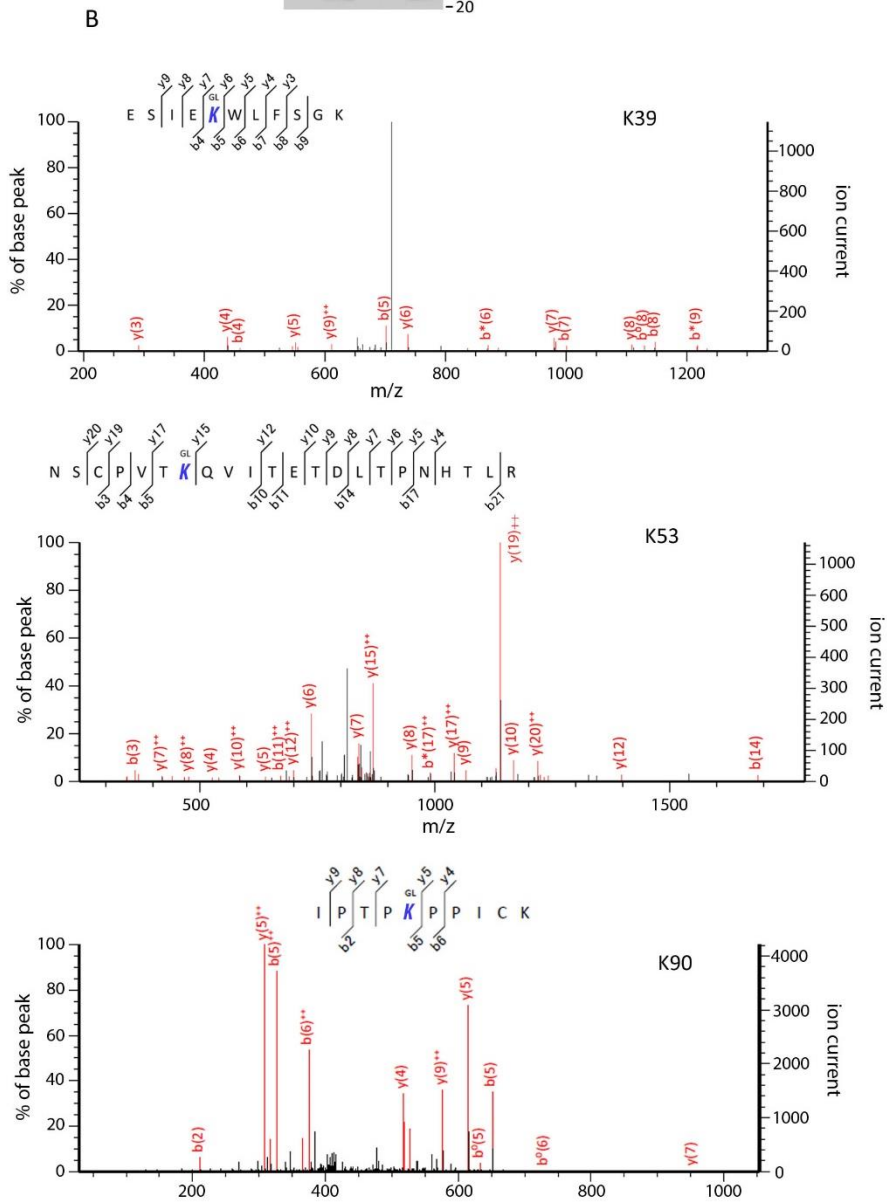
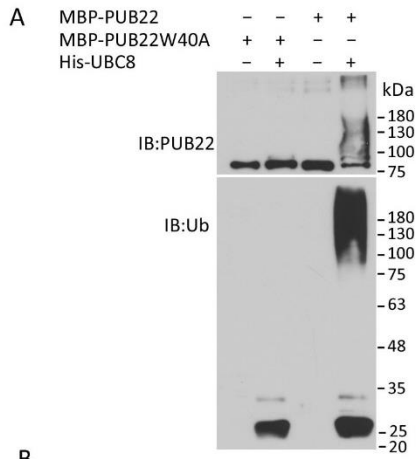
