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Supplementary Materials for

Membrane pore architecture of the CslF6 protein controls (1-3,1-4)-β-glucan structure

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This PDF file includes:

Fig. S1. Plasmid map of the *Agrobacterium* transformation vector pSJ226 and pSJ195 used for transient expression studies in *N. benthamiana*.
Fig. S2. Amino acid sequence alignment of the C-terminal region of the CslF6 proteins.
Fig. S3. The Leu-Ile amino acid change in ZmCslF6 TMH4 increases DP3/DP4 ratio.
Fig. S4. Sequence alignment of the C-terminal region of *Rhodobacter sphaeroides* BcsA and *Hordeum vulgare* CslF6 with *Oryza sativa* CesA and CslD proteins.
Table S1. Comparison of (1-3,1-4)-β-glucan abundance and structure in *N. benthamiana* leaf and in cereal wholegrain.
Table S2. Abundance of DP3-DP9 from Fig. 8.
Table S3. Primers.
References (*30–32*)

Supplementary Materials:

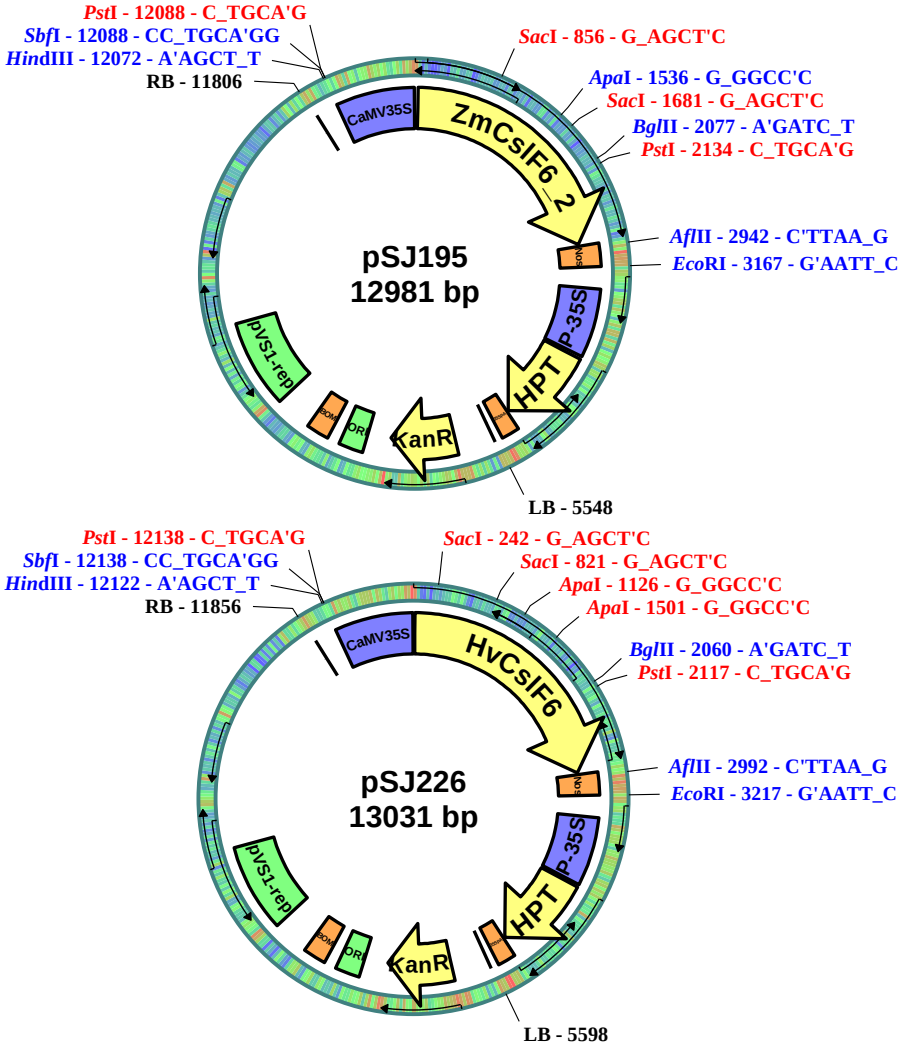

Fig. S1. Plasmid map of the *Agrobacterium* transformation vector pSJ226 and pSJ195 used for transient expression studies in *N. benthamiana*. The double lined circle is the plasmid DNA with GC content indicated in colouring (blue high GC to red AT rich content). Restriction sites are indicated on the outside (blue single cut, red double cut). RB and LB are the Agrobactrium T-DNA borders. CaM35S & Cauliflower mosaic virus 35S promoter. Nos Nopaline synthase terminator. The 35S promoter also drives the plant selectable marker HPT (hygromycin phosphotransferase with the CaMV terminator from the T-DNA but this is not used in these experiments. The plasmid backbone has a kanamycin resistance gene for selection in bacteria and high copy number origin of replications. This plasmid is based on the pCX series of vectors (*28*).

