



# Near atmospheric carbon dioxide activates plant ubiquitin cross-linking

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## ABSTRACT

**Background** Identifying CO<sub>2</sub>-binding proteins is vital for our knowledge of CO<sub>2</sub>-regulated molecular processes. The carbamate post-translational modification is a reversible CO<sub>2</sub>-mediated adduct that can form on neutral *N*-terminal  $\alpha$ -amino or lysine  $\epsilon$ -amino groups.

**Methods** We have developed triethyloxonium ion (TEO) as a chemical proteomics tool to trap the carbamate post-translational modification on protein covalently. We use <sup>13</sup>C-NMR and TEO to identify ubiquitin as a plant CO<sub>2</sub>-binding protein.

**Results** We observe the carbamate post-translational modification on the *Arabidopsis thaliana* ubiquitin  $\epsilon$ -amino groups of lysines 6, 33, and 48. We show that biologically relevant near atmospheric PCO<sub>2</sub> levels increase ubiquitin conjugation dependent on lysine 6. We further demonstrate that CO<sub>2</sub> increases the ubiquitin E2 ligase (AtUBC5) charging step via the transthiesterification reaction in which Ub is transferred from the E1 ligase active site to the E2 active site.

**Conclusions and general significance** Therefore, plant ubiquitin is a CO<sub>2</sub>-binding protein, and the carbamate post-translational modification represents a potential mechanism through which plant cells can respond to fluctuating PCO<sub>2</sub>.

## 1. Introduction

CO<sub>2</sub> was first discovered in 1757 as a component of the gas exhaled from the lung [1]. It is now broadly recognised as a vital component of various physiological processes. It has roles in, for example, metabolism, photosynthesis, chemosensing, and cellular homeostasis [2]. The fundamental importance of the gas to biology has meant that organisms across kingdoms can sense and adapt to fluctuating CO<sub>2</sub> [3]. Unfortunately, our knowledge of direct CO<sub>2</sub> targets is scant compared to our extensive knowledge of its impact on physiology. Knowledge of direct CO<sub>2</sub> targets in photosynthetic organisms is essential for exploiting them for green biotechnology and addressing crop responses to climate change.

We have a relatively large knowledge base for CO<sub>2</sub> roles in cell, tissue and organismal physiology, e.g., metabolism, acid-base homeostasis, and transport [2,4]. We have a smaller knowledge base of CO<sub>2</sub>-responsive signalling pathways. These include, for example, AMPK and Na<sup>+</sup>/K<sup>+</sup>-ATPase in mammals [5], NF- $\kappa$ B in mammals and *Drosophila* [6,7], the calpain/caspase-7/RhoA pathway in mammals [8], Ca<sup>2+</sup> signalling in mammals [9,10], Gr21a/Gr63a in insects [11,12], GC-D+

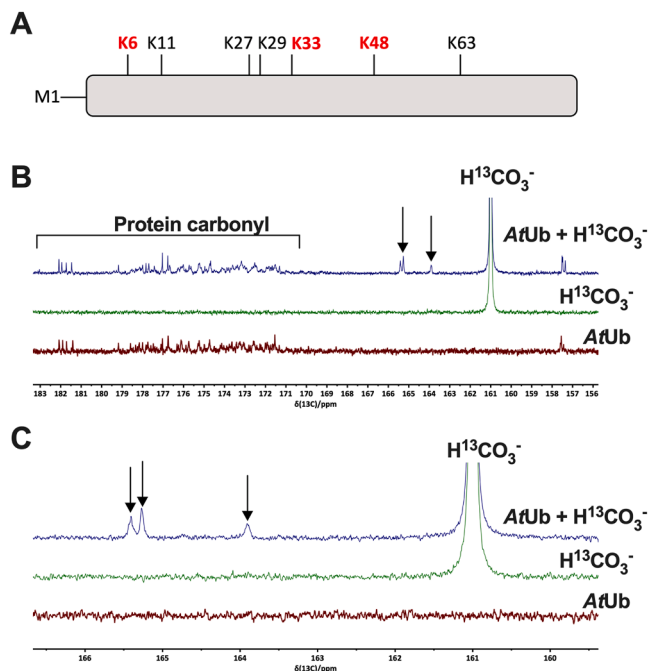
neurons in rodents [13] and SLAC1/protein kinase/ABA-dependent pathways in *Arabidopsis* [14,15]. We have very little knowledge of proteins that unambiguously respond directly to CO<sub>2</sub>. The signalling molecules unambiguously identified to signal directly in response to fluctuating inorganic carbon are the Class III nucleotidyl cyclases of animals, fungi, and prokaryotes [3], a subset of connexins (where Cx26 is the well-studied archetype) in mammals [16], receptor protein tyrosine phosphatase  $\gamma$  of mammals [17], ubiquitin in mammals [18], PII of *Synechocystis* sp. PCC 6803 [19], PP2C phosphatases of plants and fungi [20], and the MPK4/MPK12/HT1 complex of plants [21].

How might CO<sub>2</sub> regulate and interact with proteins to mediate its physiological effects? CO<sub>2</sub> can interact with protein to form the carbamate post-translational modification (PTM) on neutral *N*-terminal  $\alpha$ -amino- or lysine  $\epsilon$ -amino groups. Carbamylation (Fig. 1A) was initially discovered as a PTM regulating Rubisco [22] and haemoglobin [22] activities. It has subsequently been observed on mammalian ubiquitin [18], cyanobacterial allophycocyanin A [23] and PII protein [19]. Furthermore, a subset of proteins has a locally stabilised carbamate PTM typically utilised for catalysis. Such proteins include urease, alanine racemase, transcarboxylase 5S, class D  $\beta$ -lactamase and

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**Fig. 1.** CO<sub>2</sub> forms carbamates on AtUb. **A.** Cartoon of Ub, demonstrating the seven conserved lysine ubiquitination sites and the N-terminal M1 site. Those identified as carbamylated through MS/MS are shown in bold red type. **B.** 1D <sup>13</sup>C-NMR spectra of 5 mM AtUb wild type alone, 50 mM NaH<sup>13</sup>CO<sub>3</sub> alone, and 5 mM AtUb wild type with 50 mM NaH<sup>13</sup>CO<sub>3</sub> are shown. The background H<sup>13</sup>CO<sub>3</sub><sup>-</sup> is observed along with carbamates (arrows) and protein carbonyl resonance. **C.** Close-up of the region encompassing the carbamates.

phosphotriesterase [24]. On this basis, we have hypothesised that reversible carbamylation of neutral N-terminal α-amino groups and/or lysine ε-amino groups represents a broadly applicable method by which organisms can sense and adapt to fluctuating CO<sub>2</sub>. We have previously deployed triethyloxonium ion in conjunction with tandem mass spectrometry (TEO-MS/MS) to chemically modify carbamate PTMs and enable their identification [24]. TEO is a water-soluble reagent that traps carbamates by selective alkylation. The t<sub>1/2</sub> of TEO is approximately 6 min measured at pH 7.4 in an aqueous solution. Therefore, TEO is amenable as a trapping agent to identify protein carbamates as its properties enable its broader use in the laboratory [25]. We have used TEO to identify new CO<sub>2</sub>-binding proteins. Others have exploited the protection offered to lysine by CO<sub>2</sub> to electrophile modification to enable carbamate identification by a quantitative MS/MS strategy [19].