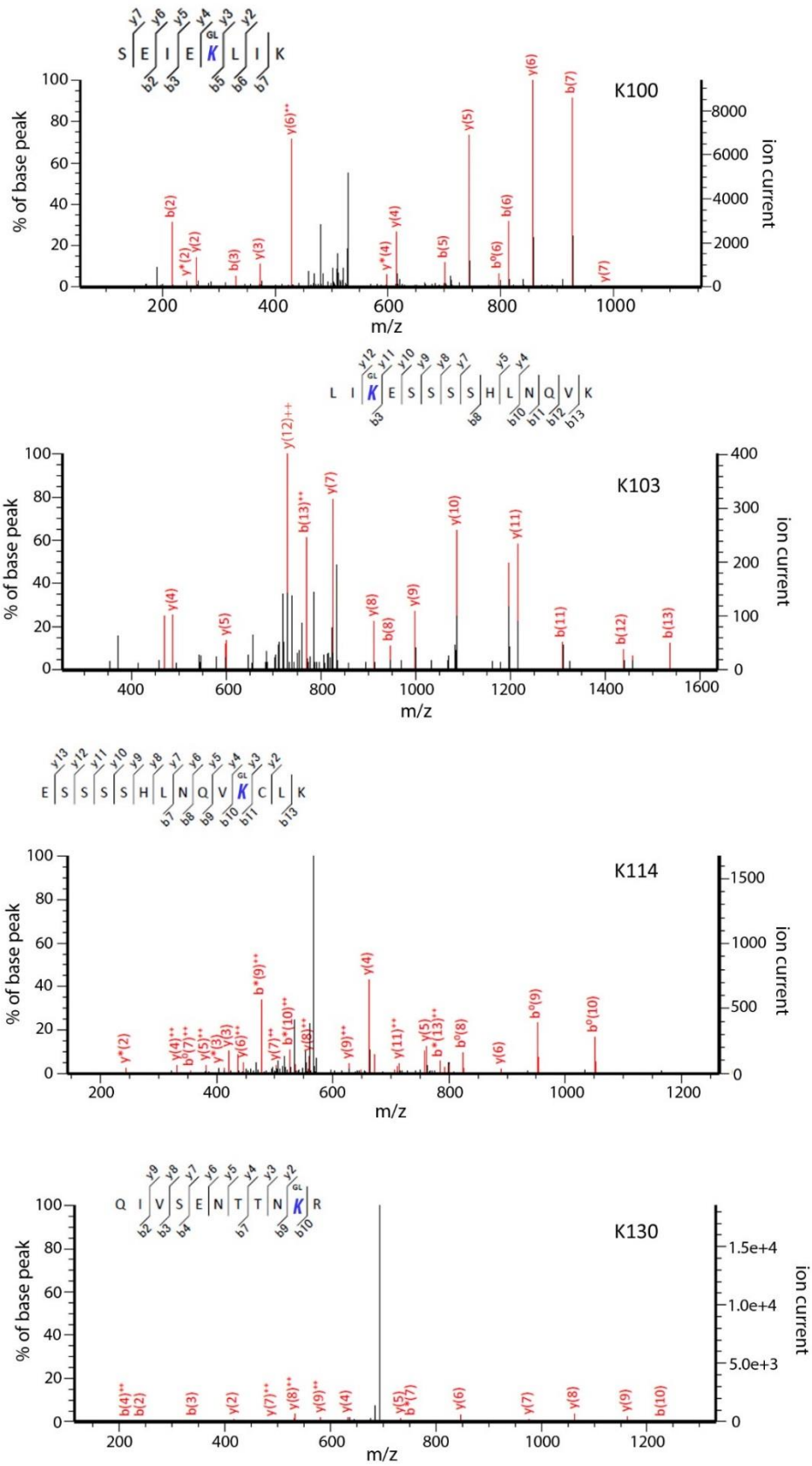
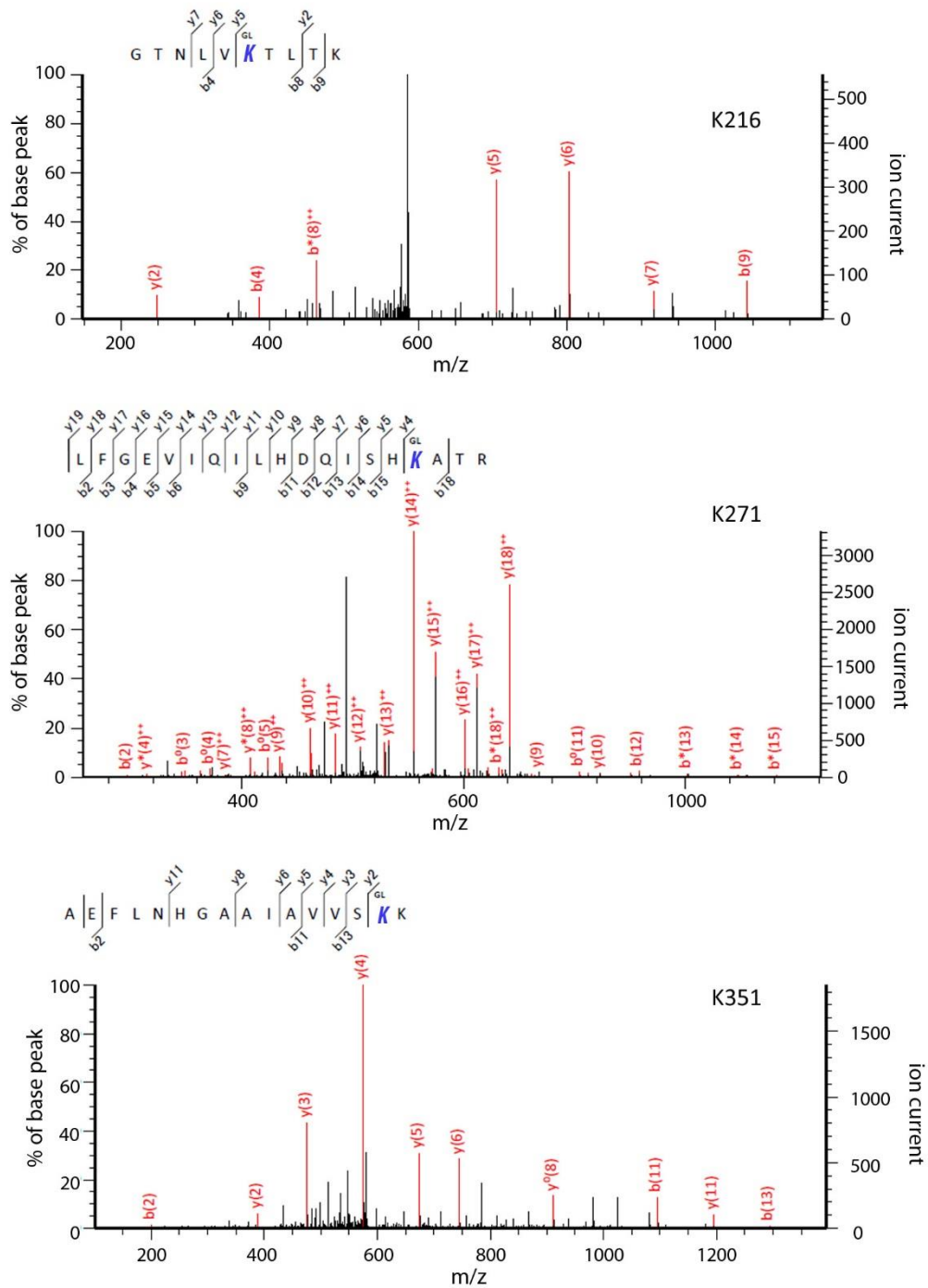