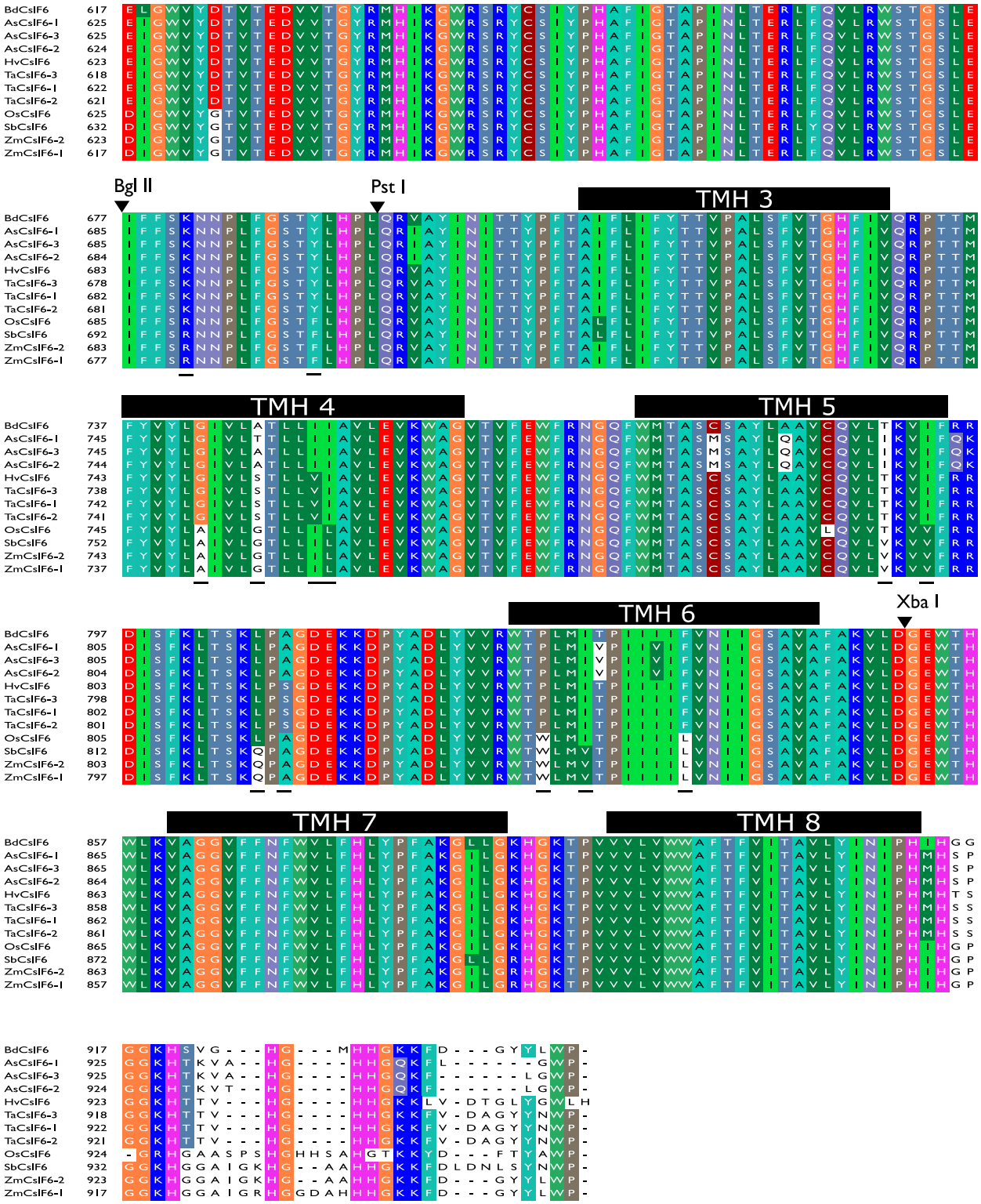

Fig. S2. Amino acid sequence alignment of the C-terminal region of the CslF6 proteins.
Full length CslF6 proteins were aligned using the default settings of Muscle in Bioedit (*30*) and

the region showing the carboxy terminal transmembrane helices (TMH) is shown. The amino acid sequences numbered on the left from the amino terminus are shown in one letter code and coloured according to physical properties (shading >50% threshold) (K,R, basic - dark blue; D,E, acidic - red; I,L,M,V small neutral - dark green; W, large hydrophobic - light green; F,Y hydrophobic - turquoise; N,Q,S,T, amine or hydroxyl - light purple; P, kink - gray; C, dark red; H pink; A small - cyan; G smallest - gold). The black