Studies in prokaryotes [19,23,26] and plants [27] demonstrate multiple proteins that bind CO<sub>2</sub> through carbamate formation. Therefore, while the identification of MPK4/12 and HT1 as a CO<sub>2</sub> sensing module that converges on CBC1 kinase in CO<sub>2</sub>-regulated stomatal movements [21] represents a significant advance, it is likely that overall plant physiology responds to CO<sub>2</sub> by a variety of mechanisms. Therefore, an extended search for plant CO<sub>2</sub>-binding proteins remains a strategically important goal.

Ubiquitin (Ub) is a highly conserved 8.5 kDa protein found in all eukaryotic cells that is conjugated to target proteins resulting in altered activity or targeted degradation in the proteasome. Several enzymes catalyse Ub conjugation. An enzyme cascade mediates Ub conjugation. First, the Ub-activating enzyme (Uba or E1) transfers Ub to the Ub-conjugating enzyme (Ubc or E2) active site, forming a thioester intermediate Ub-E2 [28]. Subsequently, the Ub is transferred from E2, in conjunction with Ub ligase (E3), to specific Lys residues on the surface of a target protein. At least one E1 enzyme, 37 E2 enzymes, and 1,400–1,500 E3 enzymes are predicted to be encoded by the *Arabidopsis* genome [29].

The Ub monomer bound to a protein surface Lys can be potentially conjugated into poly-Ub chains. Such poly-Ub chains have the potential to form on each of the seven Ub lysine side chains and the N-terminal α-amino-group, thus permitting many well-defined chain linkages to form [30]. These varying linkages underpin various physiological processes. Proteins bearing K48-conjugated Ub chains are transported to the 26S proteasome for degradation, which regulates numerous plant signalling pathways, including self-incompatibility [31], auxin [32] and jasmonate [33] signalling, and plant immunity [34]. The K63-conjugated K63 functions in apical dominance [35], the DNA damage response [25], and plant immunity [36]. There is little information on poly-Ub chains conjugated on other (M1, K6, K11, K27, K29, K33) sites in plants. The K29-linkage mediates the degradation of DELLA protein degradation [37], and the K11-linkage functions in plant growth and immunity [38].

We have previously identified mammalian Ub as a CO<sub>2</sub>-binding protein and demonstrated that it could explain how CO<sub>2</sub> regulates the NF-κB pathway [18]. Given the conservation in Ub biochemistry between mammals and plants, we investigated whether CO<sub>2</sub> regulated plant Ub conjugation. In contrast, to mammalian Ub, whose conjugation is inhibited by [CO<sub>2</sub>] in the mM range, we find that near atmospheric CO<sub>2</sub> partial pressures enhance plant Ub conjugation.

## 2. Materials and Methods

### 2.1. Ubiquitin expression and purification

The *Arabidopsis thaliana* WT Ub monomer open reading frame was cloned into the NdeI and BamHI sites of pET-23a(+) with a stop codon introduced to preclude the expression of the His<sub>6</sub> affinity tag. Single lysine to arginine mutant constructs were produced via a commercial gene synthesis/mutagenesis service (Genscript). All AtUb variants were expressed as untagged proteins from pET23a in *E. coli* BL21-AI<sup>TM</sup> (Invitrogen<sup>TM</sup>) at 37°C for 4 h with 400 μM IPTG and 0.1% (w/v) L-arabinose. Pelleted bacteria were resuspended in lysis buffer (50mM Tris-HCl pH 7.6, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.02% (v/v) NP-40 and 0.4 mg mL<sup>-1</sup> lysozyme) before being lysed by sonication (180 s on ice) and centrifuged (40,000 x g, 30 min, 4°C). The clarified lysate was transferred to a chilled beaker on ice and stirred vigorously as perchloric acid (PCA) (70% (w/v), 0.35 mL) was added dropwise. The resulting milky solution was left stirring on ice for 10 mins before being centrifuged (20,000 x g, 20 mins, 4°C). The supernatant was dialysed at 4°C for 16 h against 50 mM ammonium acetate pH 4.5 (2 L) with two buffer changes. Dialysed protein was further purified using strong cation exchange chromatography. Using an AKTA Start chromatography system (GE Healthcare), a HiTrap SP HP column (5 mL, Cytiva) was equilibrated with 50 mM ammonium acetate, pH 4.5. The partially purified protein was loaded onto the column and washed with 5 CV equilibration buffer. Protein was then eluted with a NaCl gradient elution (0–500 mM NaCl in 50 mM ammonium acetate buffer, pH 4.5). Fractions were analysed by SDS-PAGE, and the AtUb-containing fractions were dialysed into storage buffer (20 mM Tris-HCl pH 7.6, 10% glycerol) before being concentrated and snap frozen in liquid N<sub>2</sub>. AtUb aliquots were stored at -80°C.

### 2.2. AtUBA1 expression and purification

The *Arabidopsis thaliana* UBA1 open reading frame was cloned into the NdeI and XhoI sites of pET23a(+) in frame with the C-terminal His<sub>6</sub> tag by commercial gene synthesis (Genscript). AtUBA1 was expressed as a His<sub>6</sub>-tagged protein from pET23a in *E. coli* BL21-AI<sup>TM</sup> (Invitrogen<sup>TM</sup>) at 18°C for 20 h with 800 μM IPTG and 0.2% (w/v) L-arabinose. AtUBA1 was purified by Immobilised Metal Affinity Chromatography (IMAC). The protein was purified using a HiTrap HP column (1 mL, Cytiva) and an equilibration buffer of 50 mM Tris-HCl pH 7.5, 400 mM NaCl, and 20 mM imidazole. The wash and elution buffers consisted of 50 mM Tris-

HCl, 400 mM NaCl and 40 mM/200 mM imidazole. AtUBA1-containing fractions were dialysed into anion exchange start buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM DTT, 5% (v/v) glycerol) and purified using a HiTrap Q HP column (1 mL, Cytiva). The protein was eluted using a NaCl gradient (50–500 mM), and the resulting fractions were analysed by SDS-PAGE. AtUBA1 containing fractions were dialysed into size exclusion chromatography buffer (50 mM Tris-HCl, 200 mM NaCl, 1 mM DTT, 15% (v/v) glycerol), concentrated and then further purified by size exclusion chromatography on a 16/600 Superdex 200 pg column (GE Lifesciences) and an AKTA Pure chromatography system.

### 2.3. AtUBC5 expression and purification

AtUBC5 was expressed as a His<sub>6</sub>-tagged protein in *E. coli* Rosetta<sup>TM</sup> 2 (DE3) (Novagen) at 20°C for 20 h with 200 µM IPTG. AtUBC5 was purified by IMAC. The protein was purified using a HisTrap HP column (1 mL, Cytiva) and an equilibration buffer of 50 mM Tris-HCl pH 7.5, 400 mM NaCl, and 20 mM imidazole. The wash and elution buffers consisted of 50 mM Tris-HCl, 400 mM NaCl and 40 mM/200 mM imidazole. AtUBC5-containing fractions were dialysed into anion exchange start buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM DTT, 5% (v/v) glycerol) and purified using a HiTrap Q HP column (1 mL, Cytiva). The protein was eluted using a NaCl gradient elution (50–500 mM), and the resulting fractions were analysed by SDS-PAGE. AtUBC5-containing fractions were dialysed into storage buffer (50 mM Tris-HCl, 200 mM NaCl, 1 mM DTT, 15% (v/v) glycerol), concentrated and stored at -80°C.

