

Supplementary Information for

TIC236 gain-of-function mutations unveil the link between plastid division and plastid protein import

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Other supplementary materials for this manuscript include the following:

Datasets S1 to S5

Arabidopsis Escherichia	MSLRLQNPFLSTPLLHGSFNRREKRINVARRAFRSKRIYSEKKQNDWLAKVAKFSQFCGKNVQLLRKSLDSRSRMEVKCLKEPFVRSKDLVRSLAPVWEEGLFFLRCSVFFAVISGVCLL 	120 7
Arabidopsis Escherichia	VWYGQNKARVFVETKLLPSVCSVLSETIQREVDFGKVRRVSPLCITLEASSIGPHGEEFSCGEVPTMKVCVRPFASLRRGKIVVDAILSNPTVLVAQKKDFTWLGIPLSDTTLPSHLSSE SLGVVI	240 13
Arabidopsis Escherichia	EGIDFRTKTRRVSREEAGIRWDEERDNDARKAAEIGYIVPCKNYSQAKDNAVKHDRRFTEIANPNSFICMDEKMHSAEQHCMDPGVEYDVKHAELEKSFGIKIPGSGLKFLSKMLKVPRK	360 13
Arabidopsis Escherichia	YKFKWNSKSHKNSMSNISAKKRILERSASAALSYFHSLSQQKLDEPSVLSTNYDGLSLDMLLVKGDREISNQYDRHVPYGEQSLANDLDGKGYRVRGKRLLGVKKASTLDKFTVSCDPFL	480 13
Arabidopsis Escherichia	MTVDRLCALLQTKRSPSVEDIVNSSESETLSSQRGDISMNVVNQNTDDVPHGNRSGNQPRDFTFKKHEHQPVANHWRPSWPRNKKLKEAVFNILTGSSKKLTGRADPNAPHLSDELEKLP	600 13
Arabidopsis Escherichia	AVYVEKTLPVMLDSVQFKGGTLLLLAYGDTEP-REMRNVHGHVKFQNHYGRVYVQLGGNCNMWRSDVTSEDGGLLSVDVFVDTVEQNWHANLNVANFFVPIF VILLLLGSVAFLVGTTSGLHLVFKAADRWVPGLDVCKVTGGWRDLTLSDVRYEQPGVAVKAGNLHLAVGLEC-LWNSSVCINDLALKDIQVNIDSKKMPPS .:::*.*** *:::**** .:::*.*** *:::****	701 113
Arabidopsis Escherichia	ERILEIPIEWSKGRATGEVHLCMSRGESFPNLHGQLDVTGLGFHINDAPSSFSDVSASLSFRGQRIFLHNANGWFGKVPLEASGDFGIHPDEGEFHLMCQVPYVEINALMKTFKMKPLFF EQVEEEEDSGPLDLSTPYPITLTRVALDNVNIKIDDTTVSVMDFTSGLNWQEKTLTLKPTSLKGLLI *: .: .: *: .: .: .: *: .: .: .:	821 180
Arabidopsis Escherichia	PLAGSVTAVFNCQGPLDAPVFVGSCMVSRKIAYLSPDLPTSLAYEAMLKNKEAGAVAAFDRVPFSYLSANFTFNTDNCVADLYGIRATLVDGGEIRGAGNAWICPEGEVDDTALDVNFSG AL *	941 182
Arabidopsis Escherichia	NISFDKVLHRYMPEYFNIGMLKLGDLTGETKLSGALLKPRFDIKWAAPKADGSLTDARGDIVISHDNIIVNSSSVAFDLFTKLDTSYHDPCLSHQDFTQGEAMPFVVEGLDLDLRMRGFE -PKVAEVAQEEVVEPKIENPQPEEKPLGETLKDLFSRPVLPEMTDVHLPLNLNIEFK *:.::: * :::::: * :: * :: * :: * :: * :: * :: *	1061 239
Arabidopsis Escherichia	FFSLVSSYPFDSPRPTHLKATGRIKFLGKIKRHSTTKDGDVGSDKCEDAAAISSLDGDISISSLKLNQLILAPQLSGRLSVSRDHVKLDAAGRPDESLTLDFIGPLQPNSDENVQSGKLL GEQLRVTGDTDITVRTMLLKVSSIDGNTKLDALDIDSSQGIVNASGTAQ	1181 288
Arabidopsis Escherichia	SFSLQKGQLRANACFQPQQSATLEIRNFPLDELELASLRGLIQKAEIQLNLQKRRGHGLLSVIRFKFSGVLGEALDVAVRWSGDVCFMLSGRLEVMITVEKTILEQSNSRYELQGEYV 	1299 370
