

Supplementary Material

Plant Root Associated Chitinases: Structures and Functions

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1 Supplementary Tables

Table S1

S/N	Oligo	Sequence (5' – 3')
Cloning of <i>ZmChi19A</i> into p28b-SUMO vector:		
1	T-T-BasicEndoA F	ATTGGTGGATCCGAGAACCTGTACTTTTCAGGGCCAGCAGTGCAGGGCAGCAG
2	T-BasicEndoA R	GGCCGCAAGCTTTCTATCATTACTGCGCCGCCACCTCAACCGCCAGT
Cloning of <i>OsChi19A</i> into p28a vector:		
3	Oligo 6467 (O/CsChit-F)	CAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACC
4	Oligo 6468 (OsChit-R)	GTGGTGCTCGAGTTACTGCACAGCAG
Cloning of <i>CspChi18A</i> into p28a vector:		
5	Oligo 6467 (O/CsChit-F)	CAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACC
6	Oligo 6469 (CsChit-R)	GTGGTGCTCGAGTTAGGGCGTATACG
Cloning of <i>ZmChi19AΔcbd</i> into p28a vector:		
7	Oligo 6472 (p28pp-BEAnCBD-F1)	CATGAGAGGATCGCATCACCATCACCATCACGGATCCATGAGAGACCTCTTCGAGCGGC
8	T-BasicEndoA R	GGCCGCAAGCTTTCTATCATTACTGCGCCGCCACCTCAACCGCCAGT
Primer for M4 deletion of C-terminal domain of <i>ZmChi19A</i> :		
9	Oligo 6514 (S-BEA_A328*-R)	GGCCGCAAGCTTTCTATCATTACTGCGCCGCCACCTCAACTCACAGTCCGCTATTGAAGG
Site-Directed Mutagenesis: of <i>CspChi18A</i> :		
10	Forward Primer Oligo 6510 (D161A E163A-F)	CTGGATGGCATTGATTTTCGCGGACGCGTATGCCGACTATGGTAAC
11	Reverse Primer Oligo 6511 (D161A E163A-R)	GTTACCATAGTCGGCATAACGCGTCCGCGAAATCAATGCCATCCAG
p28 Vector Colony PCR & Sequencing Primers		
12	Oligo 6470 PETFOR (for pET vector)	TAATACGACTCACTATAGGG
13	Oligo 6471 PETREV (T7 Terminator for pET vector)	GCTAGTTATTGCTCAGCGG

Legend: Sequences of oligonucleotides used in this work.

Table S2

Enzyme	Prep#	FPLC-purified	% purity
SUMO	SOS155	Yes	99%
	SOS160	No	93%
SUMO- <i>ZmChi19A</i>	SOS155	Yes	97%
	SOS239	No	77%
<i>CspChi18A</i>	SOS160	Yes	100%
	SOS 240	No	76%
<i>OsChi19A</i>	SOS 114	No	95%
	SOS 129	No	97%
	SOS 124	No	73%
SUMO- <i>ZmChi19A</i> (E147A, E169A)	SOS 239	No	84%
	SOS 307	No	76%
SUMO- <i>ZmChi19A</i> (A328*)	SOS 239	No	94%
	SOS 307	No	81%
<i>CspChi18A</i> (D161A, E163A)	SOS 240	No	91%
	SOS 307	No	92%

Legend: Estimated purities of preparations used in this work. Samples of each preparation were resolved by SDS PAGE through 12% or 15% acrylamide gels depending on the molecular size of the protein (see examples in Figure 2). The relative area under each protein peak was determined by ImageJ analysis of the Coomassie blue stained gels. The % purity was determined from percent of the total quantified protein that was found in the band representing the purified protein. It should be noted that, because the gels were loaded with sufficiently high amounts of protein to detect contaminant proteins and the contaminants are at such lower levels than the purified protein, the Coomassie blue signal is likely not linear with amount of protein over the entire range of measurement. Thus, these estimates of purity are likely to be lower than the actual values and represent minimum % purity for each preparation.