rectangles above the protein sequence represent the predicted TMHs numbered from the amino terminus. Dashes indicate gaps introduced into the alignment. Restriction sites used in cloning are shown as black triangles. The thin black lines below the protein sequences show the 13 amino acid differences between the HvCslF6 and ZmCslF6 sequences between the *Bgl* II and *Xba* I sites.

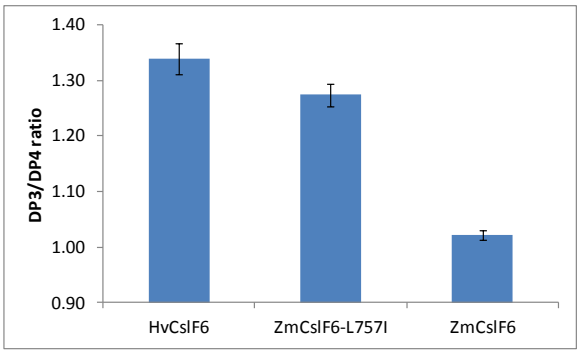

Fig. S3. The Leu-Ile amino acid change in ZmCslF6 TMH4 increases DP3/DP4 ratio
The DP3/DP4 ratio of (1-3,1-4)-β-glucan produced in *Nicotiana benthamiana* leaf from the indicated CslF6 protein is shown. The amount of (1-3,1-4)-β-glucan produced was 1.46, 3.50, and 4.23% respectively of the dry weight of the freeze dried leaf.

