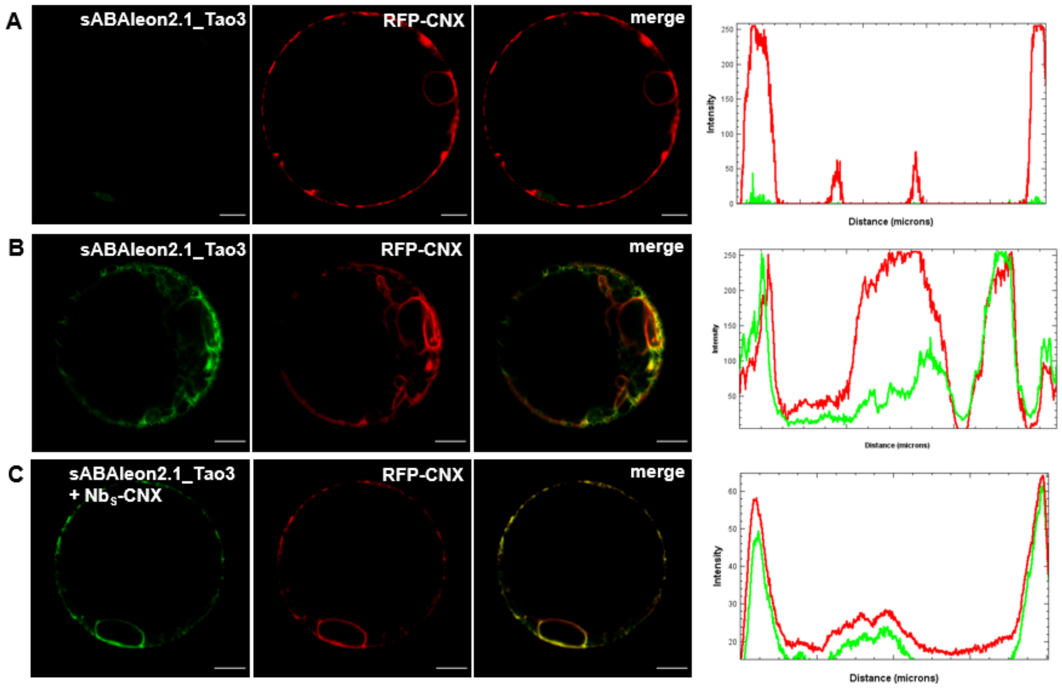
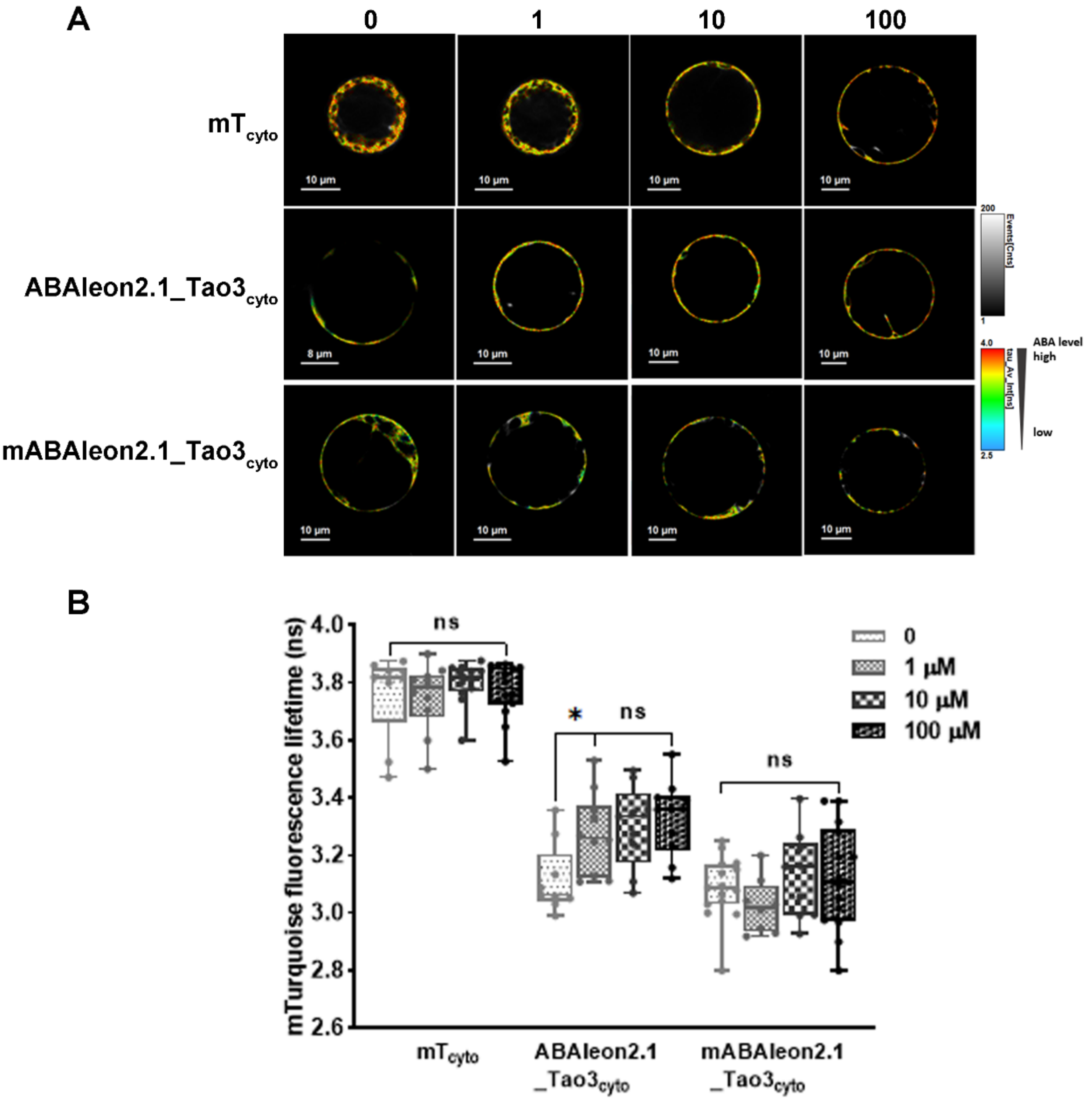