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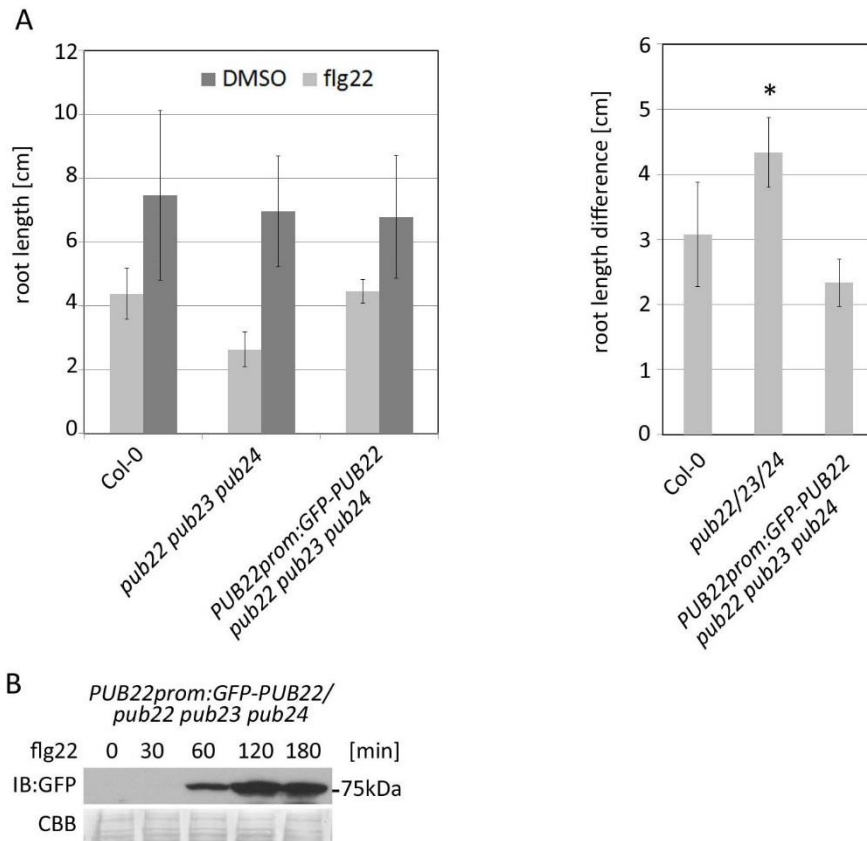
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Supplemental Figure 1. PUB22 displays autoubiquitination activity in vitro.
(A) In vitro autoubiquitination assay with MBP-PUB22 and the inactive W40A mutant using His-UBA1 and His-UBC8. MBP-PUB22 was incubated with the indicated components for 2h, resolved by PAGE and analysed by immunoblot (IB) with the indicated anti-bodies.
(B) In vitro autoubiquitination sites identified on PUB22. MS spectra of peptides with a di-glycine footprint found on GST-PUB22 after in vitro autoubiquitination assay using His-UBA and His-UBC8.



