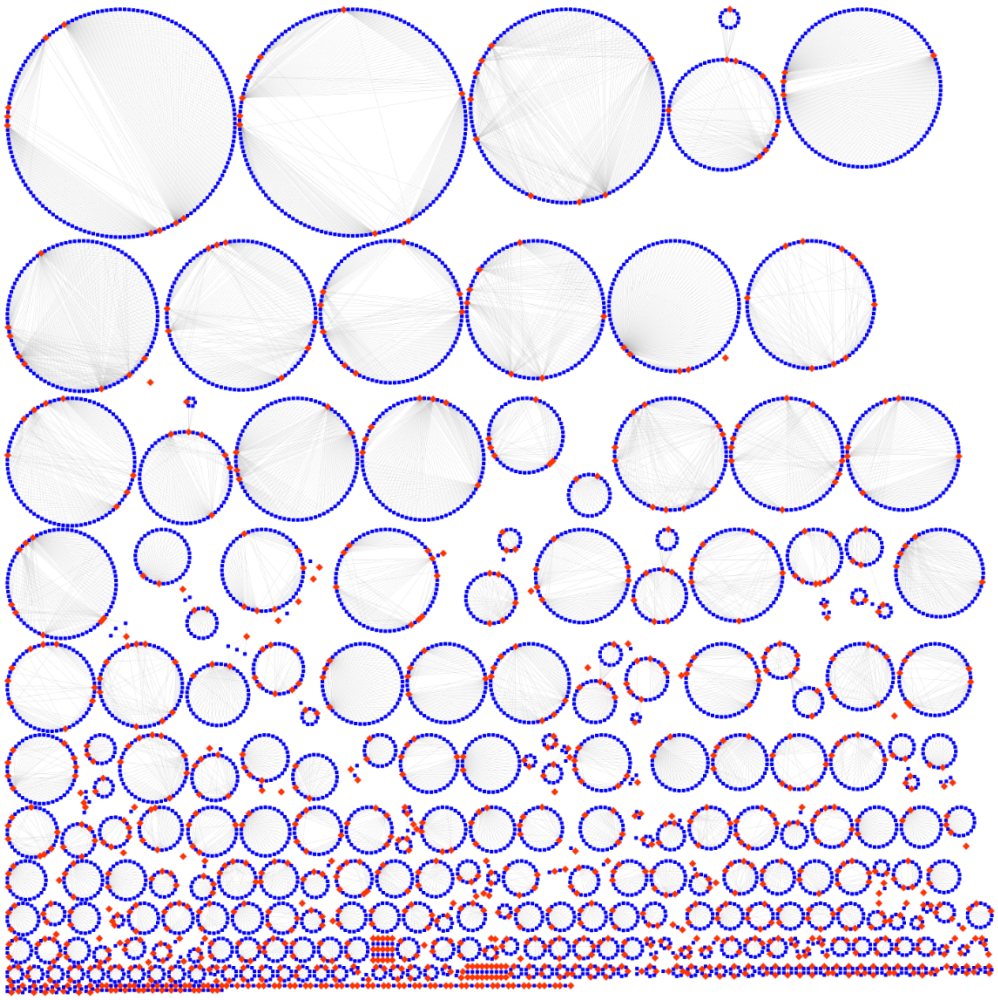
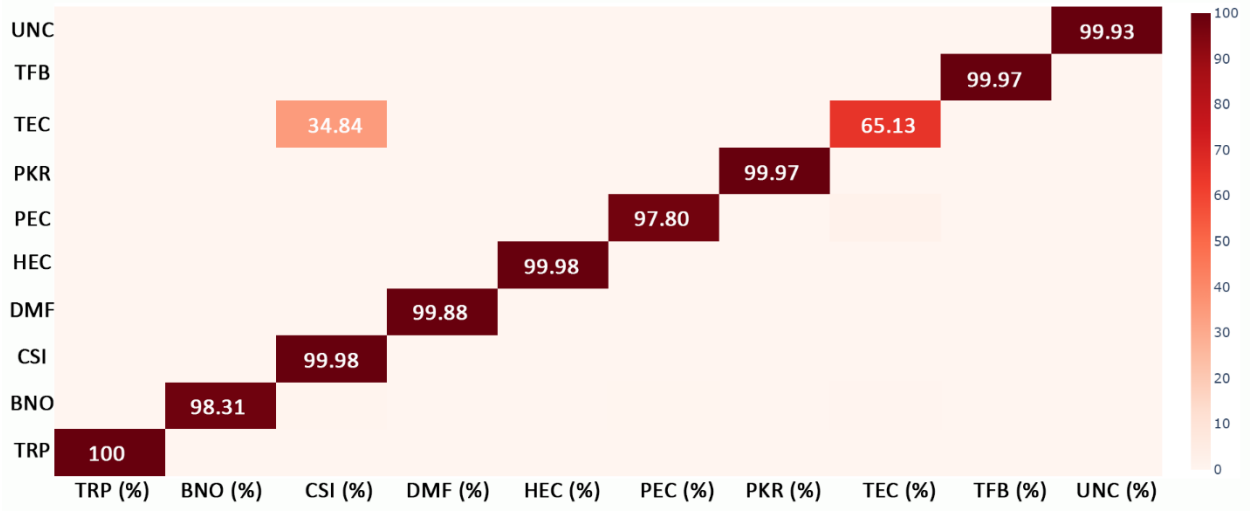
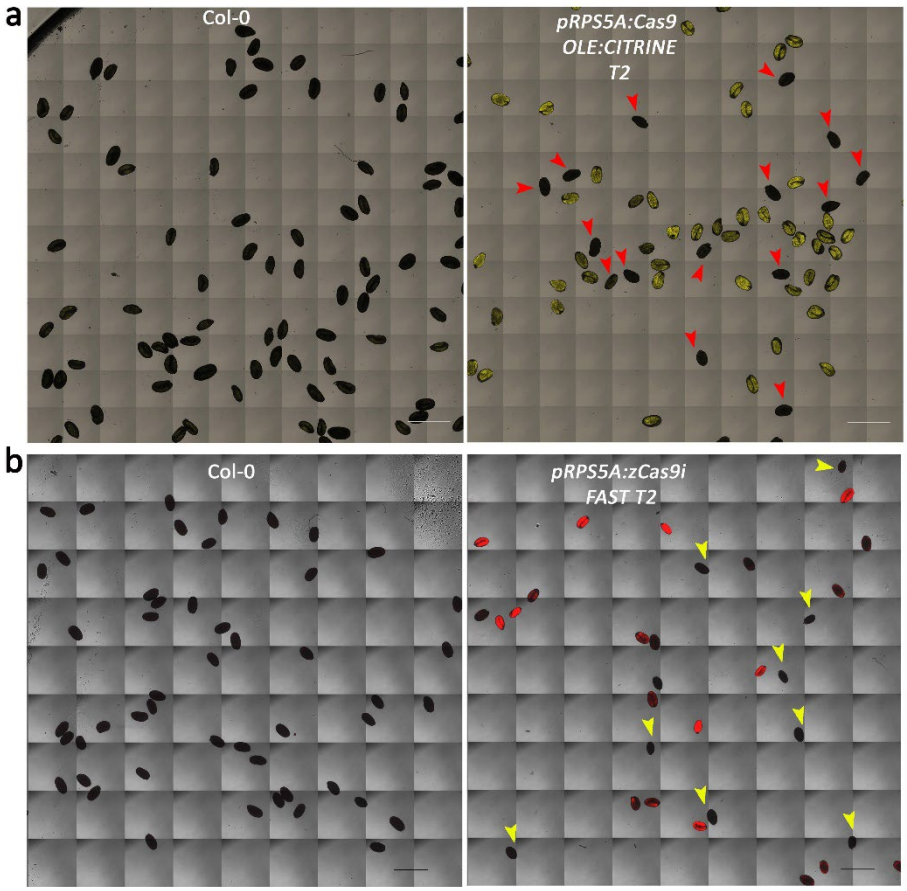