Table S3

Sample	Obtained Average	Obtained S.D	Obtained c.v.	Expected	Obtained/calculated
SUMO	19	1.0	5%	16.8	1.1
SUMO- <i>ZmChi19A</i>	42	1.0	2%	47.5	0.88
<i>OsChi19A</i>	32	1.22	4%	33.7	0.93
<i>CspChi18A</i>	31	3.94	7%	33.1	0.94

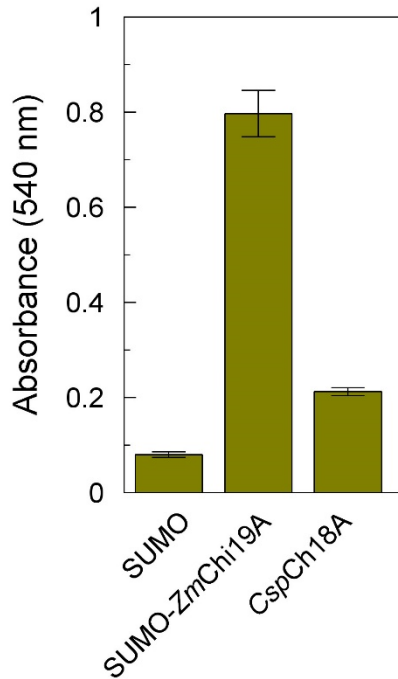
Legend: Estimated molecular weights compared with those determined from the cloned protein sequences. The obtained values for molecular weight (kDa) were determined for each of the listed proteins by from their relative mobilities (Rfs) on SDS PAGE using a set of standard proteins run on the same gels. The log MW of the standards was plotted against their Rfs and the curves fit with a polynomial (order 3). Values were determined from 3 separately run gels for each protein to provide the average estimates of the MW with standard deviation (S.D.) and coefficient of variation (c.v.).

