Supplementary information

Evolutionary tuning of molecular charge state of UBP24 shapes eukaryotic responses to high temperature

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Supplementary Table 1. Primers used in this study

Name	Sequence (5’ to 3’)	Purpose	Reference/Source
UBP24-CDS-BsaIA-F	TACCTTGAGACCTATGAGTGAAAAGAAGGTATTTGTGTTTG	Cloning	
UBP24-CDSns-BsaIG-R	CATACTGAGACCCACTTGCTTGTAGAAGAGGACATAG	Cloning	
UBP24-S360A-F	CTGTGACAAGAACACAA**GCT**TTTGTTCCGTCTGAGC	Cloning	
UBP24-S360A-R	GCTCAGACGGAACAAAA**GCT**TGTGTTCTTGTCACAG	Cloning	
UBP24-S360D-F	CTGTGACAAGAACACAA**GAT**TTTGTTCCGTCTGAGC	Cloning	
UBP24-S360D-R	GCTCAGACGGAACAAAA**TCT**TGTGTTCTTGTCACAG	Cloning	
UBP24-C206S-F	TAAACGCTGGAAACTTG**TCC**TTCCTGAATGCAACAC	Cloning	
UBP24-C206S-R	GTGTTGCATTCAGGA**AGG**ACAAGTTTCCAGCGTTTA	Cloning	
UBP24-CDS-attB3-F	GGGGACAACTTTGTATAATAAAGTTGGGATGAGTGAAAAGAAGGTATTTGTGTTTG	Cloning	
UBP24-CDSns-attB2-R	GGGGACCACTTTGTACAAGAAAGCTGGGTACACTTGCTTGTAGAAGAGGACATAG	Cloning	
UBP24p-gib-F	TCCAAGCTCAAGCTAAGCTTCCTACATGATTTTCAATAATAAGTC	Cloning	
UBP24p-gib-R	TGCTCACCATATTTGGCACATAGAACGC	Cloning	
YFP-gib-F	TGTGCCAAATATGGTGAGCAAGGGCGAG	Cloning	
YFP-gib-R	CTTTTTTGTACTTGTACAGCTCGTCCATGC	Cloning	
UBP24-linker-gib-F	GCTGTACAAGTACAAAAAAGCAGGCTTAAC	Cloning	
UBP24-gib-R	TTGAACGATCCACTTGCTTGTAGAAGAG	Cloning	
NOST-gib-F	CAAGCAAGTGGATCGTTCAAACATTTGG	Cloning	
NOST-gib-R	CCGACGTCGCATGCCTGCAGGATCTAGTAACATAGATGACAC	Cloning	

UBP24-frag-gib-F	GTCGAATCAAATGCATGATGAGTTGTTG	Cloning	
UBP24-frag-gib-R	TGTTAGCAGCCTCGAATCAACAACGCGTCTTCTATTCC	Cloning	
GST-attB1-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGTCCCCTATACTAGGTTATTGG	Cloning	
GST-attB4-R	GGGGACAACTTTGTATAGAAAAGTTGCTCATTGATTCGACCATGGACG	Cloning	
GST-gib-F	TTTAACTTTAAGAAGGAGTCAACAATGTCCCCTATACTAGGTTATTGG	Cloning	
GST-gib-R	CATCATGCATTTGATTCGACCATGGACG	Cloning	
AHA1p-gib-F	TCCAAGCTCAAGCTAAGCTTCGACGAATGATCTAATCAC	Cloning	
AHA1p-gib-R	AGGAGGCCATCTTCACCCAGAAGAAATC	Cloning	
RFP-gib-F	CTGGGTGAAGATGGCCTCCTCCGAGGAC	Cloning	
T35S_R1	CCGACGTCGCATGCCTGCAGAGGTCACTGGATTTTGGTTTTA	Cloning	
Ub-F1	ACCTTGAGACCTATGCAGATCTTCGTGAGAACCTTGACCGGCAGGACCATCACTCTCGAGGTCGAGAGCAGCGACACCATCGA	Cloning	
Ub-R1	ATACGAGACCTTAACCTCCTCTAAGCCTCAACACAAGATGAAGTGTAGATTCTCTCTGGATGTTGTAGTCAGCCAAAGTACG	Cloning	
Ub-K63-R1	ATACGAGACCTTAACCTCCTCTAAGCCTCAACACAAGATGAAGTGTAGATTCTTTCTGGATGTTGTAGTCAGCCAAAGTACG	Cloning	
Ub-F2	CAATGTCAGGGCCAGGATCCAAGACAGAGAAGGAATCCCTCCGGATCAGCAGAGATTGATCTTCGCCGGAAGGCAGCTCGAAG	Cloning	
Ub-R2	GCCATCTTCGAGCTGCCTTCCGGCGAAGATCAATCTCTGCTGATCCGGAGGGATTCCTTCTCTGTCTTGGATCCTGGCCCTGA	Cloning	
Ub-K48-F2	CAATGTCAGGGCCAGGATCCAAGACAGAGAAGGAATCCCTCCGGATCAGCAGAGATTGATCTTCGCCGGAAAGCAGCTCGAAG	Cloning	
Ub-K48-R2	GCCATCTTCGAGCTGCTTTCCGGCGAAGATCAATCTCTGCTGATCCGGAGGGATTCCTTCTCTGTCTTGGATCCTGGCCCTGA	Cloning	
Ub-F3	ATGGCCGTACTTTGGCTGACTACAACATCCAGAGAGAATCTACACTTCATCTTGTGTTGAGGCTTAGAGGAGGTTAAGGTCTC	Cloning	
Ub-K63-F3	ATGGCCGTACTTTGGCTGACTACAACATCCAGAAAGAATCTACACTTCATCTTGTGTTGAGGCTTAGAGGAGGTTAAGGTCTC	Cloning	
Ub-R3	CATTGTCGATGGTGTCGCTGCTCTCGACCTCGAGAGTGATGGTCCTGCCGGTCAAGGTTCTCACGAAGATCTGCATAGGTCTCA	Cloning	