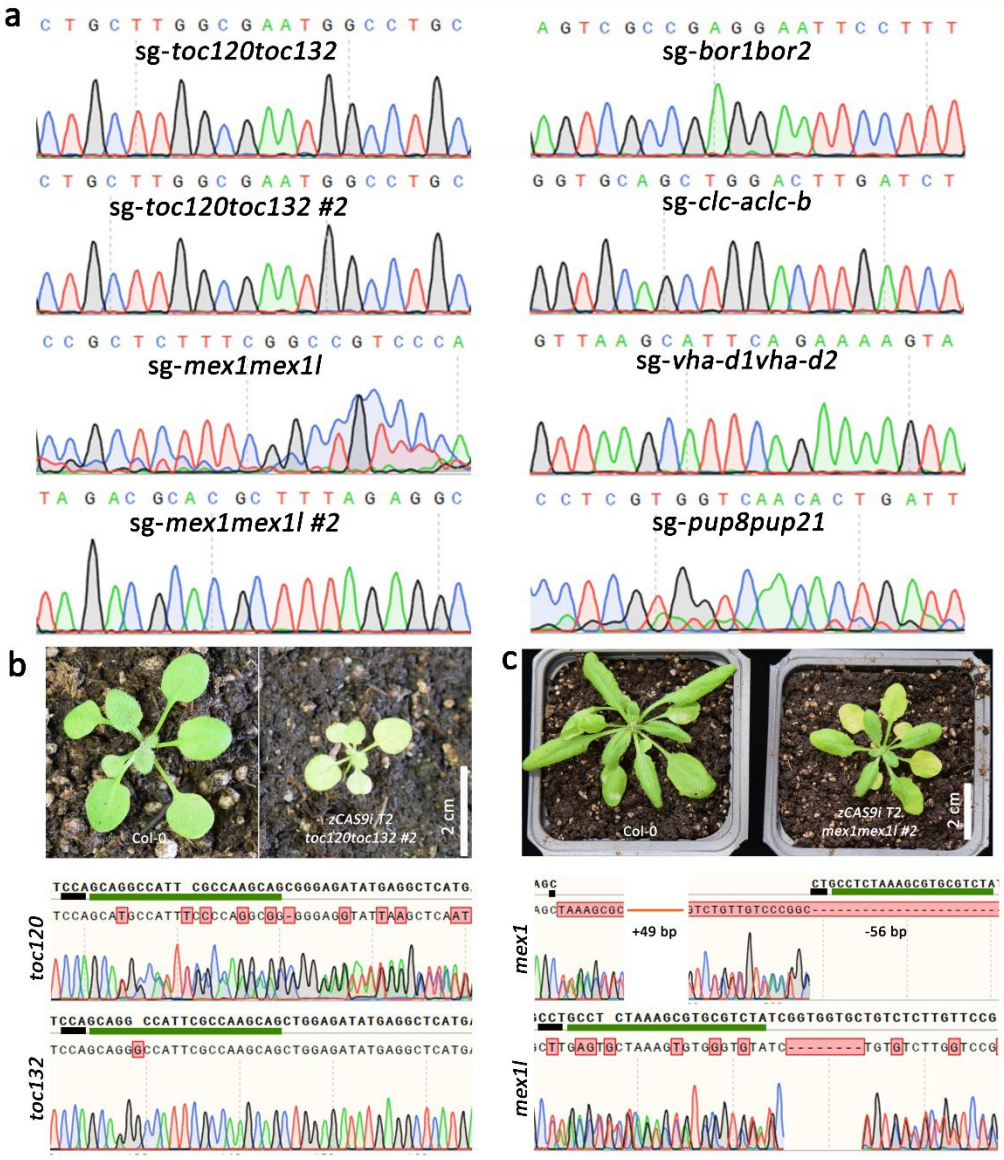
Supplementary Figure 1: Transportome Multi-Knock network. Overview of sgRNAs (blue) and the targeted genes (red) network for the CRISPR library targeting the transporters family. Shown are 1,123 transporter genes (red) and 5,635 sgRNAs (blue).



