of IAA (not CAA) treatment. Most proteins are not in the chloroplast. While chloroplast proteins have K + 114, this is most likely dicarbamidomethylation
	exp. 5. Comparative proteomics of seedlings (CDC48-WT and CDC48-DN) (IPX0004051002)	6 (3 replicates per genotype)	QE HF ^h	155,968	10 mM DTT, 8 M urea, TEAB pH 8.0 for 1 h at 60 °C (proteins by phenol, MeOH precipitation & acetone washes; SDS). 50 mM IAA for 40 min at RT. Filter-aided. Trypsin, 12 h 37 °C	upregulated proteins in the CDC48-DN lines are CHLORAD targets	3011 proteins (FDR < 1%), 47 ATCGs & 893 TargetP predicted n-encoded (31%); no alkylation detected

^aPXD031388—submitted by the Oxford group through PRIDE. ^bPXD031468 by the Shanghai group through iProX. ^cProtease inhibitor cocktail Sigma P8340—AEBSE, aprotinin, bestatin, E-64, leupeptin, and pepstatin A (in DMSO). ^dPlant protease cocktail (PPIC) Sigma P9599—AEBSE, bestatin, E-64, leupeptin, pepstatin A, and 1,10-phenanthroline (in DMSO). ^eOpen search using TPP related tools including PTMProphet in Massdiff mode. Search parameters: trypsin; maximum allowed missed cleavages 4; maximum variable modifications per peptide 3; precursor mass error tolerance −150 Da to +500 Da; precursor true tolerance 5 ppm; fragment mass error tolerance 20 ppm; variable modifications 15.994915 Da (M), and 42.010565 Da (protein Nterm); localize_delta_mass; delta_mass exclude_ranges (default). ^fClosed search parameters using the search engine MSFrager (Kong et al.⁴⁹): tryptic or semi-tryptic; missed cleavages 4; maximum variable mods per peptide 4; precursor mass error tolerance 10 ppm, MSMS mass error tolerance 20 ppm. Fixed modifications—none. Variable modifications: oxidation 15.994915 (MW), carbamidomethyl 57.021464 (C, K, M, Nterm), diGG or dicarbamidomethyl 114.042927 (C, K, Nterm), acetaldehyde 26.015650 (Nterm), deamidation 0.984016 (N, Q), carbamyl 43.005814 (K, Nterm), Gln->pyro-Glu minus 17.02650 (nQ), AEBSE aminoethylbenzenesulfonylation 183.035399 (K, Y). ^gNo timsTOF instrument was mentioned in the paper. Instead the Methods section states that this was done on a QE instrument (ThermoScientific). The iProX submission states this was done by a timsTOF Pro. ^hThe paper states that a QE was used without specifying the model.

in the published literature; here, we follow the abbreviations (IAA and CAA) as defined in ref 28.

Confident assignment of any mass modification (both post-harvest and physiological post-translational modifications—PTMs) to a specific residue, including GG to lysines, requires specific sequence coverage of the modified residue through identification of b-ion and/or y-ions and statistical significance of the assigned PTM-site through a PTM-scoring algorithm. High mass accuracy of the precursor ion (e.g., within 3–6 ppm) is needed to ensure that the peptide with the assigned PTM does not carry additional mass modifications that could provide alternative interpretation of the peptide sequence and its PTMs. Correct assignment of the monoisotopic (only C12, not C13) peak within the isotope envelope is also important to avoid additional mass accuracy errors. The mass of asparagine (N) is also 114.0429 Da and might lead to false positive identification of ubiquitination if a protein ends with K-N since a missed cleavage of K-N at the C-terminal end results in addition of 114.0429. Furthermore, the isobaric residues I and L have a mass of 113.0841 and D has a mass of 115.0269, so when combined with isotope (± 1.003355) uncertainties and/or amidation and deamidation (± 0.984016), special care and high mass accuracy are crucial. Furthermore, there are conflicting reports about whether trypsin cleaves the C-terminal of modified lysines, and therefore, assigning ubiquitination at a C-terminal K of a peptide needs to be treated with care^{29,30} but see ref 31. Nevertheless, we can conclude that MS is a very powerful tool to detect ubiquitination and indeed several large scale ubiquitination studies have been published for Arabidopsis^{32–35} as well as many non-plant species.^{22,36–39} However, as in all large-scale “omics” studies, even low false discovery rates can result in many false positive observations especially for PTMs, and a critical evaluation of the MS results is therefore needed. Because the original raw MS data for many protein MS-based publications are available through ProteomeXchange (<http://www.proteomexchange.org/>),⁴⁰ it is possible to re-evaluate reported results by re-searching these raw MS data.^{41,42} However, because many of these data sets are large and because of the need for specific expertise, many readers are not in the position to explore the raw data by themselves. Initiatives such as PeptideAtlas⁴³ are therefore important to provide access to reanalysis of published MS data sets as is available for *A. thaliana* (<http://www.peptideatlas.org/builds/arabidopsis/>).⁴⁴

METHODS

To evaluate the MS-based conclusions in Sun et al.,¹ we downloaded the raw MS files from ProteomeXchange (PXD031388 submitted through PRIDE⁴⁵ and PXD031468 submitted through iProX⁴⁶) and we carefully considered the process for generation of extracted peptides (use of proteases, reducing and alkylation agents, urea, formic acid and trifluoroacetic acid, temperature, and time) as well as the acquisition parameters (summarized in Table 1). The raw data files in PXD031388 self-identify the mass spectrometer as a “Q Exactive” (Thermo Fisher Scientific) and the data in PXD031468 self-identify as a “Q Exactive HF” (Thermo Fisher Scientific) and a timsTOF (Bruker) because this information is embedded in the raw files (this information is more reliable than the written information provided in papers or ProteomeXchange as is also the case for Sun et al.¹—see Table 1). These raw files were then converted into mzML⁴⁷

using ThermoRawFileParser⁴⁸ and processed using MSFragger 3.2⁴⁹ and the Trans-Proteomic Pipeline (TPP) software suite version 6.2.0.⁴⁰ In the following sections, we discuss the MS-based experiments and results presented in Sun et al.¹ and compare that with the results we obtained from reprocessing these raw mass spectra.

RESULTS AND DISCUSSION

Across the two PXD submissions, there are five experiments (Table 1). The summarizing Table 1 provides information on these experiments, the number of acquired tandem MS spectra (MSMS), and previously key reported findings.¹ After downloading all of the raw data, we performed first a so-called “open search” with MSFragger⁴⁹ in which the software tries to match the MSMS spectra to predicted peptides in Arabidopsis with wide precursor m/z tolerance (−150 to +500 Da). Post-analysis of the open search results to localize the discovered mass deltas was performed with the “massdiff mode” of PTMProphet.⁵⁰ The advantage of such open searches is that this allows one to detect common and less common mass modifications, including those induced by sample treatment (e.g., alkylation or urea) and in-source fragmentation. We note that most researchers do not perform such open searches because most are unfamiliar with this type of analysis, it is computationally expensive, and it is quite laborious to (manually) evaluate the results.⁵¹ These open search results can then be used to set up the search parameters for a more typical (closed) search with defined mass modifications. The results for these open searches are summarized in Figures 2 and S1 and Tables 1 and S1.

We detected more than 40 mass modifications across these five experiments ranging in frequency up to 12% of all assigned spectra in an experiment (Table S1 and Figures 2 and S1). A prominent modification was +43.006 Da from carbamylation due the urea treatment (urea breakdown at elevated temperatures produces isocyanate which reacts with N-term, Lys, and Arg) observed in the experiments 1, 2, and 4 carried out in Oxford (frequency of 1.5, 2.5, and 2.8%, respectively) but less prominent in experiments 2 and 5 carried out in Shanghai (Table S1 and Figures 2A and S1B). A surprise was the +183.228 Da modification (on K and Y) resulting from a serine protease inhibitor AEBF (4-benzenesulfonyl fluoride hydrochloride) especially in experiment 2 (4.5%) because the samples were treated with 1% plant protease inhibitor cocktail (Tables 1 and S1 and Figure 2). Finally, a few other prominent modifications were the formation of pyroglutamate (of peptide N-terminal Q and E) in all experiments (0.4–0.9%) due to N-terminal cyclization and methylation (+14.016) from the use of the acids TFA and FA, as well as oxidation and di-oxidation (+15.995 and +31.989) of M, W, H, and P (Table S1 and Figures 2 and S1).

By far the most frequent modification was +57.022 Da due to carbamidomethylation of mostly C (the intended target of alkylation treatments) but also peptide N-termini, K, S, T, D, E, Y, and H which are known off-target consequences of the alkylation treatment.²⁸ This frequency was very high at 12% in experiments 2 and 3 and 4 and 7.7% in experiments 1 and 4 (Figures 2 and S1), respectively. However, the open search clearly showed that alkylation was absent in experiment 5 (Figures 2 and S1) despite the method section in Sun et al.¹ stating alkylation with 50 mM IAA for 40 min. In contrast, Sun et al.¹ did not mention any alkylation treatment for experiment 3, whereas the samples clearly have undergone alkylation.

