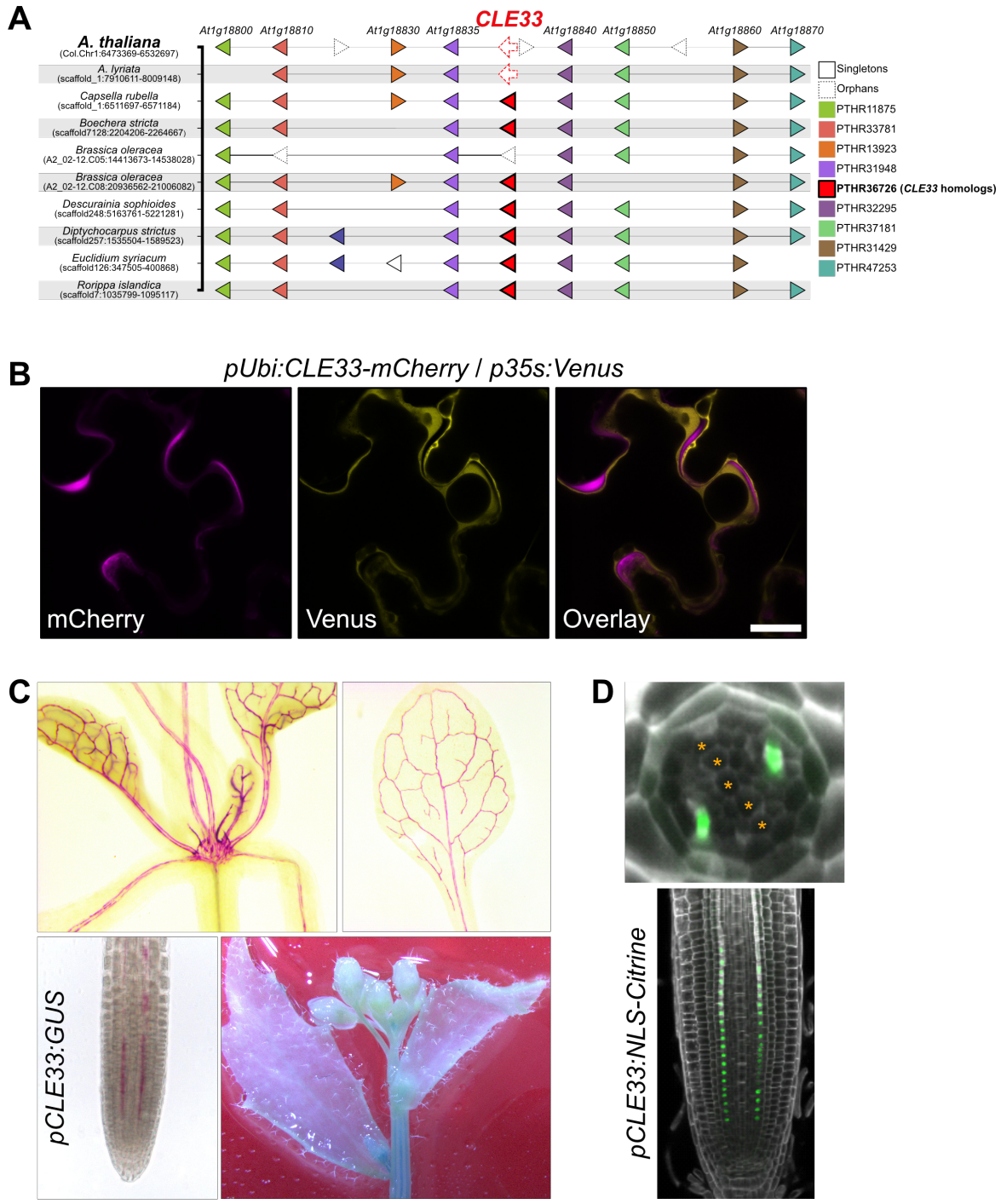
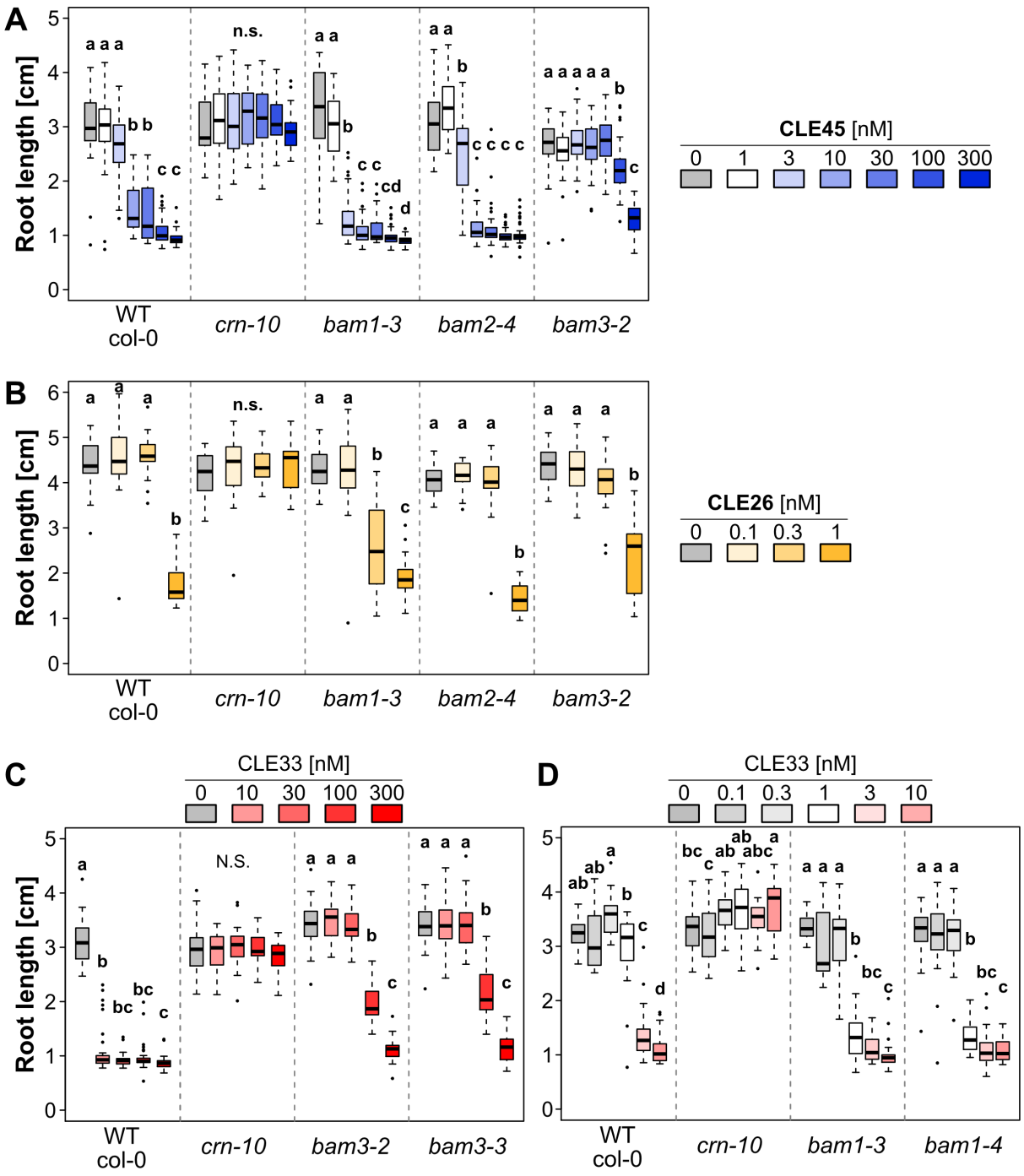
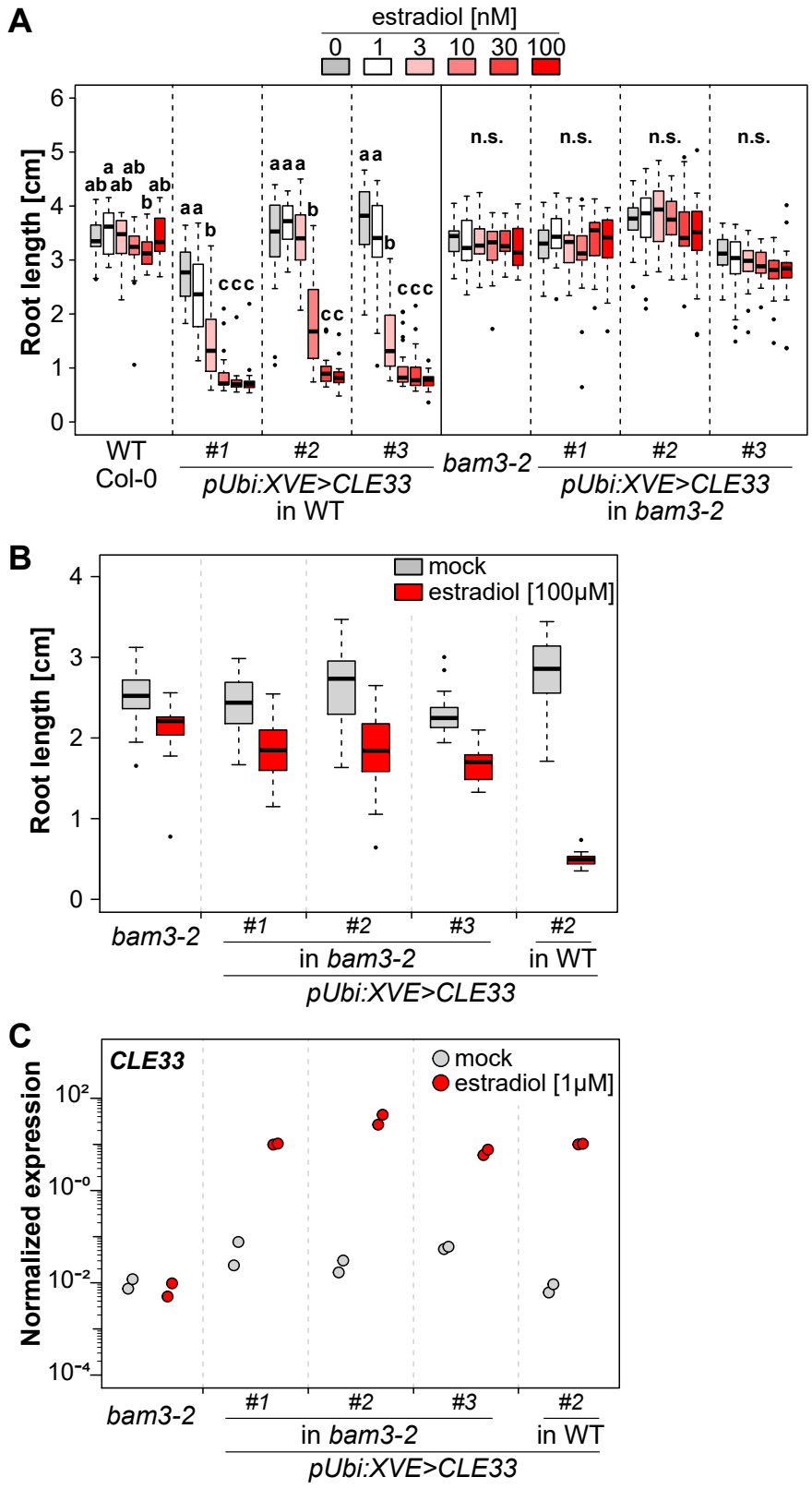
Supplemental Figures and Tables.