### 2.4. Protein CO<sub>2</sub> trapping

The recombinant protein (50–500 µg) was diluted into 2.5 mL trapping buffer (100 mM NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub>, 100 mM NaCl, pH 7.4) for CO<sub>2</sub> trapping experiments. NaHCO<sub>3</sub> dissolved in trapping buffer (0.5 mL) was added to a final concentration specified for each reaction. The required NaHCO<sub>3</sub> concentration was determined based on the desired CO<sub>2</sub> concentration and the solution pH using the Henderson-Hasselbalch equation. This solution was added to a potentiometric titrator (902 Titrando; Metrohm) and incubated at 25°C with stirring for 5 min. A freshly made solution of triethyloxonium (TEO) tetrafluoroborate (240 mg, 1.47 mmol) was prepared in trapping buffer (1 mL) and added dropwise to the solution with constant stirring. The pH was maintained at the desired set point via the automated addition of NaOH (1 M), and the reaction was left stirring for 1 h to ensure complete hydrolysis of the TEO. The trapped solution was then dialysed into dH<sub>2</sub>O (4°C, 16 h) with two buffer changes to ensure the removal of all hydrolysed TEO. The dialysed sample was then dried at room temperature using a centrifugal vacuum concentrator.

### 2.5. Mass spectrometry

S-Trap<sup>TM</sup> (Protifi) Mini digestion was performed according to the manufacturer's instructions with slight modifications. All steps used LC-MS grade reagents. Dried protein (~100–300 µg) was resuspended in 1x SDS Lysis buffer (5% (v/v) SDS, 50 mM triethylammonium bicarbonate (TEAB) pH 8.5, 50 µL). Liquid samples were diluted in an equal volume 2x SDS Lysis Buffer (10% (w/v) SDS, 100 mM TEAB pH 8.5). DTT (20 mM) was added, and the sample was boiled (10 min, 95°C) to reduce disulfide bonds. Iodoacetamide (40 mM) was added, and the sample was incubated in the dark (30 min) to alkylate the sample fully. Phosphoric acid (~1.2%) was added to the supernatant before the addition of S-Trap Binding Buffer (90% (v/v) methanol, 100 mM TEAB pH 7.55, 6x total sample volume). The resulting colloidal solution was loaded onto the S-Trap Mini spin column and centrifuged (4000 x g, 30 s) to bind the protein to the S-trap. The column was then washed with S-Trap binding buffer (400 µL) and centrifuged (4000 x g, 30 s). This washing was carried out five times before the S-Trap Mini spin column was transferred to a clean collection tube. A freshly made digestion solution

prepared of Trypsin Gold (Promega, Mass Spectrometry Grade) (1:20 (w/w) Trypsin Gold: Sample) in digestion buffer (50 mM TEAB, pH 8.5, 125 µL total volume), was added to the column. The column was briefly centrifuged (4000 x g, 2 s), any flow-through reloaded onto the column and then incubated (37°C, 16 h). Peptides were eluted from the column by adding three elution buffers, with each addition followed by a centrifugation step (1000 x g, 60 s). Elution Buffer 1 (50 mM TEAB, pH 8.5, 80 µL) was used to elute most of the aqueous peptides, followed by Elution Buffer 2 (0.2% (v/v) Formic acid, 80 µL). Finally, Elution Buffer 3 (50% (v/v) acetonitrile (ACN), 0.2% (v/v) formic acid, 80 µL) was used to elute hydrophobic peptides. All eluted peptides were combined and dried at room temperature using a vacuum centrifuge.

The digested peptides were desalted on a C18 column and analysed by ESI-MS/MS on an LTQ Orbitrap XL mass spectrometer (Thermo) coupled to an Ultimate 3000 nano-HPLC instrument. Peptides eluted from the LC gradient were injected online to the mass spectrometer (lock mass enabled, mass range 400–1800 Da, resolution 60,000 at 400 Da, 10 MS/MS spectra per cycle, collision-induced dissociation (CID) at 35% normalised CE, rejection of singly charged ions).

The LC-MS/MS raw data files (.wiff) were converted into .mgf or .mzXML files using the freeware MSConvert (ProteoWizard) and analysed using PEAKS Studio 10.5 software. An error tolerance of 15.0 ppm for the precursor mass using the monoisotopic mass and 0.2 Da for the fragment ion was used. Tryptic digests were selected using a semi-specific digest mode and a maximum of three missed cleavages per peptide. Protein modifications used were fixed (57.0215 Da@C) or variable (15.9949 Da@M, 42.0106 Da@N-term/K, 72.0211 Da@N-term/K, 73.0211 Da@N-term/K, 28.0313 Da@N-term/D/E/K, 114.0429 Da@T/S/C/K, and 383.23 Da@K).

### 2.6. <sup>13</sup>C-Nuclear magnetic resonance

Protein was exchanged into NMR sample buffer (100 mM NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub>, pH 7.6, 100 mM NaCl) using a centrifugal concentrator (Vivaspin Sartorius). The total sample volume was 0.7 mL with protein. Samples contained 10% (v/v) D<sub>2</sub>O, and inorganic carbon was added as NaHCO<sub>3</sub>. <sup>13</sup>C-NMR spectra were acquired with a Varian 600 MHz spectrometer equipped with an Agilent OneNMR Probe to deliver a maximum pulsed-field gradient strength of 62 G cm<sup>-1</sup>. A <sup>1</sup>H spectrum was acquired to examine for small molecule impurities. Thirteen <sup>1</sup>H experiments were recorded in 12 h, collecting 131072 complex points. The repetition time was 6.7 s, of which 1.7 s comprised the acquisition time. The excitation pulse angle was set to 45 degrees. The strong interfering H<sub>2</sub>O signal was eliminated using the Robust-5 pulse sequence. Thirty-two <sup>13</sup>C scans were collected, comprising 65 536 complex data points and a spectral width of 10 kHz. The repetition time was 6.3 s, of which 3.3 s comprised the acquisition time. The W5 inter-pulse delay was set to 240 µs. Rectangular 1 ms pulsed-field gradients were used in all cases with a strength of G1 = 28.3 G cm<sup>-1</sup> (first pair) and G2 = 4.9 G cm<sup>-1</sup> (second pair). The gradient stabilisation delay was 0.5 ms. The first pair of lock pre-focusing field gradients were separated from the first radio-frequency pulse by a 1.5 ms delay.