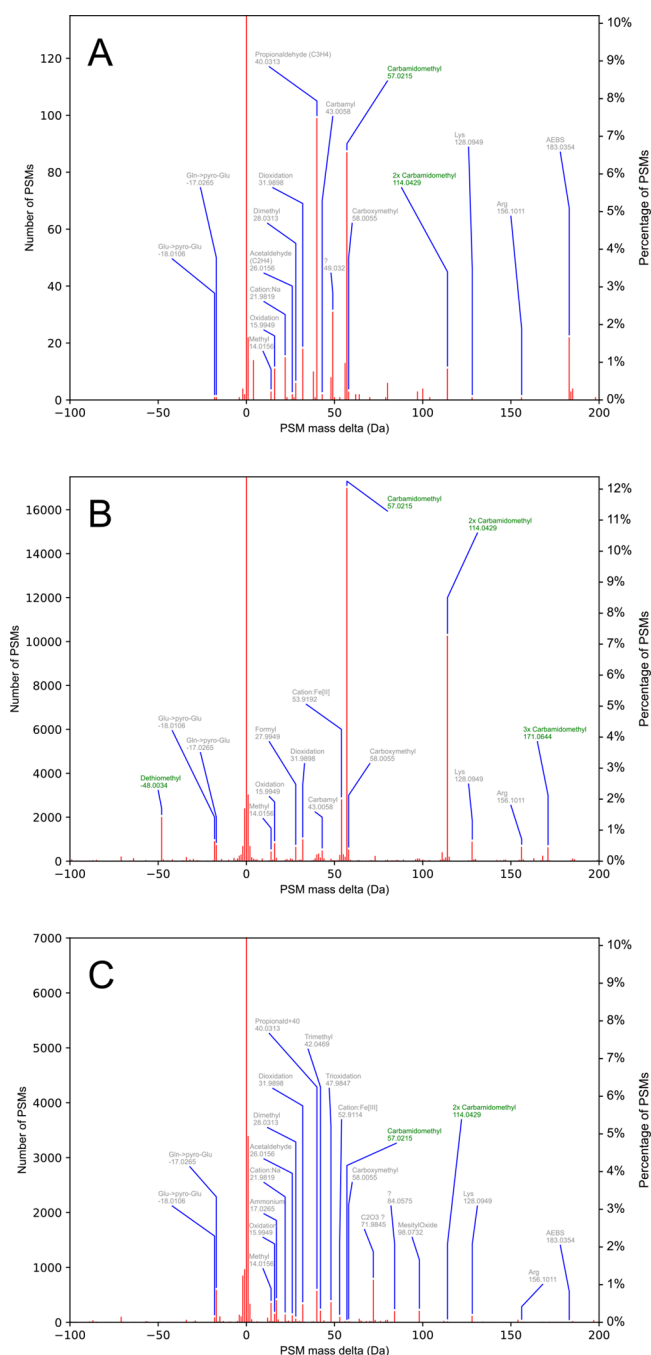


Figure 2. Detection and frequency of mass modifications by open searches of raw MS data downloaded from PXD031388 (A) and PXD031468 (B,C). Modifications marked in green can be the result of alkylation (-48.0034 , $+57.0215$, $+114.0429$, $+171.0644$) but $+114.0429$ can also be from the GG footprint (isopeptide bond to K) from ubiquitination (see Table S1 for more information). (A) Anti-K- ϵ -GG affinity eluate from isolated chloroplasts of wild-type plants by a QE instrument (exp. 2 in Table 1). (B) Anti-K- ϵ -GG affinity eluates from chloroplasts isolated from transgenic CDC48-DN plants and analyzed by timsTOF (IPX0004051001) (exp. 3 in Table 1). (C) Comparative proteomics of seedlings isolated from CDC48-WT and CDC48-DN lines analyzed by a QE instrument (exp. 5 in Table 1). Mass modifications $+71.9845$ and $+84.0575$ are unidentified.

Three other modifications were observed that are also unintended consequences of alkylation, i.e., -48.003 Da in

experiment 3 (dethiomethylation of M due to alkylation of M followed by in-source loss of the alkylated side chain), $+39.995$ Da in experiment 2 for pyro-carbamidomethylation (alkylation of C, followed by N-term S-carbamoylmethyl-cysteine cyclisation), and $+114.025$ Da (di-carbamidomethylation of K, N-term, and other residues as for $+57.022$) in particular in experiments 2 (0.9%), 3 (7.3%), and 4 (0.6%) (Figures 2 and S1 and Table S1). The -48.003 modification which was high only in experiment 3 is a known off-target consequence of (over)alkylation with iodine containing reagents (such as IAA) but not with CAA,²⁸ indicating that IAA (or iodoacetic acid) was used in experiment 3. However, as mentioned above, the $+114.025$ on K is also the main hallmark for ubiquitination due to the GG footprint—it is not possible to distinguish between these two modifications based on mass alone. Importantly, off-target alkylation patterns do provide insight as to whether the K + 114.025 is likely to result from alkylation rather than ubiquitination.^{24,28} Based on the observed modifications from these open searches, we then conducted our closed searches for each of these five experiments using the most prominent selected modifications (for closed search settings see footnotes in Table 1). The take-home message is that the reanalysis of the raw MS data from Sun et al.¹ by open searches (Figures 2 and S1 and Table S1) and closed searches strongly suggest that the surprising report of intra-chloroplast ubiquitination is due to alkylation artifacts and that IAA was used as alkylating agent in experiment 3. In the remainder of this paper, we discuss the experiments in Sun et al.¹ and compare our open and closed MS search results with the results and interpretation listed in Sun et al.¹

The paper¹ starts out with an Arabidopsis transgenic line with 35S-driven overexpression of 6xMyc-UBI (there is no chloroplast signal peptide; hence this ~ 20.5 kDa protein likely accumulated in the cytosol but no data are shown), followed by an immunoblot with anti-Myc serum of SDS-PAGE separated isolated chloroplast proteins. This showed a ~ 200 kDa band which was insensitive to treatment with the protease thermolysin (cleaves N-terminal of L, F, V, I, A, and M), which was taken as evidence that this band reflected poly-Myc6-UBI tagged proteins inside the chloroplast. Fractionation of isolated chloroplasts into membrane and soluble samples shows that this 200 kDa band is in the soluble (stromal) fraction, with weaker bands in the membrane fraction. It is not clear to us why there is only one band and why it has a mass so large (perhaps a small aggregate). Based on previous reports, polyUBI proteomes (e.g., in loss of E3 ligases, inhibition of proteasomal activity) show multiple bands across a wide mass range.^{52,53} At most, this blotting experiment provides only very circumstantial evidence for intra-chloroplast polyubiquitinated proteins. Proteins from isolated chloroplasts from this 35S-Myc6-UB line (no wild-type control) were digested with Lys-C and trypsin and analyzed by MS (Table 1—exp. 1). The paper¹ reports 359 identified chloroplast (associated) proteins and, for just one protein, stromal peptide chain release factor (PrfB3—AT3G57190), a specific ubiquitination site was detected, of which 12 proteins have an assigned K- ϵ -GG modification. PrfB3 has a reported ubiquitination at K133 (but in their Table S1,¹ there is no value for peak intensities, no MS counts, no K- ϵ -GG id; one peptide count with q-value 0.0077, score 5.8). This single spectrum, shown in their Figure S1,¹ is claimed to be evidence for the tryptic peptide AVDSL-K_(ub)DLK, but 6 of the top 10 peaks in this annotated spectrum are not assigned, and thus it appears to be a false

match. We reproduce the spectrum match in Figure 3A and manually re-evaluated this raw spectrum, and we find several

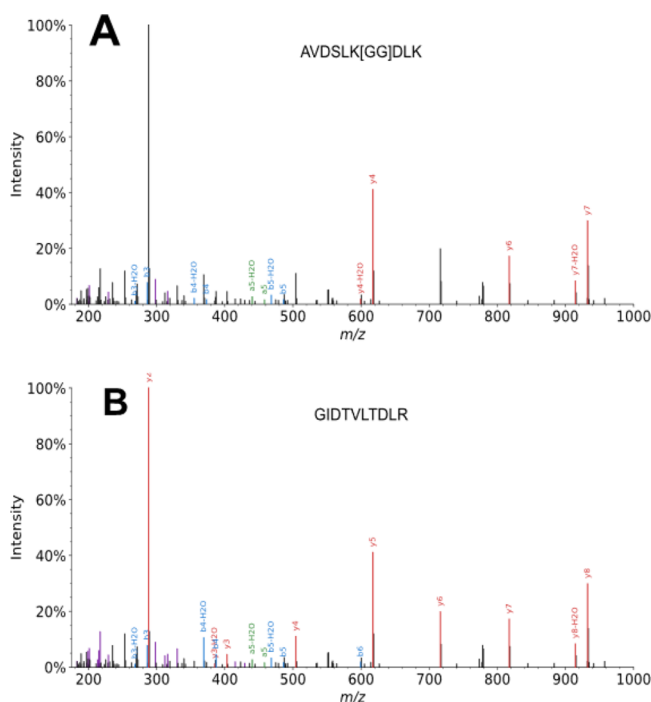


Figure 3. Alternative interpretations of the MS/MS spectrum ($z = 2^+$) with scan number 18320 for the reported ubiquitinated peptide for PrfB3. Measured experimental m/z 551.8094. (A) Reproduced match of Figure S1 from Sun et al.¹ from down-loaded raw file (USI: mzspec:PXD031388:QEX01_200129_ChloroplastMycUb_Eluate_04:scan:18320:AVDSLK[GG]DLK/2). Several crucial major peaks are unassigned. Calculated theoretical m/z 551.8088. Mass error 1.1 ppm. (B) Alternative spectrum interpretation in which nearly all major peaks are now assigned and with the same theoretical mass as the interpretation in A and Figure S1 from Sun et al.¹ (USI: mzspec:PXD031388:QEX01_200129_ChloroplastMycUb_Eluate_04:scan:18320:GIDTVLTDLR/2). The unlabeled purple peaks correspond to weak internal fragmentation ions. Note that the K + 114.0429 Da can result from either di-carbamidomethylation or the diG footprint of ubiquitination.

