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Supplementary material

TaNAC69 from the NAC superfamily of transcription factors is up-regulated by abiotic stresses in wheat and recognises two consensus DNA-binding sequences

Gang-Ping Xue^{A,C}, Neil I. Bower^{A,B}, C. Lynne McIntyre^A, George A. Riding^B, Kemal Kazan^A and Ray Shorter^A

^ACSIRO Plant Industry, 306 Carmody Rd, St Lucia, Qld 4067, Australia.

^BCSIRO Livestock Industry, 306 Carmody Rd. St Lucia, Qld 4067, Australia.

^CCorresponding author. Email: Gang-Ping.Xue@csiro.au

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TaNAC69-1 MPMG-SSAAMPALPPGFRFHPTDEELIVHYLRQQAASMPSPVPIIAEVNIYKCNPWDLPG
TaNAc69-2 MPMGSSAAMPALPPGFRFHPTDEELIVHYLGRQQAASMPSPVPIIAEVNIYKCNPWDLPG
TaNAc69-3 MPMGSSAAMPALPPGFRFHPTDEELIVHYLGRQQAASMPSPVPIIAEVNIYKCNPWDLPG
      ****

TaNAc69-1 KALFGENEWYFFSPDRKYPNGARPNRAAGSGYWKATGTDKAILSTPANESIGVKKALVF
TaNAc69-2 KALFGENEWYFFSPDRKYPNGARPNRAAGSGYWKATGTDKAILSTPANESIGVKKALVF
TaNAc69-3 KALFGENEWYFFSPDRKYPNGARPNRAAGSGYWKATGTDKAILSTPANESIGVKKALVF
      ****

TaNAc69-1 YRGKPPKGVKTDWIMHEYRLTAADNRRTTKRRGSSMRLLDDWVLCRIHKKCGNLNPFSSSDQ
TaNAc69-2 YRGKPPKGVKTDWIMHEYRLTAADNRRTTKRRGSSMRLLDDWVLCRIHKKCGNLNPFSSSDQ
TaNAc69-3 YRGKPPKGVKTDWIMHEYRLTAADNRRTTKRRGSSMRLLDDWVLCRIHKKCNLHNFSSSDQ
      ****

TaNAc69-1 EQEHEQEESSTTVEDSQNNHTVSSPKSEAFDGDGDDHLQLQQFRPMAIAKSCSLTDLNLTV
TaNAc69-2 EQEHEQEESSTTVEDSQNNHTVSSPKSEAFDGDGNDHLQLQQFRPMAIAKSCSLTDLNLTV
TaNAc69-3 EQEHEQEESSTTVEDSHNNHTVSSPKSEAFDGDGDDQLQLQQFRPMAIAKSCSLTDLNLTV
      ****

TaNAc69-1 DYAALSHLLLD--GAGASSDAGADYQLPPEENPLIYSQPPWQQLHYNNNN--GYVNNETI
TaNAc69-2 DYAALSHLLLD--GAGASSDAGADYQLPPEENPLIYSQPPWQQLHYNNNN--GYVNNETI
TaNAc69-3 DYAALSHLLLDGAGAGASSDAGADYQLPPEENPLIYSQPPWQQLHYNNNNNGYVNIIDTI
TaNAc69-4                AGADYQLPPEENPLIYSQPPWQQTVHYNNNN--GYVNNDTI
      ****

TaNAc69-1 DVPQLPEARVDDYGMNGDKYNGMKRKRSSGSLYCSQLQLPADQYSGMLIHFPFLSQQLHM
TaNAc69-2 DVPQLPEAGVDDYGMNGDKYNGMKRKRSSGSLYCSQLQLPADQYSGMLIHFPFLSQQLHM
TaNAc69-3 DVPQIPEARVDDYGMNGDRYNGMKRKRSSGSLYCSQLQLPADQYSGMLIHFPFLSQQLHM
TaNAc69-4 DVPQLPEAHVDDYGMNGDRYNGMKRKRSSGSLYCSQLQLPADQYSGMLIHFPFLSQQLHM
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Supplementary Fig. 1. Sequence alignment of three TaNAC69 proteins and TaNAC69-4 from a wheat EST TaNAC69-1 (AY625682), TaNAC69-2 (DQ022842), TaNAC69-3 (DQ022843), TaNAC69-4 (wheat EST ID: 203331236). TaNAC69-4 is an EST clone in the wheat EST database, identified by homology searching using the TaNAC69-1 sequence.

TaNAC69-1	LPPGFRFHPTDEELIVHYLRRQAASMPSPVPIIAEVNIYKCNPWDLPKALFGENEWYFF		
Hv	LPPGFRFHPTDEELIVHYLGRQAASMPSPVPIIAEVNIYKCNPWDLPKALFGENEWYFF		
Os-I	LPPGFRFHPTDEELIVHYLMNQAAASVKCPVPIIAEVNIYKCNPWDLPKALFGENEWYFF		
Os-J	LPPGFRFHPTDEELIVHYLMNQAAASIKCPVPIIAEVNIYKCNPWDLPKALFGENEWYFF		
Sb	LPPGFRFHPTDEELIVHYLMNQAAASIPCPVPIIAEVNIYQCNPWDLPKALFGESEWYFF		
Mt	LPPGFRFHPTDEELIVHYLCNQATSKPCPASIIPVVDIYKFDPEWLPDKSEFEENEWYFF		
At	LPPGFRFHPTDEELIVHYLRNQATMSKPCPVSIIPEVDIYKFDPEWLPDKTEFGENEWYFF		
St	LPPGFRFHPTDEELIMYYLRYQATSRPCPVSIIPEIDVYKFDPEWLPDKAEFGENEWYFF		
Sd	LPPGFRFHPTDEELIMYYLRYQATSRPCPVSIIPEIDVYKFDPEWLPDKAEFGENEWYFF		
Le	LPPGFRFHPTDEELIMYYLRYQATSRPCPVSIIPEIDVYKFDPEWLPDKAEFGDNEWYFF		
Mc	LPPGFRFHPTDEELIYYLKNQAMSRCPCVSIIPVVDIYKFDPEWLPDKAEFGENEWYFF		
Cs	LPPGFRFHPTDEELIVHYLRNQATSRPCPVSIIPEVDIYKFDPEWLPDKAEFGKEWYFF		
PtxPt	LPPGFRFHPTDEELVVHYLKKKAASVPLPVTIIAEIDLYKLDPEWLPKATFGEQEWYFF		
Gm	LPPGFRFHPTDEELILHYLSKKVASIPLPVSIIAEVDIYKLDPEWLPKATFGEKEWYFF		
	** .*****:::** :. * ..**.*:::** :** * * : * :.*****		
TaNAC69-1	SPRDRKYPNGARP NRAAGSGYWKATGTDKAILSTP---ANESIGVKKALVFYRKGPPKGV		
Hv	SPRDRKYPNGARP NRAAGSGYWKATGTDKAILSTP---ANESIGVKK-----		
Os-I	SPRDRKYPNGARP NRAAGSGYWKATGTDKSLSTP---TSDNIGVKKALVFYRKGPPKGV		
Os-J	SPRDRKYPNGARP NRAAGSGYWKATGTDKSLSTP---TSDNIGVKKALVFYRKGPPKGV		
Sb	SPRDRKYPNGARP NRAAGSGYWKATGTDKAIMSTP---TGQNIQVKKALVFYGGRRPPRGV		
Mt	SPREKYPNGVRPNRATLSGYWKATGTDKAIKSG----SKQIGVKKSLVFYRGRPPKGV		
At	SPREKYPNGVRPNRAAVSGYWKATGTDKAIHSG----SSNVGVKKALVFYRGRPPKGI		
St	TPRDRKYPNGVRPNRAAVSGYWKATGTDKAIYSA----NKYVGIKKALVFYRKGPPKGV		
Sd	TPRDRKYPNGVRPNRAAVSGYWKATGTDKAIYSA----NKYVGIKKALVFYRKGPPKGV		
Le	TPRDRKYPNGVRPNRAAVSGYWKATGTDKAIYSA----NKYVGIKKALVFYRKGPPKGV		
Mc	TPRDRKYPNGIRPNRATVSGYWKATGTDKAIYSG----AKYVGIKKALVFYRKGPPKGI		
Cs	SPRDRKYPNGTRPNRATVSGYWKATGTDKAIYGG----SKYLGVKKALVFYRGRPPKGI		
PtxPt	SPRDRKYPNGARP NRAATSGYWKATGTDKPIILTS---NGAQKVGKALVFYGGKPPKGI		
Gm	SPRDRKYPNGARP NRAAASGYWKATGTDKTIIVTSLQGGAEQESVGVKKALVFYRGRPPKGV		
	:** :***** ***** :***** * :***** * :***** :		
		%Ide	%Sim
TaNAC69-1	KTDWIMHEYRLT-----AADN-RTTK-RRGSS--MRLDDWVLCRIHKK	100	100
Hv	-----	99	99
Os-I	KTDWIMHEYRLTGTS---ANSTTTTKQRRASSMTMRLDDWVLCRIHKK	86	92
Os-J	KTDWIMHEYRLTGTS---ANNTTTTKQRRASSMTMRLDDWVLCRIHKK	86	91
Sb	KTDWIMHEYRLVTAD---ADGKTTAAKRRGGS--MRLDDWVLCRIHKK	84	89
Mt	KTDWIMHEYRLI-----GSQKQT-SKHIGS--MRLDDWVLCRIYKK	74	83
At	KTDWIMHEYRLH-----DSRKAS-TKRNGS--MRLDEWVLCRIYKK	75	83
St	KTDWIMHEYRLS-----DSKSQTYSKQSGS--MRLDDWVLCRIYKK	74	86
Sd	KTDWIMHEYRLS-----DSKSQTYSKQSGS--MRLDDWVLCRIYKK	74	86
Le	KTDWIMHEYRLS-----DSKSQT-SKQSGS--MRLDDWVLCRIYKK	75	87
Mc	KTDWIMHEYRLT-----ESKHQP-IKQHGS--MRLDNWVLCRIYKK	73	83
Cs	KTDWIMHEYRLN-----DPTRQP-YKHNGS--MKLDDWVLCRIYKK	74	81
PtxPt	KTDWIMHEYRLIENSSSRPPAADSATKKGGS--LRLDDWVLCRIYKK	72	84
Gm	KTNWIMHEYRLV-----DN-NKPIKLDSS--MRLDDWVLCRIYKK	75	85
	** .***** :* *****:***** .**		

Supplementary Fig. 2. NAC proteins from other plant species that share the highest homology with TaNAC69-1 in the NAC domain among wheat NAC proteins present in the wheat EST database. Only one NAC protein per plant species sharing the highest homology and > 70% identity with the TaNAC69 NAC domain is presented. Hv, *Hordeum vulgare* NAC (gi: 21182621); Os-I, *Oryza sativa* indica NAC (AAAA02030644); Os-J, *Oryza sativa* japonica NAC (gi: 29674907); Sb, *Sorghum bicolor* NAC (gi: 45949087); Mt, *Medicago truncatula* NAC (gi: 11910349); At, *Arabidopsis thaliana* NAP (AJ222713); St, *Solanum tuberosum* NAC (gi: 21920677); Sd, *Solanum demissum* NAC (AC154033); Le, *Lycopersicon esculentum* NAC (gi: 5604792); Mc, *Mesembryanthemum crystallinum* NAC (gi: 26564003); Cs, *Citrus sinensis* NAC (gi: 38051081); PtxPt, *Populus tremula* × *Populus tremuloides* NAC (gi:24106131); Gm, *Glycine max* NAC (the sequence derived from three EST sequences gi:17962878, 9987195, 21677753). Ide, identity; Sim, similarity. Note: to ascertain whether the above non-cereal NAC genes are the most homologous to TaNAC69 among wheat NAC genes, the NAC domain sequences of these non-cereal genes were used to search the wheat EST database that currently contains 561 100 sequences, using the 'tblastn' program. The NAC genes from these non-cereal species exhibited the highest homology with a wheat 5'-sequence EST (gi:23401647), which contains the partial sequence of TaNAC69-1.

A

TaNAC69-selected oligonucleotides - DNA-binding site I	RBA (%)
S01 gagat CGT gcacagt ACG taactgttaca	100 ± 2.4
S02 tcaagtgg CGT atggtat ACG taactacag	98.1 ± 3.0
S03 gacctgat gCGT atccatt ACG taactac	96.0 ± 3.8
S04 ggagtCGT gtgcgct ACG taacctcctgca	95.3 ± 2.7
S05 ctg gagagaCGT ataggac ACG tcaactcgaa	94.2 ± 2.9
S06 ggggtgt ggagaCGT gtttct ACG gcactg	89.1 ± 2.5
S07 ggccctg agagagaCGT acctgac ACG gcatc	86.4 ± 1.8
S08 ggaatt gatagCGT atcgat ACG caacct	82.0 ± 3.4
S09 ggg aaagCGT aatggat ACG caaccacggc	79.3 ± 2.8
S010 agctg gaaagCGT atgata ACG taatc	77.5 ± 1.5
S011 gagtgaCGT atgaggt ACG taatcaggcgc	74.6 ± 2.3
S012 aagatgCGT acacgat ACG taaccagctcc	70.2 ± 4.0
S013 gatggCGT acatcat ACG taacttatcgct	68.0 ± 1.9
S014 gcaattCGT gtgtgt ACG tcaactgcac	67.4 ± 2.2
S015 aggtagCGT agctcat ACG taaccgtgagta	65.0 ± 1.7
S016 taaagaCGT gattacc ACG taactccatgac	63.2 ± 2.8
S017 agagaCGT ggacatc ACG tctcaggactat	60.9 ± 3.1
S018 ggttgcCGT aaggtct ACG tattcctagt	58.8 ± 1.4
S019 caag attgCGT aatcaat ACG caactc	57.0 ± 1.8
S020 ggatagCGT aggtgt ACG taattgtagt	56.2 ± 2.3
S021 gatgcCGT gtcttat ACG caagtgtgaatc	55.7 ± 2.0
S022 gccc ggttgcCGT acagtgc ACG tcatcgt	54.2 ± 1.6
S023 acct gagtgaCGT caggttc ACG taactggt	44.8 ± 2.4
S024 gctagCGT atggagc ACG taactctatggtcaactc	42.7 ± 1.3
S025 gctagCGT atgcatt ACG caactaatcgtctgctc	41.2 ± 1.8
S026 cac gaaggCGT gaggtac ACG taatcattgc	38.6 ± 1.7
S027 gatcgccaacatg CGT aaagtat ACG tcaacc	38.3 ± 1.9
S028 caa agagcCGT atgtgct ACG caacagtgc	35.0 ± 1.4
S029 cggattCGT atcgatg ACG taaccgaagct	31.8 ± 1.8
S030 caga atatCGT gttgetc ACG tacttggga	31.3 ± 1.3
S031 tgc gttatCGT ggttcat ACG taacagtca	24.9 ± 1.5
S032 tcgaaggCGT atcctac ACG gatctgttcg	23.2 ± 1.2
S033 caac gatagCGT aaaggcct ACG tatcgaat	19.9 ± 0.9
S034 agtaaCGT gccgtac ACG tctgtactctgc	14.0 ± 1.2
S035 gacgcCGT acatgat ACG tcaactcgttggt	13.1 ± 1.5
S036 gagggaCGT ggcacgt ACG gaactcaagcc	12.6 ± 1.4
S037 acggaaCGT ggatcca ACG taattcctag	11.2 ± 0.7
S038 cgacaatatCGT tgacct ACG taaccagct	9.1 ± 0.8
Consensus rrwdnCGTrnnnnnyACGtaayy	

	r	r	w	d	n	C	G	T	r	n	n	n	n	n	y	A	C	G	t	a	a	y	y
A	16	15	16	13	9	0	0	0	22	9	10	3	8	17	1	38	0	0	0	27	34	0	2
C	0	3	1	0	5	38	0	0	1	7	9	10	9	5	15	0	38	0	6	9	0	26	16
G	22	18	1	17	18	0	38	0	14	6	13	15	9	6	1	0	0	38	4	0	0	1	1
T	0	2	20	8	6	0	0	38	1	16	6	10	12	10	21	0	0	0	28	2	4	11	19

B

TaNAC69-selected oligonucleotides - DNA-binding site II	RBA (%)
S039 ggaggtg ttt aatg ttt ac ACG tctctagt	93.3 ± 3.4
S040 cgg gt ttt gtctatt ccACG ctctttcat	54.9 ± 2.2
S041 catgctag ttc acctggg cACG ttctctgga	39.1 ± 1.8
S042 agactggtcaaat tttagcACG caactactg	33.4 ± 1.5
S043 agagacttaggct ttt at ACG tcaactttag	31.0 ± 1.7
S044 tga aaagagtc ggttcc ccattcACG taacc	29.1 ± 1.6
S045 cga agact ctatgctccg tACG tcaattgca	27.3 ± 1.2
S046 cct taatt atcatag gtaacACG taatcct	17.5 ± 1.1
S047 tcgatag tggtgctg tttACG gcactgaac	10.7 ± 1.2
Consensus rgtskttnnnnnnnyACGtmwyy	

Supplementary Fig. 3. Sequence alignment of TaNAC69-selected oligonucleotides and their binding activity. (A) TaNAC69 binding site I. Oligonucleotides were cloned from the fourth and fifth round of binding site selection. The consensus binding sequence is highlighted in bold. The lower-case letters of the consensus sequence indicate the bases which are present in > 50% of TaNAC69-selected oligonucleotides. Relative binding activity (RBA) is measured as fluorescent units and is relative to the binding activity of SO1. Values are means ± s.d. of triplicate assays. (B) TaNAC69 binding site II. RBA is relative to the binding activity of SO1. Values are means ± s.d. of triplicate assays. d = agt, m = ca, s = gc, k = gt, r = ag, w = at, y = ct. Note: initially, 15 TaNAC69-selected oligonucleotides, cloned from the fifth round of

binding site selection, were analysed for TaNAC69-binding activity. Fourteen of them exhibited binding activity and were sequenced. Sequence alignment of these oligonucleotides revealed a binding sequence spanning 23 bp [these oligonucleotide sequences were presented for supporting the methodology paper (Xue, 2005)]. As TaNAC69 binds to a relatively long DNA sequence, many more selected oligonucleotides are required for the identification of consensus binding sequences. Thus, 15 more oligonucleotides cloned from the fifth round selection and 35 from the fourth round selection were analysed for binding activity. Thirty-three oligonucleotides of 50 with significant binding activity were sequenced. The sequence alignment of a total of 47 oligonucleotides revealed the two consensus binding sites above.