Table S4

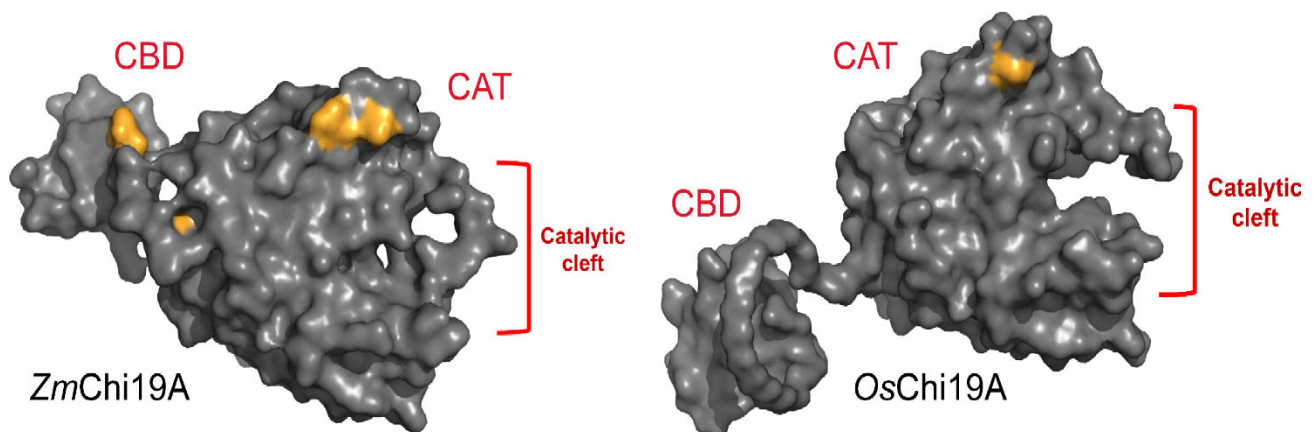
Enzyme, Substrate	K _m (μM)	V _{max} (μMmin ⁻¹)	K _{cat} (Sec ⁻¹)	pH			Temperature (°C)			Source
				Optima	Activity Range	Stability Range	Optima	Activity Range	Stability Range	
Plant chitinases										
<i>Zea mays</i> : ZmChi19A 4-MU-(GlcNAc) ₃	30 ± 6.6	14 ± 2.2	2.1 ± 0.33	8.0	5.0 – 9.0	4.0 – 11.0 (1 h)	45	20 - 60	0 - 50 (1 h)	This work
<i>Oryza sativa</i> : OsChi19A 4-MU-(GlcNAc) ₃	22 ± 2.6	20 ± 1.5	1.9 ± 0.86	5.5	4.0 – 7.0	4.0 – 11.0 (1 h)	35	20 - 50	0 - 50 (1 h)	This work
Barley chitinase, 4-MU-(GlcNAc) ₃	33	12	0.006	5.0	4.0 – 7.0	-	-	-	-	(Hollis et al., 1997)
Barley chitinase, 4-MU-(GlcNAc) ₄	3	1.2	0.58	5.0	4.0 – 7.0	-	-	-	-	(Hollis et al., 1997)
Rubber (<i>Hevea brasiliensis</i>) chitinase, 4-MU-(GlcNAc) ₃	100	-	-	5.0	4.0 – 7.0	4.0 – 7.0 (20 min)	45	30 - 40	30 - 40 (20 min)	(Sukprasirt and Wititsuwannakul, 2014)
<i>Ipomoea carnea</i> chitinase, p-np-(GlcNAc)	500	0.025	29	6.0	5.0 – 9.0	5.0 – 9.0 (24 h)	60	60 - 80	20 - 80 (1 h)	(Patel et al., 2009)
<i>Drosera rotundifolia</i> chitinase, FITC-Chitin	1660	88.50	77	6.0	5.0 – 6.0	-	40	20 - 40	-	(Rajninec et al., 2020)
<i>Agave tequilana</i> chitinase, CC	-	-	-	5.0	2.0 – 7.0	2.0 – 9.0 (24 h)	-	-	-	(Sierra-Gómez et al., 2019)
Fungal chitinase										
<i>Aspergillus niger</i> chitinase, 4-MU-(GlcNAc) ₃	50 ± 6.9	-	16± 0.9	-	-	-	-	-	-	(van Munster et al., 2015)
Bacterial chitinases										
<i>Chitinophaga oryzae</i> I303, CspCh18A 4-MU-(GlcNAc) ₃	37 ± 10	10 ± 2.5	1.1 ± 0.28	8.0	6.0 – 10.0	4.0 – 11.0 (1 h)	50	20 - 70	0 - 70 (1 h)	This work
<i>Pseudoalteromonas aurantia</i> Chi23, 4-MU-(GlcNAc) ₂	65± 12	0.011	5.4 ± 0.5	5.0	4.0 – 5.0	3.0 – 10.0 (1 h)	60	50 - 70	50 - 70 (1 h)	(Wang et al., 2019)
<i>S. marcescens</i> ChiA, 4-MU-(GlcNAc) ₂	59	-	35	-	-	-	-	-	-	(Honda et al., 2003)
<i>S. marcescens</i> ChiB, 4-MU-(GlcNAc) ₂	42	-	12	-	-	-	-	-	-	(Honda et al., 2003)
<i>Thermococcus chitonophagus</i> , Tc-ChiD(ΔS), CC	-	-	-	4.0	3.5 – 4.5	-	90	90 - 95	80 - 100 (30 min)	(Horiuchi et al., 2016)
MetaChi18A chitinase, 4-MU-(GlcNAc) ₂	2.7	40	3.2	5.0	4.0 – 9.0	4.0 – 11.0 (1,24 h)	50	40 - 60	20 - 50 (1,3,24 h)	(Thimoteo et al., 2017)
<i>Thermococcus chitonophagus</i> , Tc-ChiD(ΔBD): CC	-	-	-	4.5	4.5 – 5.0	-	80	80 - 85	80 - 90 (30 min)	(Horiuchi et al., 2016)

Legend: Survey of kinetic parameters and enzyme properties from published work and this work. A comparison of kinetic parameters and the effects of pH and temperature on chitinase activities and stabilities as reported in the literature and in this work. CC, colloidal chitinase

2 Supplementary Figures

**FIGURE S1**

Colloidal chitin cleavage activities of *CspChi8A* and SUMO-*ZmChi18A*. The colloidal chitin cleavage activities of *Zea mays* Basic-endochitinase (*ZmChi19A*) and *Chitinophaga* chitinase sp. (*CspChi18A*) are compared with SUMO as a negative control.