much better matching peptide sequences (without the need to assign any mass modifications) in which the major peaks are now assigned (Figure 3B). None of the potential sequences match PrfB3 and in fact do not seem to match to Arabidopsis proteins at all. ~1% of assignable spectra match to ordinary contaminants (human and bovine proteins) and another 1% of assignable spectra match to *Escherichia coli*. The most plausible match for this spectrum is the peptide GIDTVLTDLR, matching to the rpmB (50S ribosomal protein L28) protein found in many bacteria, including *Ectothiorhodospira* and *Vibrio* species, although not the *E. coli* version of rpmB. The universal spectrum identifiers (USIs)⁵⁴ are provided for both interpretations of the same spectrum (USIs may be examined interactively at <http://proteomecentral.proteomexchange.org/usi/>). Our open search finds that only 0.85% of identified spectra show evidence for a +114.0249 mass shift (Figure S1 and Table S1) and most on the N-terminus (due to di-alkylation). Our closed search identified 387 proteins, including 41 chloroplast-encoded proteins and 273 TargetP-predicted n-encoded proteins (81%); this indicated that the chloroplast isolation was successful. About 5.5% of the PSMs

included a +57.00215 mass modification (nearly exclusively on cysteine and the rest mostly on peptide N-termini) but only 0.17% for the +114.0429 modification, mostly on the peptide N-terminus. Hence, our closed search of the raw MS data in experiment 1 does not support ubiquitination of chloroplast proteins. We also note that there is no negative control in this experiment such as an affinity purification using isolated chloroplasts from wt. Hence, this first experiment does not provide support for ubiquitination within the chloroplast.

The Sun et al. paper¹ then continues with another ubiquitination experiment but this time using isolated chloroplasts from wild-type plants and peptide affinity enrichment using anti K-ε-GG antibody beads (Table 1—exp. 2). The paper claims that MS identified 57 unique UBI sites in 40 proteins. However, results with the MaxQuant software reported in their Table S2¹ show only peptides in which two lysines each carry a +57.022 mass, likely from off-target alkylation with CAA. Indeed, in our open search, we find only a very low frequency (0.85%) of the +114.025 modification (Figures 2A and S1B and Table S1). Also comparing the open search results of just the affinity eluate and the combined results of input, flow-through, and eluate showed no difference in frequency of the +114.0249 peak (0.85% vs 0.83%, in total vs eluate only) (Table S1). This was confirmed by our closed search (Figure 4) which showed that 0.24% of

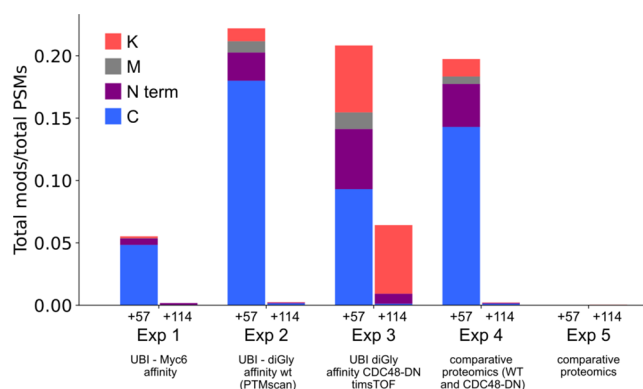


Figure 4. Bar diagram with the frequency of mass modifications +57.0215 and +114.0429 Da in the five experiments based on closed searches of the raw MS data. PSM FDR values were < 0.002 without PTM site scoring. Other search parameters are listed in the footnote of Table 1. Color coding indicates if the mass modification was localized on cysteine (blue), lysine (salmon), the peptide N-terminus (purple), or methionine (gray). Mass modifications of +57.0215 and +114.0429 on other amino acids that were not searched (S, T, E, D, Y, and H) are likely assigned to C, K, M, and N termini, whichever are physically nearest.

the PSMs includes a +114.025 modification (0.03% on K). Their Table S2¹ lists two GG-labeled peptides for chloroplast-encoded RBCL—but in one case the mass error is 25 ppm, which make it insignificant (errors should be < ~6 ppm on a QE instrument as used here). Also, out of the 40 proteins, the plant proteome data base (PPDB)⁵⁵ annotated only eight as plastid localized and most do not have a predicted chloroplast transit peptide (cTP); hence, most of these 40 are not plastid proteins. At 0.73% protein FDR, there are 1268 proteins and there are 15 PSMs with a potential K + 114. Six PSMs are for ubiquitin (five at K48 and one at K63), but upon manual inspection of the spectra, none of the other nine have credible K + 114 modifications. Furthermore, the closed search shows a