Arabidopsis Escherichia	LPGSRDRDLGQKEAGSFLMRAMTGHLGSVISSMGRWRMRLEVPKAEVAEMLPLARLLSRSTDPAVHSRSKDLFIQSVQNLCLQA-ENLRDLLEEIRGYYTPPSEVVLEDLSLPGLAELKG YQADDLKLKLTGKMTDYTLSMRTAVKGLEIPPATITLDAKGNEQQVNLDKLTVAALEGKTELKALLDWQQAI ** :**:: . ** **: :: :: :: :: :: :*: **: :: :: ::	1418 442
Arabidopsis Escherichia	HWHGSLDASGGGNGDTLAEFDFHGDDWEWGTYKTQRVLATGSYNNDDGLRLKEMLIQKGNATLHADGTLLGPKTNLHFAVLNFPVSLIPTLVEVVES	1515 543
Arabidopsis Escherichia	-SATDIVHSLRKLLSPIKGILHMEGDLRGSLEKPECDVQVRLLDGAVGGIDLGRAEVFASLTSNSRFLFNSNFEPFVQNGHVHIQGSVPVSFSQKNMSEGEVSETDRGGAV INAPGLDNALPGLGGTAKGLVKVRGTVEAPQLLADITARGLRWQELSVAQVRVEGDIKSTDQIAGKLDVRVEQISQPDVNINLVTLNAKGSEKQHELQL .* .: ::* * . **:::: **:: :::::::::::::	1625 642
Arabidopsis Escherichia	KIPSWAKEKEDDEKRTSRDRSEERWDSQLAESLK	1659 761
Arabidopsis Escherichia	ADIKDGGMTLLTAISPYANWLQGNADIRLQVGGTVDHPVL DTTKEGLPQGSITLSGRNVQVTQTVNDAALPVAFQTLNLTAELRNNRAELGWTIRLTNNGQFDGQVQVTDPQGRRNLGGNVNIRNFNLAMINPIFTRGEKAAGMVSANLRLGGDVQSPQL * *.* :.: ::: .::::::::::::::::::::::::	1714 881
Arabidopsis Escherichia	DGSASFHRASISSPVLRKPLTNFGGTLHVKSNRLCITSLESRVSRKGKLVVKGNLPLRSNEASAGDGIELKCEVLEVRAKNFLSCQV-DTQLQITGSMLQPTISGNIKLSQGEAYLPHDK FGQLQVTGVDIDGNFMPFDMQPSQLAVNFNGMRSTLAGTVRTQQGEIYLNGDA ***.: *** : :** : :** : :: *::** ** *	1833 934
Arabidopsis Escherichia	GGGAAPLNRLAANQYSIPGAAINQAVSSRYFARFFGTERASSGMKFSQSTGKSNSVEKEIEEVKMKPNMDIRLSDMKLVLGPELRIMYPLILNFAVSGELELDGMAHPK DWSQIENWRARVTKGSKVRITVPPMVRMDVSPDVVFEATPNLFTLDGRVDVPWA * * * * * * * * * * * * * * * * * *	1942 989
Arabidopsis Escherichia	FIKPKGVLTFENGDVNLVATQVRLKREHLNVAKFEPEHGLDPL-LDLALVGSEWQFRVQSRASNWQDKLVV RIVVHDLPESAVGVSSDVVMLNDNLQPEEPKTASIPINSNLIVHVGNNVRIDAFGLKARLTGDLNVVQDKQGLGLNGQINIPEGRFHAYGQDLIVRKGELLFSGPPD-QPYLNIEAI *. *:: .**:* : * * * * * * * * * * * * *	2012 1105
Arabidopsis Escherichia	TSTRSVEQDALSPSEAAKVFESQL-AESILEGDGQLAFKKLATATLGTIMPRIEGKGEFGQARWRLVYAPQIPSLLSVDPTVDPLKSLA-SNISFG RNPDATEDDVIAGVRVTGLADEPKAEIFSDPAMSQQAALSYLLRCQGLESDQSDSAAMTSMLIGLGVAQSGQIVGKIGETFGVSNLALDTQGVGDSSQVVVSGYVLPG . :.*:* .*: *::*:*:*: *:* .* *::* **::* **::* **::* **::*	2106 1213
Arabidopsis Escherichia	TEVEVQLGKRLQASVVRQMKDSEMAMQWTLIYQLTSRLRVLLQSAPSKRLLFEYSATSQD 2166 LQVKYGVGIFDSIATLTLRYRLMPKLYLEAVSGVDQALDLLYQFEF 1259 :*: * : * ** *:* : * : * : * : *:::	