### 2.7. In vitro ubiquitin conjugation assays

Assay components were degassed via sparging with N<sub>2</sub>, and assays were carried out in an inert atmosphere of N<sub>2</sub> within an anaerobic chamber (BelleTechnology). Ub conjugation assays were performed in 50 µL reactions in a reaction buffer consisting of 200 mM HEPES pH 7.9, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 2 mM adenosine 5'-triphosphate (ATP), 1 mM DTT, 25 µM AtUb, 24 µM AtUBC5 and 0.1 µM AtUBA1. C<sub>i</sub> was added as HCO<sub>3</sub><sup>-</sup>/CO<sub>2</sub>, with [anion] maintained with supplemental Cl<sup>-</sup>. Assays were initiated by adding either AtUBA1 or ATP. Following initiation, assays were incubated (30 min, 25°C) before termination with 2x Laemlli sample buffer. Time course assays were performed as above but in 100 µL reactions. 10 µL aliquots were removed at each time point and

terminated as above. Assays were resolved via SDS-PAGE on 15% (w/v) polyacrylamide gels and, following imaging, were analysed using ImageJ software. For normalisation, independent paired experiments (0 versus 100  $\mu\text{M}$   $\text{CO}_2$ ) were processed by SDS-PAGE. For each SDS-PAGE gel, the AtUBC5-AtUb conjugate and (AtUBC5-AtUb + free AtUb) were quantified. The ratio obtained for 100  $\mu\text{M}$   $\text{CO}_2$  was normalised to 0  $\mu\text{M}$   $\text{CO}_2 = 1$ . The datasets presented represent the independent paired experiments. Di-Ub linkage analysis was performed by LC-MS/MS following a gel band digest of the di-Ub band. Data were analysed using PEAKS searching for the DiGly variable modification (+114.04 Da) on lysine residues.

## 2.8. Statistical analysis

All data are presented as the distribution of independent data points representing independent experiments. All statistics and graphical analyses were performed using GraphPad Prism 8 (GraphPad Software, inc.).

## 3. Results

### 3.1. $\text{CO}_2$ forms carbamates on plant ubiquitin

Carbamate formation on mammalian Ub at physiologically relevant  $\text{CO}_2$  partial pressure ( $\text{PCO}_2$ ) down-regulates Ub cross-linking [18]. A key feature of Ub is its seven conserved lysine residues (K6, K11, K27, K29, K33, K48, K63) and an N-terminal  $\alpha$ -amino group, which can serve as ubiquitination sites in the formation of poly-Ub chains. These sites are conserved between mammals and plants (Fig. 1A). Therefore, we investigated whether this phenomenon was conserved in a plant Ub of *Arabidopsis thaliana* typically exposed to lower  $\text{PCO}_2$ . The open reading frame of a single Ub monomer from *Arabidopsis* was cloned into the expression vector pET23a and purified as a recombinant protein (AtUb) (Supplementary Fig. 1).

$^{13}\text{C}$  NMR is a spectroscopic technique used to analyse the environment of carbon atoms within a molecule. 1D  $^{13}\text{C}$  NMR has been used to report the formation of the carbamate groups on proteins [39,40]. Protein carbonyl resonances are found in the 170–185 ppm region, whereas carbamate resonances are located upfield of these values (163–166 ppm) as the carbon atom experiences relatively high shielding due to the delocalised nature of the negative charge between the heteroatoms of the carbamate group.

$^{13}\text{C}$  NMR spectra supported the formation of carbamates on AtUb (Fig. 1B–C). Within the range of chemical shifts observed, the total inorganic carbon ( $\text{CO}_2 + \text{HCO}_3 = \text{Ci}$ ) control (green) spectrum consists of a strong  $\text{HCO}_3^-$  resonance at 161 ppm, while the Ub control spectrum (red) shows the protein carbonyl resonances between 171–182 ppm that are observed in the absence of additional  $\text{C}_i$ . No resonances are observed between 163–166 ppm, the region associated with carbamate groups, in these spectra. The AtUb +  $\text{C}_i$  spectrum (blue) contains three additional peaks within this range, at 163.9, 165.2 and 165.4 ppm chemical shifts, respectively. The absence of these peaks in the control spectra is evidence that they are derived from interactions between AtUb and  $\text{CO}_2$ . Furthermore, the spectra were normalised based on the intensity of the protein carbonyl peaks (171–182 ppm). Therefore, the carbamate resonances have not arisen from a difference in the concentration of the Ub samples. The presence of three distinct resonances suggests the formation of more than one distinct carbamate group. There are eight predicted potential sites of carbamate formation on Ub: the  $\epsilon$ -amino groups of the seven lysine residues and the  $\alpha$ -amino group of the N-terminus. However, this spectrum alone cannot assign the carbamate resonances to any of these groups.

### 3.2. Plant ubiquitin binds near atmospheric $\text{CO}_2$

Following the observation of carbamate groups in their native form

on AtUb by  $^{13}\text{C}$  NMR, an orthologous approach was used to identify the specific sites of carbamate formation. Carbamate trapping uses TEO, a Meerwein reagent, to ethylate carbamate groups so they are stable for electrospray ionisation and LC-MS/MS analysis [24]. The carboxyethyl group is identified as a 72.0211 Da mass shift associated with a given lysine residue when analysed by MS/MS.

A TEO-MS/MS trapping experiment was carried out on recombinant AtUb at elevated  $\text{C}_i$  (1.47 mM  $\text{CO}_2$ ). These conditions were have previously been used to screen the *Arabidopsis* proteome for  $\text{CO}_2$ -binding sites. Trypsin was used to digest the trapping reaction mixture, and LC-MS/MS was used to analyse samples, followed by data analysis using Peaks (Bioinformatics Solutions Inc). The data were interrogated for modifications on the N-terminus and lysine with masses of 72.0211 Da (trapped carbamate) and 28.0313 Da (O-ethylation on glutamate and aspartate side chains). The carbamate modification was observed on two AtUb lysine residues: K33 and K48 (MS-MS peptide amino acids 30–42 IQDKEGIPPDQQR, proposed carbamylation on K33; MS-MS peptide amino acids 43–54 LIFAGKQLEDGR, proposed carbamylation on K48) (Fig. 2A–B). Within the data sets presented here, the carbamates were observed on both peptides on internal lysine residues, exhibiting a so-called missed cleavage. The missed cleavage occurs because carboxyethylation removes the cationic charge on the lysine essential for cleavage site recognition by trypsin. This observation supports the identification of carbamates on both AtUb K33 and K48 as a missed cleavage is an otherwise rare event. Peptides covering four of the remaining lysines (all but K6) were identified, suggesting carbamates do not form on these lysines at 1.47 mM  $\text{C}_i$ . However, we cannot eliminate the possibility that carboxyethylated peptides at these sites are refractory to MS/MS.

As an additional control, the experiment was repeated with the same concentration of  $^{13}\text{C}_i$ , in which the carboxyethylation modification shows a 73.0211 Da m/z shift due to the additional +1 Da m/z of the  $^{13}\text{C}$  isotope. Peptides containing a  $^{13}\text{C}$ -carboxyethylated lysine were observed for both the K33 and K48 residues, validating both sites (Fig. 2C–D).