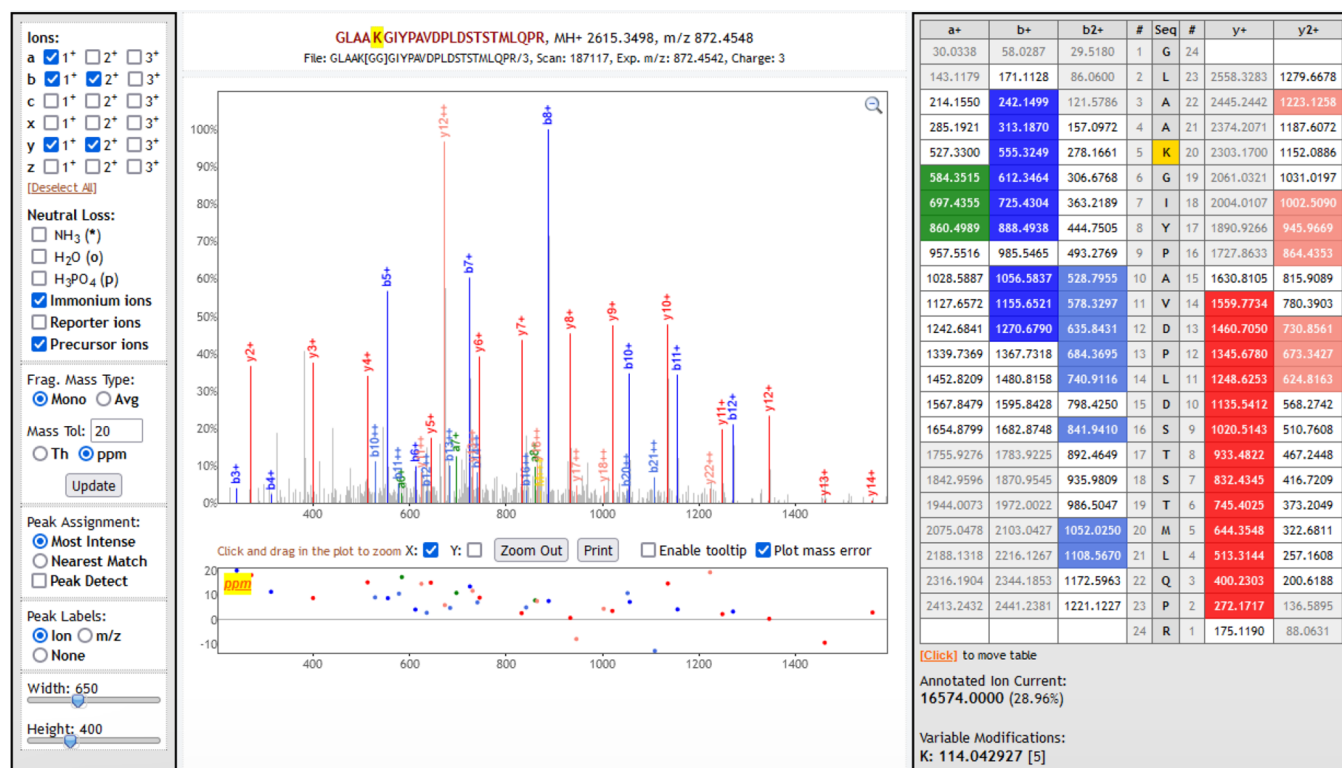


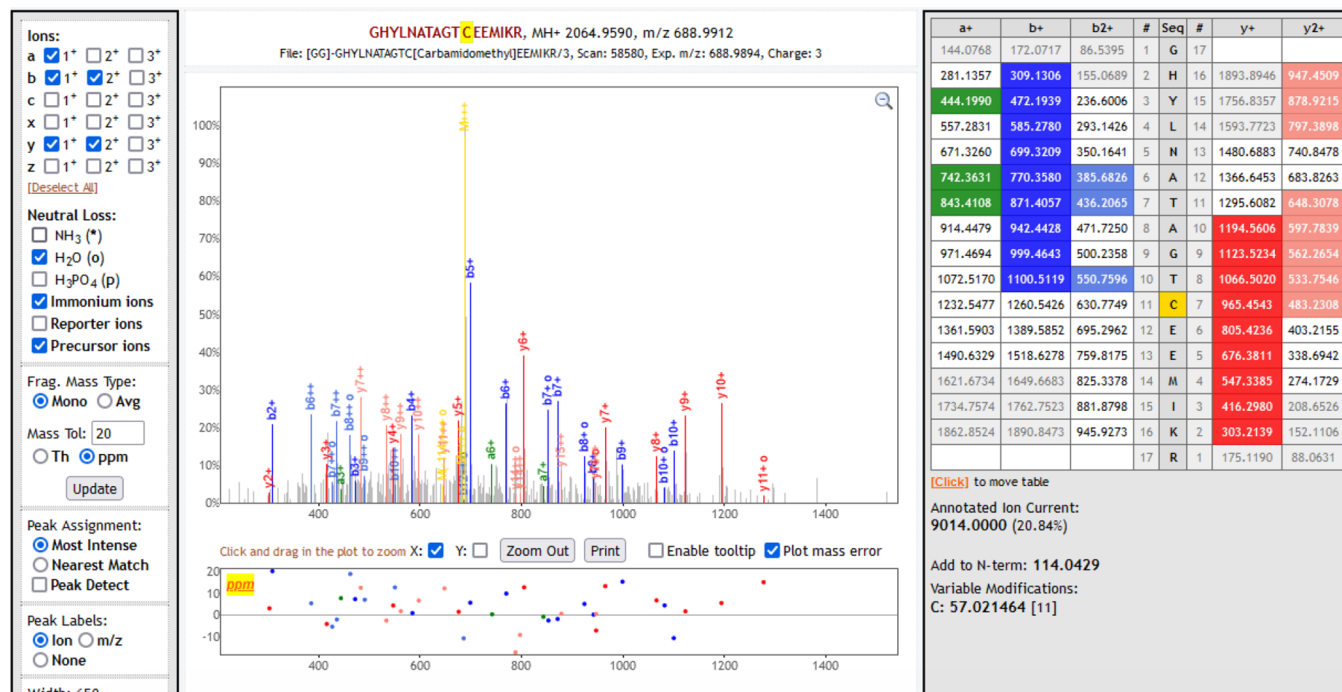
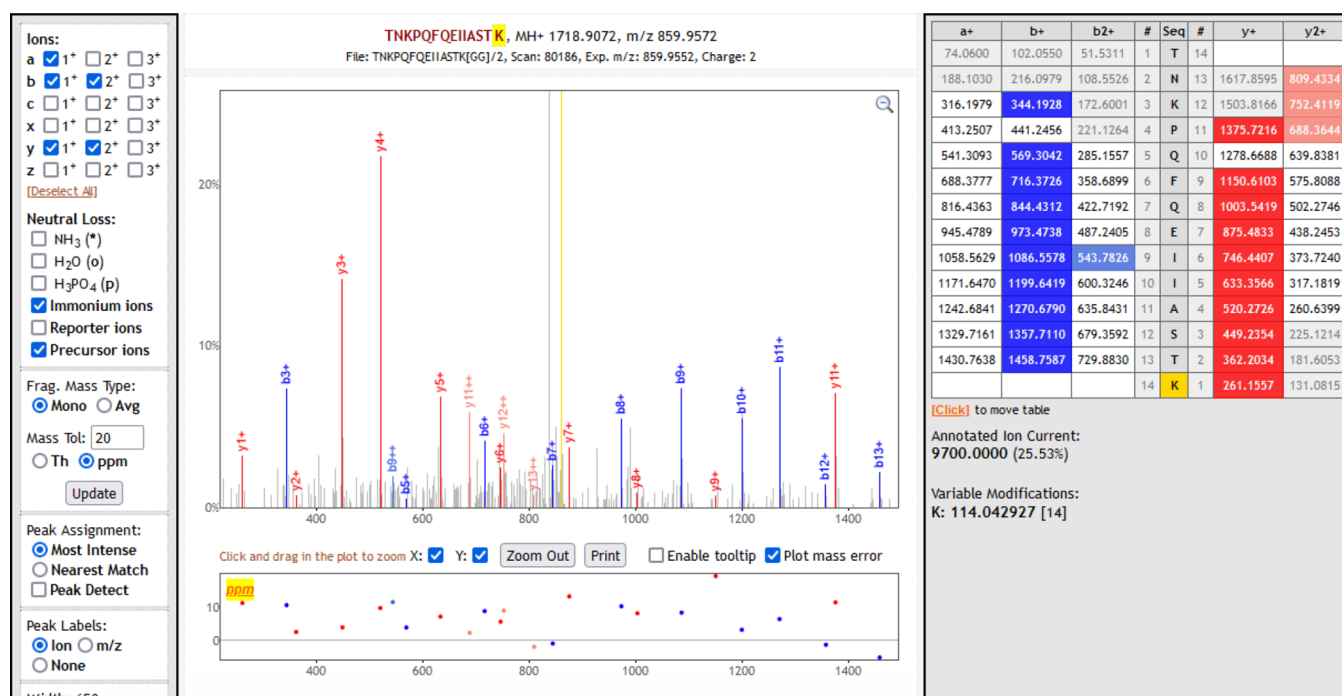
Figure 5. Example of a K + 114 detection in peptide GLAAK[GG]GIYPAVDPLDSTSTMLQPR/3 from ATCG00480.1 (CF1 β) from anti-K- ϵ -GG affinity eluates from chloroplasts isolated from transgenic CDC48-DN plants and analyzed by timsTOF (IPX00040S1001). The corresponding USI is [http://proteomecentral.proteomexchange.org/us1/?usi=mzspec:PXD031468:P20210200988_DN_Slot1-72_1_319:scan:187117:GLAAK\[GG\]GIYPAVDPLDSTSTMLQPR/3](http://proteomecentral.proteomexchange.org/us1/?usi=mzspec:PXD031468:P20210200988_DN_Slot1-72_1_319:scan:187117:GLAAK[GG]GIYPAVDPLDSTSTMLQPR/3). This PSM has reasonably good coverage of the putative K + 114 with good b5–b7 ions although the b3 and b4 ions are a bit weak. Unannotated peaks at 326.1710, 383.1869, 413.2027, 441.1976, and 546.252 correspond to b-type internal fragmentation ions for fragments PLD, PAVD, PLDS, DPLD, and YPAVD, respectively (and there are many more less prominent ones) (exp. 3 in Table 1). Note that the K + 114.0429 Da can result from either di-carbamidomethylation or the diG footprint of ubiquitination.

high percentage (22%) of PSMs that include peptides with a +57.0215 mass modification (for cysteine, but also N-termini and lysine indicative of off-target alkylation) but very few of the +114.0429 modification (0.24%) (Figure 4). Hence, also this second experiment does not provide support for ubiquitination within the chloroplast. A brief summary is given in Table 1.

The main MS-based argument for intra-chloroplast ubiquitination stems from an ubiquitination experiment with the dominant negative line CDC48-DN in which expression of the endogenous cytosolic CDC48 is suppressed (Table 1, exp. 3). The paper¹ states that the MS was done by a QE instrument, but based on the data sets we found in the repositories, the MS analysis was done using a timsTOF (Bruker) instrument (there is no mention of a timsTOF anywhere in the Sun et al.¹ text or Supplemental Information). The paper¹ reports that 768 unique UBI sites were identified in 316 chloroplast or “chloroplast-associated” proteins. There was no requirement for observation in more than one replicate. We evaluated the predicted subcellular localization of these 316 proteins based on the subcellular localization predictor TargetP⁵⁶ and curated localization from PPDB. Including 13 ATCG proteins and 20 outer envelope proteins that do not have a cTP, only 127 out of these 316 proteins have a predicted chloroplast localization (note that TargetP overestimates chloroplast localization) and based on PPDB, only 112 out of 316 have a curated plastid location. This shows that only ~40% of the reported chloroplast proteins are in fact chloroplast-localized, including

envelope proteins. The number of non-chloroplast proteins is probably higher because the authors searched only 4174 *Arabidopsis* chloroplast(plastid)-associated protein sequences using UniProtKB identifiers⁵⁷ rather than the full predicted proteome with ~28,000 proteins in Araport11 or UniProtKB.^{44,58} Because >85% of the predicted proteome was not searched, this increased the potential for false positive identification of peptides and proteins; this limited search space is unusual and should be avoided.^{42,59} Indeed, our closed search of the same raw MS data files identified 5556 proteins (protein FDR <0.67%), including 56 ATCG, with nearly 40 K distinct identified peptides and 848 K PSMs (Table 1). Localization predictions and curated annotations in PPDB indicate that at most ~25% of these 5500 nuclear-encoded proteins are chloroplast proteins even if the authors state that chloroplasts were isolated for this experiment. This very large percentage of non-chloroplast proteins showed that chloroplast isolation was not successful.