**FIGURE S2**

Predicted locations of O- glycosylation sites in the 3D structures of full-length enzymes **Left:** *Zea mays* Basic-endochitinase (*ZmChi19A*) and **Right:** *Oryza sativa* chitinase (*OsChi19A*). The glycosylation sites were predicted by NetOGlyc version 4, and the images were created using PyMOL version 2.5.2. O-Glycosylation sites shown in orange. *ZmChi19A* is predicted to have two potential glycosylation sites in the chitin-binding domain, and 2 in the catalytic domain (CAT). *OsChi19A* is predicted to have one potential glycosylation site in the catalytic domain (CAT). No N-glycosylation sites were predicted by NetNGlyc for either protein.

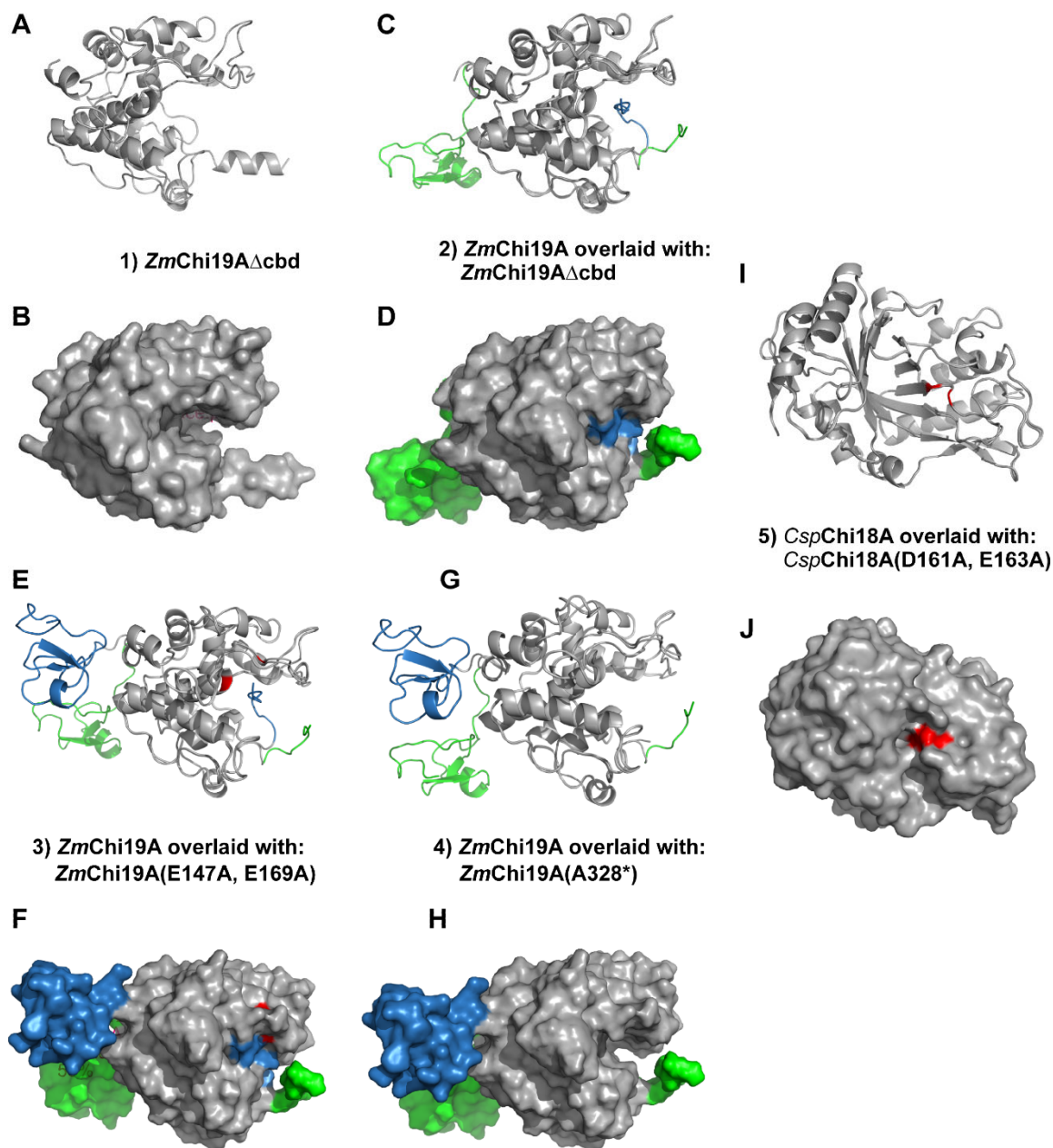


FIGURE S3

Structural alignment of wild-type and mutant chitinases. 1) *ZmChi19A*Δcbd: A) secondary structure, B) surface structure, 2) Alignment of *ZmChi19A* with *ZmChi19A*Δcbd [RMSD = 0.93]: C) aligned secondary structures, D) aligned surface structures. 3) Alignment of *ZmChi19A* with *ZmChi19A*(E147A, E169A) [RMSD = 1.32]: E) aligned secondary structures, F) aligned surface structures. 4) Alignment of *ZmChi19A* with *ZmChi19A*(A328*) [RMSD = 1.48]: G) aligned secondary structures, H) aligned surface structures. 