RAF20KD-gib-F	TGTACAAAAAAGCAGGCTTACTTAGGACGCCGGATTCTGAAC	Cloning	
RAF20KD-gib-R	TTGTACAAGAAAGCTGGGTTCATTTGGAAGCCCTGTGAGCTG	Cloning	
OST1-S175A-F	TCGCAACCAAAA**GCA**ACTGTTGGA	Cloning	
OST1-S175A-R	TCCAACAGT**TGC**TTTTGGTTGCGA	Cloning	
OST1-frag-BsaIB-F	TAGGCTTGAGACCGTCAGGAGTTAGTTACTGTCATGC	Cloning	
OST1-frag-BsaIG-R	CGATACTGAGACCAATTCTTTGGTTCCTCGGGAT	Cloning	
UBP24-gRNA1-604-F	ATTGGATGCCTCTGTGACCCCCAT	CRISPR-gRNA	
UBP24-gRNA1-604-R	AAACATGGGGGTCACAGAGGCATC	CRISPR-gRNA	
UBP24-gRNA3-1812-F	ATTGGTTAAAGACTTTACACCAAG	CRISPR-gRNA	
UBP24-gRNA3-1812-R	AAACCTTGGTGTAAAGTCTTTAAC	CRISPR-gRNA	
UBP24-gRNA5-2416-F	ATTGGAATTCTAAAGACAAGTGTG	CRISPR-gRNA	
UBP24-gRNA5-2416-R	AAACCACACTTGTCTTTAGAATTC	CRISPR-gRNA	
sgUBP3_upstream_guide_F	/5Phos/GATCGATGGAAAGGAAAGCGGTACCG	CRISPR-gRNA	
sgUBP3_upstream_guide_R	/5Phos/AAAACGGTACCGCTTTCCTTTCCATC	CRISPR-gRNA	
sgUBP3_CDS_guide_F	/5Phos/GATCGAACAATCTCGACAGTCCTCTG	CRISPR-gRNA	
sgUBP3_CDS_guide_R	/5Phos/AAAACAGAGGACTGTCGAGATTGTTC	CRISPR-gRNA	
UBP3_KO_RT_F	AGAAAGTCATTTTAATAGTAAGTCAGACTCGTCTGCTACCATCATGACGGGGGGTGGTAT	Repair template	
UBP3_KO_RT_R	CTATATTATTTTTTATGTATTTTGTCTATAATACCACCCCCCGTCATGATGGTAGCAGAC	Repair template	
UBP3_E691A_RT_F	AGCAAAAGAGGCAAGAAAACTAAGACCGCTGCCAAGAGGACTGTCgccATTGTTCCATCA	Repair template	
UBP3_E691A_RT_R	GAACTGGCCACCGAAAAGTTTGGAGATTGGTGATGGAACAATggcGACAGTCCTCTTGGC	Repair template	