Neither in the method section in the paper¹ nor in the iProX⁴⁶ submission record is there any mention of alkylation in this timsTOF data set. Yet our open search (Figure 2B and Table S1) clearly shows extensive monoalkylation (+57.022 Da—12% of all identified spectra) whereas also our closed search finds high levels of monoalkylation in particular on cysteine (9.3%), N-termini (4.8%), and lysine (5.4%) (Figure 4). Furthermore, the method section in the paper¹ states that the search parameters included fixed C-carbamidomethylation (the desired effect of alkylation of cysteine is to prevent the



sulfhydryl from making disulfide bonds that can interfere with protein identification and is the only reason proteomics samples are treated with alkylating agents), variable K- ϵ -GG, and M oxidation. In contrast, the text files in the PXD (the file “modification-specific” peptides, the parameter file, and the “evidence file”) indicated that protein N-terminal acetylation was also allowed, but there was no mention anywhere of cysteine carbamidomethylation. Furthermore, neither variable carbamidomethylation on K and N-termini nor variable di-carbamidomethylation of other residues than lysine (+114.025 similar as GG) was included in the search; this is problematic as our open search (and also closed search) shows that these modifications are very prominent. The conflicting information around alkylation and inadequate search parameters raises serious concern to what extent the +114.025 Da modification on K represents the GG footprint of ubiquitination.

We then compared our search results to the data displayed in Figure 2 of Sun et al.,¹ in particular for the chloroplast-encoded proteins (ATCG identifiers), and we corroborated most (~80%) of the claimed K + 114.043 detections at specific sites in the ATCG proteins and spectrum identifiers. One example is shown in Figure 5. Here, peptide GLAAK[+114] GIYPAVDPLDSTSTMLQPR from ATCG00480 (CF1 β) is quite clearly shown to have a K + 114 modification. A second example (Figure 6) shows convincing detection of +114.043 on a C-terminal K (TNKPQFQEIIASTK[+114]) from ATCG00120 (CF1 α). However, there are conflicting reports about whether trypsin is able to cleave C-terminal of ubiquitinated modified lysine,^{29,30} but a recent paper suggested trypsin can cleave such modified lysines.³¹ A third example however shows a +114.043 on the N-terminus as well as a single C-carbamidomethylation (+57.0215) of peptide [+114]-GHYLNATAGTC[+57]EEMIKR in ATCG00490 (RBCL) (Figure 7). It is unclear if this evidence of N-terminal +114.043 is from di-carbamidomethylation or non-canonical N-terminal ubiquitination. This confirms our concerns about the possibility of extensive off-target alkylation and over-alkylation in this data set.

The authors state that the chloroplast ubiquitinome has 2.7 ubiquitination sites per protein. For thylakoid proteins, 17 are on the stromal side and 11 on thylakoid luminal side. It would be amazing that E3 ligases could reach even into the thylakoid lumen compartment. Moreover, they claim a unique consensus ubiquitination motif for chloroplast proteins (xxxxxxKxxxPxx), leading to the suggestion that there is a specific ubiquitination process inside chloroplasts (Figure 2E in Sun et al.¹). Given the observed high amounts of off-target alkylation and our observation that only 25% of the ~5500 identified proteins in this experiment are located in chloroplasts, it is most likely that this motif is artificial.

Ubiquitin attachment to the substrate always occurs via a ϵ -isopeptide bond between the C-terminal glycine of ubiquitin and the side chain of a lysine in the substrate (Figure 1C). However, the attached ubiquitin monomer itself can also be ubiquitinated leading to polyubiquitination of the substrate (Figure 1C). Polyubiquitination chains are formed by linking several ubiquitin monomers to each other through any of the seven lysines (K6, 11, 27, 29, 33, 48, and 63) present in ubiquitin in addition to the ubiquitin N-terminus; these are referred to as linkage types.⁶⁰ These polyubiquitination chains can be homotypic (i.e., ubiquitin units are connected by the same linkage type) or heterotypic (i.e., ubiquitin units are connected by different linkage types), and chains can be linear

or branched. Chain position, linkage type, and topology determine the fate of a ubiquitylated substrate. The linkage polyubiquitination leading to proteasomal degradation typically occurs on K48-linked chains.^{60,61} The determination of the nature of the substrate ubiquitination is technically challenging.^{36,37,61} Figure 2D of Sun et al.¹ showed a linkage analysis indicating that 32% was from ubiquitin K48 (no further information was provided on this analysis). It was stated that “because K48 was the most abundant linkage type, much of the chloroplast ubiquitinome can be assumed to be primed for proteasomal degradation.” However, there are two issues with this analysis: (i) we identified 5500 proteins in these samples of which only ~25% was chloroplast-localized (Table 1) and (ii) off-target lysine di-carbamidomethylation is confounding this analysis. From our closed search of the raw MS data in this experiment (exp. 3 in Table 1), we collected all peptides matching to the ubiquitin monomer family that contained a K + 114.0429 mass modification (PSM FDR < 0.002, site PTMProphet localization $P > 0.90$ or “no-choice” ($P = 1.0$)). This identified 487 PSMs across the seven lysines, with 62% of the K48 linkage type (Figure S2). Interpretation of such linkage analysis is difficult due to off-target lysine alkylation but nevertheless supports the presence of polyubiquitinated proteins in the affinity pull-down. However, because only 25% of the identified proteins are located in the chloroplast, no conclusions can be drawn about intra-chloroplast ubiquitination.

Follow-up experiments using transient expression of Flag-UBI in Arabidopsis protoplasts (wt, CDC48-WT or CDC48-DN) and immunoblotting claim to show that overexpressed transgenic Pfb3 as well as endogenous chloroplast-encoded PsbC (CP43) are polyubiquitinated.¹ However, it is not clear how Flag-UBI is imported into chloroplasts, and either PrfB3-HA or CP43 appears to shift to higher masses (the expected effect of polyubiquitination). Hence these follow-up experiments do not provide support for intra-chloroplast ubiquitination.

Finally, Sun et al.¹ described two comparative proteomics experiments in which overexpression CDC48-wt and the CDC48-DN lines (estradiol-induced) using either isolated chloroplasts or leaf extracts (Table 1, exp. 4 and 5). The authors again did not search the MS data against the complete predicted Arabidopsis proteome but only a subset of chloroplast-associated sequences. As we pointed out earlier, this will result in false discoveries in particular for proteins that are part of multi-gene families; instead, the MS data should be searched to all possible Arabidopsis proteins after which subsets of identified proteins could be analyzed in more detail. The authors report that these experiments suggest that proteins increased in CDC48-DN represent targets of the CHLORAD pathway. However, it is not clear to us how to untangle pleiotropic effects of suppressing the CDC48 activity and direct effects. Our closed search for experiment 4 identified 1394 proteins (protein FDR < 0.14%), including 52 ATCGs and 1086 chloroplast-predicted n-encoded; hence, these samples were clearly mostly isolated chloroplasts. Our closed search for experiment 5 identified 3016 proteins (protein FDR < 0.4%), including 47 ATCGs and 893 chloroplast predicted n-encoded (31% of total), consistent with unfractionated seedling samples. Search criteria for these closed searches are listed in the footnote of Table 1. As pointed out earlier, we did not detect evidence for alkylation of the samples in experiment 5 (lack of carbamidomethylation

(+57) or di-carbamidomethylation (+114), in conflict with the stated method, alkylation with IAA (50 mM, 40 min at RT).

Additional experiments in Sun et al.¹ to further assess the role of CDC48 do not involve protein MS analysis and are therefore beyond the scope of this study but will be addressed in a future publication.