5) Alignment of *CspChi18A* with *CspChi18A*(D161A, E163A) [RMSD = 1.17]: I) aligned secondary structures, J) aligned surface structures. Aligned regions shown in grey, unaligned regions shown in green for the native chitinases and blue for the mutants. Mutated amino acids shown in red. The proteins were folded with AlphaFold and mutants were aligned with the native enzymes to evaluate the effects of the mutations on structure.

Supplementary Data

Maize: *Zea mays*

ZmChi19A NP_001150560.1, NM_001157088.2

0.542 ROOT QQC___

Atgtcgacaccaggagccccgagcctggccacgacagcggcggtgtcctgtgctgctcctggccgcggtgctcgcggtcac
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MSTPGAPSLATTA¹⁰AAVLCVL²⁰AAALAVTVAS³⁰GQQCGQQAGGATCRDCLCCSRFGFCGDTSEYCGAGCQSQC⁷⁰T
 GVASVVRDL⁸⁰FERLLLHRNDAAC⁹⁰PARGFYTYDAFLAAAA¹²⁰FPAGTTGGDEQRKREVA¹³⁰FLGQTSHE¹⁴⁰TGGWPTAPDGP
 SWGYCFKQERNPPSDYCEPRPQWPCAPGKKYFGRGPIQISFN¹⁵⁰NYGYPAGRAIGVDLLNNDLVATDPVIS¹⁶⁰FKTALWFWMN
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 LGQTSHE¹⁵⁰TGGWPTAPDGP¹⁶⁰SWGYCFKQER¹⁷⁰NPPSDYCEPR¹⁸⁰PQWPCAPGKK¹⁹⁰YFGRGPIQIS²⁰⁰FN
 NYGYPAGR²¹⁰AIGVDLLNND²²⁰DLVATDPVIS²³⁰FKTALWFWMN²⁴⁰ARDNKPSCHA²⁵⁰VITGQWTP²⁶⁰TAADRAAGRGAP²⁷⁰GYG
 VITNIIN²⁸⁰GGIECGHGT²⁹⁰PRVTDRI³⁰⁰GFYKRYCDVFRIG³¹⁰YGSNLDCDQ³²⁰RPFNSGLAVE³³⁰VAAQ