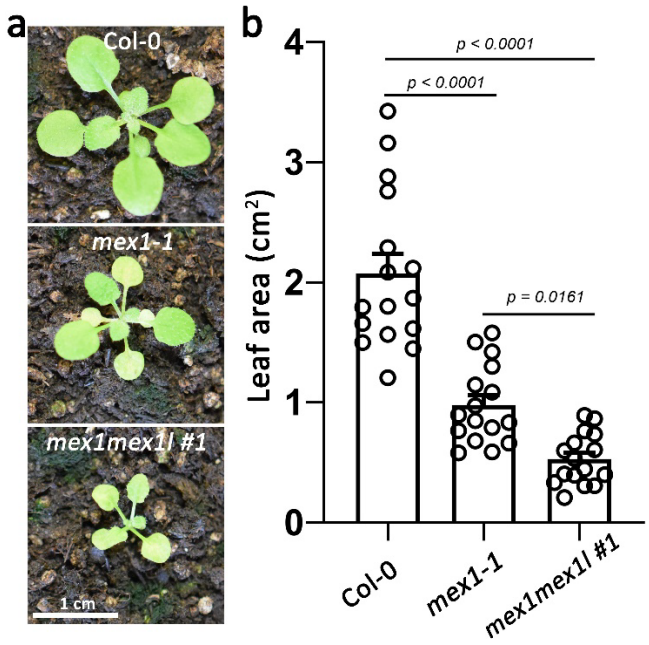
Supplementary Figure 2: sgRNAs cross-contamination analysis of the ten sub-libraries based on next-generation sequencing. Heatmap illustrating the percentage of sgRNAs for each individual sub-library. The color scale bar reflects the relative percentage. All libraries are highly specific, except for TEC which shows significant amplification of the CSI sub-library. TRP (transporters); PKR (protein kinases, protein phosphatases, receptors, and their ligands); TFB (transcription factors and other RNA and DNA binding proteins); BNO (proteins binding small molecules); CSI (proteins that form or interact with protein complexes including stabilizing factors); HEC (hydrolytic enzymes); TEC (metabolic enzymes and enzymes that catalyze transfer reactions); PEC (catalytically active proteins, mainly enzymes); DMF (proteins with diverse functional annotations not found in the other categories); and UNC (proteins of unknown function or cannot be inferred).