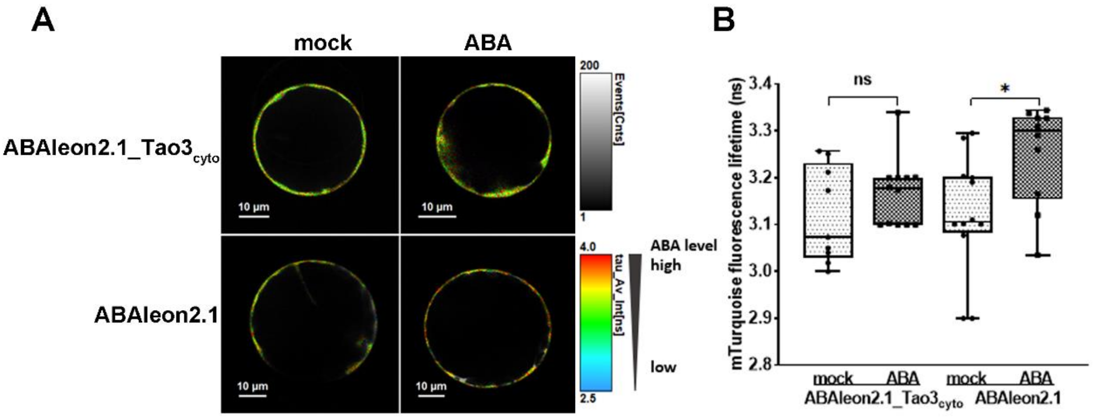