Figure S1. Alignment of protein sequences of Arabidopsis TIC236 and *E. coli* **TAMB.** The amino acid sequences of TIC236 (protein accession number: NP_180137) and TAMB (protein accession number: NP_418642) were obtained from NCBI (https://www.ncbi.nlm.nih.gov/protein/) and used for alignment using the Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov). Asterisks refer to conserved amino acid residues between proteins, and the mutated residues are highlighted in red. The identity of the two sequences is ~29%.



Figure S2. TIC236-GF residues in different plant species and genotyping of *tic236-gf* **mutants. A,** The TIC236-like protein sequences from 27 plant species reported in Phytozome v12.1 were aligned using ClustalW. The three mutated residues in *spcrl* mutants are indicated with blue arrowheads. Aligned regions were visualized using WEBLOGO. **B**, Sanger DNA sequencing result of *TIC236* mutations in the plants shown in Fig. 1*D*.

A	wт	crl C	RL-GFP crl	В	In	out	anti	-GF	P IP		
		***		kDa	TW C	GRL-GFP crl	WT	GFP	CRL-GFP crl		
				70 - 55 - 40 -		-	-		-	CRL-GFP Heavy cha	in
				35 - 25 -			_	-	_	anti-GFP ■ GFP/Light	chain
	A	19 C	(1)				10	10	77		
	V		20							CBB	
С											
Exclusive unique peptide count											
ld F	Identified Proteins	MW (kDa)	GFP				CRL-GFP				
	1 Totolilo		rep1 r	ep2	rep	3 I	rep1		rep2	rep3	
			•	•						4.4	
	GRL	30 kDa	0	0	0		13		27	11	
	URL TOC75	30 kDa 89 kDa	0	0	0 4		13 3		27 19	18	
	TOC75 TOC34	30 kDa 89 kDa 35 kDa	0 0 0	0 0 0	0 4 0		13 3 3		27 19 3	11 18 2	
	TOC75 TOC34 TOC132	30 kDa 89 kDa 35 kDa 132 kDa	0 0 0 0	0 0 0 0	0 4 0 0		13 3 3 0		27 19 3 3	18 2 3	

Figure S3. The biologically active CRL-GFP fusion protein unveils CRL-associated proteins. A, Top: images represent 21-day-old WT, *crl*, and 35S::*CRL-GFP crl* (*CRL-GFP crl*) plants. Scale bar, 5 mm. Middle: confocal images of chlorophyll autofluorescence of 5-day-old cotyledons (scale bar, 10 µm). Bottom: localized cell death in 10-day-old cotyledons, as visualized by TB staining (scale bar, 0.5 mm). **B**, Western blot of Co-IP using 14-day-old WT, 35S::*GFP* (*GFP*), and *CRL-GFP crl* plants. GFP-conjugated Dynabeads were used to pull down CRL-GFP and its associated proteins. The proteins were subjected to MS analysis after digestion. In parallel, equal amounts of proteins were used for Western blot analysis. Heavy and light chains of the GFP antibody are indicated. Equal protein loading is shown by CBB staining. **C**, List of proteins showing TOC and TIC components, together with CRL, which were detected at least twice in the eluates from *CRL-GFP crl* but not in *GFP* samples.





Figure S4. CRL interacts with transit peptides of FTSZ proteins and the amounts of FTSZ2 family proteins were reduced in *crl* in 2-day-old seedlings. A and B, Split-Venus constructs of CRL and the transit peptide (tp) lacking mature protein region (A) or tp-deleted mature protein (m) (B) of either RBCS, FTSZ1, FTSZ1, FTSZ2-1, or FTSZ2-2 were transiently coexpressed in *N. benthamiana* leaves. Fluorescence signal of the integrated Venus protein was monitored by confocal microscopy. Scale bar, 10 μ m. (C) Steady-state levels of FTSZ2 family proteins. Total proteins were extracted from wild-type (WT) and *crl* mutant seedlings grown on medium (1x MS and 2% sucrose and 16 h light/8 h dark) for 2 days or 5 days and were used for immunoblotting. Plastid PGL35 (PLASTOGLOBULIN 35, also called Fibrillin, At4g04020) was detected as a control.