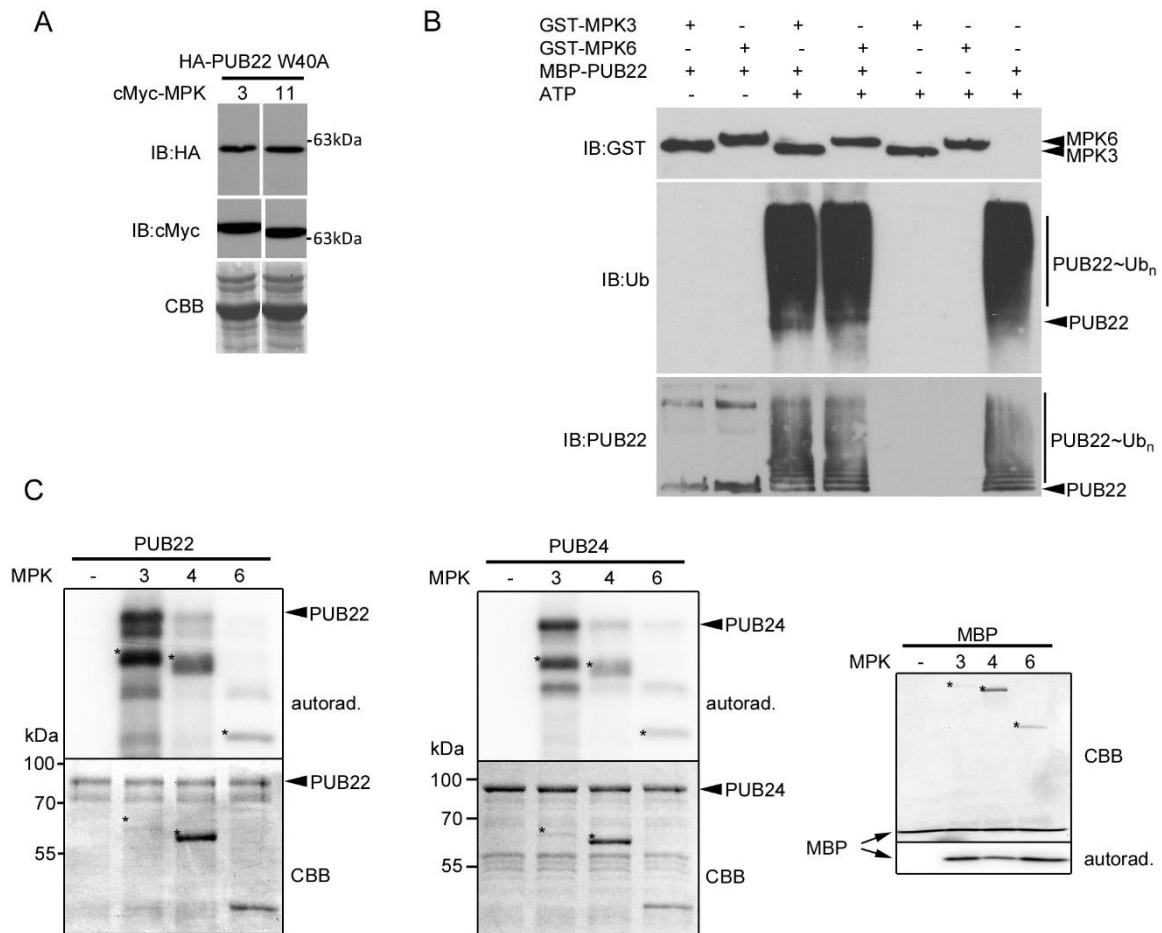
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2 **Supplemental Figure 2.** *PUB22prom:GFP-PUB22* complements the *pub22 pub23 pub24*
3 phenotype.