ubp24-1_LP	TTTTGGGTCCTAAAATCCCAC	Genotyping	
ubp24-1_RP	TCGAGCTCTAAATTTCAAGCG	Genotyping	
ubp24-crispr_LP	CTCTCTAACTTAGCTCTCCTCCAAATTCGG	Genotyping	
ubp24-crispr_RP	CGCCTTCTACTTGATTGATTGAACCATG	Genotyping	
UBP3_KO_check_F	AACCCATCGGGAAACACATA	Genotyping	
UBP3_KO_check_R	GCTGCTAACGCAAGAGCAAT	Genotyping	
UBP3_seq1	AACGGCAACGGTTCTTCG	Genotyping	
UBP3_seq2	CCGTAGTGTTTATGAACAGTGGC	Genotyping	
UBP3_seq3	CCAGTCCATTCCATTATTCCAAGAG	Genotyping	
UBP3_seq4	CGACTCTTGAATTGGTATTCCG	Genotyping	
UBP3_seq5	CATTGAAAAACAACACCTCAGGG	Genotyping	
Actin-qPCR-F	GGCTCCTCTTAACCCAAAGGC	qPCR	[1]
Actin-qPCR-R	CACACCATCACCAGAATCCAGC	qPCR	[1]
UBP24-qPCR-F1	AAGCAAGTGTAAGAGATT	qPCR	
UBP24-qPCR-R1	TATAAGATGAGAACCAGATTA	qPCR	
UBP24-qPCR-F2	TCGGATAAGAACAATGAT	qPCR	
UBP24-qPCR-R2	TCAGACATAGACAAGTTAT	qPCR	
AHA1-qPCR-F1	GGAAAGAGGGGGAGAAAATG	qPCR	[2]
AHA1-qPCR-R1	GGAGCCAATATCCTTGACGA	qPCR	[2]
AHA1-qPCR-F2	CATCGACAGTAGCGGAGAGA	qPCR	[2]

AHA1-qPCR-R2	GCCAACTCAATAGCAAGCAA	qPCR	[2]
Optimised *UBP24 CDS* without a stop codon	ATGAGTGAAAAGAAGGTATTTGTGTTTGGATCCTTTACAGAACATGAAACTAGGTCATTTTTTGAGCAGAAA CCCACTAAGGATCCTCAGAATTCTAAAGACAAGTGTGTGGGAAGTATTCAATTTGGCTCCTTGAATCTTGCT GCGGAAAATTCTTCAGTAAATACTAATGGCGAGTTGAAGAAAGGTGAGGCTGATGGGACAGTTAAATCTGCT GGGAGTCAAGAGAGACTTGATGCTTCTAGACCTGCCAGCTCGGATAAGAACAATGATAGTGATGCAAAACTC CCAAGAAAGAACTCTTTGAGAGTTCCAGAGCATGTGGTACAAAATGGAATCATAAAAGAGATCTCTGAAAGC AACAAATCTCTCAACAATGGTGTGGCGGTGAAAACAGATCCTATTGGTTTGGATAACTTGTCTATGTCTGAT GGTGAAAGTGATCCTGTGTATAAAGCTTCGAGCTCTAAATTTCAAGCGCTGGACAATGAAGATTTCTCAAGT GACTCTTCGTCTGGAAGCATTCAAAGAAAGAAAAACCTGAAGGTTCCTACTGAATCTGTTCCACCTGTTAAA GACTTTACACCAAGAGGATTAATAAACGCTGGAAACTTGTGCTTCCTGAATGCAACACTACAAGCGTTGCTT TCATGTTCTCCTTTTGTGCAGCTCCTCCAGAAAATTCAACTCCAAGATATTCCAAAGGCTGACTCTCCAACC TTAGCTGCATTTTCCGAGTTCATCTCCGAATTGGATGTGCCAAGCAGTTCCAGCATCAGAAATAACGTTACT GTTGTTGAGGCTGGTAGACCTTTTAGACCTGCCATGTTCGAAGGAGTCCTTAGAAATTTTACCCCAGATGTA CTCAACAACATGTCTGGTAGGCCAAGGCAGGAAGATGCTCAAGAGTTTTTGAGTTTTATAATGGACCAAATG CATGATGAGTTGTTGAAACTCAAGGAACAGTCCCCCAAAGTCACCGCTTCTAAGTCATCGGTTATTTCTTCT GCCAACGATGATGGTGATGAATGGGAAACAGTTGGACCCAAAAACAAATCAGCTGTGACAAGAACACAAAGT TTTGTTCCGTCTGAGCTTAGTGAGATATTTGGCGGGCAGCTTAAAAGCGTAGTGAAGGCAAAAGGAACTAAA GCTTCGGCTACTGTACAGCCGTATCTCTTGCTACACCTTGATATTCATCCGGACGGTGTGCAAGGAATAGAA GACGCGTTGCATTTGTTTTCTGCCCAAGAAGATCTTGAAGGATATAGAGCCTCGGTTACTGGGAAGACTGGT GTAGTGAGTGCTAGTAAGTCGATAAAGATACAGAAACTCTCAAAGATAATGATACTGCACCTAATGCGTTTT AGCTACGGAAGCCAAGGGAGTACTAAGCTGCGTAAAGGCGTTAAATTTCCCCTTGAACTCAACTTAAACCGC AGCCATCTTGTTTCTCTATCCAATGAGAGTTTGAGATATGAACTTGTGGCAACAATTACCCACCACGGATGG GATCCCTCAAAAGGACACTACACCACAGATGCTCGACGAAAGAATGGTCAATGGCTAAGGTTTGATGATGCC TCTGTGACCCCCATAGGGACAAAACTGGTGTTGCACGATCAAGCCTATGTCCTCTTCTACAAGCAAGTG	DNA manipulation	Twist Bioscience