Conclusions and Recommendations

In this study, we set out to evaluate claims based on MS data that intra-chloroplast proteins are polyubiquitinated within intact chloroplasts (with intact chloroplast double envelope membranes) and subsequently exported into the cytosol, with the cytosolic chaperone CDC48 playing a central role in this chloroplast extraction process.¹ Using the deposited raw MS data (from ProteomeXchange), we carried out both open and closed searches to evaluate the evidence for the +114 modification of lysines (and possibly other residues) that constitutes the only MS-based evidence in ref 1 for possible protein ubiquitination. We did not find sound support for ubiquitination in the 6Myc-UB affinity experiments nor evidence in the K- ϵ -GG affinity experiments on chloroplast proteins isolated from a transgenic 35S-CDC48-WT line. We do find solid support for lysine +114 modifications on chloroplast-encoded and nuclear-encoded chloroplast proteins in the timsTOF experiment isolated from a transgenic 35S-CDC48-DN line. However, there are a range of concerns with how this experiment was carried out, thereby calling into question the cause of the +114 modification. First and foremost, the +114 modification can be a consequence of double alkylation (di-carbamidomethylation) during incubation with alkylating agents (here likely IAA). The relationship between the +114 modification and alkylation treatment is very clearly demonstrated by one of the MS experiments (comparative proteomics of seedlings CDC48-WT and CDC48-DN) where there was no monoalkylation (+57 - carbamidomethylation) of cysteines or other residues (mostly K, N-terminus), nor any evidence for a +114 modification. There is another pressing concern in this ubiquitination experiment 3 (timsTOF), and in that, it was claimed that affinity purification was done on isolated chloroplasts; yet we find more than 5500 Arabidopsis proteins in these samples of which only 25% were chloroplast proteins. We do not understand this discrepancy but clearly calls into question the notion that this experiment provides evidence for intra-chloroplast ubiquitination *in vivo*. IAA is well known for resulting in unintended alkylation of lysines, thus making IAA inappropriate for use in MS-based ubiquitination experiments.^{24,26,28} CAA is considered a good alternative as it results in fewer off-target alkylation (even though it results in unwanted mono-, di-, and tri-oxidation of several amino acids). Indeed the Sun et al.¹ paper described the use of CAA for the first two ubiquitination experiments (1 and 2) in which we did not find much evidence for the K + 114 modification of chloroplast proteins (Oxford submission). However, the method in Sun et al.¹ for the experiment 3 (timsTOF), where we corroborated the presence of K + 114 on chloroplast proteins, did not detail or mention any alkylation treatment, whereas we find overwhelming evidence for cysteine alkylation (the intended target of alkylation treatments). The comparative proteomics experiment carried out by the same collaborator (in Shanghai) states that IAA was used for alkylation, yet there was clearly no alkylation done.

CONCLUSIONS

Based on our own analysis presented here, the most recent reviews on “ubiquitinomics”,^{22,23,25,36,37,61,62} and the primary literature, we recommend the following for the MS-based determination of possible *in vivo* ubiquitination of intra-chloroplast proteins of viable and intact chloroplasts:

- Ensure that the plants are not under significant chloroplast stress (e.g., due to high light treatment) as this will result in (a) rapid release of chloroplast proteins into the cytosol as demonstrated by confocal microscopy of plants expressing chloroplast-targeted green fluorescent protein (GFP) in the *flu* mutant^{63,64} and (b) ATG-dependent and independent autophagy of swollen or otherwise damaged chloroplasts or selected chloroplast content and delivery and degradation in the vacuole.^{7–13,65–67}
- Use low concentration of appropriate (e.g., CAA but not IAA) alkylating agents (e.g., 20 mM) for in-solution digests and ensure removal or quenching of alkylating agent prior to addition of trypsin (or other proteases) to avoid N-terminal peptide and lysine alkylation. An alternative method to block the cysteine sulphydryl group without the introduction of any potentially confounding acetamide at all is by treatment with S-methyl methanethiosulfonate (MMTS) with a +45.9877 modification or acrylamide resulting in a +71.037 modification.²⁸ One could also consider omitting sample alkylation since this will avoid di-carbamidomethylation of lysines (and other amino acid residues) while still obtaining relatively high PSM match rates.²⁸
- Include appropriate negative affinity-enrichment controls to determine the true false discovery rate of the K- ϵ -GG footprint (or K + 114) and that affinity enrichment for K- ϵ -GG containing peptides is highly selective.
- Use parallel alternative methods to enrich for peptides with ubiquitination; there are several independent methods now available.^{22,36,37,62}
- Perform an open search on the acquired MS data to ensure that the mass modifications in the data are consistent with the intended sample handling.
- For the analysis of MS data, include only the most relevant (variable) modifications (ideally based on open searches) and site scoring algorithms to reduce false discovery rate of ubiquitination (in particular +57.022 and +114.0429 Da on lysine, cysteine, and peptide N terminus).
- The sequence database used for MS data analysis should include all proteins of the target organism (e.g., Arabidopsis predicted protein sequences in Araport11 or TAIR10) as well as additional proteins that may be in the sample (including contaminants such as keratin, trypsin, and *E. coli* proteins) not just proteins of interest.

Finally, if indeed *in vivo* intra-chloroplast ubiquitination in intact chloroplasts does occur, the challenge will be to identify how ubiquitin is imported into chloroplasts, how intra-chloroplast ubiquitin is activated (are there E1 and E2 like enzymes within chloroplasts? or is perhaps activated ubiquitin imported as activated monomers?), and how chloroplast proteins are selected for (poly)ubiquitination (are there ubiquitin ligases in chloroplasts?). Given the inconsistencies and pitfalls in Sun et al.,¹ as well as the biological challenges for

intra-chloroplast ubiquitination in intact chloroplasts, we have great reservations that such a speculative pathway contributes to chloroplast (and other plastid types) proteostasis.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jproteome.3c00178>.

Detection and frequency of mass modifications by open searches of raw MS data acquired on a QE instrument from PXD031388 and Ubiquitin linkage analysis for experiment 3 (PDF)

Mass modifications identified in open searches across the five experiments as shown in Figures 2 and S1 (XLSX)

■ AUTHOR INFORMATION

Corresponding Authors

Klaas J. van Wijk – Section of Plant Biology, School of Integrative Plant Sciences (SIPS), Cornell University, Ithaca, New York 14853, United States; orcid.org/0000-0001-9536-0487; Email: kv35@cornell.edu

Eric W. Deutsch – Institute for Systems Biology (ISB), Seattle, Washington 98109, United States; orcid.org/0000-0001-8732-0928; Email: edeutsch@systemsbiology.org

Authors

Tami Leppert – Institute for Systems Biology (ISB), Seattle, Washington 98109, United States

Zhi Sun – Institute for Systems Biology (ISB), Seattle, Washington 98109, United States; orcid.org/0000-0003-3324-6851

Complete contact information is available at:

<https://pubs.acs.org/doi/10.1021/acs.jproteome.3c00178>

Author Contributions

T.L. and Z.S. carried out all MS searches, supervised by E.W.D., and assembled the search results. E.W.D. and K.J.V.W. wrote the paper.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was funded in part by the National Science Foundation MCB 1940961 (K.J.V.W.), National Science Foundation IOS-1922871 (K.J.V.W. and E.W.D.) and DBI-1933311 (E.W.D.), and the National Institutes of Health grant R01 GM087221 (E.W.D.).

■ REFERENCES

- (1) Sun, Y.; Yao, Z.; Ye, Y.; Fang, J.; Chen, H.; Lyu, Y.; Broad, W.; Fournier, M.; Chen, G.; Hu, Y.; Mohammed, S.; Ling, Q.; Jarvis, R. P. Ubiquitin-based pathway acts inside chloroplasts to regulate photosynthesis. *Sci. Adv.* **2022**, *8*, No. eabq7352.
- (2) Li, J.; Yuan, J.; Li, Y.; Sun, H.; Ma, T.; Huai, J.; Yang, W.; Zhang, W.; Lin, R. The CDC48 complex mediates ubiquitin-dependent degradation of intra-chloroplast proteins in plants. *Cell Rep.* **2022**, *39*, No. 110664.
- (3) Kwon, Y. T.; Ciechanover, A. The Ubiquitin Code in the Ubiquitin-Proteasome System and Autophagy. *Trends Biochem. Sci.* **2017**, *42*, 873–886.
- (4) Guerra, D. D.; Callis, J. Ubiquitin on the move: the ubiquitin modification system plays diverse roles in the regulation of endoplasmic reticulum- and plasma membrane-localized proteins. *Plant Physiol.* **2012**, *160*, 56–64.
- (5) Vierstra, R. D. The expanding universe of ubiquitin and ubiquitin-like modifiers. *Plant Physiol.* **2012**, *160*, 2–14.
- (6) Shu, K.; Yang, W. E3 Ubiquitin Ligases: Ubiquitous Actors in Plant Development and Abiotic Stress Responses. *Plant Cell Physiol.* **2017**, *58*, 1461–1476.
- (7) van Wijk, K. J. Protein maturation and proteolysis in plant plastids, mitochondria, and peroxisomes. *Annu. Rev. Plant Biol.* **2015**, *66*, 75–111.
- (8) Fu, Y.; Li, X.; Fan, B.; Zhu, C.; Chen, Z. Chloroplasts Protein Quality Control and Turnover: A Multitude of Mechanisms. *Int. J. Mol. Sci.* **2022**, *23*, 7760.
- (9) Woodson, J. D. Control of chloroplast degradation and cell death in response to stress. *Trends Biochem. Sci.* **2022**, *47*, 851–864.
- (10) Nishimura, K.; Kato, Y.; Sakamoto, W. Essentials of Proteolytic Machinery in Chloroplasts. *Mol. Plant* **2017**, *10*, 4–19.
- (11) Izumi, M.; Nakamura, S.; Li, N. Autophagic Turnover of Chloroplasts: Its Roles and Regulatory Mechanisms in Response to Sugar Starvation. *Front. Plant Sci.* **2019**, *10*, 280.
- (12) Otegui, M. S. Vacuolar degradation of chloroplast components: autophagy and beyond. *J. Exp. Bot.* **2018**, *69*, 741–750.
- (13) Zientara-Rytter, K.; Sirko, A. To deliver or to degrade: an interplay of the ubiquitin-proteasome system, autophagy and vesicular transport in plants. *FEBS J.* **2016**, *283*, 3534–3555.
- (14) Hand, K. A.; Shabek, N. The Role of E3 Ubiquitin Ligases in Chloroplast Function. *Int. J. Mol. Sci.* **2022**, *23*, 9613.
- (15) Ling, Q.; Broad, W.; Trosch, R.; Topel, M.; Demiral Sert, T.; Lymperopoulos, P.; Baldwin, A.; Jarvis, R. P. Ubiquitin-dependent chloroplast-associated protein degradation in plants. *Science* **2019**, *363*, No. eaav4467.
- (16) Ling, Q.; Jarvis, P. Regulation of Chloroplast Protein Import by the Ubiquitin E3 Ligase SP1 Is Important for Stress Tolerance in Plants. *Curr. Biol.* **2015**, *25*, 2527–2534.
- (17) Ling, Q.; Huang, W.; Baldwin, A.; Jarvis, P. Chloroplast biogenesis is regulated by direct action of the ubiquitin-proteasome system. *Science* **2012**, *338*, 655–659.
- (18) Zhang, Y.; Xia, G.; Zhu, Q. Conserved and Unique Roles of Chaperone-Dependent E3 Ubiquitin Ligase CHIP in Plants. *Front. Plant. Sci.* **2021**, *12*, No. 699756.
- (19) Nakamura, S.; Izumi, M. Chlorophagy does not require PLANT U-BOX4-mediated ubiquitination. *Plant Signal. Behav.* **2020**, *16*, No. 1861769.
- (20) Kikuchi, Y.; Nakamura, S.; Woodson, J. D.; Ishida, H.; Ling, Q.; Hidema, J.; Jarvis, R. P.; Hagihara, S.; Izumi, M. Chloroplast Autophagy and Ubiquitination Combine to Manage Oxidative Damage and Starvation Responses. *Plant Physiol.* **2020**, *183*, 1531–1544.
- (21) Jeran, N.; Rotasperti, L.; Frabetti, G.; Calabritto, A.; Pesaresi, P.; Tadini, L. The PUB4 E3 Ubiquitin Ligase Is Responsible for the Variegated Phenotype Observed upon Alteration of Chloroplast Protein Homeostasis in Arabidopsis Cotyledons. *Genes* **2021**, *12*, 1387.
- (22) Trulsson, F.; Vertegaal, A. C. O. Site-specific proteomic strategies to identify ubiquitin and SUMO modifications: Challenges and opportunities. *Semin. Cell Dev. Biol.* **2022**, *132*, 97–108.
- (23) Kellsall, I. R. Non-lysine ubiquitylation: Doing things differently. *Front. Mol. Biosci.* **2022**, *9*, No. 1008175.
- (24) Nielsen, M. L.; Vermeulen, M.; Bonaldi, T.; Cox, J.; Moroder, L.; Mann, M. Iodoacetamide-induced artifact mimics ubiquitination in mass spectrometry. *Nat. Methods* **2008**, *5*, 459–460.
- (25) Svozil, J.; Baerenfaller, K. A Cautionary Tale on the Inclusion of Variable Posttranslational Modifications in Database-Dependent Searches of Mass Spectrometry Data. *Methods Enzymol.* **2017**, *586*, 433–452.