Rice: *Oryza sativa*

GenBank: AAL34318.1, GenBank: L40337.1

0.744 ROOT & LEAF EQC___

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 gaccagagggcgttcaacagcgggttgccggcggtgacgtga

(Codon modified for IDT)

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MKATTTAVAL LVAAAAAMVAQ VVAEQCGSQAGGALCPNCLC CSSYGWCGST SDYCGDGCQS QCDGCGGGGG G
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 YGP AGEAIGEDLL NNPELVASDP VVSFKTALWF WMT PQSPKPS CHDVITGQWT PSSGDIAAGR VPG
 YGVITNI INGGLECGFG PDDR VANRIG FYQRYCNVLG IGYGSNLDCY DQRPFN SGLA AVQ*

Bacteria: Chitinophaga sp. 1303 (2832517...2833428)

GenBank: QJB42510.1, GenBank: CP051204.2 0.706 ROOT & SOIL CKK

Tcagggcggtatacgttacgctgctgccatagaggatgttgacacaccggagaaatacacatgtttgctggtgctggtga
 ggttgtagccacaggtaaataccatagccgtgtgttttggctgctgggtggccagccagttggcggtgctttgactggttagcc
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 40 AFAQQLADTA NYYGLDGIDF DDEYADYGNN GLPQPNDSSF VMLLDELRLK MPTKIIISFY YGPAASR
 LSW GGKKAGDFIN YSWNAMYGTYS VVPNVAGLTK ANLGPAAVDI QATSQSTANW LATQTKNNGY GIY
 LWYNLTS TDKHVYFSGV SNILYGSSVT YTP*

p28b-SUMO-TTBasic-Endochitinase-A: [BC: 138.3.2] 6557 bp (bp = 390: 918)
 (a.a = 130: 306) [with SUMO-&His-tag = 47.53 kDa, no tag = 32.54 kDa]
 KanR

tggcgaaatgggacgcgcctgtagcggcgcatgaagcgcggcggtgtggtggttacgcgcagcgtgaccgctacacttg
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Original pET28b-10xHis-SUMO vector: 5636 bp

[BC: 139.8.1]

(bp = 444)

(a.a = 148)

[with His-tag = 16.84 kDa]

KanR

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Nucleotide Sequence (Wild-type) :

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Nucleotide Sequence (M1 + M2) :

ATTGGTGGATCCGAGAACCCTGTACTTTTCAGGGCCAGCAGTGC GGCCAGCAGGCCGGCGGCGCAACGTGCCGCGACTGCCCT
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GGCC

G-block Nucleotide Sequence (M1 + M2_IDT Codon modified):

ATTGGTGGATCCGAGAACCTGTACTTTTCAGGGCCAGCAGTGCAGGGCAGCAGGCAGGCGGTGCGACGTGCAGAGACTGCCCT
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 ACCTTGATTGTGATGGGCAACGTCCTTTCAATAGCGGACTGCGCGTTGAGGTGGCGGCGCAGTAATGATAGAAAGCTTGC
 GGCC

Cloned Nucleotide Sequence:

ATGAGAGGATCGCATCACCATCACCATCACGGATCCATGTGTAAAAAAGACGAAGCCAGGCCCGACAACCCGCAAACCAA
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 GAAATATGCCGACTATGGTAACAATGGGCTGCCACAACCCAATGACAGCTCCTTTGTCTATGCTCCTCGATGAGCTGCGGAAG
 CTGATGCCCAACCAAATAATATCGTTCTATTATTACGGTCCGGCGGCTTCCCGTTTGTCTGGGGCGGCAAAAAAGCAGGC
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 GGCCCCGAGCTGTGGATATTTCAGGCTACCAGTCAAAGCACCGCCAATGGCTGGCCACCAGACCAAAAAACAACGGCTAT
 GGTATTTACCTGTGGTACAACCTCACCAGCACCGACAAACATGTGTATTTCTCCGGTGTGTCCAACATCCTCTATGGCAGC
 AGCGTAACGTATACGCCCATAA

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