Note. Bold letters indicate the introduction of missense mutations. Underlined letters indicate restriction enzyme recognition sites or overlapping regions for Gateway or Gibson recombination.

Supplementary Table 2. Key resources used in this study

Type	Name	Identifier	Information	Reference
Gene	UBP24	AT4G30890		
Gene	OST1	AT4G33950		
Gene	ABA2	AT1G52340		
Gene	PYR1	AT4G17870		
Gene	PYL1	AT5G46790		
Gene	PYL4	AT2G38310		
Gene	PYL5	AT5G05440		
Gene	PYL8	AT5G53160		
Gene	RAF20	AT1G79570		
Gene	RAF24	AT2G35050		
Gene	AHA1	AT2G18960		
Gene	UBQ1	AT3G52590		
Strain	FY4xFY5 (diploid prototrophic S288C)	GA763		[3]
Strain	*Δubp3*	GA1709	Generated in this study	
Strain	$ubp3^{E691A}$	GA1713	Generated in this study	
Chemical	BbsI-HF	NEB, R3539L	Restriction enzyme for cloning	
Chemical	BsaI-HFv2	NEB, R3733L	Restriction enzyme for cloning	
Chemical	BsmBI-v2	NEB, R0739L	Restriction enzyme for cloning	

Chemical	T4 DNA ligase	NEB, M0202L	Ligase for cloning	
Chemical	EndoLysC	SignalChem, L585-31N	Protease for mass spectrometry analysis	
Chemical	Sequencing grade modified trypsin	Promega, V5111	Protease for mass spectrometry analysis	
Chemical	Tris(hydroxymethyl) aminomethane (Tris base)	AnalytiChem, CL00.2042		
Chemical	Sodium hypochlorite solution	AnalytiChem, CL02.1438		
Chemical	Murashige and Skoog (MS) medium	Duchefa Biochemie, M0222		
Chemical	Sucrose (Saccharose, D[+])	AnalytiChem, CL00.1927		
Chemical	MES monohydrate	Duchefa Biochemie, M1503		
Chemical	Agar No. 4 - plant tissue culture grade	Neogen, NMC0250A		
Chemical	Sodium hydroxide (NaOH)	Supelco, 106498		
Chemical	Potassium hydroxide (KOH)	Supelco, 105012		
Chemical	Potassium chloride (KCl)	Supelco, 104936		
Chemical	Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	Sigma-Aldrich, C3306		
Chemical	Magnesium chloride hexahydrate ($MgCl_2 \cdot 2H_2O$)	Supelco, 105833		
Chemical	Manganese(II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$)	Supelco, 105927		
Chemical	Calcium sulfate dihydrate ($CaSO_4 \cdot 2H_2O$)	Sigma-Aldrich, C3771		
Chemical	Dithiothreitol (DTT)	Duchefa Biochemie, D1309		
Chemical	Ethylenediaminetetraacetic acid (EDTA)	Duchefa Biochemie, E0511		
Chemical	Glycine	Supelco, PHR1111		
Chemical	Glycerol	Sigma-Aldrich, P12352200		

Chemical	Sodium dodecyl sulfate (SDS)	Sigma-Aldrich, 822050		
Chemical	IGEPAL CA-630 (NP-40)	Sigma-Aldrich, I8896		
Chemical	Hydrochloric acid (HCl)	Supelco, 100317		
Chemical	Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, D4540		
Chemical	Abamine SG oxalic acid salt	Chiralix, CX94224		
Chemical	Abscisic acid (ABA)	Sigma-Aldrich, A1049		
Chemical	Cycloheximide (CHX)	Sigma-Aldrich, 01810		
Chemical	Concanamycin A (ConcA)	Santa Cruz Biotechnology, SC-202111A		
Chemical	MG132	Sigma-Aldrich, M8699		
Chemical	Isopropyl β-D-thiogalactopyranoside (IPTG)	Sigma-Aldrich, I6758		
Chemical	adenosine-5′-o-(3-thio)-triphosphate (ATPγS)	Sigma, A1388		
Chemical	p-Nitrobenzyl mesylate (PNBM)	Sigma, SML3810		
Chemical	Difco skim milk	Becton Dickinson, 232100		
Chemical	cOmplete ULTRA Tablets, Mini, EDTA-free, EASYpack protease inhibitor cocktail	Roche, 05892791001		
Chemical	PhosSTOP	Roche, 4906837001		
Chemical	Miller’s LB broth base	Invitrogen, 12795027		
Chemical	NEBuilder HiFi DNA Assembly kit	NEB, E2621L	Enzyme for Gibson cloning	
Chemical	Gateway LR Clonase II Enzyme mix	Invitrogen, 11791	Recombinase for Gateway cloning	
Chemical	Gateway BP Clonase II Enzyme mix	Invitrogen, 11789	Recombinase for Gateway cloning	

Chemical	Urea	AnalytiChem, CL00.2102		
Chemical	1 M Triethylammonium bicarbonate buffer	Sigma-Aldrich, T7408		
Chemical	Tris(carboxyethyl)phosphine (TCEP)	Thermo Scientific, 20490		
Chemical	Iodoacetamide	Sigma-Aldrich, I1149		
Chemical	Acetonitrile (ACN)	Biosolve, 012078		
Chemical	Trifluoroacetic acid (TFA)	Biosolve, 202341		
Chemical	30% Ammonium hydroxide solution (NH_4OH)	Sigma-Aldrich, 221228		
Chemical	Formic acid	Supelco, 100264		
Chemical	Bromocresol Purple	Sigma-Aldrich, 114375		
Commercial kit	4–20% mini-PROTEAN TGX stain-free precast gels	Biorad, 4568093		
Commercial kit	Trans-Blot Turbo mini PVDF transfer packs	Biorad, 1704156		
Commercial kit	SuperSignal West Femto maximum sensitivity substrate	Thermo Scientific, 34095		
Commercial kit	4x NuPAGE LDS sample buffer	Invitrogen, NP0007		
Commercial kit	10x NuPAGE sample reducing agent	Invitrogen, NP0009		
Commercial kit	InstantBlue Coomassie protein stain	Abcam, ab119211		
Commercial kit	GFP-Trap magnetic agarose	Chromotek, gtma		
Commercial kit	RFP-Trap magnetic agarose	Chromotek, rtma		
Commercial kit	Pierce anti-HA magnetic beads	Thermo Scientific, 88837		
Commercial kit	Glutathione Sepharose 4B	Cytiva, 17-0756-01		
Commercial kit	PureCube 100 Ni-INDIGO agarose	Cube Biotech, 75105		

Commercial kit	Pierce 660 nm protein assay kit	Thermo Scientific, 22662		
Commercial kit	C18 ODS SampliQ solid phase extraction (SPE) column	Agilent, 5982-1165	Protein desalting	
Commercial kit	MagReSyn Ti-IMAC magnetic microspheres	ReSyn Biosciences, MR-TIM0010	Phosphopeptide enrichment	
Commercial kit	OMIX C18 pipette tips	Agilent, A57003100	Protein desalting	
Commercial kit	ReliaPrep RNA miniprep systems	Promega, Z6112	Total RNA extraction	
Commercial kit	qScript cDNA synthesis kit	Quantabio, 95047	Complementary DNA synthesis	
Commercial kit	LightCycler 480 SYBR Green I master	Roche, 04887352001		
Commercial kit	QIAprep Spin Miniprep Kit	Qiagen, 27104	Plasmid extraction	
Commercial kit	SapphireAmp Fast PCR Master Mix	Takara, RR350A		
Antibody	Anti-GFP HRP	Miltenyi Biotec, 130-091-833	1:5000, 5% skim milk in PBST or TBST	
Antibody	Anti-RFP	Chromotek, 6G6	1:2000, 5% skim milk in PBST or TBST	
Antibody	Anti-mouse HRP	Cytiva, NXA931	1:5000, 5% skim milk in PBST or TBST	
Antibody	Anti-HA HRP	Abcam, ab1190	1:10000, 5% skim milk in PBST	
Antibody	Anti-thiophosphate ester	Abcam, ab92570	1:5000, 5% skim milk in TBST	
Antibody	Anti-rabbit HRP	Cytiva, NA934	1:10000, 5% skim milk in TBST	
Software	MaxQuant (version 2.1.4.0)		Protein spectrum searching	[4]
Software	Perseus (version 2.0.10.0)		Phosphoproteome data analysis	[5]
Software	PhosR		Data imputation	[6]
Software	BLASTp (web interface)		Protein BLAST	[7]
Software	Clustal Omega (web interface)			[8]

Software	Interactive Tree Of Life (iTOL, version 6, web interface)			[9]
Software	AlphaFold2 and AlphaFold3 server (web interface)		Protein structure prediction	[10]
Software	NAMD (version 3.0b6)		Molecular dynamics (MD) simulation	[11]
Software	CHARMM36m force field		MD simulation parameters	[12]
Software	CHARMM-GUI (web interface)		System manipulation for MD simulation	[13]
Software	MDAnalysis (version 2.9.0)		Analysis for MD simulation results	[14]
Software	Matplotlib (version 3.10.1)		Figure plotting	[15]
Software	Seaborn (version 0.13.2)		Figure plotting	[16]
Software	R (version 4.2)			
Software	Python (version 3.11)			
Software	Fiji (ImageJ, version 1.54g)		Image analysis	[17]

Supplementary methods

Constructs, mutants and transgenic lines

To generate the CRISPR-edited *ubp24* mutant (*ubp24-cripsr*), three guide RNA oligomer pairs were respectively annealed and assembled with pGG-A-AtU6-26-BbsI-ccdB-BbsI-B, pGG-B-AtU6-26-BbsI-ccdB-BbsI-C, and pGG-C-AtU6-26-BbsI-ccdB-BbsI-D[18] by BbsI-HF followed by T4 DNA ligase-mediated ligation. The three entry vectors and pJET-D-linkerII-G were introduced into pFASTRK-AG[18] by BsaI-HFv2 and T4 DNA ligase.

To generate lines expressing *UBP24:YFP-UBP24*, *UBP24:YFP-UBP24(S360A)*, *UBP24:YFP-UBP24(S360D)* in *ubp24-1*, the wild-type *UBP24* coding sequence (CDS) was optimised and synthesised to remove BsaI cutting sites (Twist Bioscience). The synthesised *UBP24* CDS fragment was first assembled into BsaI-linearised pEN-L1-AG-L2[19] by BsaI-HFv2 digestion followed by T4 DNA ligase-mediated ligation. The S360A and S360D mutation on *UBP24* CDS were generated by overlapping polymerase chain reaction, assembled into pEN-L1-AG-L2[19] by same method described above. The 1072 bp *UBP24* promoter cloned from wild-type Col-0 genomic DNA, *YFP* cloned from pGG-D-YFP-E[20], *UBP24* variant CDS cloned from vectors described previously, and NOS terminator cloned from pGG-E-NOST-F[18] were assembled into BsaI-linearised pGGB-AG[18] by NEBuilder HiFi DNA Assembly kit. The optimised UBP24 coding sequence for synthesis is provided in Supplementary Table 1.

To generate wild-type and ubiquitylation-deficient lines expressing *AHA1:RFP-AHA1(WT)* and *AHA1:RFP-AHA1(5KR)* in wild-type and *ubp24-1*, the *AHA1* CDS cloned from wild-type complementary DNA was assembled into BsaI-linearised pEN-L1-AG-L2[19] by NEBuilder HiFi DNA Assembly kit.. The synthesised *AHA1(5KR)* DNA fragment was assembled with pTwist ENTR (Twist Bioscience). The resulting entry vectors were then recombined into the destination vector pB7WGR2 by Gateway LR Clonase II Enzyme mix to generate *35S:RFP-AHA1(WT)* and *35S:RFP-AHA1(5KR)*. The 3417 bp *AHA1* promoter cloned from wild-type Col-0 genomic DNA, and *RFP-AHA1* variants-*35S* terminator cloned from pB7WGR2 containing *AHA1* variant sequences were assembled into BsaI-linearised pGGH-AG[18] by NEBuilder HiFi DNA Assembly kit.