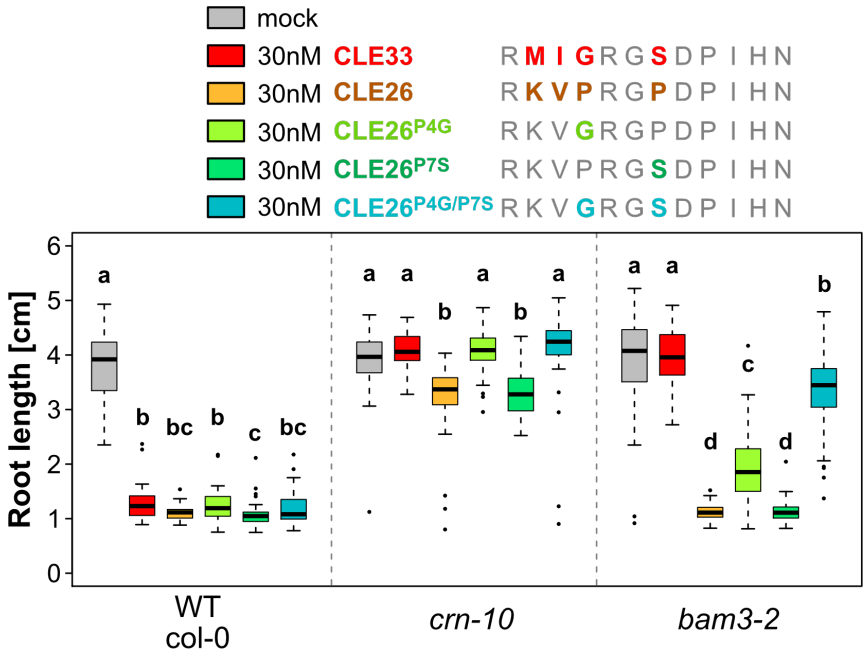
Supplemental Figure 1. A. Syntenic context of the *CLE33* locus on chromosome 1 in other Brassicaceae species. In *Arabidopsis thaliana* and *lyriata CLE33* genes position and orientation are represented by a doted red arrow. The family ID PTHR36726 refers to *CLE33* orthologs. **B.** Confocal images of *CLE33-mCherry* transiently expressed in *Nicotiana benthamiana* leaves, showing apoplast localization. **C.** Vascular *CLE33* expression revealed by transcriptional-GUS fusion in rosette junction (upper-left), cotyledon (upper-right), root apex (lower-left), inflorescence (lower-right). **D.** *CLE33* is expressed in developing protophloem sieve element cells. Orange asterisks indicate xylem cells.