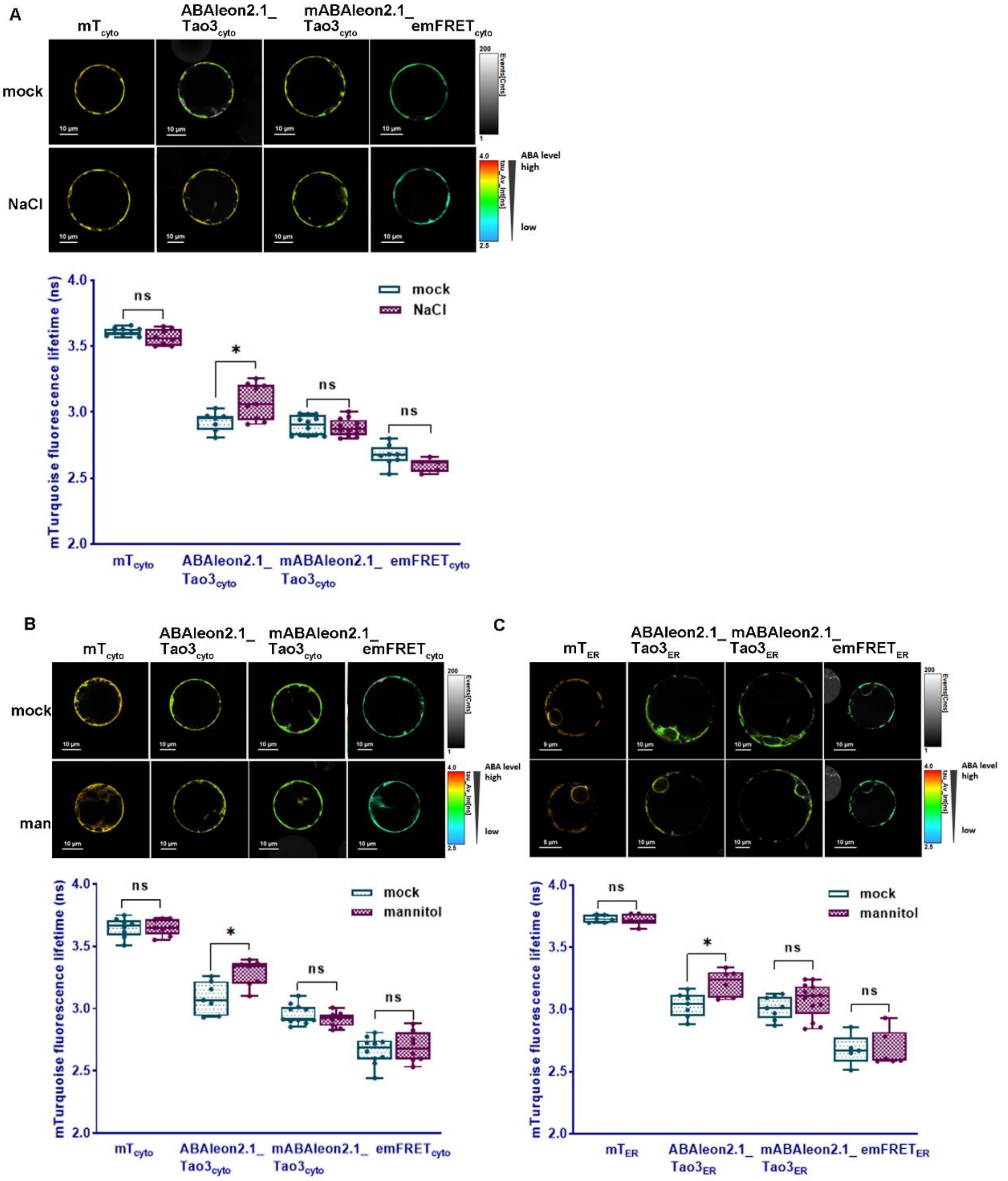
Supplemental Figure S1 The secretion and retainment of sABAleon2.1_Tao3 in the ER. **A,B** Expression of sABAleon2.1_Tao3 alone resulted in the sensor either secreted (**A**) or expressed in transit compartments (**B**), as shown by the different fluorescent signals indicated by the green line (sABAleon2.1_Tao3) or the red line (RFP-CNX) in the intensity plot. **C** Coexpression of sABAleon2.1_Tao3 with Nb$_S$-CNX resulted in the colocalization of sABAleon2.1_Tao3 with the ER marker RFP-CNX, as indicated by the overlapping signal peaks in the intensity plot. Scale bars, 5 µm.



Supplemental Figure S2 Cytosolic ABAleon2.1_Tao3 shows ABA-induced specific increases in τ_{mT}. **A,B** Representative FLIM images (**A**) and data (**B**) show specific increases in τ_{mT} in ABAleon2.1_Tao3$_{cyto}$, but not in that of donor-only (mT$_{cyto}$) and the mutated ABAleon2.1_Tao3 (mABAleon2.1_Tao3$_{cyto}$), in which the ABA receptor PYR1 was mutated (R116G). FLIM data are presented as box bars with all data points. Statistics were performed using one-way ANOVA followed by a Duncan's multiple range test (*$P < 0.05$ comparing different ABA concentrations treated ABAleon2.1_Tao3$_{cyto}$ with mock treated ABAleon2.1_Tao3$_{cyto}$; ns, not significant).



Supplemental Figure S3 ABAleon2.1_Tao3$_{cyto}$ shows a lower sensitivity of ABA compared with ABAleon2.1. **A,B** Representative FLIM images (**A**) and data (**B**) of the ABAleon2.1_Tao3$_{cyto}$ and ABAleon2.1 in response to 100 nM ABA treatment. The fluorescence lifetime values are shown as box bars with all data points. n = 9/12. Significance was determined by students' t-test ($*P < 0.05$; ns, not significant).



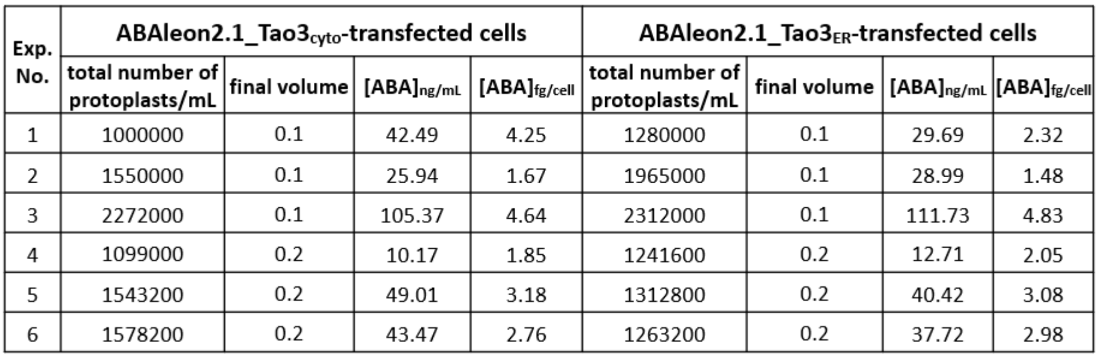
Supplemental Figure S4 Osmotic stresses triggered specific increases in the τ_{mT} of ABAleon2.1_Tao3s but not in that of donor-only controls (mT_{cyto} and mT_{ER}), mutated ABAleon2.1_Tao3s (mABAleon2.1_Tao3$_{cyto}$ and mABAleon2.1_Tao3$_{ER}$), empty FRET controls (emFRET$_{cyto}$ and emFRET$_{ER}$). **A** Shown are representative FLIM images and data of cytosol-localized constructs in response to NaCl (10 mM). **B, C** FLIM images and data of cytosolic (**B**) and ER (**C**)-resided constructs in response to mannitol (50 mM). Data are presented as box bars with all data points. Statistics was performed using students' t-test (*$P < 0.05$ compared with mock treatment; ns, not significant).

Supplemental Table S1 Biochemical properties of ABA probes used in this study.

titration range	ABA probes	linear regression		τ_{min}	τ_{max}	DR (%)	K'dN (4 Parameter logistic)	
		Y-intercept $[ABA_{exo=0}]$ (nM)	slope				nM	fg/cell
0 - 100 μM	ABAleon2.1	129.1	1.845	3.05	3.45	-13.1	194.2	-
	ABAleon2.1_Tao3$_{cyto}$	38.5	3.068	3.03	3.49	-15.2	398.4	-
	aABAleon2.1_Tao3$_{cyto}$	217.6	2.282	2.95	3.35	-13.6	114.1	19.19 ± 1
	ABAleon2.1_Tao3$_{ER}$	48.1	1.546	3	3.4	-13.3	103.6	17.73 ± 1.05

Biochemical properties of ABAleon2.1 and ABAleon2.1_Tao3s in ABA titration assays using tobacco protoplasts. Shown are titration range, different ABA probes, linear regression models, the minimum (τ_{min}) and maximum (τ_{max}) of mTurquoise fluorescence lifetime in each assay, the dynamic range (DR) calculated as (τ_{min}-τ_{max}) ×100/τ_{min}, the original ABA affinity (K'd) (in nM) calculated from a four-parameter logistic fit and the K'dN that was normalized by linear regression model. The protoplasts that had been treated with ABA or mock were harvested and extraction for measuring endogenous ABA by LC/MS. The endogenous ABA level (ng/mL) was calculated by the equation $[ABA]_{ng/mL}$ = (Peak area of ABA) × [ABA-d6]/ (Peak area of ABA). The endogenous ABA level (nM) was calculated by the equation $[ABA]_{nM}$= $[ABA]_{ng/mL}$ ×1000/264.32. The endogenous ABA level (fg/cell) was calculated by the equation [ABA]fg/cell=([ABA]ng/mL × the sample volume) × 1000/the total (alive) number of protoplasts in each sample.

Supplemental Table S2 Basal level of endogenous ABA in individual cells.



Exp. No.	ABAleon2.1_Tao3$_{cyto}$-transfected cells				ABAleon2.1_Tao3$_{ER}$-transfected cells			
	total number of protoplasts/mL	final volume	[ABA]$_{ng/mL}$	[ABA]$_{fg/cell}$	total number of protoplasts/mL	final volume	[ABA]$_{ng/mL}$	[ABA]$_{fg/cell}$
1	1000000	0.1	42.49	4.25	1280000	0.1	29.69	2.32
2	1550000	0.1	25.94	1.67	1965000	0.1	28.99	1.48
3	2272000	0.1	105.37	4.64	2312000	0.1	111.73	4.83
4	1099000	0.2	10.17	1.85	1241600	0.2	12.71	2.05
5	1543200	0.2	49.01	3.18	1312800	0.2	40.42	3.08
6	1578200	0.2	43.47	2.76	1263200	0.2	37.72	2.98

The total alive number of protoplasts that had been transfected with cytosolic ABAleon2.1_Tao3 (ABAleon2.1_Tao3$_{cyto}$) or ER-targeted ABAleon2.1_Tao3 (ABAleon2.1_Tao3$_{ER}$) was counted before harvest and extraction for measuring endogenous ABA by LC/MS. The endogenous ABA level (ng/mL) was calculated by the equation $[ABA]_{ng/mL}$ = (Peak area of ABA) × [ABA-d6]/ (Peak area of ABA). The endogenous ABA level (fg/cell) was calculated by the equation $[ABA]_{fg/cell}$ = ($[ABA]_{ng/mL}$ × the final volume of each sample) × 1000000/the total alive number of protoplasts in each sample. Data are from six independent experiments.

Supplemental Table S3 Oligonucleotides and plasmids used in this study.

New fragment (vector)	Primers	Sequence (5'-3')	Template	Recipient Vector
CNX-HA-Nbs (pYLZ06)	NheI-HA-S	CGgctagcatgtatccttatgatgttcctgat	pSF087*	pYLZa06 (CNX-GFP) cut NheI/BamHI
	HA-Nbs-BamHI-AS	GCTGATggatccctagctgctcacggtcacctgggtg		
cytRFP (pYLZ13)	35S-EcoRI-S	TGCAGCgaattcctacgcagcaggtctcatcaagacg	pFK098*	pSF074 cut EcoRI/BamHI
	RFP-BamHI-AS	GCTGATggatcctctagattatgctccagtactgtggc		
CNX-RFP (pYLZ18)	NheI-RFP-S	CGgctagcatggcctcctccgaggac	pYLZ13	pYLZa06 (CNX-GFP) cut NheI/BamHI
	BamHI-RFP-AS	GCTGATggatcctctagattatgctccagtactgtggc		
RFP-CNX (pYLZ14)	35S-EcoRI-S	TGCAGCgaattcctacgcagcaggtctcatcaagacg	pSF074*	pSF074 cut EcoRI/BamHI
	35S-ClaI-AS	GCTGATatcgatatagagagagagatagatttatag		
	spRFP-ClaI-S	TGCACGatcgatgaggctttgtaaattcacagctctcttcttctactattttctctcctactgctttctgctagcatggcctcctccgaggacgtcatc	pYLZ13	
	RFP-XmaI-AS	GCTGATcccgggtgctccagtactgtggcggccctcgg		
	CNX-XmaI-S	GCTGATcccgggcatatggaactgattgagaaagccg	pSF087*	

	CNX-BamHI-AS	TGCACGggatccctaattatcacgtctcggttgcct		
smT-PYR1 (pYLZ21)	35S-EcoRI-S	TGCAGCgaattcctacgcagcaggtctcatcaagacg	pSF074*	pSF074 cut EcoRI/BamHI
	35S-ClaI-AS	GCTGATatcgatatagagagagagatagatttatag		
	spmT-ClaI-S	TGCACGatcgatgaggctttgtaaattcacagctctctcttctctactattttctctcctactgctttctgctagcgccatggtgagcaagggcgaggagctgt	h2.1-L (mT-PYR1)	
	PYR1-BamHI-AS	GCTGATggatcccgtcacctgagaaccacttccgtcacc		
mT-PYR1 (pYLZ25)	EcoRI-35S-S	TGCAGCgaattcctacgcagcaggtctcatcaagacg	pSF074*	pYLZ21 cut EcoRI/NheI
	NheI-35S-AS	GCGgctagcatatagagagagagatagatttatagagag		
sABAleon2.1_Tao3 (pYLZ24)	linker3-ABI1-BamHI-S	GCTGATggatccggtggaggcgttgatcctgataatgaagcatacgaaatgccttctgaagaaggctatcaagattatgaaccggaggctggtggaggcagtgtgcctttgtatggttttac	h2.1-S (ABI1-cpV)	pYLZ21 cut BamHI
	cpV-BamHI-AS	TGCAGCggatccctactcgatgttgtggcggatcttgaag		
ABAleon2.1_Tao3 (pYLZ23)	35S-EcoRI-S	TGCAGCgaattcctacgcagcaggtctcatcaagacg	pSF074*	pYLZ24 cut EcoRI/NheI
	35S-NheI-AS	GCGgctagcatatagagagagagatagatttatagagag		

smT-PYR1-linker3 (pYZW81)	Psp-PYR1-F	GACGAGCTGTACAAGGGGCCCATGCCTTCGGAGTTAACACCAGAAGAAC	pYLZ24	pYLZ21 cut Psp/BamHI
	BamHI-linker3-PYR-R	TGAACGATCTGCTTCGGATCCCTAGCCTCCACCAGCCTCCGG		
mT-PYR1-linker3 (pYZW82)	EcoRI-35S-S	TGCAGCgaattcctacgcagcaggtctcatcaagacg	pSF074*	pYZW81 cut EcoRI/NheI
	NheI-35S-AS	GCGgctagcatatagagagagagatagatttatagagag		
sRFP-PYR1 (pYLZa02)	PYR1-XmaI-S	ATGCcccgggatgccttcggagttaacaccagaaga	pYLZ21	pYLZ14 cut NheI/XmaI
	PYR1-BamHI-AS	GATCggatccctacgtcacctgagaaccacttccg		
sRFP-PYR1 (R116G) (pYLZa05)	RFP3'-XmaI-PYR1-S	cacagtactggagcacccgggatgccttcggagttaacac	pYLZ21	pYLZa02 cut XmaI/BamHI
	PYR1 (R116G)-AS	attcgtcagcccatgttcgcctccgatgatactgaatcc		
	PYR1 (R116G)-S	gcgaacatgggctgacgaattacaaatccgttacgacggt		
	3'nos-BamHI-PYR1-AS	gaacgatctgcttcggatccctacgtcacctgagaaccac		
smT-PYR1 (R116G) (pYLZ33)	sp-NcoI-mT-S	tgctttctgctagcgccatggtgagcaagggcgaggagctgt	pYLZ21	pYLZ40 cut NcoI/BamHI
	GC-mT-AS	catgggccccttgtacagctcgtccatgccgagag		

	GC-PYR1-S	tacaaggggcccatgccttcggagttaacaccagaag	pYLZa05	
	3'nos-BamHI-PYR1'-AS	gaacgatctgcttcggatcccgtcacctgagaaccac		
sABAleon2.1_Tao3 (R116G) (pYLZ34)	Linker3-ABI1-BamHI-S	GCTGATggatccggtggaggcgttgatcctgataatgaagcatacgaaatgccttctgaagaaggctatcaagattatgaaccggaggctggtggaggcagtgtgcctttgtatggttttac	h2.1-S (ABI1-cpV)	pYLZ33 cut BamHI
	cpV-BamHI-AS	TGCAGCggatccctactcgatgttgtggcggatcttgaag		
ABAleon2.1_Tao3 (R116G) (pYLZ35)	35S-EcoRI-S	TGCAGCgaattcctacgcagcaggtctcatcaagacg	pSF074*	pYLZ34 cut EcoRI/NheI
	35S-NheI-AS	GCGgctagcatatagagagagagatagatttatagagag		
smT (pYLZ40)	EcoRI-35S-S	TGCAGCgaattcctacgcagcaggtctcatcaagacg	pYLZ21	pSF074 cut EcoRI/BamHI
	mT-BamHI-AS	GCTGATggatcccttgtacagctcgtccatgccgagag		
smT-linker3-cpV (pYLZ41)	linker3-cpV-BamHI-S	GCTGATggatccggtggaggcgttgatcctgataatgaagcatacgaaatgccttctgaagaaggctatcaagattatgaaccggaggctggtggaggcatggacggcggcgtgcagctc	h2.1-S (ABI1-cpV)	pYLZ40 cut BamHI
	cpV-BamHI-AS	TGCAGCggatccctactcgatgttgtggcggatcttgaag		

mT-linker3-cpV (pYLZ46)	35S-EcoRI-S	TGCAGCgaattcctacgcagcaggtctcatcaagacg	pSF074*	pYLZ41 cut EcoRI/NheI
	35S-NheI-AS	GCGgctagcatatagagagagagatagatttatagagag		

[*], plasmids kindly provided by Dr. Peter Pimpl.

h2.1, ABAleon2.1, kindly provided by Dr. Waadt.