Figure S5. The *ftsh11-2* allele of *ftsh11* mutants also significantly suppresses *crl* phenotypes. **A**, Schematic representation of the FTSH11 gene (accession number: AT5G53170). Exons and introns are shown as black boxes and black lines between exons, respectively. The inverted triangles indicate the T-DNA insertion sites of the *ftsh11-1* (SALK_033047) and *ftsh11-2* (SALK_012285) mutants. **B**, Top: representative plant images of 21-day-old WT, *crl*, *ftsh11-2*, and *crl ftsh11-2* are shown (top panel). Middle: confocal images of chlorophyll signals from mesophyll cells (scale bar, 10 µm). Bottom: cell death in cotyledons, as visualized via TB staining (Scale bar, 0.5 mm).



Figure S6. The *crl* **mutant phenotypes were not caused by reduced translocon protein levels.** Immunoblot analyses of total proteins extracted from 5-day-old seedlings. The amount of proteins loaded is indicated above the lane. The same amounts of proteins were loaded to examine the relative abundance of TIC110, TOC75, TIC40, and TOC33 proteins. H3 protein was used as a loading control.



Figure S7. Label-free protein quantitation of TOC75 and OEP80 proteins in *tic236-gf* plants. iBAQ intensities are shown as mean \pm SD (n=3). Ribulose-bisphosphate carboxylase large-chain (RBCL) was chosen as the control. All *P*-values are from two-tailed Student's *t*-tests. **P* < 0.05, ***P* < 0.01.

ID	Gene	Name	Primer sequence (5' to 3')	Purpose	
AT5G51020	CRI	crl-LP	CCGAGAGACGTGAGATCAGT		
A13031020	UKL	crl-RP	ggtcacCTGTTCGAGATACA		
AT5G53170	ETSH11	ftsh11-1-LP	CTCCTCTCCATACTTCTTCGT		
	FISHII	ftsh11-1-RP	agtcatggtaacaatacCAGT		
AT5G53170	ETQU11	ftsh11-2-LP	AAGCAACCACTGCATGTTACC	Constrains	
	FISHII	ftsh11-2-RP	TGTCTTTCCAGTACCAGGTGC	Genotyping	
AT1G63900	SD1	sp1-3-LP	TATTCGCTGAATCGAGCAAAC		
	5P1	sp1-3-RP	GCTGCCATGTATAACAGGCTG		
AT2G25660	TICODE	tic236-2-LP	GTTGTGTAATCCACGCACATG		
	110236	tic236-2-RP	ACGAAACGCCCTTCTAGCTAC		
4.700.05000	TICODO	M4 seq F	GTTGCTCTCCTTTTCTCTTC	Sequencing to identify	
A12G25660	110230	M4 seq R	CGAATTACAGACAACAGGCCA	TIC236-4GF mutation	
A.TO.O.O.C.000	TIOOOO	M5 seq F	CTAAATCTTCAGAAGCGAAGAG	Sequencing to identify	
A12G25660	110236	M5 seq R	GGCAATACATATTCACCTTGAA	TIC236-5GF mutation	
	TIOGOG	M6 seq F	TTGACTTCCATGGAGATGATTGG	Sequencing to identify	
A12G25660	HC236	M6 seq R	TGGTGACAAGAGTTTCCTCAAAGA	TIC236-6GF mutation	
		Q ACTIN2 F	CTGTTGACTACGAGCAGGAGATGG		
A13G18780	AC12	Q ACTIN2 R	CAAACGAGGGCTGGAACAAGACT		
		Q AKR4C8 F	CGGCTGTTAACCAAGTTGAATG		
AT2G37760	AKR4C8	Q AKR4C8 R	CCCAAAGGAGAGTAACCAGATAAG		
		Q ADH1 F	GAGTGTGTGAACCCGAAAGA		
AT1G77120	ADH1	Q ADH1 R	GTAGTTCCCGAAGAAAGTACCC	qRT-PCR	
		Q SMR7 F	CGCCAAAACATCGATTCGGG		
AT3G27630	SMR7	Q SMR7 R	TCGAAATCTGAAGGAGGCACA		
	SRO3	Q SRO3 F	CGGTTGGTACTCTGGTTCTAAA		
AT1G70440		Q SRO3 R	GCGAGAGAGTAACGATGATGAA		
	CRL	attb-CRL-F	GGGGACAAGTTTGTACAAAAAGCAGGCTtc ATGGGTACCGAGTCGGGTTC	Generate CRL fragment for	
AT5G51020		attb-CRL-R	GGGGACCACTTTGTACAAGAAAGCTGGGTc GTCTTGCAAGATGAGGGACCC	BiFC construct	
AT1G02280	TOC33	attb-TOC33-F	GGGGACAAGTTTGTACAAAAAGCAGGCTtc ATGGGGTCTCTCGTTCGTGA	Generate TOC33 fragment	
		attb-TOC33Ter-R	GGGGACCACTTTGTACAAGAAAGCTGGGTc TTAAAGTGGCTTTCCACTTGT	for BiFC construct	
AT5G05000	TO 00 (attb-TOC34-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTT CATGGCAGCTTTGCAAACGCTTCGT	Generate TOC34 fragment	
	TOC34	attb-TOC34Ter-R	GGGGACCACTTTGTACAAGAAAGCTGGGTC TCAAGACCTTCGACTTGCTAAACC	for BiFC construct	
AT1C67090	PBCS1A	attb-mRbcSatg-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTT CATGGAATTGGCTAAGGAAGTTGACT	Generate mRBCS fragment	
AT 1007 090	RDUSIA	attb-mRbcS-R	GGGGACCACTTTGTACAAGAAAGCTGGGTC ACCGGTGAAGCTTGGTGGC	for BiFC construct	
AT5G55280	FTSZ1	attb-mFtsZ1atg-F	GGGGACAAGTTTGTACAAAAAGCAGGCTT CATGTCCTTCTCCGATGGAATCTGCG	Generate mFTSZ1 fragment	
A15G55280		attb-mFtsZ1-R	GGGGACCACTTTGTACAAGAAAGCTGGGTC GAAGAAAAGTCTACGGGGAGAAGACGA	for BiFC construct	
AT2G36250	FTSZ2-1	attb-mFtsZ2-1atg-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTT CATGGCCGCTCAGAAATCTGAATC	Generate mFTSZ2-1	
		attb-mFtsZ2-1-R	GGGGACCACTTTGTACAAGAAAGCTGGGTC GACTCGGGGATAACGAGAG	fragment for BiFC construct	
AT3G52750	FTS72-2	attb-mFtsZ2-2atg-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTT CATGGCTTCTCATAAGTACGAGTCTTC	Generate mFTSZ2-2	
A13G3273U	AT3G52750	1 1322-2	attb-mFtsZ2-2-R	GGGGACCACTTTGTACAAGAAAGCTGGGTC GAGGCGAGGATAGCGAGAGC	fragment for BiFC construct