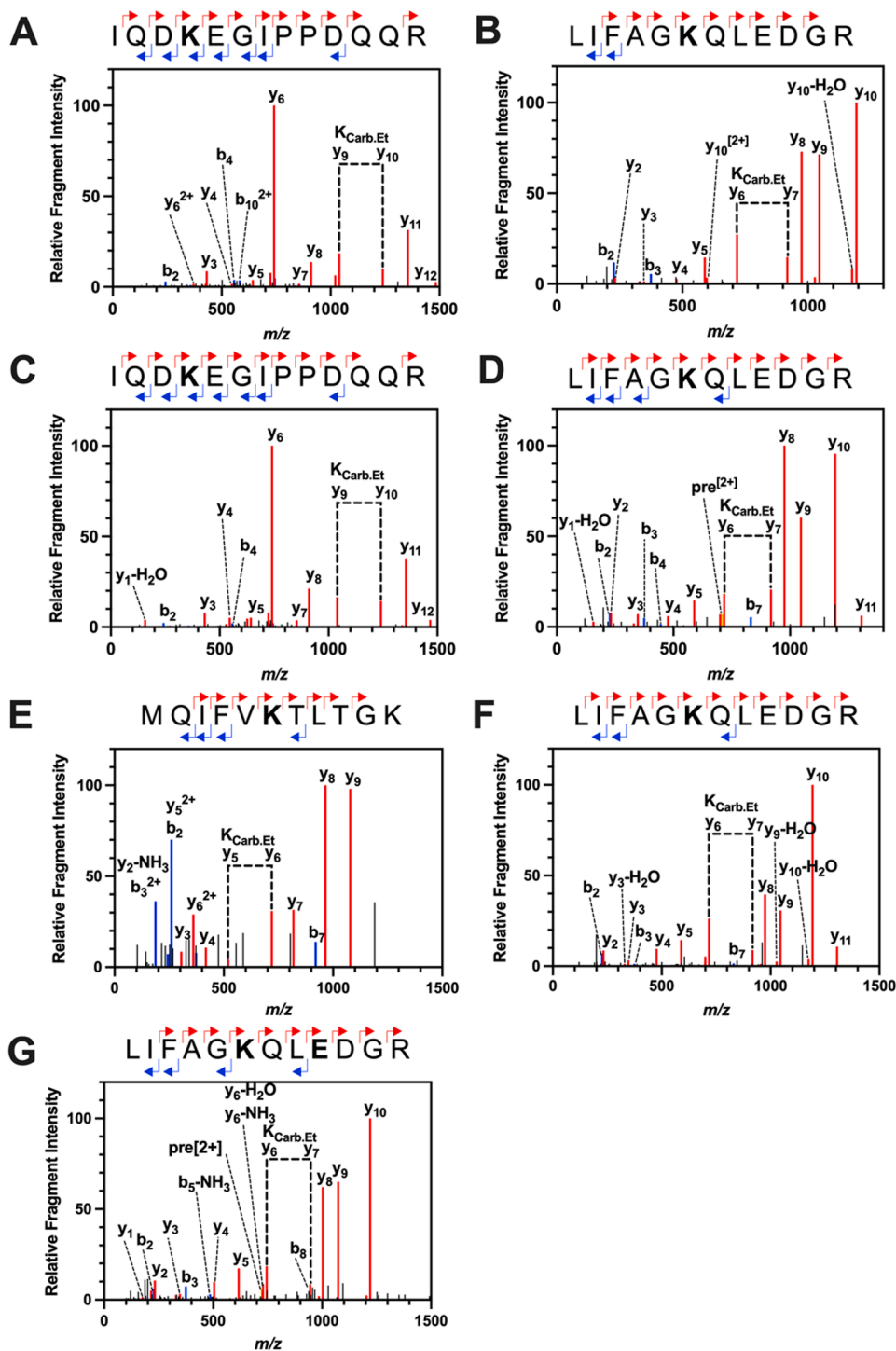
Despite observing the carbamate modification on K33 and K48 residues at 1.47 mM  $\text{CO}_2$ , this is orders of magnitude higher than physiologically relevant  $\text{PCO}_2$  for *Arabidopsis*. Cellular  $[\text{CO}_2]$  within typical C3 plants is much closer to atmospheric  $\text{CO}_2$  ( $\sim 10 \mu\text{M}$ ) [41], and thus AtUb was trapped under these conditions. Independent experiments identified the carboxyethyl modification on the K6 and K48 residues of AtUb (Fig. 2E–F). The carboxyethyl modification was not observed on the K33 residue under these conditions, while unmodified K33-containing peptides were identified. This finding suggests that the K33 residue is less sensitive to  $\text{CO}_2$  than the K6 and K48 residues and that the K33 carbamate group only forms at elevated  $\text{PCO}_2$ .

A subsequent trapping experiment with  $^{13}\text{C}_i$  identified the  $^{13}\text{C}$  carboxyethyl modification on K48 (Fig. 2G). The K6 residue was not identified with near atmospheric  $^{13}\text{CO}_2$ . However, we note that the AtUb N-terminal peptide was typically unreliable to observe by MS/MS, suggesting that the modified and unmodified forms of this peptide exhibit relatively poor flyability [42].

### 3.3. Near-atmospheric $\text{CO}_2$ stimulates plant Ub conjugation

Having identified carbamylation sites at K6 and K48 of AtUb sensitive to micromolar  $\text{CO}_2$ , we developed an *in vitro* assay system to test the potential biochemical impact of these modifications. There are two Ub-activating E1 enzymes in *A. thaliana*: AtUBA1 and AtUBA2, which are reported to display broadly similar expression profiles across the plant [43]. AtUBA1 has previously been recombinantly expressed and widely used in plant *in vitro* ubiquitination assays and thus was selected as the E1 enzyme for this work. The *A. thaliana* E2 enzyme AtUBC5 is one of nine reported to function in an E3-dependent manner [44]. AtUBA1 and AtUBC5 were produced as purified recombinant proteins (Supplementary Fig. 2), and we confirmed the formation of di-AtUb was dependent