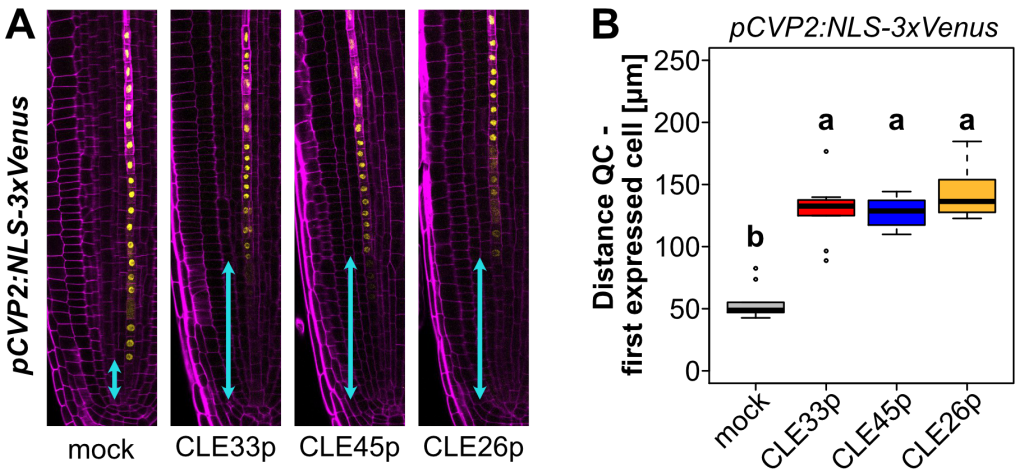
Supplemental Figure 2. CLE33/CLE45 *BAM3*-dependent and CLE26 *BAM3*-independent root growth arrest. A-D. Dose dependent root growth response to synthetic CLE45 (**A**), CLE26 (**B**) and CLE33 peptides (**C-D**). Letters indicate different statistical group (ANOVA, post-hoc Tukey test).



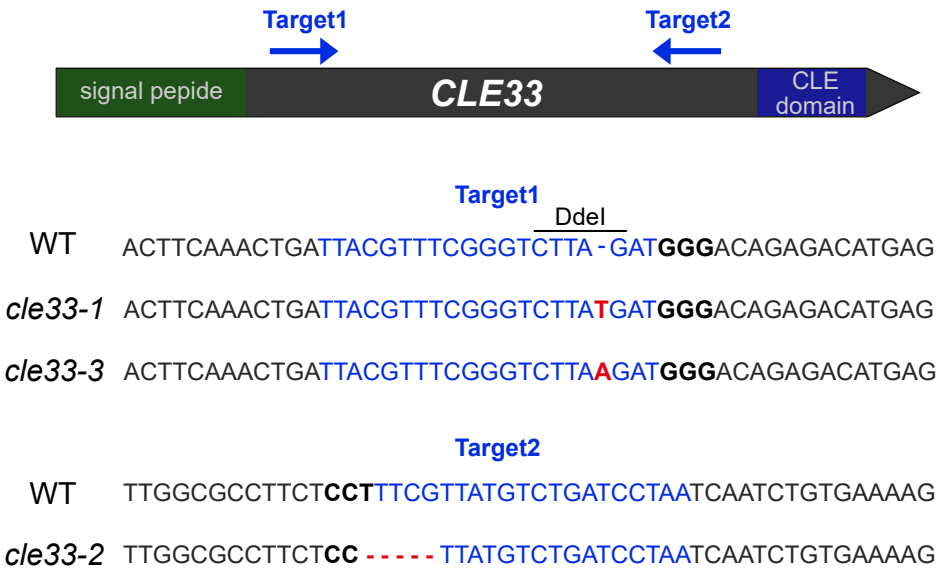
Supplemtal Figure 3. Dose-dependent root growth arrest by inducible over-expression of *CLE33* requires *BAM3*. A-B. Dose dependent root growth response to estradiol-inducible lines expressing *CLE33* in wild-type and *bam3-2* mutant. Letters show different statistical group (ANOVA, post-hoc Tukey test). **C.** Transcriptional induction of *CLE33* by overnight estradiol treatment detected by qPCR (n=2).