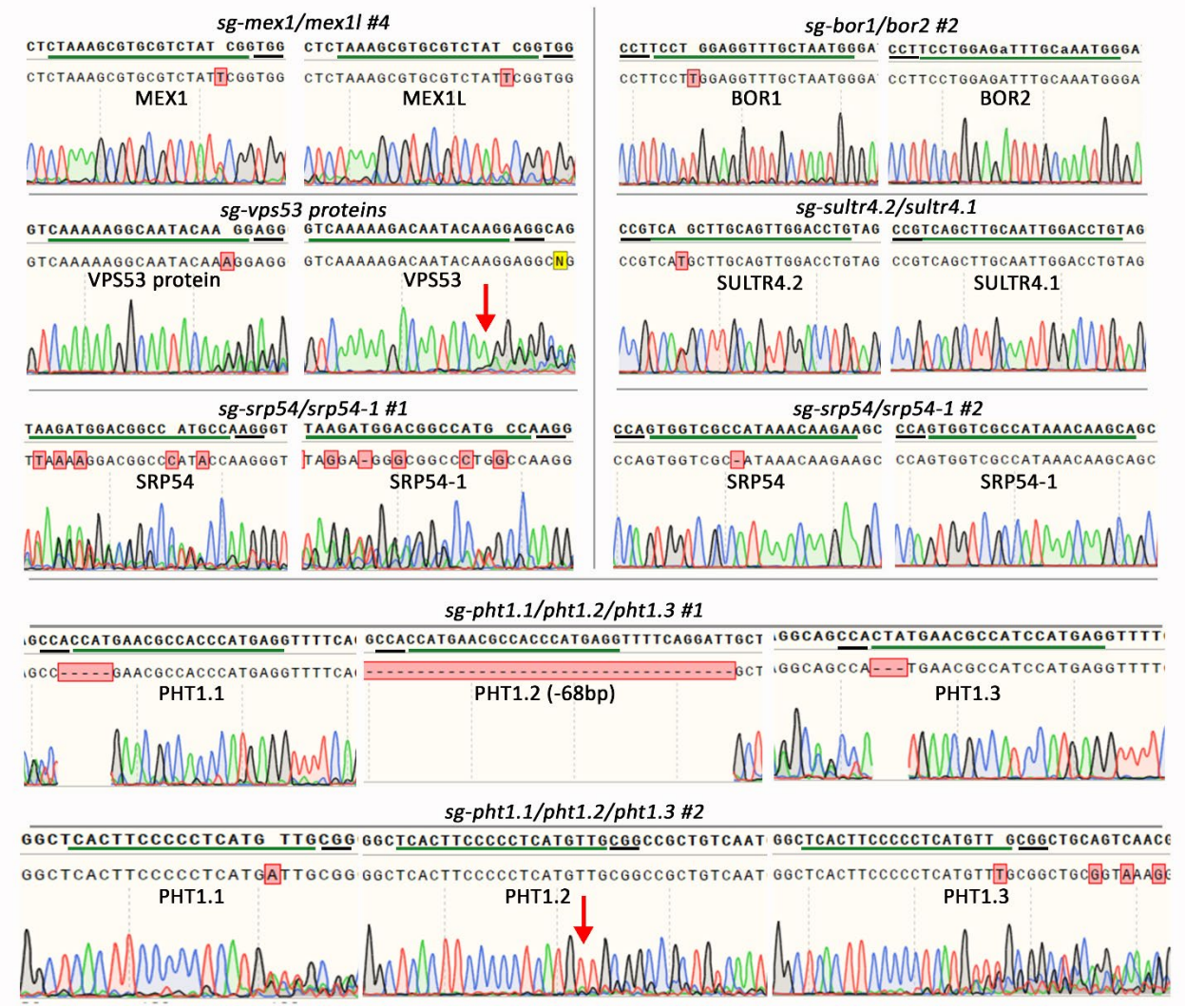
Supplementary Figure 3. Visual fluorescence segregation for Cas9-free seeds. a, Cas9-free seeds selection in pRPS5A:Cas9 OLE:CITRINE T2 seeds. Yellow signal in seeds indicates for OLE:CITRINE. The Cas9-free seeds, which do not produce the yellow fluorescence, are marked by red arrows. Scale bar = 1 mm. **b,** Cas9-free seeds selection in pRPS5A:zCas9i *FAST* T2 seeds. The Cas9-free seeds, which do not produce the red fluorescence, are marked by yellow arrows. FAST (pOLE1:tagRFP) [1]. Scale bar = 1 mm.



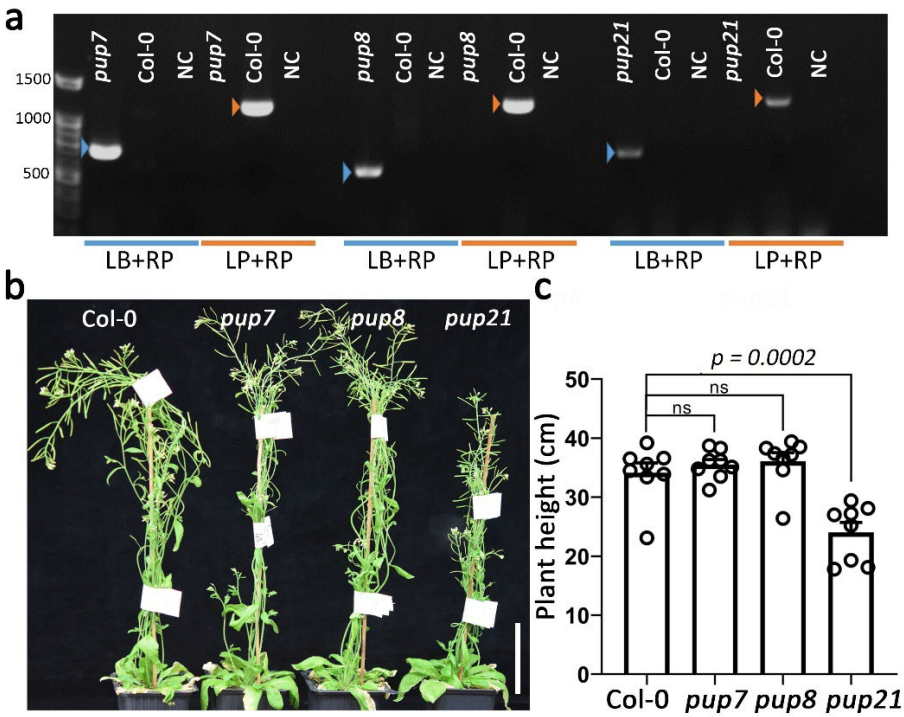
Supplementary Figure 4. Sequencing data for multiple sgRNA and their putative target genes. a, Chromatograms show sgRNA sequences of the indicated TRP Multi-Knock lines. Lines are presented in Fig 4. Two additional lines are presented for *toc132toc120* and *mex1mex1l* (#2). **b,c,** Shown are *toc132toc120* (**b**) and *mex1mex1l* (**c**) knockout alleles, which are additional to the lines presented in Fig 4. Photographs show an independent line for *toc120toc132* #2 (left). Chromatograms indicate for the types of mutation (left). Images show a similar independent line for *mex1mex1l* #2 (right). Chromatograms indicate for the types of mutation (right). Scale bar = 2 cm; PAM is marked with a black underline; 20-bp gRNA is underlined in green.



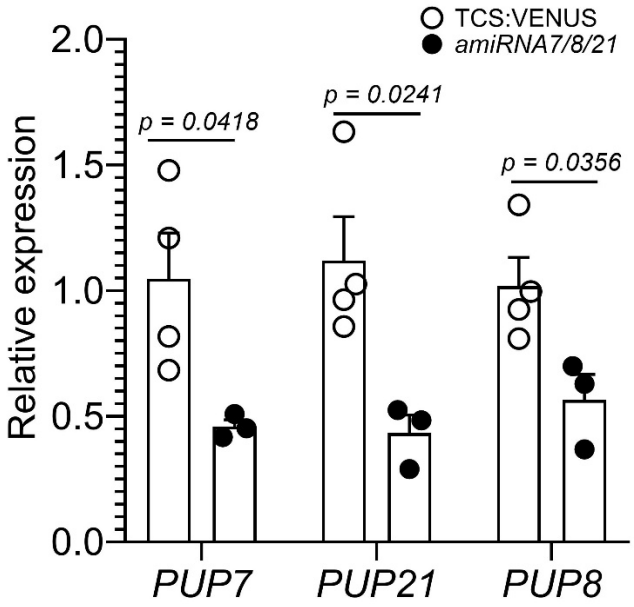
Supplementary Figure 5. *mex1* and *mex1l*, single and double knockout phenotypes. a, Representative images showing shoot phenotypes of 18-day-old of the wild type (Col-0), single (*mex1-1*) and double mutant homozygous plants (*mex1mex1l T3 #1*). Scale bar = 1 cm. **b,** Quantification of phenotypes shown in (a). Shown are means (±SE), n = 16 plants, *p* values were determined by One-way ANOVA.



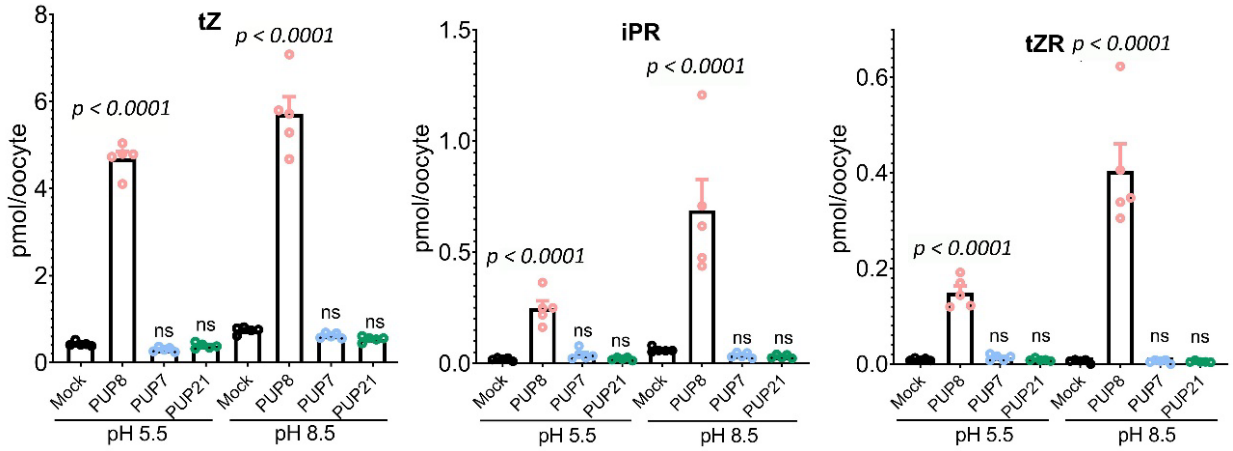
Supplementary Figure 6. Single, double, and triple mutants generated by Multi-Knock. Shown are Sanger sequencing chromatograms indicating the type of mutation in T2 lines. mex1mex1l #4 and bor1bor2 #2 are additional lines (independent lines) to the ones presented in Fig. 4 and Sup. Fig. 4. Two similar independent lines with different sgRNAs targeting the same targets (Phosphate transporters: PHT1.1, PHT1.2, and PHT1.3) show different mutation types. The ambiguous peak is indicated by a red arrow. PAM is marked with a black underline; 20-bp gRNA is underlined in green.



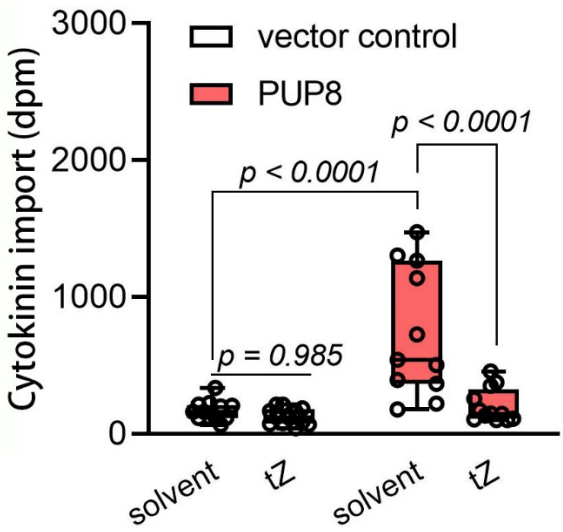
Supplementary Figure 7: Characterization of single T-DNA insertion mutants. a, Genotyping of the *pup7*, *pup8*, *pup21* single mutant plants compared to Col-0. Orange arrows indicate for WT amplification bands, blue arrows indicated for T-DNA insertion amplification bands. LB: left border primer of T-DNA insertion, LP: left genomic primer, RP: right genomic primer, NC: negative control. **b**, Plant height of 58-day-old plants of the indicated genotypes. **c**, Quantification of plant height. Shown are means (±SE), n = 8 plants, *p* values were determined by One-way ANOVA. Scale bar = 5 cm.



Supplementary Figure 8: *PUP7*, *PUP8*, and *PUP21* are down-regulated in *amiR7/8/21*. Relative expression of the indicated *PUP amiR7/8/21*-targeted genes in 15-day-old seedlings, quantified by qRT-PCR. *amiRNA7/8/21* stands for amiRNA triple knockdown *PUP7/8/21*. Shown are averages (±SE), n = 4 (Control), n = 3 (*amiR7/8/21*); *p* value two tailed *t* test is indicated for each analysis.



Supplementary Figure 9: PUP8 promotes cytokinin uptake in *Xenopus laevis* oocytes. 60 min transport assay in 10 µM cytokinin at pH = 5.5 and pH = 8.5. n = 5 ±SE, *p* values were determined by One-way ANOVA.



Supplementary Figure 10: PUP8 tZ competition assays. 3H-tZ import into microsomal fractions prepared from tobacco leaves infiltrated with vector control and 35S:YFP-PUP8 (PUP8), conducted in the absence (solvent) and presence of a 1,000-fold access of non-labelled tZ. Significant differences of means to solvent control were determined by ordinary One-way ANOVA, n = 12 (vector control - solvent and tZ; and PUP8 - tZ), n = 11 (PUP8 - solvent). Box plots represent 25th-75th percentiles; whiskers represent min-max (with all points), central bands in the box plot show the medians.

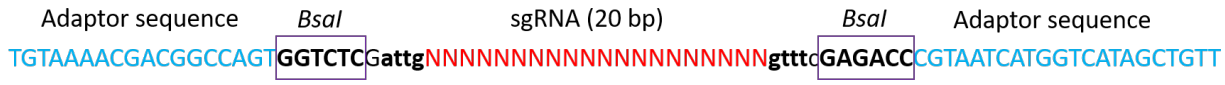
Supplementary Table 1. Overview of sgRNAs and gene numbers per family.

Families	Targeted genes per family (Coverage %)	Genes per family	sgRNAs per family
BNO	1443 (76.1%)	1896	5899
CSI	1399 (82.7%)	1692	4919
DMF	1343 (71.0%)	1891	5000
HEC	1438 (84.3%)	1706	6215
PEC	1252 (82.6%)	1515	4975
PKR	1190 (84.7%)	1405	6161
TEC	1041 (86.0%)	1211	4145
TFB	2042 (78.2%)	2611	6010
TRP	1123 (84.6%)	1327	5635
UNC	3881 (59.3%)	6544	10170

Supplementary Table 2. Adaptor sequences for each Multi-Knock group.

Adaptor sequences are marked in blue.

Pools	Pool description	Adaptor sequence + sgRNA
PKR	Protein kinases, protein phosphatases, receptors, and their ligands	CTGGTCATCATCCTGCCTTTggtctcGattgNNNNNNNNNNNNNNNNNNN GTTTcGAGACCTGTTATATGGAGGGGGCAAA (82 bp)
BNO	Protein binding small molecules	GGATTATTCATACCGTCCCAggtctcGattgNNNNNNNNNNNNNNNNNNN GTTTcGAGACCAATCAGCCATACCACATTTG (82 bp)
CSI	Proteins that form or interact with protein complexes including stabilization of those	AGAGCTCGTTTAGTGAACCGggtctcGattgNNNNNNNNNNNNNNNNNNN GTTTcGAGACCGATGAGTTTGGACAAACCAC (82 bp)
TFB	Transcription factors	CGTTGGCTACCCGTGATATTggtctcGattgNNNNNNNNNNNNNNNNNNN GTTTcGAGACCCTATTCGGCTATGACTGGGC (82 bp)
TEC	Metabolic and other enzymes catalyzing transfer reactions	TGTAAAACGACGGCCAGTggtctcGattgNNNNNNNNNNNNNNNNNNN GTTTcGAGACCCGTAATCATGGTCATAGCTGTT (82bp)
PEC	Active catalytic proteins, mainly enzymes	GGAAACAGCTATGACCATGggtctcGattgNNNNNNNNNNNNNNNNNNN GTTTcGAGACCGTCGTGACTGGGAAAACC (79 bp)
UNC	Genes for which the function is not known or cannot be inferred	CGACTCACTATAGGGAGAGCGGCggtctcGattgNNNNNNNNNNNNNNNNNNN GTTTcGAGACCCTGCCATGGAAAATCGATGTTCTT (89 bp)
DMF	A protein with diverse functional annotation not found in the other categories	TCCTCCGCTTATTGATATGCggtctcGattgNNNNNNNNNNNNNNNNNNN GTTTcGAGACCCCTTGTTACGACTTTTACTTCC (84 bp)
HEC	Hydrolytic enzymes, excluding protein phosphatases	GCGAGAGTAGGGAACTGCggtctcGattgNNNNNNNNNNNNNNNNNNN GTTTcGAGACCGTGTCTCAAAATCTCTGATGTT (82 bp)
TRP	Transporters	CCAAGCTATTTAGGTGACACggtctcGattgNNNNNNNNNNNNNNNNNNN GTTTcGAGACCTAGAAGGCACAGTCGAGG (80 bp)



Example of the sgRNAs Multi-Knock oligonucleotides design. The 20-bp sgRNA is shown in red letters and two *BsaI* sites are marked by purple rectangles. Adaptors are shown in blue.

Supplementary Table 3. Primers for amplification of subgroups of sgRNA library

Pools	Forward primer（5'-3'）	Reverse primer（5'-3'）
PKR	CTGGTCATCATCCTGCCTTT	TTTGCCCCCTCCATATAACA
BNO	GGATTATTCATACCGTCCCA	CAAATGTGGTATGGCTGATT
CSI	AGAGCTCGTTTAGTGAACCG	GTGGTTTGTCCAAACTCATC
TFB	CGTTGGCTACCCGTGATATT	GCCCAGTCATAGCCGAATAG
TEC	TGTAAAACGACGGCCAGT	AACAGCTATGACCATGATTACG
PEC	GGAAACAGCTATGACCATG	GGTTTTCCCAGTCACGAC
UNC	CGACTCACTATAGGGAGAGCGGC	AAGAACATCGATTTTCCATGGCAG
DMF	TCCTCCGCTTATTGATATGC	GGAAGTAAAAGTCGTAACAAGG
HEC	GCGAGAGTAGGGAACTGC	AACATCAGAGATTTTGAGACAC
TRP	CCAAGCTATTTAGGTGACACGG	CCTCGACTGTGCCTTCTAGG

Supplementary Table 4. Primers for NGS PCR amplification and sgRNAs genotyping in transgenic plants.

Cas9 expression constructs	Forward primer（5'-3'）	Reverse primer（5'-3'）
pRPS5A:zCas9i	TACTAGATCGACGCTACTAG	CCGACTCGGTGCCACTTTT
pUBI:Cas9	CCCCTGGGAATCTGAAAGAAG	CCGACTCGGTGCCACTTTT
pEC:Cas9	CCCCTGGGAATCTGAAAGAAG	CCGACTCGGTGCCACTTTT
pRPS5A:Cas9 OLE:CITRIN	CCCCTGGGAATCTGAAAGAAG	CCGACTCGGTGCCACTTTT

Supplementary Table 5. Primers used for PCR-based genotyping.

Targets	Forward primer（5'-3'）	Reverse primer（5'-3'）
TOC120	CTCTTACGCACCATCACTG	GCTTCGGCAAGAATCTTGG
TOC132	TCTCCTGCGCACCATAAGT	TCACCATACTGCTGTTCAG
MEX1	AGGGAAATCCAGGTTTGGG	CTGCCACAGCAATACCAAAAG
MEX1L	AGGGAAATCCAGGTTTGGG	GAGTAGAGACCACTCCCAG
BOR1	GCTTTTCATGCAGCAAGCCA	GAACATCAAGCATCTCCTGC
BOR2	GGCCTTCTTATAGCTATGCTC	GGAACTTGAAGCATCTCCTGA
CLC-a	GACACTCCCAATATGTTTGG	CAGAGGCAGATCCACATGT
CLC-b	GGAATCCCTGAGATCAAAGC	AGTACACCTCCAACAGGTG
VHA-d1	CCGCATCAACCTTTTCCATC	GAGGGTGTTCCTCATAATCTC
VHA-d2	CCCTTAGCTGTTTCATAACCC	CAAGGTATGCTTTGTAGAGGG
PUP7	CTCTTTTGCCAGCCACTAGCT	CCACAAGAAGAGCAGAGGATACAG
PUP8	CCCAAACCAAAAATCCCAAACC	CCAGGGATAGCAGCAATCCAA
PUP21	TTTCAGTACCTCAAACGAAGAACTGT	AGTACCACACAAGTTGCAACTAG
VPS53	GCTTGTGGCCCAAGTACATC	GGTAGCCTAGCGTTAAATTTG
VPS53-P	GATGTTGCTGGTTAGCCTCTT	GGGAGAGTAATCTGCTAGGTAG
SULTR4.1	GAAGTACTCCTGCTCTTGG	AGACGCAACATGACACAAC
SULTR4.2	GGCTTCAACCAATATACGG	CGAATAAGCCATCCAAGCC
SRP54	TACTGAGTTGTGATTCTTGC	AATGCTAAATCAATGCCATGA
SRP54-1	GCTGACAGGAAAAATGCTAAT	CTCATCCATATGCTCTCCTGTTC
PHT1.1	CTCTAGGAAATGGCCGAA	CCATTTAGTTGGATCTTAAACC
PHT1.2	GAGAGGCTTAGATGGCTGA	CCATTTAGTTGGATCTTAAACC
PHT1.3	GCGTACGATCTCTTTTGTGT	CATTAGTTGGATCGCAAACC

Supplementary Table 6. Genotyping primers for T-DNA lines

Gene	T-DNA Line	Primer name	Primer sequence (5'-3')
PUP7	SALK_084103	LP	GTAGAGCCGCCATTCAAAAG
		RP	GAGAACTACACAACTCGCAAC
		LB	ATTTTGCCGATTTCGGAAC
PUP8	SALK_137526	LP	TCGTTGTTGACTTCCAAATCC
		RP	TGAGGATCAGTTGTACCAGGG
		LB	ATTTTGCCGATTTCGGAAC
PUP21	GK-288E11	LP	AGCAAAAGGTACCTGATGACG
		RP	CTTTTCGTCGAGGTAGTGCTG
		LB	ATAATAACGCTGCGGACATCTACATT

Supplementary Table 7. *PUP* cloning

Cloning primers. Gene	Forward primer（5'-3'）	Reverse primer（5'-3'）
PUP7 genomic	caccATGGACAGATCTCAAGAACACTATGCC	TCACACACTTTGTATGTTTGTGTGACC
PUP8 CDS	caccATGGAAATAACTCAAGTAATCTATGT	TCATACACTATGTATGTTTGTGTGACCTTCC
PUP21 CDS	caccATGGGCATATCTCAAGTACACTATTGC	TCATAAAGTTTGTGTTTCTTCCTCAACAGGTT

Supplementary Table 8. MRM transitions for LC-MS/MS analysis.

Analyte	Retention Time [min]	Q1 [*m/z*]	Q3 [*m/z*]	CE [eV]	Reference
tZ $[M+H]^+$	1.25	220.0	136.0^{Qt}	15	(Ionescu et al. 2017)
tZR $[M+H]^+$	1.40	352.0	220.0^{Qt}	15	(Ionescu et al. 2017)
iPR	1.60	336.1	204.0^{Qt}	15	(Ionescu et al. 2017)
$[M+H]^+$		336.1	136.0	27	
		336.1	148.0	23	

Qt = quantifier ion, additional transitions were used for identification only; CE=collision energy; Q=quadrupole.

References:

1. Shimada, T. L., Shimada, T. & Hara-Nishimura, I. A rapid and non-destructive screenable marker, FAST, for identifying transformed seeds of Arabidopsis thaliana: TECHNICAL ADVANCE. *Plant J.* **61**, 519–528 (2010).