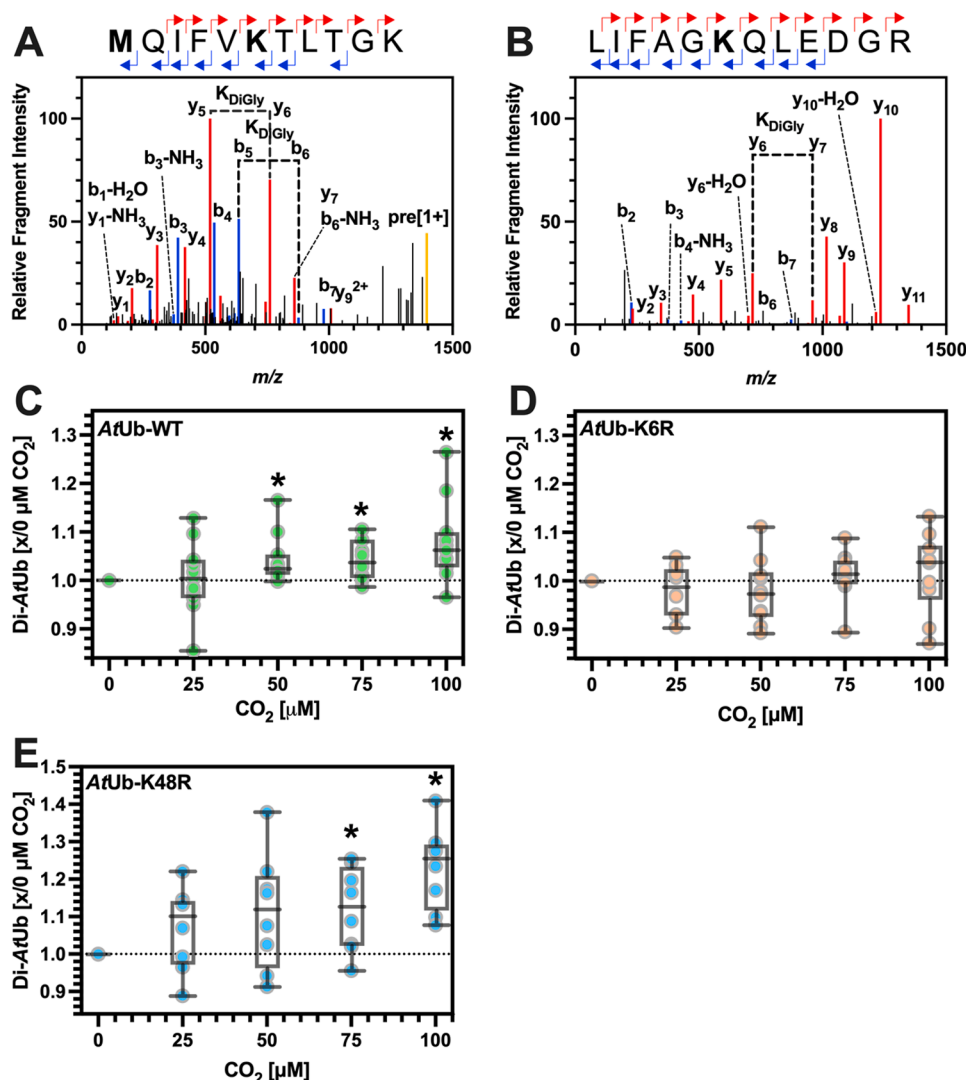


**Fig. 2.** CO<sub>2</sub> binds AtUb. Plots of relative fragment intensity versus mass/charge ratio (m/z) for fragmentation data from MS/MS identifying ethyl-trapped carbamate on recombinant AtUb. A. AtUb K33 with 20 mM <sup>12</sup>CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. B. AtUb K48 with 20 mM <sup>12</sup>CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. C. AtUb K33 with 20 mM <sup>13</sup>CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. D. AtUb K48 with 20 mM <sup>13</sup>CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. E. AtUb K6 with 130 μM <sup>12</sup>CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. F. AtUb K48 with 130 μM <sup>12</sup>CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. G. AtUb K48 with 130 μM <sup>13</sup>CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. Peptide sequences indicate predominant +1y (red) +1b (blue) ions identified by MS/MS shown in the plot. The modified residue is indicated in bold. K\_Carb.Et indicates the molecular weight difference between ions diagnostic of the modified Lys.

on AtUBA1, AtUBC5, AtUb, and ATP (Supplementary Fig. 3).

We determined the di-AtUb linkage type in assays by LC-MS/MS and searched for a diglycine +114.04 Da mass shift formed after trypsin cleavage at the AtUb C-terminus (Supplementary Fig. 4). LC-MS/MS analysis of trypsin-digested AtUBC5-synthesised di-AtUb identified the +114.04 Da di-glycine modification on K6 and K48 (Fig. 3A-B). Unmodified peptides containing all the other AtUb lysine residues were identified, indicating AtUBC5 does not conjugate AtUb at these residues. Repeated experiments demonstrated that the +114.04 Da di-glycine modification on K6 was not observed using AtUb-K6R protein. However, the +114.04 Da di-glycine modification on K48 was still observed

using AtUb-K6R protein. The +114.04 Da di-glycine modification on K48 was not observed using AtUb-K48R protein. The +114.04 Da diglycine modification on K6 was still observed using AtUb-K48R protein. Therefore, K6R and K48R AtUb mutant proteins were used to analyse the relative contributions of these two linkage types to di-AtUb formation. Di-AtUb production was measured as a proportion of the sum of mono- and di-AtUb. For each mutant, this was then normalised to di-AtUb production with the WT protein. For AtUb-K6R, the mean normalised intensity of the di-AtUb band was 88.1% of the WT di-AtUb band. For AtUb-K48R, the mean normalised intensity of the di-AtUb band was 4.4% of the WT di-AtUb band. We conclude that under the



**Fig. 3.** CO<sub>2</sub> increases AtUb conjugation. **A-B.** Plots of relative fragment intensity versus mass/charge ratio ( $m/z$ ) for fragmentation data from MS/MS identifying the di-Gly modification on recombinant AtUb at K6 (**A**) and K48 (**B**). Peptide sequences indicate predominant +1y (red) +1b (blue) ions identified by MS/MS shown in the plot. The modified residue is indicated in bold.  $K_{DiGly}$  indicates the molecular weight difference between ions diagnostic of the modified Lys. **C-E.** Box and whisker (5-95%) plots of the ratio of di-AtUb formed in the presence of added CO<sub>2</sub> compared to zero CO<sub>2</sub>. **C.** AtUb-WT conjugation (\*  $p < 0.05$ , one-sample t-test, theoretical mean=1.000; 25  $\mu$ M CO<sub>2</sub>  $p = 0.8531$ ,  $t = 0.1900$ ,  $df = 10$ ; 50  $\mu$ M CO<sub>2</sub>  $p = 0.0163$ ,  $t = 2.884$ ,  $df = 10$ ; 75  $\mu$ M CO<sub>2</sub>  $p = 0.0059$ ,  $t = 3.486$ ,  $df = 10$ ; 100  $\mu$ M CO<sub>2</sub>  $p = 0.0121$ ,  $t = 3.056$ ,  $df = 10$ ). **D.** AtUb-K6R conjugation (one-sample t-test, theoretical mean=1.000; 25  $\mu$ M CO<sub>2</sub>  $p = 0.3027$ ,  $t = 1.093$ ,  $df = 9$ ; 50  $\mu$ M CO<sub>2</sub>  $p = 0.3440$ ,  $t = 0.9988$ ,  $df = 9$ ; 75  $\mu$ M CO<sub>2</sub>  $p = 0.4587$ ,  $t = 0.7742$ ,  $df = 9$ ; 100  $\mu$ M CO<sub>2</sub>  $p = 0.5219$ ,  $t = 0.6664$ ,  $df = 9$ ). **E.** AtUb-K48R conjugation (\*  $p < 0.05$ , one-sample t-test, theoretical mean=1.000; 25  $\mu$ M CO<sub>2</sub>  $p = 0.1246$ ,  $t = 1.744$ ,  $df = 7$ ; 50  $\mu$ M CO<sub>2</sub>  $p = 0.0904$ ,  $t = 2.043$ ,  $df = 7$ ; 75  $\mu$ M CO<sub>2</sub>  $p = 0.0199$ ,  $t = 3.002$ ,  $df = 7$ ; 100  $\mu$ M CO<sub>2</sub>  $p = 0.0006$ ,  $t = 5.900$ ,  $df = 7$ ).

standard conditions of the assay, AtUBC5 exhibits a strong preference for forming K48-conjugated di-AtUb *in vitro*. The enzyme further produces a relatively small population of K6-conjugated di-AtUb. Therefore, AtUBC5 is suitable for investigating the impact of carbamate formation at K6 and K48 at near atmospheric PCO<sub>2</sub>.