Final expression vectors were transformed into *Agrobacterium tumefaciens* strain C58C1 by heat shock. The *Arabidopsis* transformation was conducted by floral dipping. All primers used are provided in Supplementary Table 1.

For co-IP, to generate *35S:GFP-UBP24*, the entry vector pEN-L1-AG-L2[19] containing *UBP24* CDS was recombined into the destination vector pB7WGF2 by Gateway LR Clonase II Enzyme

mix. The *35S:3×HA-OST1* was described previously[2]. The *35S:RFP-AHA1* was described above in this study.

To generate constructs for recombinant protein expression, the wild-type and S360A UBP24 fragments (311 to 411 amino acids) were cloned from pEN-L1-AG-L2[19] containing *UBP24* CDS variants. The *GST* CDS was cloned from pDEST15 (Invitrogen). The *UBP24* CDS fragment and *GST* CDS were assembled with BsaI-linearised pDEST-RC-BG by NEBuilder HiFi DNA Assembly kit. The wild-type and S175A *OST1* fragments (125 to 225 amino acids) were cloned from pDEST-HisMBP containing *OST1* CDS[2]. The OST1 fragments replaced the linker II of pGG-C-linkerII-G by BsaI-HFv2 and T4 DNA ligase. The *OST1* fragment from pGG-C-OST1-G and *GST* from pGG-B-GST-C were assembled into pDEST-RC-BG by BsaI-HFv2 and T4 DNA ligase. The CDS of RAF20 kinase domain were cloned from wild-type *Arabidopsis* complementary DNA and assembled with BsaI-linearised pEN-L1-AG-L2[19] by NEBuilder HiFi DNA Assembly kit. The entry vector containing CDS of RAF20 kinase domain were then recombined into the destination vector pDEST-HisMBP[21] by Gateway LR Clonase II Enzyme mix. All primers used are provided in Supplementary Table 1.

To generate HA-tagged ubiquitin, *UBQ1* CDS was synthesised (Twist Bioscience) and assembled into linearised pDONR221 by NEBuilder HiFi DNA Assembly kit. The entry vector together with pEN-L4-3×HA-R1 was recombined into the destination vector pB7m24GW2 by Gateway LR Clonase II Enzyme mix. To generate an enzyme-dead mutation of UBP24, the C206S mutation on *UBP24* CDS were generated by overlapping polymerase chain reaction, assembled into pEN-L1-AG-L2[19] by BsaI-HFv2 digestion followed by T4 DNA ligase-mediated ligation. Final expression vectors were transformed into *Agrobacterium tumefaciens* strain C58C1 by heat shock. The *35S:GFP*-UBP24 and *35S:RFP-AHA1* was described above in this study. To generate constructs for HA-tagged single lysine K48 and K63 ubiquitin, the ubiquitin CDS fragments were generated by three pairs of annealed primers with mutations and assembled into BsaI-linearised pEN-L1-AG-L2[19] by NEBuilder HiFi DNA Assembly kit. The entry vector together with pEN-L4-3×HA-R1 was recombined into the destination vector pB7m24GW2 by Gateway LR Clonase II Enzyme mix. Final expression vectors were transformed into *Agrobacterium tumefaciens* strain C58C1 by heat shock.All primers used are provided in Supplementary Table 1.

For BiFC, *UBP24* CDS, *AHA1* CDS and *GST* CDS cloned from pDEST15 (Invitrogen) were firstly recombined into pDONR221 P3-P2, pDONR221 P1-P4 and pDONR221 P1-P4, respectively, by Gateway BP Clonase II Enzyme mix. The pDONR221 P1-P4 containing *OST1* CDS was described previously[2]. To generate constructs for BiFC, two indicated protein CDSs

from the entry vectors were recombined into the destination vector pBiFCt-2in1-NN[22] by Gateway LR Clonase II Enzyme mix. All primers used are provided in Supplementary Table 1.