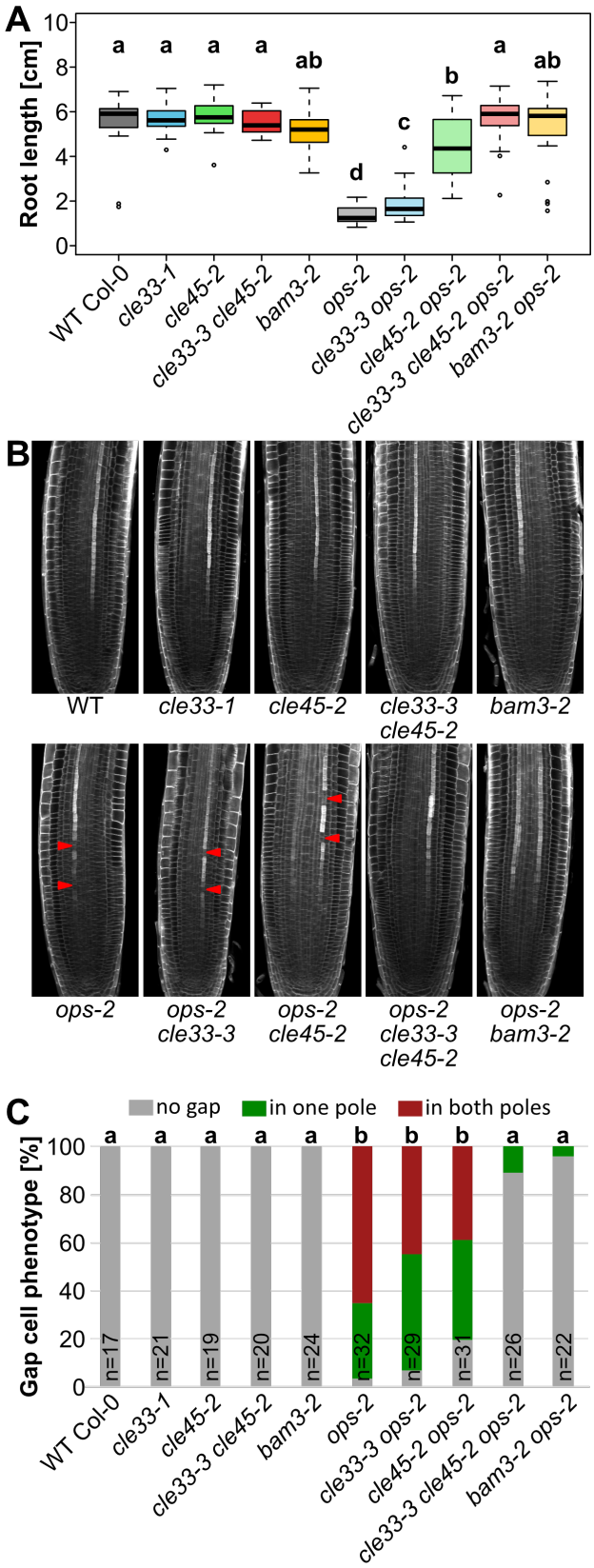
Supplemental Figure 4. Substitution of proline residues in CLE26 in positions 4 and 7 to glycine and serine leads to *BAM3*-dependent root responses. Letters indicate different statistical group (ANOVA, post-hoc Tukey test).