AT5G55280	FTSZ1	attb-FtsZ1tp-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTT CATGGCGATAATTCCGTTAGCA	Generate tpFTSZ1 fragment	
		attb-FtsZ1tp-R	GGGGACCACTTTGTACAAGAAAGCTGGGTC TGTTGAATCGCTTCTTCGTTT	for BiFC construct	
AT2G36250	FTSZ2-1	attb-FtsZ2-1tp-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTT CATGGCAACTTACGTTTCACC	Generate tpFTSZ2-1	
		attb-FtsZ2-1tp-R	GGGGACCACTTTGTACAAGAAAGCTGGGTC AACAACAACTCGGTTTTTCTTAC	fragment for BiFC construct	
AT3G52750	FT070 0	attb-FtsZ2-2tp-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTT CATGGCAGCTTATGTTTCTCC	Generate tpFTSZ2-2	
	A13G52750	F15ZZ-Z	attb-FtsZ2-2tp-R	GGGGACCACTTTGTACAAGAAAGCTGGGTC AACAACACGATATCTTTTAGTAG	fragment for BiFC construct
AT1G67090		1G67090 RBCS1A	attb-RbcStp-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTT CATGGCTTCCTCTATGCTCTCT	Generate tpRBCS fragmen
	AT 1G67090		attb-RbcStp-R	GGGGACCACTTTGTACAAGAAAGCTGGGTC GGAATCGGTAAGGTCAGGAAG	for BiFC construct

Table S1: List of primers used in this study.

Dataset S1 (separate file). The expression levels of genes upregulated in 5-day-old seedlings of *crl* versus WT are shown in *spcrl1*.

Dataset S2 (separate file). The expression levels of genes downregulated in 5-day-old seedlings of *crl* versus WT are shown in *spcrl1*.

Dataset S3 (separate file). List of the proteins that were found to be associated with CRL.

Dataset S4 (separate file). List of TOC, TIC, and plastid division proteins that may associate with CRL.

Dataset S5 (separate file). iBAQ intensity of TOC and TIC proteins in *tic236gf* mutants versus WT.