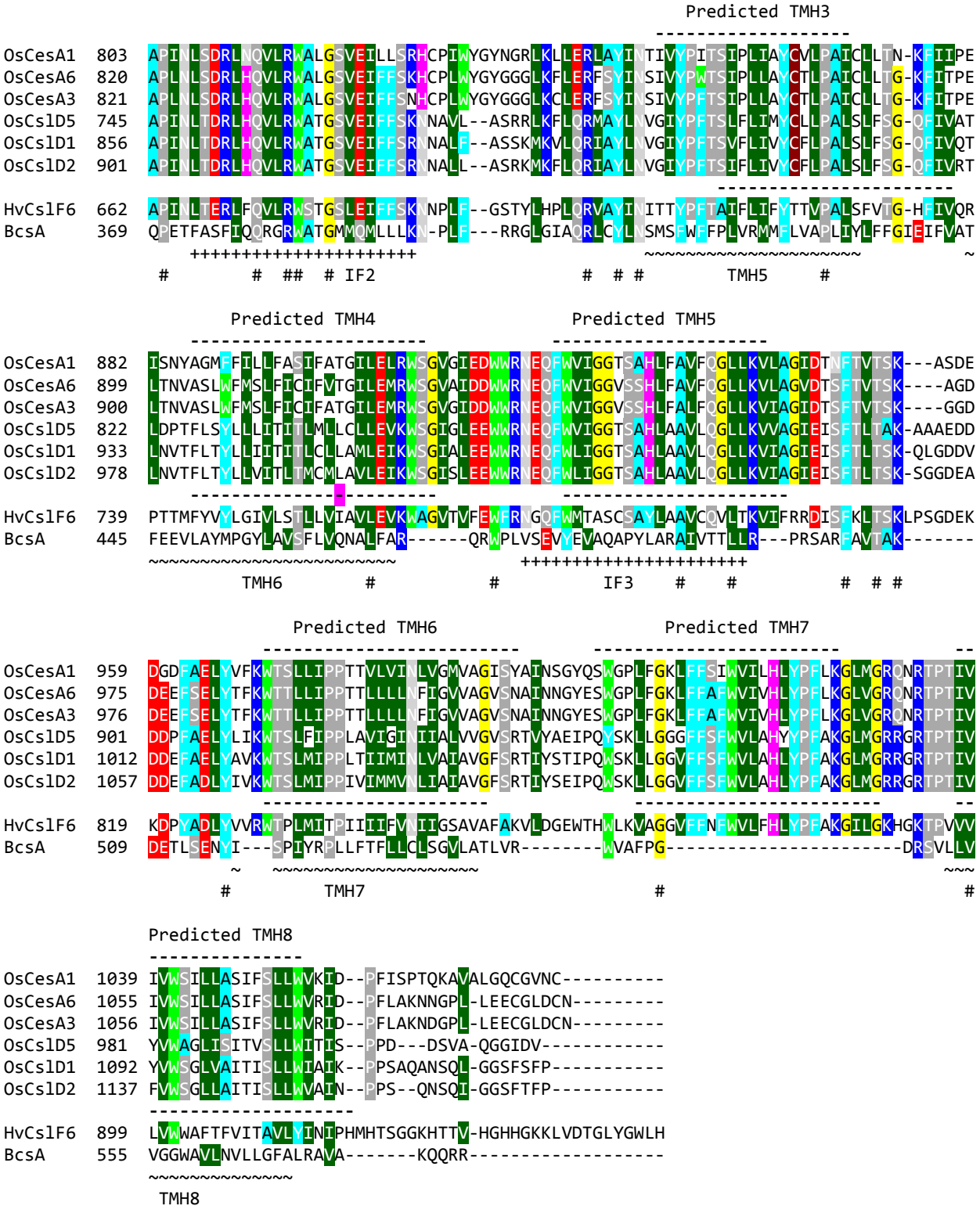

Fig. S4. Sequence alignment of the C-terminal region of *Rhodobacter sphaeroides* BcsA and *Hordeum vulgare* CslF6 with *Oryza sativa* CesA and CslD proteins. Full length CslF, CesA and CslD proteins and the *R.sphaeroides* BcsA protein 1-580 aa (omitting the PilZ domain) were aligned using the default settings of ClustalW algorithm (*31*) in Bioedit (*30*) and the region

showing the carboxy terminal transmembrane helices (TMH) and interfacial domains (IF) is shown. The amino acids (numbered on the left) are highlighted in colour according to similar physical properties (cutoff 64%). Dashed lines above the CesA and CslF sequences indicate the position of predicted TMHs numbered from the amino terminus, whereas tilde (~) or plus (+) characters below the BcsA sequence indicate TMHs or interfacial domains respectively as determined from the crystal structure. Fully conserved amino acids are indicted with a hash # mark beneath the BcsA protein sequence. The Ile757 in HvCslF6 predicted TMH4 shown to affect the structure of (1-3,1-4)-β-glucan is indicated by pink highlight. Note the alignment shown here differs from previous CesA and BcsA alignments (*20*, *32*) in that there is no gap between TMH5 and TMH6 of BcsA but is now between TMH6 and IF3. Uniprot ID numbers (OsCesA1, Q6AT26; OsCesA6, Q6YVM4; OsCesA3, Q69V23; OsCslD5, Q5Z6E5; OsCslD1, Q8W359, OsCslD2, Q9LHZ7). HvCslF6, ABZ01578.1 Genbank Acession number).

Table S1. Comparison of (1-3,1-4)-β-glucan abundance and structure in *N. benthamiana* leaf and in cereal wholegrain.