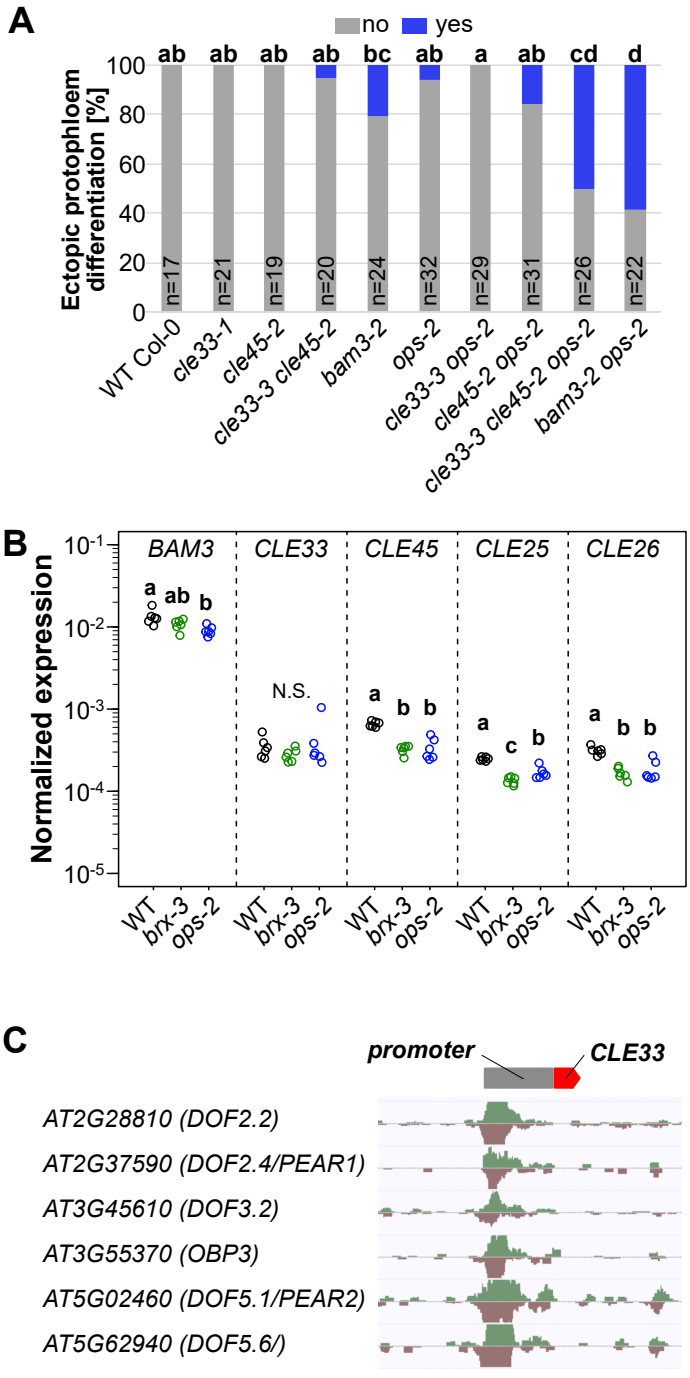
Supplemental Figure 5. CLE33 inhibits protophloem development. A. Confocal images of the protophloem cell identity marker *pCVP2:NLS-3xVenus* upon treatment with phloem peptides. Cyan arrows indicate the distance between the first fluorescent cells and the quiescent centre, quantified in (**B**). Letters indicate different statistical group (ANOVA, post-hoc Tukey test).



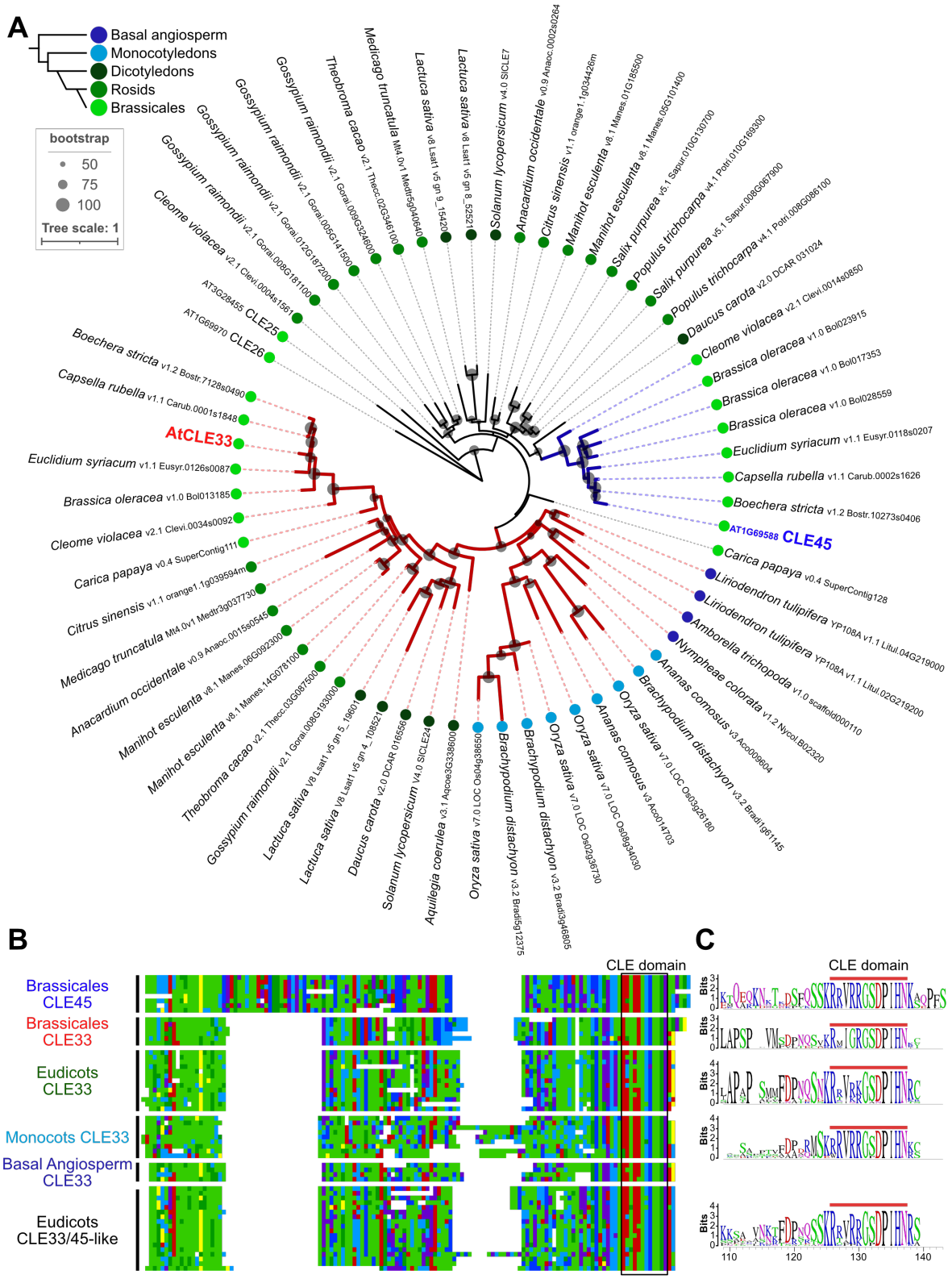
Supplemental Figure 6. CRISPR-Cas9 targeted regions in *CLE33*. Blue arrows indicate the single-guide RNAs. Protospacer-adjacent motifs (PAM) are in bold. Indels are indicated in bold red for each mutant. The endonuclease DdeI recognition site is indicated by a black line.



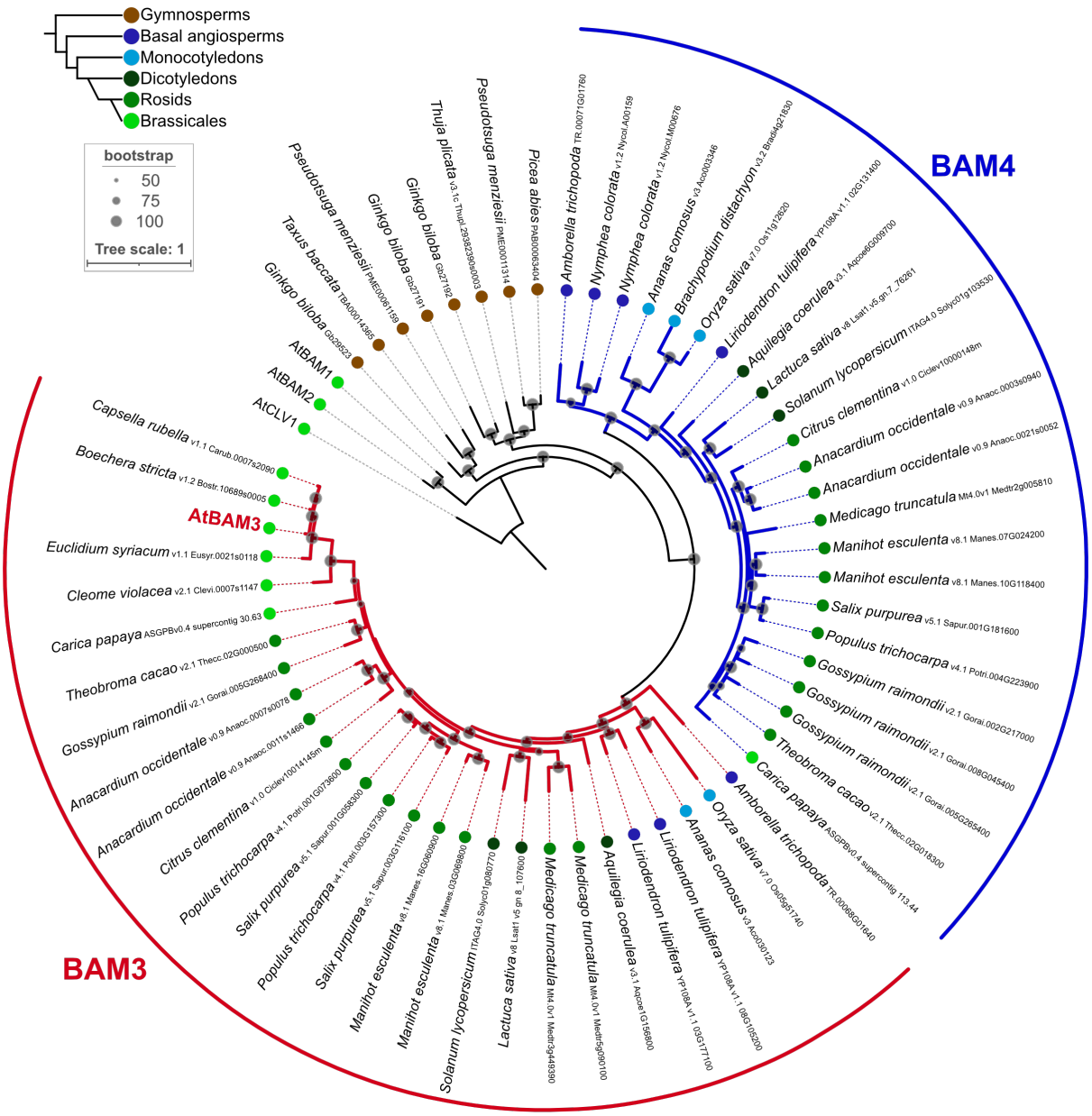
Supplemental Figure 7. *CLE33* and *CLE45* act in concert in *OPS*-mediated protophloem development. A. Seedling root length at 10 days post germination. Letters indicate different statistical group (ANOVA, post-hoc Tukey test). **B.** Representative confocal images of calcofluor white stained roots presenting protophloem differentiation continuity. Red arrows indicate protophloem gap cells. **C.** Quantification of the gap cell phenotype frequency. Letters indicate different statistical group (χ^2 test with Benjamini-Hochberg correction).



Supplemental Figure 8. CLE33/45-BAM3 module repress ectopic protophloem differentiation. A. Percentage of roots with non-regular protophloem cells presenting a clear cell wall reinforcement. Letters indicate different statistical group (χ^2 test with Benjamini-Hochberg correction). **B.** Expression levels of phloem-specific *CLE*s in the root tissues of *brx-3* and *ops-2* mutants tested by q-PCR **C.** DAP-seq peaks (in-vitro binding sites) of selected DOFs transcription factors (Plant Cistrome database, http://neomorph.salk.edu). Cloned promoter for transcriptional fusions and *CLE33* coding sequence are indicated in grey and red, respectively.



Supplemental Figure 9. *CLE33* orthologs are present in basal angiosperms. A. Phylogenetic tree of the *CLE33* and *CLE45* homologs. *CLE33* and *CLE45* orthologs are shown in red and blue branches, respectively. Tree is rooted with *AtCLE25* and *AtCLE26*. **B.** Multi sequence alignment of *CLE33/45* homolog sequences deriving out of the phylogenetic tree shown in A. Group of eudicots *CLE33/45*-like genes corresponds to the other sequences not being identified as *CLE33* or *CLE45* orthologs. **C.** Conservation LOGO of the CLE propeptide highlighting the C-terminal part according to their evolutionary group shown in B.



Supplemental Figure 10. Phylogenetic tree of the *BAM3* and *BAM4* orthologs. Remarkable absence of *BAM4* in *Brassicaceae*, a copy that emerged from a duplication with BAM3 in early angiosperms. Tree is rooted with AtCLV1.

Supplemental Table 1. Primers used in this study.

Cloning		
LI Esp3I A-B *pAtCLE33*	C62	TATCGTCTCAGCGGGTATAATGAACTAATTATCCTTAAGCTCCTCATATGT
	C63	TATCGTCTCACAGAGTTAACTAATATGTAGATACTACAGCGTTTTGTTTCT GAAAG
LI Esp3I A-B *pAtCLE45*	E42	TATCGTCTCAGCGGTTGATTAATCAAGAATGCACAATTG
	E43	TATCGTCTCACAGATTCTGCTCTTAGGCAGACAAG
LI Esp3I A-B *pAtCLE26*	E40	TATCGTCTCAGCGGCGAGCAAATTTTCTCGTTGG
	E41	TATCGTCTCACAGAGGTTTCTAGCCTTTGTGGATA
LI Esp3I C-D *AtCLE33*	H33	TATCGTCTCACACCATGGGTGTTTCAATTGGTCATATAGGAATGA
	H34	TATCGTCTCACCTTTCAGCATTTGTTGTATTTGTTGTGAATAGGAT
LI BsaI C-D *AtCLE33 noStop*	C64	TATGGTCTCACACCATGGGTGTTTCAATTGGTCATATAGGAATGATTC
	C65	TATGGTCTCACCTTGCATTTGTTGTATTTGTTGTGAATAGGATCAGATC
LI BsaI C-D *AtCLE33*	C64	TATGGTCTCACACCATGGGTGTTTCAATTGGTCATATAGGAATGATTC
	C66	TATGGTCTCACCTTTCAGCATTTGTTGTATTTGTTGTGAATAGGATCAG
LI B-C *NLS_H2B*	C60	TATGGTCTCATCTGATGGCGAAGGCAGATAAGAAA
	C61	TATGGTCTCAGGTGAGAGAACTCGTAAACTTCGTAACC
LI C-D *Citrine*	C58	TATGGTCTCACACCATGGTGAGCAAGGGCGAGG
	C59	TATGGTCTCACCTTTTACTTGTACAGCTCGTCCATGCC
LI A-G FastRed	PP494	ATGAAGACTTTACGGGTCTCAGCGGAATGTCGCGGAACAAATTTTAAAAC
	PP495	TAGAAGACAATCGAGAAGTCACGTGTCAATG
	PP496	ATGAAGACTTTCGACTCCTTTCTTAATATATCTAACAAAC
	PP497	ATGAAGACTTCAGAGGTCTCAGACAACTAAATGGAGCAACCTACTG
LI B-C *XVE*	G58	ATGAAGACTTTACGGGTCTCATCTGATGAAAGCGTTAACGGCCAGG
	G59	GAAGACTGGACTCGTCTGGCGCTCCATG
	G60	GAAGACCGAGTCCAATCATCAGGATCTCTAG
	G61	GAAGACAGGAAACGGACCAAAGCCACTTG
	G62	GAAGACCGTTTCCTCCACGGATGCCC
	G63	ATGAAGACTTCAGAGGTCTCAGGTGTGCTAGAGTCGACTAGCTTCAGCG

Guide RNAs		
gRNA1	C67	ATTGTTACGTTTCGGGTCTTAGAT
	C68	AAACATCTAAGACCCGAAACGTAA
gRNA2	C69	ATTGTTAGGATCAGACATAACGAA
	C70	AAACTTCGTTATGTCTGATCCTAA

qPCR		
AtCLE33	J77	ACGTTTCGGGTCTTAGATGGG
	J78	AGGCGCCAAGTTTCCTTTTG
AtActin	E08	CCGATCCAGACACTGTACTTCCTT
	E09	CTTGCACCAAGCAGCATGAA

New mutants genotyping			
cle33-1 & *cle33-3*	C64	TATGGTCTCACACCATGGGTGTTTCAATTGGTCATATAGGAATGATTC	DdeI (cut WT)
	J11	GAACACGCAAAGGATGAACA	
cle33-2	H31	TGGGACAGAGACATGAGGATGCA	Sequencing
	H34	TATCGTCTCACCTTTCAGCATTTGTTGTATTTGTTGTGAATAGGAT	
bam3-3	J16	TCCCGAGGTGAAATCTCCGCAA	WT
	J17	TGAACAAAAAGCGAGCAATCTCA	
	J16	TCCCGAGGTGAAATCTCCGCAA	T-DNA insertion
	E17	ATTTTGCCGATTTCGGAAC	

Supplemental Table 2. Cloning

Name	**Description**
Golden Gate Level 0	
L0 B-C XVE A	PCR amplification from pMDC7 with G58+G59 primers. Assembled by blunt ligation into BB01
L0 B-C XVE B	PCR amplification from pMDC7 with G60+G61 primers. Assembled by blunt ligation into BB01
L0 B-C XVE C	PCR amplification from pMDC7 with G62+G63 primers. Assembled by blunt ligation into BB01
Golden Gate Level I	
LI B-C XVE	Assembled by BpiI cut-ligation from: L0 B-C XVE A + L0 B-C XVE B + L0 B-C XVE C, into BB03
LI Esp3I A-B *pAtCLE33*	PCR amplification of *A. thaliana* genomic DNA with C62+C63 primers. Assembled by blunt ligation into BB02
LI Esp3I A-B *pAtCLE45*	PCR amplification of *A. thaliana* genomic DNA with E42+E43 primers. Assembled by blunt ligation into BB02
LI Esp3I A-B *pAtCLE26*	PCR amplification of *A. thaliana* genomic DNA with E40+E41 primers. Assembled by blunt ligation into BB02
LI Esp3I C-D *gAtCLE33*	PCR amplification of *A. thaliana* genomic DNA with H33+H34 primers. Assembled by blunt ligation into BB02
LI BsaI C-D *gAtCLE33*	PCR amplification of *A. thaliana* genomic DNA with C64+C66 primers. Assembled by blunt ligation into BB02
LI BsaI C-D *gAtCLE33* noSTOP	PCR amplification of *A. thaliana* genomic DNA with C64+C65 primers. Assembled by blunt ligation into BB02
LI B-C *NLS_H2B*	PCR amplification from pENTRY H2B with C60+C61 primers. Assembled by blunt ligation into BB01
LI C-D *Citrine*	PCR amplification from pENTRY Citrine with C58+C59 primers. Assembled by blunt ligation into BB02
LI A-G FastRed	PCR amplifications from pAGM55261 with PP494+PP495 and PP496+PP497. Assembled by BpiI cut ligation into BB03
Golden Gate Level II	
LII F1-2 FastRed	Assembled by BsaI cut-ligation from: LI A-G FastRed into LIIc F1-2 (BB30)
LII F3-4 *pOI:Gus*	Assembled by BsaI cut-ligation from: LI A-B pOI (G082) + LI B-E Gus (G79) + LI E-F HSP-term (G45) + LI F-G dy (BB09) into LIIc F3-4 (BB33)
LII F3-4 *pOI:H2B-Citrine*	Assembled by BsaI cut-ligation from: LI A-B pOI (G082) + LI B-C NLS_H2B + LI C-D Citrine + LI D-E dy (BB08) + LI E-F NOS-term (G06) + LI F-G dy (BB09) into LIIc F3-4 (BB33)

LII F3-4 *pOI:XVE>GOI*	Assembled by BsaI cut-ligation from: LI A-B pOI (G082) + LI B-C XVE + LI C-D GOI (G83)+ LI D-E dy (BB08) + LI E-F HSP-term (G45) + LI F-G dy (BB09) into LIIc F3-4 (BB33)
LII F3-4 *pUbi:CLE33-mCherry*	Assembled by BsaI cut-ligation from: LI A-B pAtUbi + LI B-C dy (BB06) + LI C-D gAtCLE33 noStop + LI D-E mCherry (G25) + LI E-F HSP-term (G45) + LI F-G dy (BB09) into LIIc F3-4 (BB33)
LII F5-6 *p35s:Venus*	Assembled by BsaI cut-ligation from: LI A-B p35s (G05) + LI B-C dy (BB06) + LI C-D Venus (G20) + LI D-E dy (BB08) + LI E-F NOS-term (G06) + LI F-G dy (BB09) into LIIc F5-6 (BB36)
Golden Gate Level III	
LIII FastRed / *pOI:Gus*	Assembled by BpiI cut-ligation from: LII F1-2 FastRed + LII 2-3 ins (BB44) + LII F3-4 *pOI:Gus* + LII 4-6 dy (BB41) into LIIIβ Fin (BB52)
LIII FastRed / *pCLE33:Gus*	Assembled by Esp3I cut-ligation from: LI A-B *pAtCLE33* into LIII FastRed / pOI:Gus
LIII FastRed / *pOI:H2B-Citrine*	Assembled by BpiI cut-ligation from: LII F1-2 FastRed + LII 2-3 ins (BB44) + LII F3-4 *pOI:H2B-Citrine* + LII 4-6 dy (BB41) into LIIIβ Fin (BB52)
LIII FastRed / *pCLE33:H2B-Citrine*	Assembled by Esp3I cut-ligation from: LI A-B Esp3I *pAtCLE33* into LIII FastRed / *pOI:H2B-Citrine*
LIII FastRed / *pCLE45:H2B-Citrine*	Assembled by Esp3I cut-ligation from: LI A-B Esp3I *pAtCLE45* into LIII FastRed / *pOI:H2B-Citrine*
LIII FastRed / *pCLE26:H2B-Citrine*	Assembled by Esp3I cut-ligation from: LI A-B Esp3I *pAtCLE26* into LIII FastRed / *pOI:H2B-Citrine*
LIII *pAtUbi:CLE33-mCherry / p35s:Venus*	Assembled by BpiI cut-ligation from: LII 1-3 dy (BB38) + LII F3-4 *pUbi:CLE33-mCherry* + LII 4-5 ins (BB43) + LII F5-6 *p35s:Venus* into LIIIβ Fin (BB52)
LIII FastRed / *pOI:XVE>GOI (LacZ)*	Assembled by BpiI cut-ligation from: LII F1-2 FastRed + LII 2-3 ins (BB44) + LII F3-4 *pOI:XVE>GOI* + LII 4-6 dy (BB41) into LIIIβ Fin (BB52)
LIII FastRed / *pOI:XVE>GOI (ccdb)*	Assembled by Esp3I cut-ligation from: LI A-B pOI (G84) + LI C-D GOI (G85) into LIII FastRed / *pOI:XVE>GOI*(LacZ)
LIII FastRed / *pUbi:XVE>CLE33*	Assembled by BsaI cut-ligation from: LI A-B pAtUbi + LI BsaI C-D *gAtCLE33* into LIII FastRed / *pOI:XVE>GOI*(LacZ)
pAGM55261_AtCLE33gRNA1	Assembled by BsaI cut-ligation from primer dimer of gRNA1 into pAGM55261
pAGM55261_AtCLE33gRNA2	Assembled by BsaI cut-ligation from primer dimer of gRNA2 into pAGM55261