4 (A) Primary root lengths seedlings measured seven days after transplanting onto media
5 containing 1 μ M flg22 or DMSO as control. A homozygous T3 line carrying a single copy of
6 the *PUB22prom:GFP-PUB22* construct in the *pub22 pub23 pub24* background was used.
7 Data shown as mean of three independent experiments +/- SD (n \geq 60). Asterisk indicates
8 significantly different values between wild-type and *pub22 pub23 pub24* with p<0.05 (one
9 way ANOVA, Tukey post hoc test).

10 (B) PUB22 accumulation is induced by flg22 in the *PUB22prom:GFP-PUB22/pub22 pub23*
11 *pub24* line. Protein expression was induced by flg22 (1 μ M) treatment in two week-old
12 seedlings carrying a *PUB22prom:GFP-PUB22* construct. Total protein samples were
13 analysed by IB using anti-GFP. Coomassie brilliant blue (CBB) is shown as loading control.

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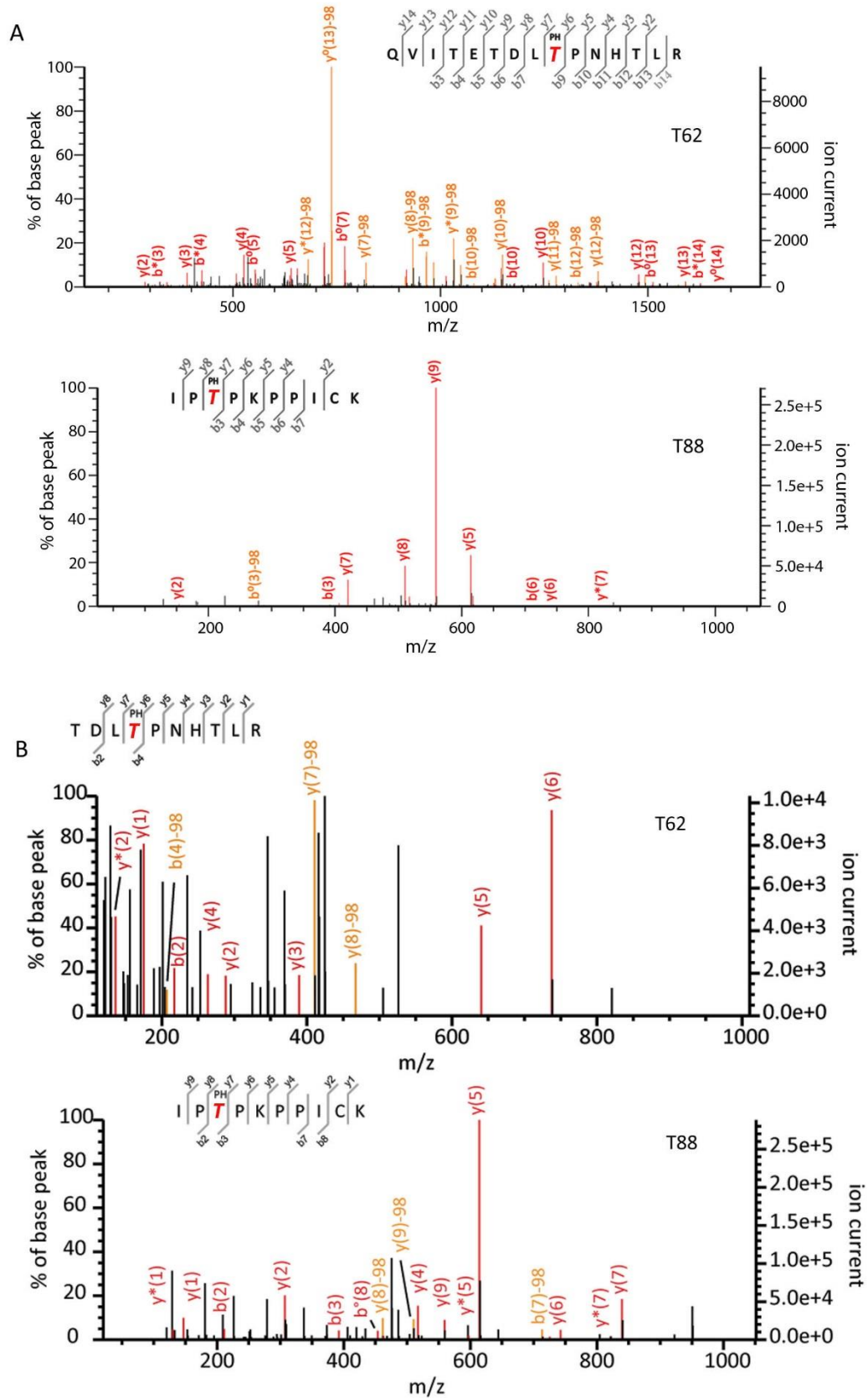
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2 **Supplemental Figure 3.** Post-translational modification of PUB22 and PUB24.

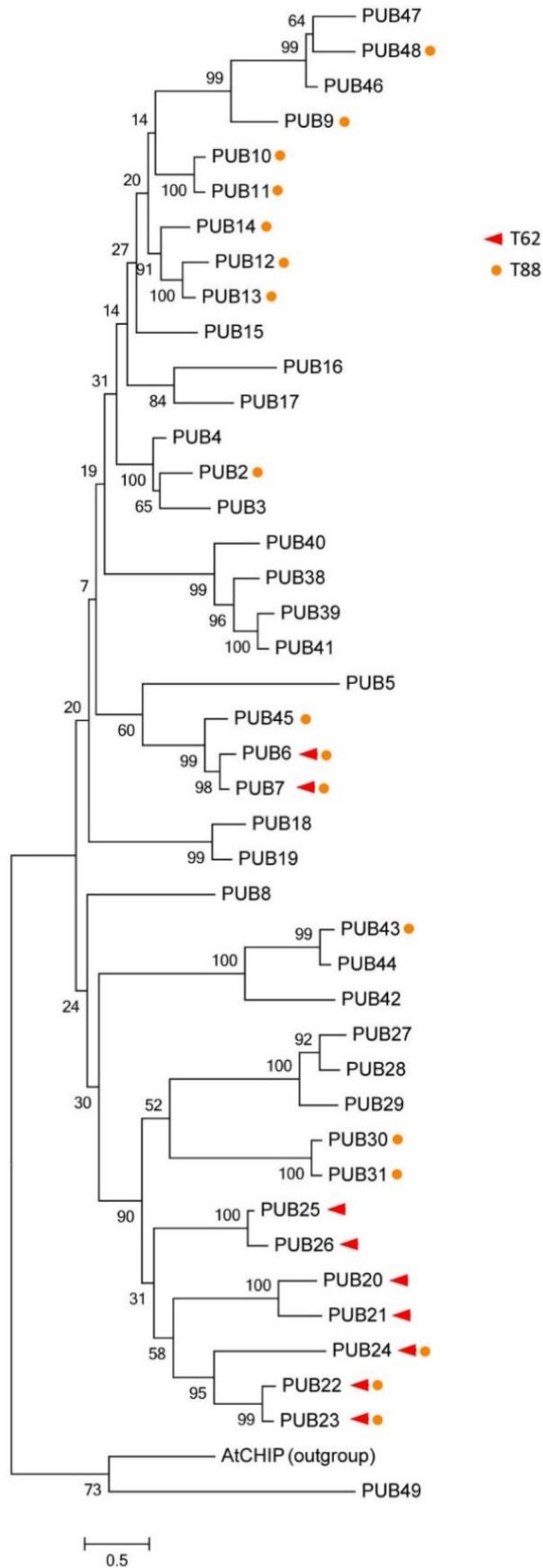
3 (A) Protein expression from experiments in Figure 2a. cMyc-nYFP-MPK3 or cMyc-nYFP-
 4 MPK11 were coexpressed with HA-cYFP-PUB22 W40A as indicated in Figure 2A. Total
 5 proteins were analysed by IB with anti-cMyc and anti-HA. Equal loading shown by coomassie
 6 brilliant blue (CBB).