The pH sensitivity of the AtUBA1/AtUBC5 system was measured across a range of pH values (pH 6.5–8.3), and we observed a clear pH dependence (**Supplementary Fig. 5**). Degassing CO<sub>2</sub> from an assay can increase pH and give the appearance of a stimulated activity. Therefore, we performed assays to assess the role of CO<sub>2</sub> at pH 7.9, close to the observed pH optimum. Further, we monitored assay pH over a PCO<sub>2</sub> range (1–4 mM total C<sub>i</sub>) and demonstrated no difference in final assay pH (7.8). We could, therefore, eliminate altered pH as an explanation for any observations. To probe the effects of AtUb carbamylation on di-AtUb formation, AtUBC5-catalysed di-AtUb formation was measured at increasing PCO<sub>2</sub> (**Fig. 3C**). We observed a statistically significant increase in the mean rate of di-AtUb formation with increasing PCO<sub>2</sub>. This surprising result was in direct contrast to previous observations, where significantly higher PCO<sub>2</sub> inhibited mammalian Ub conjugation.

Having observed this unexpected stimulation in di-AtUb formation using WT AtUb, K6R and K48R mutant AtUb were used to determine whether carbamylation at either residue was responsible for this effect (**Fig. 3D-E**). AtUb-K6R ablated the stimulation in di-AtUb formation across the same PCO<sub>2</sub> range. There was no statistically significant

difference in the mean of the normalised values at each PCO<sub>2</sub> relative to the zero PCO<sub>2</sub> values. This observation suggests that the CO<sub>2</sub>-stimulated increase in di-AtUb formation depends on K6. Conversely, the CO<sub>2</sub>-stimulated increase in di-AtUb formation was still observed using AtUb-K48R, presumably through an increase in conjugation at the K6 site.

To control for CO<sub>2</sub>-mediated effects via carbamylation of AtUBA1 and AtUBC5, both enzymes were TEO-trapped at 100  $\mu$ M CO<sub>2</sub>, the highest [CO<sub>2</sub>] used within the *in vitro* assays. No carboxyethyl modifications were observed on AtUBC5 under these conditions (89 peptides contributing to 83% protein coverage at a 1% false discovery rate). The only lysine outside of the covered region was the AtUBC5 K5 residue. The absence of carboxyethyl modifications suggests that any observed effects are not due to carbamate formation on AtUBC5. However, it is not possible to formally exclude AtUBC5 K5 carbamylation. Similarly, no carboxyethyl modifications were observed on AtUBA1 under these conditions (299 peptides contributing to 91% protein coverage at a 1% false discovery rate). Unfortunately, multiple lysine residues were not covered by the identified peptides due to the proximity of these lysine residues to other lysine and arginine residues. Where lysine and arginine residues are in close proximity in a protein, very small peptides result after trypsin digest. Such small peptides are very difficult to identify by MS/MS.

### 3.4. Near-atmospheric CO<sub>2</sub> stimulates plant UBC5 charging

Incomplete MS/MS coverage of AtUBA1 and AtUBC5 cannot formally exclude a CO<sub>2</sub> effect on either or both enzymes. However, the observation that AtUb K6R ablates the CO<sub>2</sub> response makes such a possibility unlikely. Therefore, we investigated whether the AtUBA1 and AtUBC5 charging steps explained the observations dependent on AtUb carbamylation. The charging reaction was investigated in which AtUBA1 adenylates the AtUb C-terminus, activating it for transfer to a cysteine at the AtUBA1 active site via a thioesterification reaction. Even at 4°C, by the first assay time point (at 5s), the concentration of the AtUBA1-AtUb conjugate had reached a steady state (**Supplementary Fig. 6**). Thus, the rate of this step relative to the others within the reaction made it unlikely it could be responsible for a pronounced change in di-AtUb conjugation in response to CO<sub>2</sub>.

The AtUBC5 charging step occurs via a transthioesterification reaction in which the AtUb is transferred from the E1 active site cysteine to the E2 active site cysteine via forming an E1-E2-Ub complex. The exact mechanism remains uncharacterised, but recent structural studies using a stable Cdc34-Ub mimetic in complex with UBA1 have shed light on two distinct conformations the complex can occupy, potentially providing two snapshots of the transthioesterification mechanism [45]. In di-AtUb formation, the AtUb-AtUBC5 conjugate can be considered the active form E2 enzyme. Therefore, we investigated whether AtUb carbamylation influences AtUb-AtUBC5 conjugate formation.

The rate of AtUBC5-AtUb conjugation was measured at 0 and 100 μM CO<sub>2</sub> (the upper and lower bounds of the concentrations used in the di-AtUb conjugation reaction (**Fig. 3**)). We observed a statistically significant increase (~9%) in the mean ratio of AtUBC5-AtUb conjugate formation at 100 μM CO<sub>2</sub> relative to 0 μM (**Fig. 4**). Therefore, AtUb carbamylation at K6 increases di-AtUb formation by enhancing AtUBC5-

AtUb formation.

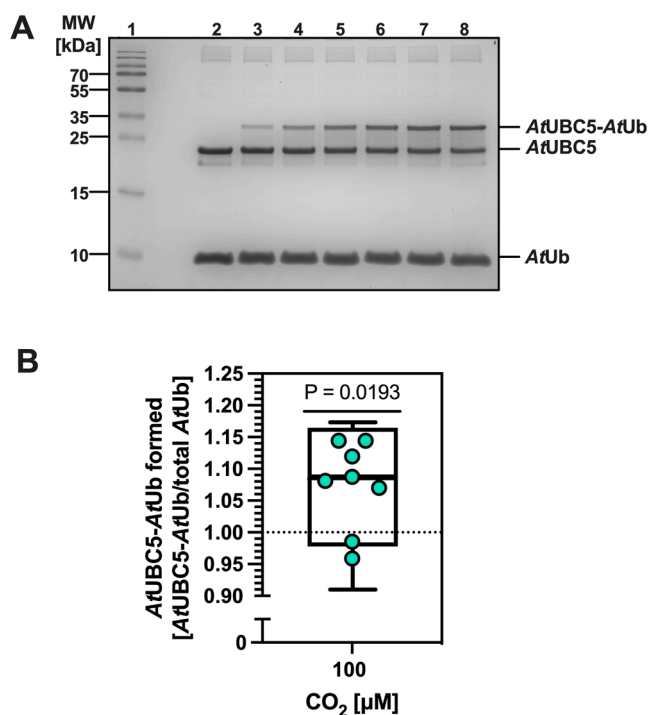
## 4. Discussion

TEO-MS/MS with low micromolar CO<sub>2</sub> concentrations identified the AtUb K48 and K6 residues as CO<sub>2</sub>-binding targets. Observing these sites under physiologically relevant CO<sub>2</sub> partial pressures suggests they may be biochemically relevant post-translational modifications within a plant. The identification of the carbamate PTM on the K6 residue is supported by analysis of Ub lysine side chain pK<sub>a</sub> values via HSQC experiments that predicted the K6 residue to have a moderately suppressed pK<sub>aH</sub> relative to other Ub lysine residues [46] (where pK<sub>aH</sub> represents the pK<sub>a</sub> of the conjugate acid used to determine amine basicity). The Ub K48 residue had the second lowest predicted pK<sub>aH</sub>. These two sites were identified at atmospheric CO<sub>2</sub>, indicating a correlation between side chain pK<sub>aH</sub> and sensitivity to carbamylation, as previously postulated [22]. However, while these pK<sub>aH</sub> values are suppressed relative to the other lysine residues, they are not strikingly low (~10.5). This value is close to the reported pK<sub>aH</sub> of a free L-lysine side chain. It would still equate to a minor fraction of the ε-amino group existing in the deprotonated state at a physiological pH. Inspection of Ub crystal structures shows that both the K6 and K48 residues are broadly solvent exposed [47,48]. While this solvent exposure would favour interactions with CO<sub>2</sub>, subsequent carbamate stabilising interactions, such as those observed in haemoglobin, appear less plausible. Early studies on Ub acetylation using *p*-nitrophenyl acetate reported complete acetylation of the K6 residue [49]. While carbamylation and acetylation are two distinct processes, they are both non-enzymatic and are underpinned by the nucleophilicity of the ε-amino group. Given the measured pK<sub>aH</sub>, it seems there are additional underpinning factors that determine modification.