- (26) Hains, P. G.; Robinson, P. J. The Impact of Commonly Used Alkylating Agents on Artifactual Peptide Modification. *J. Proteome Res.* **2017**, *16*, 3443–3447.
- (27) Kuznetsova, K. G.; Solovyeva, E. M.; Kuzikov, A. V.; Gorshkov, M. V.; Moshkovskii, S. A. Modification of cysteine residues for mass spectrometry-based proteomic analysis: facts and artifacts. *Biomed. Khim.* **2020**, *66*, 18–29.
- (28) Muller, T.; Winter, D. Systematic Evaluation of Protein Reduction and Alkylation Reveals Massive Unspecific Side Effects by Iodine-containing Reagents. *Mol. Cell. Proteomics* **2017**, *16*, 1173–1187.
- (29) Shi, Y.; Xu, P.; Qin, J. Ubiquitinated proteome: ready for global? *Mol. Cell. Proteomics* **2011**, *10*, No. R110.006882.
- (30) Burke, M. C.; Wang, Y.; Lee, A. E.; Dixon, E. K.; Castaneda, C. A.; Fushman, D.; Fenselau, C. Unexpected trypsin cleavage at ubiquitinated lysines. *Anal. Chem.* **2015**, *87*, 8144–8148.
- (31) Sun, Z.; Xiao, W.; Li, N.; Chang, L.; Xu, P.; Li, Y. Large-Scale Profiling of Unexpected Tryptic Cleaved Sites at Ubiquitinated Lysines. *J. Proteome Res.* **2023**, *22*, 1245.
- (32) Walton, A.; Stes, E.; Cybulski, N.; Van Bel, M.; Inigo, S.; Durand, A. N.; Timmerman, E.; Heyman, J.; Pauwels, L.; De Veylder, L.; Goossens, A.; De Smet, I.; Coppens, F.; Goormachtig, S.; Gevaert, K. It's Time for Some "Site"-Seeing: Novel Tools to Monitor the Ubiquitin Landscape in Arabidopsis thaliana. *Plant Cell* **2016**, *28*, 6–16.
- (33) Grubb, L. E.; Derbyshire, P.; Dunning, K. E.; Zipfel, C.; Menke, F. L. H.; Monaghan, J. Large-scale identification of ubiquitination sites on membrane-associated proteins in Arabidopsis thaliana seedlings. *Plant Physiol.* **2021**, *185*, 1483–1488.
- (34) Romero-Barrios, N.; Monachello, D.; Dolde, U.; Wong, A.; San Clemente, H.; Cayrel, A.; Johnson, A.; Lurin, C.; Vert, G. Advanced Cataloging of Lysine-63 Polyubiquitin Networks by Genomic, Interactome, and Sensor-Based Proteomic Analyses. *Plant Cell* **2020**, *32*, 123–138.
- (35) Berger, N.; Demolombe, V.; Hem, S.; Rofidal, V.; Steinmann, L.; Krouk, G.; Crabos, A.; Nacry, P.; Verdoucq, L.; Santoni, V. Root Membrane Ubiquitinome under Short-Term Osmotic Stress. *Int. J. Mol. Sci.* **2022**, *23*, 1956.
- (36) Steger, M.; Karayel, O.; Demichev, V. Ubiquitinomics: History, methods, and applications in basic research and drug discovery. *Proteomics* **2022**, *22*, No. e2200074.
- (37) Sun, M.; Zhang, X. Current methodologies in protein ubiquitination characterization: from ubiquitinated protein to ubiquitin chain architecture. *Cell Biosci.* **2022**, *12*, 126.
- (38) Trullsson, F.; Akimov, V.; Robu, M.; van Overbeek, N.; Berrocal, D. A. P.; Shah, R. G.; Cox, J.; Shah, G. M.; Blagoev, B.; Vertegaal, A. C. O. Deubiquitinating enzymes and the proteasome regulate preferential sets of ubiquitin substrates. *Nat. Commun.* **2022**, *13*, 2736.
- (39) Hansen, F. M.; Tanzer, M. C.; Bruning, F.; Bludau, I.; Stafford, C.; Schulman, B. A.; Robles, M. S.; Karayel, O.; Mann, M. Data-independent acquisition method for ubiquitinome analysis reveals regulation of circadian biology. *Nat. Commun.* **2021**, *12*, 254.
- (40) Deutsch, E. W.; Mendoza, L.; Shteynberg, D. D.; Hoopmann, M. R.; Sun, Z.; Eng, J. K.; Moritz, R. L. Trans-Proteomic Pipeline: Robust Mass Spectrometry-Based Proteomics Data Analysis Suite. *J. Proteome Res.* **2023**, *22*, 615.
- (41) Martens, L.; Vizcaino, J. A. A Golden Age for Working with Public Proteomics Data. *Trends Biochem. Sci.* **2017**, *42*, 333–341.
- (42) Knudsen, G. M.; Chalkley, R. J. The effect of using an inappropriate protein database for proteomic data analysis. *PLoS One* **2011**, *6*, No. e20873.
- (43) Desiere, F.; Deutsch, E. W.; King, N. L.; Nesvizhskii, A. I.; Mallick, P.; Eng, J.; Chen, S.; Edde, J.; Loevenich, S. N.; Aebersold, R. The PeptideAtlas project. *Nucleic Acids Res.* **2006**, *34*, D655–D658.
- (44) van Wijk, K. J.; Leppert, T.; Sun, Q.; Boguraev, S. S.; Sun, Z.; Mendoza, L.; Deutsch, E. W. The Arabidopsis PeptideAtlas: Harnessing worldwide proteomics data to create a comprehensive community proteomics resource. *Plant Cell* **2021**, *33*, 3421–3453.
- (45) Perez-Riverol, Y.; Csordas, A.; Bai, J.; Bernal-Llinares, M.; Hewapathirana, S.; Kundu, D. J.; Inguganti, A.; Griss, J.; Mayer, G.; Eisenacher, M.; Perez, E.; Uszkoreit, J.; Pfeuffer, J.; Sachsenberg, T.; Yilmaz, S.; Tiwary, S.; Cox, J.; Audain, E.; Walzer, M.; Jarnuczak, A. F.; Ternent, T.; Brazma, A.; Vizcaino, J. A. The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Res.* **2018**, *47*, D442–D450.
- (46) Ma, J.; Chen, T.; Wu, S.; Yang, C.; Bai, M.; Shu, K.; Li, K.; Zhang, G.; Jin, Z.; He, F.; Hermjakob, H.; Zhu, Y. iProX: an integrated proteome resource. *Nucleic Acids Res.* **2019**, *47*, D1211–D1217.
- (47) Martens, L.; Chambers, M.; Sturm, M.; Kessner, D.; Levander, F.; Shofstahl, J.; Tang, W. H.; Rompp, A.; Neumann, S.; Pizarro, A. D.; Montecchi-Palazzi, L.; Tasman, N.; Coleman, M.; Reisinger, F.; Souda, P.; Hermjakob, H.; Binz, P. A.; Deutsch, E. W. mzML—a community standard for mass spectrometry data. *Mol. Cell. Proteomics* **2011**, *10*, No. R110.000133.
- (48) Hulstaert, N.; Shofstahl, J.; Sachsenberg, T.; Walzer, M.; Barsnes, H.; Martens, L.; Perez-Riverol, Y. ThermoRawFileParser: Modular, Scalable, and Cross-Platform RAW File Conversion. *J. Proteome Res.* **2020**, *19*, 537–542.
- (49) Kong, A. T.; Leprevost, F. V.; Avtonomov, D. M.; Mellacheruvu, D.; Nesvizhskii, A. I. MSFragger: ultrafast and comprehensive peptide identification in mass spectrometry-based proteomics. *Nat. Methods* **2017**, *14*, 513–520.
- (50) Shteynberg, D. D.; Deutsch, E. W.; Campbell, D. S.; Hoopmann, M. R.; Kusebauch, U.; Lee, D.; Mendoza, L.; Midha, M. K.; Sun, Z.; Whetton, A. D.; Moritz, R. L. PTMProphet: Fast and Accurate Mass Modification Localization for the Trans-Proteomic Pipeline. *J. Proteome Res.* **2019**, *18*, 4262–4272.
- (51) Geisler, D. J.; Kong, A. T.; Avtonomov, D. M.; Yu, F.; Leprevost, F. D. V.; Nesvizhskii, A. I. PTM-Shepherd: Analysis and Summarization of Post-Translational and Chemical Modifications From Open Search Results. *Mol. Cell. Proteomics* **2021**, *20*, 100018.
- (52) Zhou, J.; Liu, D.; Wang, P.; Ma, X.; Lin, W.; Chen, S.; Mishev, K.; Lu, D.; Kumar, R.; Vanhoutte, I.; Meng, X.; He, P.; Russinova, E.; Shan, L. Regulation of Arabidopsis brassinosteroid receptor BRI1 endocytosis and degradation by plant U-box PUB12/PUB13-mediated ubiquitination. *Proc. Natl. Acad. Sci. U. S. A.* **2018**, *115*, E1906–E1915.
- (53) Zhang, Y.; Chen, M.; Zhou, S.; Lou, Y.; Lu, J. Silencing an E3 Ubiquitin Ligase Gene OsJMJ715 Enhances the Resistance of Rice to a Piercing-Sucking Herbivore by Activating ABA and JA Signaling Pathways. *Int. J. Mol. Sci.* **2021**, *22*, 13020.
- (54) Deutsch, E. W.; Perez-Riverol, Y.; Carver, J.; Kawano, S.; Mendoza, L.; Van Den Bossche, T.; Gabriels, R.; Binz, P. A.; Pullman, B.; Sun, Z.; Shofstahl, J.; Bittremieux, W.; Mak, T. D.; Klein, J.; Zhu, Y.; Lam, H.; Vizcaino, J. A.; Bandeira, N. Universal Spectrum Identifier for mass spectra. *Nat. Methods* **2021**, *18*, 768–770.
- (55) Sun, Q.; Zybailov, B.; Majeran, W.; Friso, G.; Olinares, P. D.; van Wijk, K. J. PPDB, the Plant Proteomics Database at Cornell. *Nucleic Acids Res.* **2009**, *37*, D969–D974.
- (56) Emanuelsson, O.; Brunak, S.; von Heijne, G.; Nielsen, H. Locating proteins in the cell using TargetP, SignalP and related tools. *Nat. Protoc.* **2007**, *2*, 953–971.
- (57) Breuza, L.; Poux, S.; Estreicher, A.; Famiglietti, M. L.; Magrane, M.; Tognolli, M.; Bridge, A.; Baratin, D.; Redaschi, N.; UniProt, C. The UniProtKB guide to the human proteome. *Database* **2016**, *2016*, No. bav120.
- (58) Cheng, C. Y.; Krishnakumar, V.; Chan, A. P.; Thibaud-Nissen, F.; Schobel, S.; Town, C. D. AraPort11: a complete reannotation of the Arabidopsis thaliana reference genome. *Plant J.* **2017**, *89*, 789–804.
- (59) Sticker, A.; Martens, L.; Clement, L. Mass spectrometrists should search for all peptides, but assess only the ones they care about. *Nat. Methods* **2017**, *14*, 643–644.
- (60) Sadanandom, A.; Bailey, M.; Ewan, R.; Lee, J.; Nelis, S. The ubiquitin-proteasome system: central modifier of plant signalling. *New Phytol.* **2012**, *196*, 13–28.

- (61) Deol, K. K.; Strieter, E. R. The ubiquitin proteoform problem. *Curr. Opin. Chem. Biol.* **2021**, *63*, 95–104.
- (62) Vere, G.; Kealy, R.; Kessler, B. M.; Pinto-Fernandez, A. Ubiquitomics: An Overview and Future. *Biomolecules* **2020**, *10*, 1453.
- (63) Kim, C.; Meskauskiene, R.; Zhang, S.; Lee, K. P.; Lakshmanan Ashok, M.; Blajecka, K.; Herrfurth, C.; Feussner, I.; Apel, K. Chloroplasts of Arabidopsis are the source and a primary target of a plant-specific programmed cell death signaling pathway. *Plant Cell* **2012**, *24*, 3026–3039.
- (64) Danon, A.; Miersch, O.; Felix, G.; Camp, R. G.; Apel, K. Concurrent activation of cell death-regulating signaling pathways by singlet oxygen in Arabidopsis thaliana. *Plant J.* **2005**, *41*, 68–80.
- (65) Zhuang, X.; Jiang, L. Chloroplast Degradation: Multiple Routes Into the Vacuole. *Front. Plant Sci.* **2019**, *10*, 359.
- (66) Nakamura, S.; Hidema, J.; Sakamoto, W.; Ishida, H.; Izumi, M. Selective Elimination of Membrane-Damaged Chloroplasts via Microautophagy. *Plant Physiol.* **2018**, *177*, 1007–1026.
- (67) Dominguez, F.; Cejudo, F. J. Chloroplast dismantling in leaf senescence. *J. Exp. Bot.* **2021**, *72*, S905–S918.

Recommended by ACS

Machine Learning on Large-Scale Proteomics Data Identifies Tissue and Cell-Type Specific Proteins

Tine Claeys, Lennart Martens, *et al.*

MARCH 24, 2023
JOURNAL OF PROTEOME RESEARCH

READ 

Assessing Multiple Evidence Streams to Decide on Confidence for Identification of Post-Translational Modifications, within and Across Data Sets

Oscar M. Camacho, Andrew R. Jones, *et al.*

APRIL 26, 2023
JOURNAL OF PROTEOME RESEARCH

READ 

Interactomes of Glycogen Synthase Kinase-3 Isoforms

Kevin W. Cormier, James R. Woodgett, *et al.*

FEBRUARY 13, 2023
JOURNAL OF PROTEOME RESEARCH

READ 

Comprehensive Interactome Mapping of the DNA Repair Scaffold SLX4 Using Proximity Labeling and Affinity Purification

Camila M. Aprosoff, Haley D.M. Wyatt, *et al.*

APRIL 18, 2023
JOURNAL OF PROTEOME RESEARCH

READ 

Get More Suggestions >