7 (B) PUB22 does not ubiquitinate MPK3 or MPK6 in vitro. In vitro ubiquitination assay with
 8 MBP-PUB22 and GST-MPK3 or GST-MPK6 using His-UBA1 and His-UBC8 and incubated
 9 for 2h. Proteins were analysed by IB with anti-GST, anti-ubiquitin and anti-PUB22.

10 (C) PUB24 is also phosphorylated by MPK3 in vitro. Recombinant MBP-PUB22 or MBP-
 11 PUB24 were incubated alone or with activated MPK3, MPK4 and MPK6 and separated by
 12 PAGE (MAPKs indicated by asterisk). Autophosphorylation and trans-phosphorylation were
 13 visualized with radioactive ATP and autoradiography. The artificial kinase substrate, myelin
 14 basic protein (MBP), was used to adjust equal MPK activities (right panel). The protein
 15 loading control is shown by CBB staining.



- 1 **(A)** LC-MS/MS analysis spectra of PUB22 phosphorylated tryptic peptides from in vitro
- 2 phosphorylation assay with MPK3. To obtain phosphorylated PUB22, GST-PUB22 was
- 3 incubated alone or with activated GST-MPK3, GST-MPK4 and untagged MPK6.
- 4 **(B)** LC-MS/MS analysis spectra of PUB22 phosphorylated tryptic peptides from in vivo
- 5 samples. *UBQ10prom:GFP-PUB22* transgenic seedlings grown for 14 days in liquid media
- 6 were treated with 1 μ M flg22 or water (mock) and harvested after 30min. GFP-PUB22 was
- 7 immune-purified resolved by PAGE and gel bands were analysed by LC-MS/MS.
- 8 Phosphorylated peptides were not detected in the control samples.
- 9

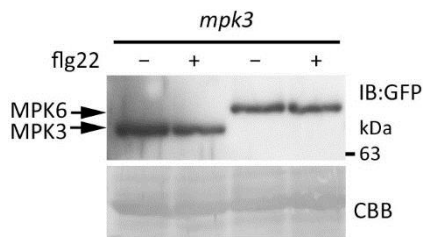


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2 **Supplemental Figure 5.** Phylogenetic relations between Arabidopsis PUB proteins class II
3 and III.

1 Alignment was performed using MAFFT and manually edited and used for phylogenetic
2 analysis using maximum likelihood with 1000 random drawn samples (bootstraps). Bootstrap
3 values greater than 0.5 are shown at the corresponding node. The phylogenetic tree was
4 rooted using AtCHIP as outgroup. Highlighted are PUB proteins with a conserved threonine
5 followed by proline at position 62 (orange circle) and 88 (red arrowhead). Scale bar denotes
6 0.3 amino acid substitutions per site.

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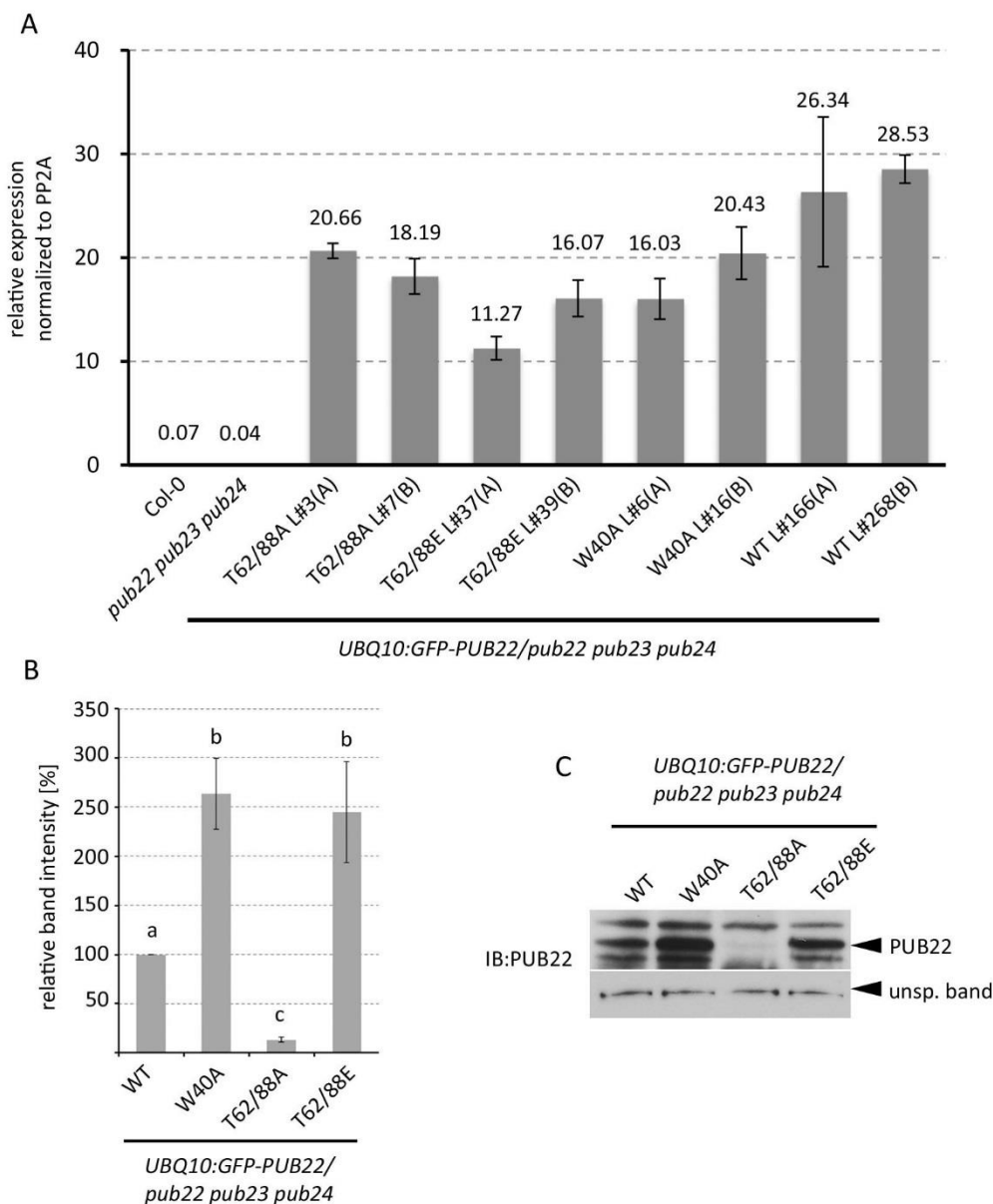