Yeast strain construction

The sgRNA/Cas9 vector pV1382 (Addgene plasmid #111436) was obtained from Gerald Fink[23]. The plasmid was linearised using BsmBI-v2. 20-nucleotide guide RNAs (sgRNAs) targeting the UBP3 locus (upstream and inside UBP3 coding sequence) were designed using CRISPOR web tool[24], flanked by homology arms matching the overhangs generated via the linearization, and synthesised as complementary 5'-phosphorylated oligonucleotides (Supplementary Table 1) by Integrated DNA Technologies. The sgRNA oligonucleotides were hybridised and ligated into the linearised plasmid using T4 DNA ligase, followed by heat-shock transformation into *E. coli* strain DH5α. Plasmids were extracted using QIAprep Spin Miniprep Kit. For the *UBP3* deletion, a 90-bp repair DNA template was synthesised, containing 45-bp homology arms flanking the *UBP3* coding sequence and the Cas9-targeted site located upstream of the gene. To introduce the *ubp3*^{E691A} mutation, the repair template was designed to contain homology arms flanking the Cas9 cleavage site and to bear required mutation (GAG codon substituted with GCC). *S. cerevisiae* cells were co-transformed with the sgRNA/Cas9 plasmid and the repair template using high-efficiency DMSO/lithium acetate/polyethylene glycol protocol[25]. Transformants were selected on YPD medium (1% yeast extract, 2% peptone, 2% glucose) containing nourseothricin (cloNAT, 100 μg/mL), and successful edits were verified via PCR using SapphireAmp Fast PCR Master Mix and Sanger sequencing. The strains employed in the present work are summarised in Supplementary Table 2.

Stomatal bioassay by imprinting

The procedure was described previously[26,27] with some modification. 21-day-old *Arabidopsis* seedlings were firstly incubated at 21°C under darkness for 2 hours, and then transferred to a 42°C dark incubator for 2 hours. The abaxial epidermis of the third true leaves was negatively imprinted by the mixture of the catalyst and polysiloxane (3M ESPE Express light body). After totally solidification, the mixture was departed from the leaves and made a positive imprint with transparent nail varnish. The positive imprints were imaged by a TM1000 scanning electron microscope (Hitachi). The stomatal apertures (stomatal width divided by length) were measured from the images by Fiji (ImageJ) software.

MG132 treatment

The 10-day-old Arabidopsis seedlings grown on ½ MS plates containing 1% sucrose, 0.5% MES and 0.8% (w/v) plant agar, pH adjusted to 5.7 with KOH were transferred to 12-well plates containing ½ MS medium containing 1% sucrose, 0.5% MES and 50 μM MG132, pH adjusted to 5.7 with KOH. After incubation for 16 hours, the seedlings were ground by liquid nitrogen, homogenised in 1x Laemmli sample buffer (62.5 mM Tris base, 2% SDS and 10% glycerol, pH adjusted to 6.8 with HCl) and incubated at 70°C for 10 minutes prior to SDS-PAGE and western blotting.

Gene expression

10-day-old *Arabidopsis* seedlings grown at 21°C under the long day condition were harvested for gene expression. Total RNA was extracted using ReliaPrep RNA miniprep systems. The synthesis of complementary DNA was performed with 1 μg of RNA with a qScript cDNA synthesis kit. Quantitative reverse transcription polymerase chain reaction was performed on a LightCycler 480 (Roche Diagnostics) with LightCycler 480 SYBR Green I master. All steps were followed according to the manufacturer's guidelines. All primers used are provided in Supplementary Table 1.

Root acidification assay

The procedure was described previously[28] with some modification. Seeds were germinated on regular ½ MS plates containing 1% sucrose, 0.5% MES and 0.8% (w/v) plant agar, pH adjusted to 5.7 with KOH. After 10-day growing under the long day condition at 21°C, the seedlings were washed with Milli-Q water and transferred to a ½ MS plate containing 1 mM $CaSO_4$, 0.8% (w/v) plant agar and 0.006% bromocresol purple, pH adjusted to 5.7 with HCl and KOH. The images were taken after additional 10-day growing.

Root growth assay

Seeds were germinated on regular ½ MS plates containing 1% sucrose, 0.5% MES and 1% (w/v) plant agar, pH adjusted to 5.7 with KOH. After 7-day vertically growing under the continuous light condition at 21°C, the seedlings on plates were imaged with scanner Expression 11000XL (Epson). The primary root length was measured by Fiji (ImageJ) software.

Ubiquitylation assay for chain type determination

35S*:RFP-AHA1* or 35S*:RFP-AHA1(5KR)* was co-infiltrated with *35S:*3×*HA-UBQ1*, *35S:*3×*HA-Ub(K48)* or *35S:*3×*HA-Ub(K63)* into *Nicotiana* leaves. One day before harvesting, the leaves were treated with 1 μM concanamycin A. The materials were ground with liquid nitrogen and homogenised with extraction buffer. The soluble protein extract was incubated with Pierce anti-HA magnetic beads at 4°C for 1 hour. After washing three times with the extraction buffer, the beads were resuspended in 1x NuPAGE LDS sample buffer and then incubate at 70°C for 10 minutes. The supernatants were loaded on the gel for SDS-PAGE and western blotting.

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