TN69L29	LPPGFRFHPTDEELIVHYLRRQAASMPSPVPIIAEVNIYKCNPWDLP GKALFGENEWYFF			
NAP	LPPGFRFHPTDEELIVYLRNQTMSPKCPVSIPEVDIYKFDPWQLPEKTEFGENEWYFF			
ANAC025	LPPGFRFHPTDEELVHYLKKKADSVPLPVSIIAEIDLKFDPWELPSKASFGEHEWYFF			
ANAC056	LPPGFRFHPTDEELVHYLKRKAASAPLPVAIIAEVDLYKFDPWELPAKASFGEQEWYFF			
AtNAM	LPPGFRFHPTDEELVIHYLKRKADSVPLPVAIIADVDLYKFDPWELPAKASFGEQEWYFF			
	A	B		
TN69L29	SPRDRKYPNGARPNRAAGSGYWKATGTDKAILSTPANES--IGVKKALVFYRGPCKGKVK			
NAP	SPREKYPNGVRPNRAAVSGYWKATGTDKAIHSGSSN---VGVKKALVFYKGRPPKGIK			
ANAC025	SPRDRKYPNGVRPNRAATSGYWKATGTDKPIFTCN---HKVGVKKALVFYGGKPPKGIK			
ANAC056	SPRDRKYPNGARPNRAATSGYWKATGTDKPVLASDGNQ--KGVKKALVFYSGKPPKGVK			
AtNAM	SPRDRKYPNGARPNRAATSGYWKATGTDKPVISTGGGSKKVGKALVFYSGKPPKGVK			
	C	D		
		%Ide	%Sim	
TaNAC69-1	<u>TDWIMHEYRLTAADNRT-----TKRRGSSMRLLDDWVLCRIHKK</u>	100	100	
NAP	<u>TDWIMHEYRLHDSRKAS-----TKRSGS-MRLDEWVLCRIYKK</u>	75	83	
ANAC025	<u>TDWIMHEYRLTDGNLSTAAKPPDLTTRKNSLRLLDDWVLCRIYKK</u>	70	81	
ANAC056	<u>SDWIMHEYRLIENKPNRPPGCDFGN-KKNSLRLLDDWVLCRIYKK</u>	71	82	
AtNAM	<u>SDWIMHEYRLTDNKPTHI---CDFGN-KKNSLRLLDDWVLCRIYKK</u>	71	84	
	E			

Supplementary Fig. 4. Sequence alignment TaNAC69-1 NAC domain with *Arabidopsis* NAC proteins. Only NAC proteins with amino acid identity at 70% or higher are shown. In terms of sequence homology in five conserved subdomains, the homology level of these four *Arabidopsis* NAC proteins with TaNAC69 is very similar. Relatively lower percentages in the amino acid identity with TaNAC69 in ANAC025, ANAC056 and AtNAM are attributed to linker length. NAP (AJ222713, At1g69490), ANAC025 (At1g61110), ANAC056 (At3g15510) and AtNAM (AF123311, At1g52880). Ide, identity; Sim, similarity.

Supplementary Table 1. A list of primers used for cloning or RT-PCR

The sequences underlined are restriction sites for cloning into the DBP-CELD vector

Primer name	Sequence
NAC69S1	5'-GCAGCATT <u>TTTT</u> TATGCAGTAGCCA
NAC69S2	5'-ATTGATTCCGTTCC <u>CC</u> ACCTG
TaNAC69F	5'-CCATTTGAACTTGCTGTAGATCGA
TaNAC69R	5'-TTCTGAAATTTGTCCGTTCTTATCG
TaNAC69-1F	5'-TGCCTCCCGAAAACCCA
TaNAC69-1R	5'-TTGTTCACGTAGCCGTTGTTGT
TaNAC69-2F	5'-CAGCTCCAGCGGATCAGT
TaNAC69-2R	5'-CTTTTCGATCCAAGCTCTTTGG
TaNAC69-3F	5'-AACAAATGGCTACGTGAACATCGA
TaNAC69-3R	5'-AAACTGCCGCTGGACCTCTT
TaNAC69-4F	5'-CACTATAACAATAACAACGGGTACGTG
TaNAC69-4R	5'-TCTACATGCGCCTCGGGTA
TaCCFF	5'-GCTCTAACCCACTCGGCCTAA
TaCCFR	5'-TCTACACCCAGTACACATATGACATAA
C12B07F	5'-GAACTGTCTGGATTGTCCCATCA
C12B07R	5'-ACAGTAGGCCACACCAATGTAC
ANAC029S	5'-CCAGGCTAGCGAAGTAACTTCCCAATCTACCCT
ANAC029A	5'-CCGGATCCAAACTTAAACATCGCTTGACGATGATGGT

Supplementary Table 2. The final concentrations of components in the association reactions

Component	High stringency	Low stringency
Hepes	25 mM	25 mM
KCl	100 mM	50 mM
EDTA	0.16 mM	0.25 mM
MgCl ₂	5 mM	4.5 mM
Bovine serum albumin	1 mg mL ⁻¹	0.9 mg mL ⁻¹
DTT	1.1 mM	1.2 mM
Triton X-100	0.067%	–
Nonidet P-40	–	0.02%
Glycerol	–	9%