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12 **Supplemental Figure 6.** PUB22 stabilization is MPK3 dependent.

13 Luc-PUB22 fusion was coexpressed with MPK3 or MPK6 in *mpk3* protoplasts. Protein levels
14 of YFP-MPK3 and YFP-MPK6 of experiments shown in Figure 3B. Proteins were analyzed
15 by IB using anti-GFP.

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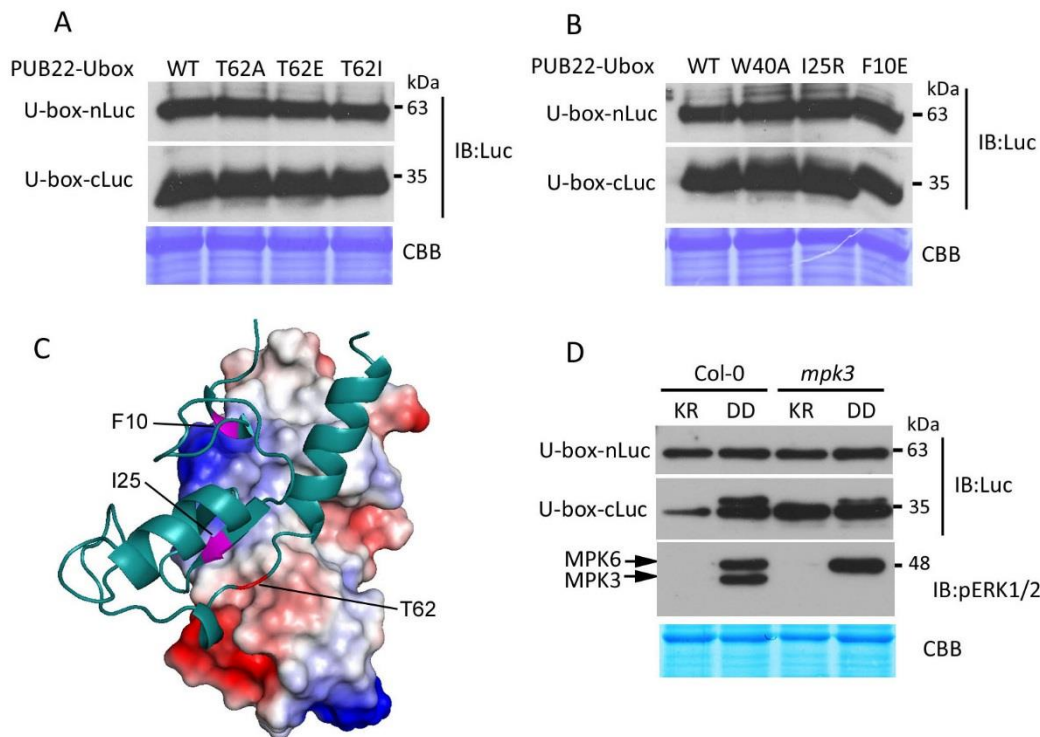
2 **Supplemental Figure 7.** Transgenic lines express comparable levels of transcript of PUB22
3 WT and mutant variants.

4 **(A)** Quantitative real-time PCR of selected T3 homozygous transgenic lines carrying
5 *UBQ10prom:GFP-PUB22* WT and variants W40A, T62/88A and T62/88E. Samples were
6 taken from adult plants and *PP2A* (AT1G13320) was used as a reference gene. Data shown
7 as mean +/- SD (n=3).

8 **(B)** Analysis of the relative amounts of PUB22 and mutant variants (line A). The band
9 intensity of PUB22 WT was determined, set to 100% and the relative amounts for the other
10 lines were calculated. Data shown as mean +/- SEM of four independent experiments.
11 Letters indicate significantly different values at p<0.05 (one way ANOVA, Tukey post hoc
12 test).

13 **(C)** Representative immunoblot showing accumulation of PUB22 WT and W40A, T62/88A
14 and T62/88E variants in transgenic lines A used to calculate relative protein amounts in (B).

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2 **Supplemental Figure 8.** PUB22 homo- and hetero-oligomerizes. (Supports Figure 5.)

3 (A) Protein levels of split luciferase experiments shown in Figure 5F. N- and C-terminal
4 fragments of luciferase fused to PUB22 U-box WT, T62A, T62W and T62I variants were
5 analyzed by IB with anti-Luc.

6 (B) Protein levels of split luciferase experiments shown in Figure 5G. N- and C-terminal
7 fragments of luciferase fused to PUB22 U-box WT, W40A, I25R and F10E variants were
8 analyzed by IB with anti-Luc.

9 (C) Model of the PUB22 U-box dimer highlighting exchanged residues. Front protomer
10 shown in cartoon style (turquoise) and back protomer shows electrostatic surface charges.

11 (D) Protein levels of split luciferase experiments shown in Figure 5H. N- and C-terminal
12 luciferase fusion proteins of PUB22 U-box coexpressed with MKK5 KR (inactive) or MKK5
13 DD (constitutive active) were analyzed by IB with anti-Luc. Activation of MPK3 and MPK6
14 was confirmed using anti-ERK1/2.

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1 **Supplemental Tables**2 **Table 1: Primers for genotyping**

Gene	Locus	5`-Forward-3`	5`-Reverse-3`
<i>PUB22</i>	At3G52450	ATGTCCATGGGGAAGGAATAG	AATGCCCGTCGTGGATATC
<i>PUB23</i>	At2G35930	CAATCTTGGTGCACCCTAAAC	TTTTCATCAGCAGGGATATGC
<i>PUB24</i>	At3G11840	GACGACGTCGTATCAAAGGAC	TCGATTGAGGATTGATCGATC
<i>MPK3</i>	At3G45640	ATTTTTGTCAACAATGGCCTG	TCTGCCTTTTCACGGAATATG

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4 **Table 2: Primers used for SDM on PUB22 and PUB22 U-box in entry vectors**

Mutation	5`-Forward-3`	5`-Reverse-3`	Type II enzyme	Diagnostic
F10E	ATATATGAAGACGAGTTCC TTTGTCCAATCTCTCT	ATATATGAAGACGAACTC GGAAGGAATCTCTATCT	Bpil	none
I25R	ATATATGAAGACCGAGTTT CCACCGGAATAAC	ATATATGAAGACGAACTC CGCACCGGATCT	Bpil	none
W40A	ATATATGAAGACGCGCTCT TTCCGGTAAGA	ATATATGAAGACAAGAG CGCCTTCTCGATG	Bpil	HaeII
T62I	ATATATGAAGACATTCCAA ACCACAC	ATATATGAAGACTTTGGA ATAAGATCAGTT	Bpil	none
T62A	ATATATGGTCTCGGCGCC GAACCACACTCTTCGCC	ATATATGGTCTCGGCGC CAGATCAGTTTCGGTTAT G	Bsal	HaeII
T62E	ATATATGGTCTCGAGCCC AACCACACTCTTC	ATATATGGTCTCGGGCT CAAGATCAGTTTCG	Bsal	BanII
T88A	ATATATGAAGACAGCTCCA AAACCTCCGATC	ATATATGAAGACTTGGAG CTGGGATCCTCTC	Bpil	none
T88E	ATATATGAAGACGAGCCC AAACCTCCGATCTG	ATATATGAAGACTTGGG CTCTGGGATCCTCTCT	Bpil	BanII

5

6 **Table 3: Primers for cloning**

Gene	Plasmid	5`-Forward-3`	5`-Reverse-3`
<i>PUB22</i> <i>Ubox</i> <i>no stop</i>	pENTR3C	GGCATTUATGGATCAAGAGAT AGAGA	GGTGATTUUCTAGATGCGAAGA TGAC
<i>PUB22</i>	pMal	CATGTCGACATGGATCAAGAGA TAGAGA	CATCTGCAGTCAAGCAGGATA CGAAT
<i>PUB24</i>	pMal	CATGAATTCATGAATATATAC GTACA	CATCTGCAGTTAGATCTTTGGC CCTTTG
<i>Exo70B2</i>	pET38b	GAATTCGATGGCTGAAGCCGGT GACGA	CTCGAGTCAACTTGAGCTTTCC TTGA

7

8 **Table 4: qRT-PCR**

Gene	5`-Forward-3`	5`-Reverse-3`
<i>PP2A</i>	TAACGTGGCCAAAATGATGC	GTTCTCCACAACCGCTTGGT
<i>PUB22</i>	TGCAATTGGGAGTTGTAGCA	GATTCCCTCCAAACCTAGC

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