The E2 enzyme, AtUBC5, was observed to form free Ub chains conjugated primarily via K48 and K6. Demonstrating AtUBC5 as a K48-specific plant E2 is intriguing as there is little literature on fully characterised plant E2 enzymes. K48-specific E2 enzymes such as the mammalian E2-25k and the yeast Cdc34 have attracted particular attention due to the canonical role of K48-linked Ub in the 26S-proteasomal degradation pathway [50]. AtUBC5 could provide a further case study across kingdoms, helping to unpack the mechanisms which determine the linkage specificity by which E2 enzymes synthesise polyubiquitin chains.

*In vitro* assays using the AtUBC5 indicated that di-AtUb formation was stimulated by increasing PCO<sub>2</sub> in a K6-dependent manner. Limitations to the *in vitro* system meant that while assay CO<sub>2</sub> concentrations were near atmospheric, they still slightly exceeded those that might be observed within a plant. The molar ratio of AtUb to CO<sub>2</sub> resembled that of theoretical conditions based on mammalian tissue-free Ub concentrations, as the cellular concentration of free Ub in plant tissues is unreported. Therefore, it is hard to perfectly ascribe the *in vitro* observations in this study to a plant cellular environment. However, all reasonable steps have been taken to mimic likely conditions. Nonetheless, plant dark respiration can see rapid rises in leaf intercellular [CO<sub>2</sub>]. Therefore, it is possible that the response to CO<sub>2</sub> underpins a diurnal regulation of Ub conjugation.

The work of Linthwaite et al. demonstrated that mammalian Ub K48 carbamylation inhibited E2-25k catalysed K48 poly-Ub chain synthesis *in vitro* [18]. This observation was hypothesised to occur due to the carbamylation PTM directly blocking access to the K48 residue of acceptor Ub. That carbamylation could have the opposite effect in the plant K48-specific system appears contradictory but can be explained by distinctions between the assays. Firstly, the finding that inhibition of K48-linked poly-AtUb synthesis was not observed during this work can be explained by the significantly lower CO<sub>2</sub> concentrations used, which do not resemble the concentration range across which Linthwaite et al. observed inhibition. Secondly, that Linthwaite et al. did not observe an allosteric stimulation via the K6 residue may be due to



**Fig. 4.** CO<sub>2</sub> increases AtUBC5 charging. **A.** Example of SDS/PAGE analysis and Coomassie Blue staining showing the time course of AtUBC5-AtUb conjugate formation performed at atmospheric CO<sub>2</sub>. Lanes are 1. Molecular mass standards; 2. Time zero; 3. 1 min; 4. 2 mins; 5. 3 mins; 6. 4 mins; 7. 5 mins; 8. 6 mins. **B.** Box and whisker (5-95%) plot of AtUBC5-AtUb conjugate formed at 100 μM CO<sub>2</sub> when AtUBC5-AtUb conjugate formation at zero CO<sub>2</sub> is normalized to 1 ( $p = 0.0193$ , one-sample t-test, theoretical mean=1.000; 100 μM CO<sub>2</sub>,  $t=3.025$ ,  $df=7$ ). Assays were run for two minutes.

differences between the mammalian E1-E2 and plant E1-E2 combinations used in the studies. Consistent with this, Linthwaite et al. identified a Ub carbamate site at K63 by  $^{13}\text{C}$ -NMR but did not observe inhibition of polyubiquitin chain synthesis with a K63-specific system across the same range of  $\text{CO}_2$  concentrations.

As poly-Ub chain synthesis is a complex multi-step reaction, individual steps were probed to establish how a K6-mediated stimulation in activity might occur. The initial AtUBA1-AtUb (E1-AtUb) charging reaction was rapid and unlikely to have a rate-determining effect on di-AtUb formation.  $k_{\text{cat}}$  values for E1-catalyzed reaction steps have previously been reported in the  $1\text{--}2\text{ s}^{-1}$  range, over 10-fold greater than reported  $k_{\text{cat}}$  values for E2-catalysed ubiquitin conjugation and chain extension reactions [51]. The subsequent Ub transthioesterification step appeared a better candidate. AtUBC5-AtUb thioester conjugate formation was enhanced by increasing  $\text{CO}_2$ . How might this occur? Structural studies of mimetic *S. cerevisiae* UBA1-Cdc34-Ub complexes adopted during the transthioesterification reaction displayed hydrogen-bonding interactions between a modified K6R ubiquitin residue and both the UBA1 and Cdc34 enzymes in respective open and closed conformations [45]. While the specific mechanistic features of how K6 carbamylation would stimulate the reaction are unknown, perturbations of these hydrogen-bonding interactions by K6 carbamate formation are a plausible starting point. The proximity of the K6 residue to the Ile44 hydrophobic patch, an essential recognition domain which mediates many of the Ub protein-protein interactions [52,53], might reveal how the K6-dependant  $\text{CO}_2$ -mediated stimulation can occur at the Ub-Ub conjugation level. The K6 residue is important in donor Ub docking to the K11-specific E2 Ube2S, forming an ionic interaction with a glutamic acid residue on the Ube2S docking surface [54].

Similarly, in Cdc34, the K6 residue of acceptor Ub interacts with the Cdc34 enzyme via hydrogen bonding to orient the K48 residue towards the E2-Ub thioester bond [55]. A K6D Ub mutation decreased the Ub release rate from the Cdc34-Ub complex. However, this observation was attributed to the length of the aspartic acid side chain and the loss of the polar contact with Cdc34 S71 as opposed to the introduction of the anionic charge, as would be observed for carbamylation. Such allosteric effects have been observed for acetylated Ub residues. Acetyl-K6 and acetyl-K48 Ub were present in low concentrations in human 293F cells, and *in vitro*, both inhibited free poly-Ub chain formation (as catalysed by multiple E2 enzymes) [56]. The authors observed broad repression with acetyl-K6, but acetylation differs from carbamylation in that it irreversibly modifies the cationic lysine residue with a neutrally charged group instead of the transient, anionic carbamate group.

K6 linkages are generally considered atypical in plants [57]. Analysis of the *A. thaliana* ubiquitylome indicated they were present at a low abundance [58,59]. The cellular functions of K6-linked poly-Ub chains also remain uncharacterised in plants, with functional studies limited to other eukaryotes where they have been associated with processes such as DNA repair and mitophagy [60]. Future work should be directed towards understanding the extent to which Ub K6 carbamylation impacts E2 charging and the impact on plant physiology.

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## CRedit authorship contribution statement

**Harry G Gannon:** Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **Martin J Cann:** Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.bbadv.2023.100096](https://doi.org/10.1016/j.bbadv.2023.100096).

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