	Nicotiana benthamiana leaf		Wholegrain	
CslF6	(1-3,1-4)-β-glucan (% w/w)	DP3/DP4 ratio	(1,3–1,4) β-glucan (% w/w)	DP3/DP4 ratio
Bd	3.0-4.0	1.72	38	8.02
Ta	0.6-2.0	1.60	0.6	2.80
Hv	0.6-2.5	1.40	4.1*	2.55
As	0.2-0.5	1.09	4.6	1.74
Zm-1	5.9	1.13	trace	n.d †
Zm-2	1.2-2.8	1.10	trace	n.d.
Os	0.8-3.0	0.89	0.02	1.18
Sb	3.8-5.9	0.93	0.28	3.40‡

The range of (1-3,1-4)-β-glucan content between experiments in *Nicotiana benthamiana* leaf is shown. Husks were removed from the wholegrains if present and possible.

* Source barley control flour, Megazyme (1-3,1-4)-β-glucan assay kit.

† n.d. not detectable

‡ Due to the small seed size and greater seedcoat/endosperm ratio this (1-3,1-4)-β-glucan with a high DP3/DP4 ratio most likely originates from the outer seed tissues and not the endosperm.

Table S2. Abundance of DP3-DP9 from Fig. 8.

	BdCslF6		HvCslF6		TaCslF6		AsCslF6		BdCslF6-IL	
	av	sd	av	sd	av	sd	av	sd		
DP3	59.21	0.049	54.77	0.122	55.47	0.052	47.98	0.133	52.49	0.302
DP4	33.64	0.022	37.16	0.123	36.55	0.098	44.10	0.154	37.78	0.085
DP5	4.12	0.043	4.60	0.023	4.69	0.028	4.09	0.014	5.85	0.127
DP6	1.42	0.001	1.58	0.013	1.52	0.005	1.61	0.035	1.99	0.036
DP7	0.62	0.030	0.62	0.018	0.55	0.005	0.90	0.071	0.59	0.037
DP8	0.40	0.000	0.41	0.023	0.39	0.023	0.50	0.044	0.57	0.017
DP9	0.59	0.003	0.87	0.042	0.84	0.050	0.81	0.069	0.72	0.001
	HvCslF6 IL		AsCslF6 IL		ZmCslF6		OsCslF6		SbCslF6	
	av	sd	av	sd	av	sd	av	sd	av	sd
DP3	47.45	0.541	42.26	0.085	48.97	0.238	43.46	0.219	43.40	0.499
DP4	41.31	0.253	47.55	0.054	41.29	0.214	44.15	0.112	45.80	0.494
DP5	6.39	0.090	5.48	0.052	6.31	0.047	7.18	0.058	7.10	0.069
DP6	2.42	0.075	2.17	0.062	1.84	0.029	2.80	0.030	2.01	0.030
DP7	0.77	0.047	0.94	0.013	0.33	0.014	0.44	0.004	0.33	0.000
DP8	0.58	0.042	0.64	0.021	0.42	0.018	0.57	0.018	0.44	0.013
DP9	1.07	0.033	0.97	0.076	0.83	0.011	1.40	0.042	0.93	0.021

Table S3. Primers.

Primer	Gene Orientation	Sequence
SJ69	Os F	TCCCCCACGTACTTTACGAC
SJ77	Hv R	GATGGATGCATGCACTGACT
SJ116	Hv,Ta,Bd,As F	CATGGCGCCAGCGGTGG
SJ156	Ta R	GCACTGTTCAGTGGATGACTTGTTG
SJ243	As R	ACAGCTCAGCGGAAGACTTG
SJ277	Hv,Ta,Bd,As F	AAGATGGCTAGCATGACTGGTGGACAGCAAATGGGTATGGCGCCAGCGGT
SJ324	Os R	CTCATGGCCAGGCGTAGGTGAA
SJ357	Bd R	GTCGATCTTCTTCGTCCCGAT
SJ387	Sb F	GAGGGCGCAGCCGGCATTATGG
SJ388	Sb R	CTTCACGGCCAGTTGTAGGAGAGGTTG
SJ391	Zm1 F	CCGCCAGGCAGGCAGAGAGG
SJ392	Zm R	TCACGGCCAGAGGTAGTAGCCGT
SJ393	Zm2 F	GCCAGGCAGGCAGGCATTATGG
RoRidT 17	R	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTTT