Functional characterization of *BjCET3* and *BjCET4,* two new cation-efflux transporters from *Brassica juncea* L.

Minglin Lang, Mengyu Hao, Qiangwang Fan, Wei Wang, Shaojing Mo, Weicheng Zhao, Jie Zhou

Supporting information

Figure legends

Fig. S1. Multiple alignment analysis of deduced amino acid sequences of the CE proteins. Clustal X software were used for multiple alignments of deduced amino acid sequences of the CE proteins. Conserved amino acid and putative zinc binding site were indicated. Accession numbers and species names of each gene are shown in supplemental Table S2.

Fig. S2. Testing the effect of Cd stress on the growth of *B. juncea*. A serial of Cd concentration (0, 25, 200, 500, 1000, 5000 μM $CdCl_2$) and 0-16 days time course were used for the treatment. The photographs showed no significant harmful effect on the *B. juncea* plants in the two days of Cd treatment (A, B). After 16 d Cd treatment, plants treated with 25 μM $CdCl_2$ grew normally as the control (without Cd treatment), plants treated with 200 and 500 μM $CdCl_2$ grew similar to each other, and a little more worse than control and 25 μM $CdCl_2$ treated plants (C). Plants treated with 1000 and 5000 μM $CdCl_2$ grew much worse than the others (C).

Fig. S3. A representative of zinc-tolerance assay plates. The image shows growth of vector control and *BjCET4* expressing *cot1& zrc1* strains. Disks 1–5 containing 1.2, 2.4, 4, 6, and 8 μmol of Zn, respectively.

Fig. S4. Analysis of the effect of expressing BjCET3 and BjCET4 in *cot1* mutant yeast with over supplied metal ions. Expression of BjCET3 and BjCET4 restore growth of empty vector (pYES2) transformed *cot1* yeast strain under metal stressed condition. Growth on SMM-Uracil liquid medium (6.7 g yeast nitrogen base without amino acids, 20 mg histidine and 4 % (w/v) galactose per liter) with or without metal supplementation is shown. Control shows yeast growth on medium without metal supplementation.

Fig. S5. The photographs show representative performance of BiCET3, BjCET4 and empty vector (control) transgenic tobaccos at day 50 of growing at normal condition.

Fig. S6. Testing the exporting activity of BjCET:GFP proteins. The BjCET3:GFP and BjCET4:GFP proteins were expressed in the *zrc1* mutant yeast (YK41) and cultured with oversupplied Zn. Metal tolerance was quantified by using the assay system described in Persans *et al.*, 2001. Data are means ± SD of three biological repeats. *zrc1* yeast expressing BjCET3:GFP or BjCET4:GFP show high tolerance to Zn stress than control (*zrc1* expressing pYES2:GFP).

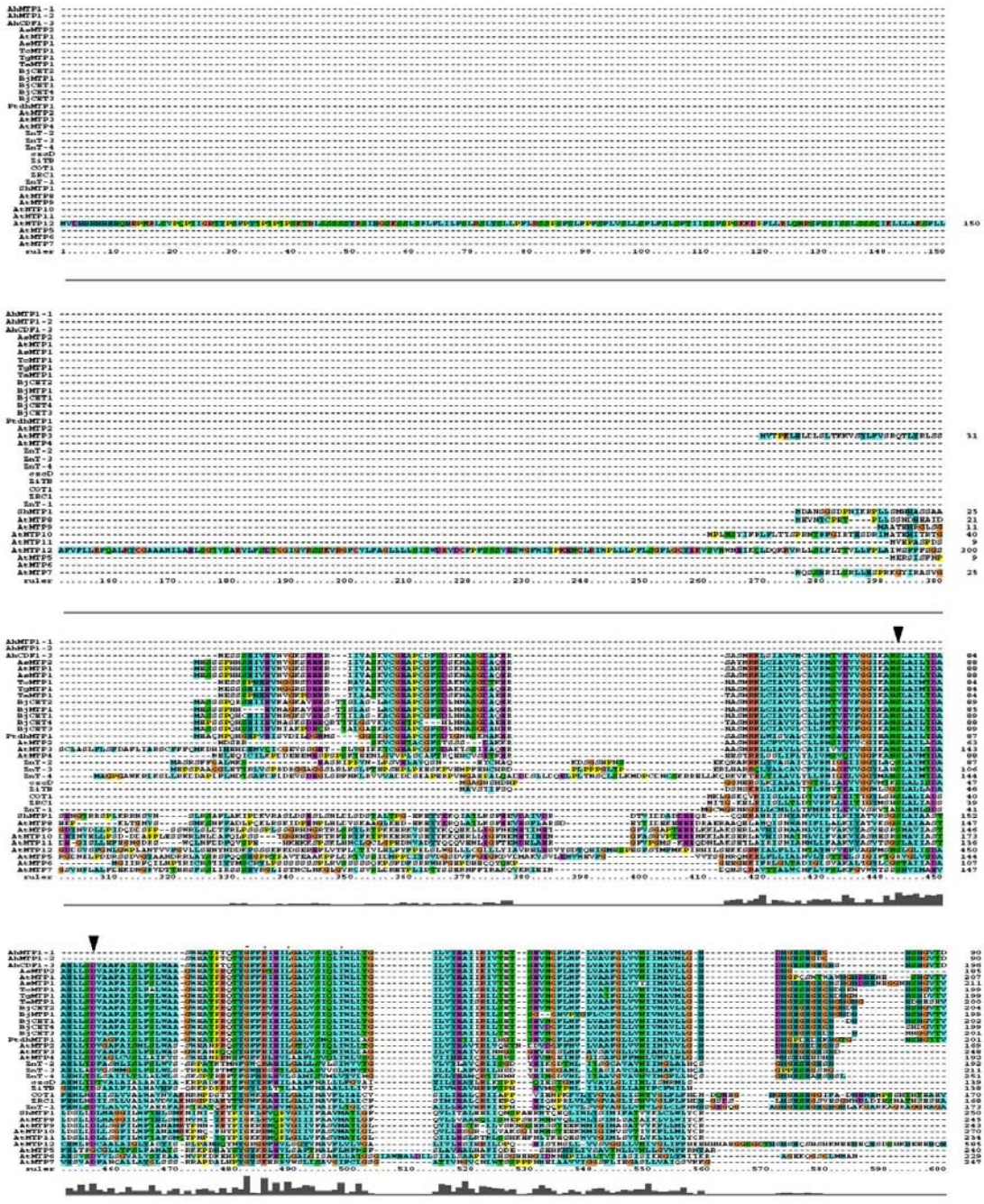


Fig S1-1

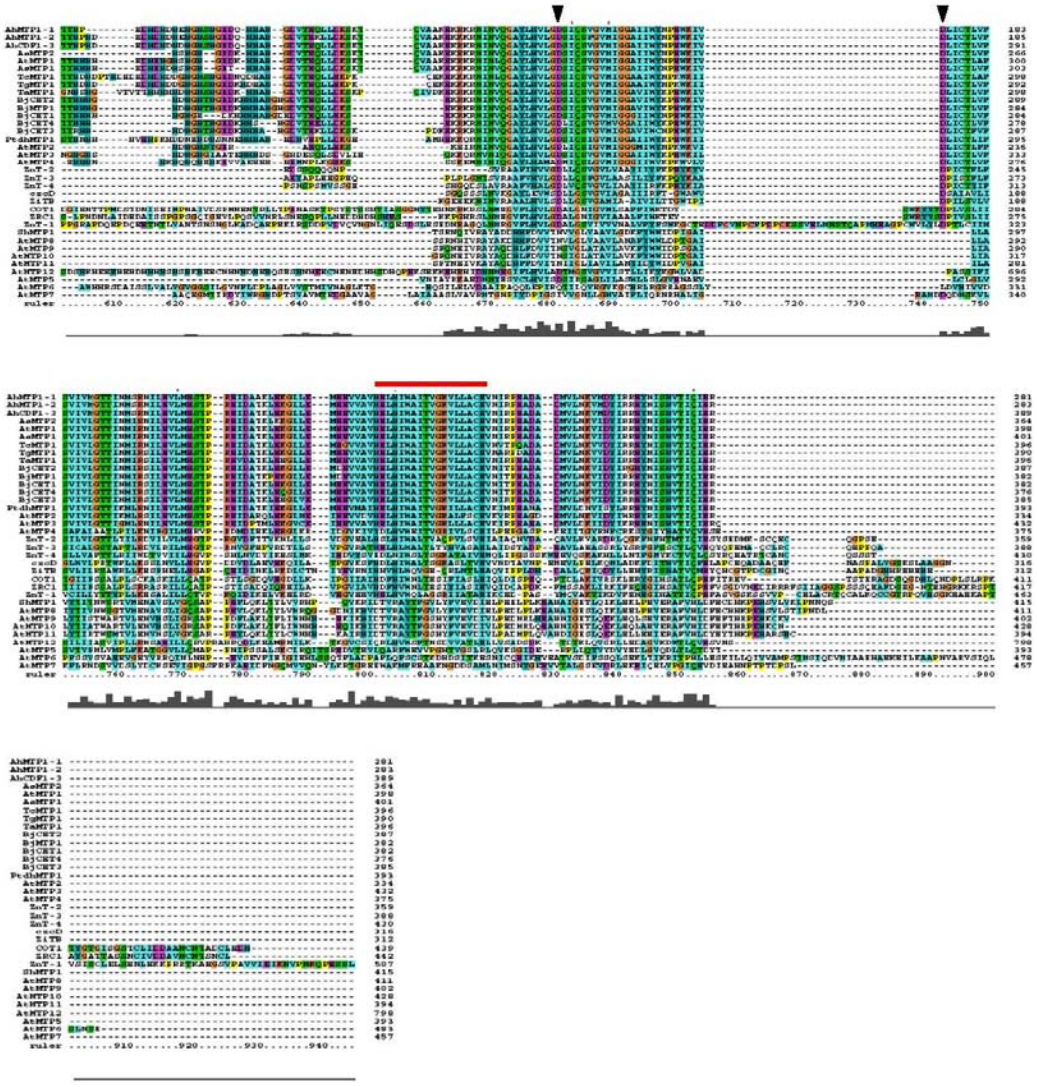
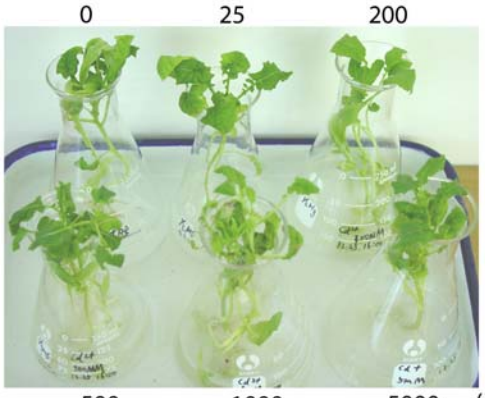


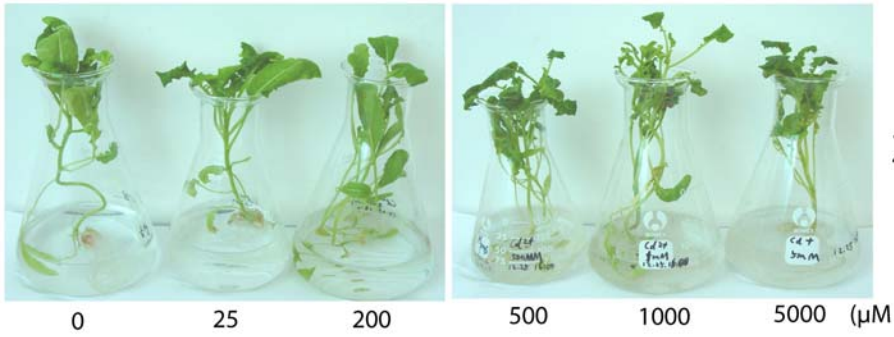
Fig S1-2

A



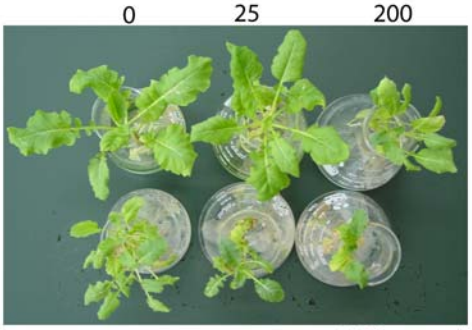
0 day

B



2 days

C



16 days

Fig. S2

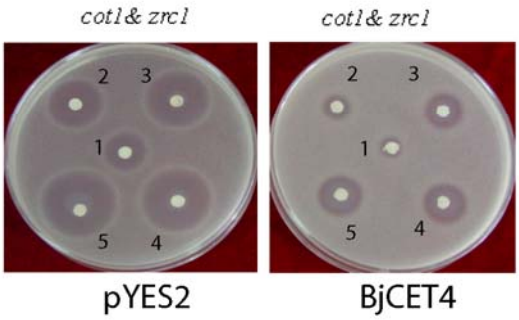


Fig. S3

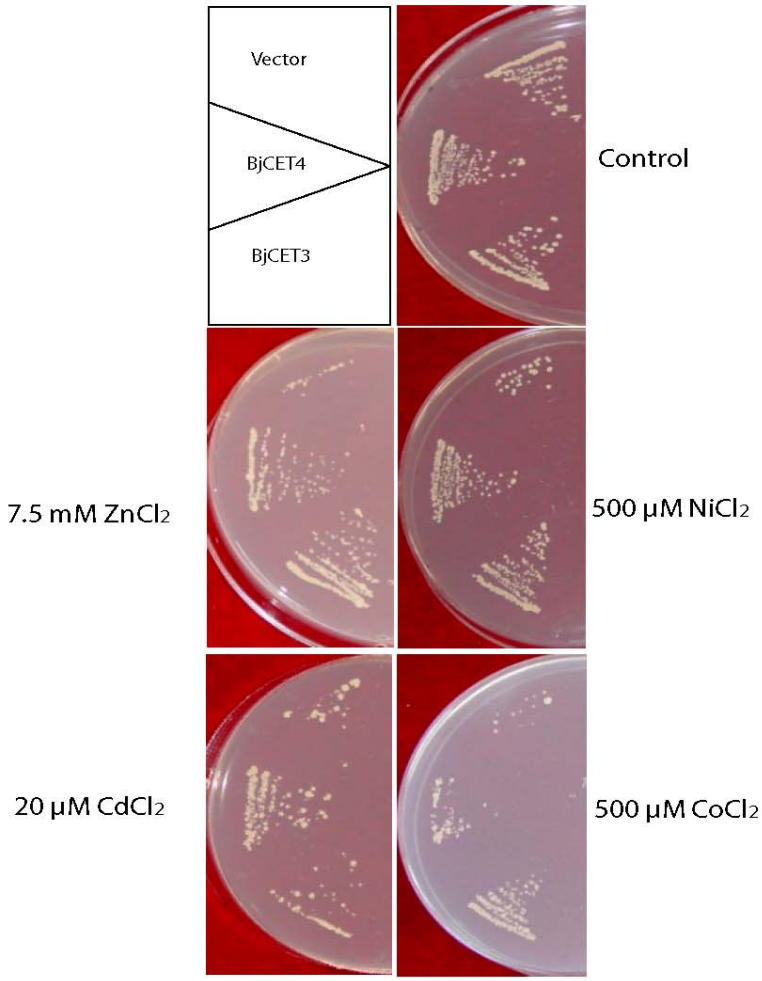

Vector
BjCET4
BjCET3
Control
7.5 mM ZnCl2
500 µM NiCl2
20 µM CdCl2
500 µM CoCl2

Fig. S4

50 days in normal medium

Fig. S5

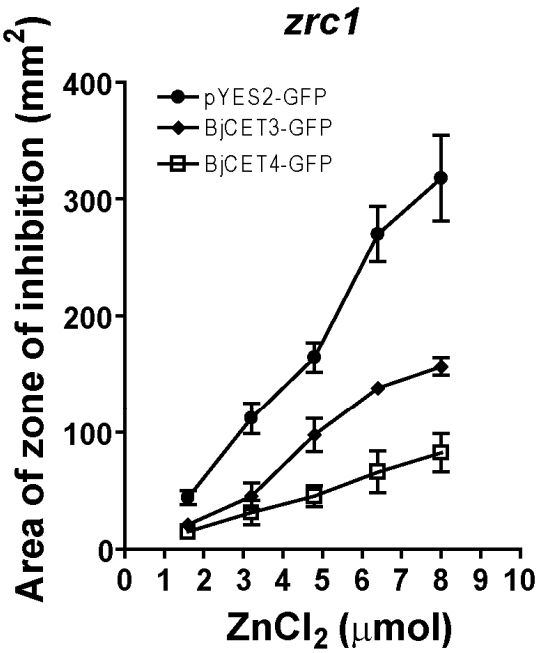

zrc1
Area of zone of inhibition (mm2)
400
300
200
100
0
pYES2-GFP
BjCET3-GFP
BjCET4-GFP
0 1 2 3 4 5 6 7 8 9 10
ZnCl2 (μmol)

Fig. S6

Table S1 Topology and hydrophile analysis of putative proteins of CE members from *B. juncea* and other species CSS, conserved N-terminal signature sequence; PSS, partial N-terminal signature sequence; TM,

Gene name	Species	GenBank Accession no.	Residues	N-terminal signature sequence	Transmembrane-spanning regions					
					No.	TM1	TM2	TM3	TM4	TM5
BjCET1	*B. juncea*	AY187082	382	SLAILTDAAHLLSD (CSS)	6	59 -76	92-111	126-145	161-182	248-269
BjCET2	*B. juncea*	AY188449	388	SLAILTDAAHLLSD (CSS)	6	59 - 76	92-111	126-145	161-182	255-274
BjCET3	*B. juncea*	AY188450	385	SLAILTDAAHLLSD (CSS)	6	59 - 76	92-111	126-145	161-182	253-272
BjCET4	*B. juncea*	***AY188451***	376	SLAILTDAAHLLSD (CSS)	6	58 -75	91-110	125-144	160-181	244--263
BjMTP1	*B. juncea*	AY483149	382	SLAILTDAAHLLSD (CSS)	6	55 -72	88-107	122-141	156-177	250-269
ZAT	*A.thaliana*	NM180128	398	SLAILTDAAHLLSD (CSS)	6	58 -77	91-110	125-144	160-180	266-285
AtMTP6	*A.thaliana*	NP182304	483	STAIIADAAHSVSD (CSS)	5	89 -108	107-130	147-163	191-212	237-257
AhCDF1-2	*A. halleri*	AJ556183	389	SLAILTDAAHLLSD (CSS)	6	54 -71	87-106	121-140	156-177	257-276
TgMTP1	*T.goesingense*	AAK91869	390	SLAIMTDAAHLLSD(CSS)	6	54 -73	87-106	121-140	156-177	258-277
ShMTP1	*Stylosanthes hamata*	AY181256	415	SVNSLRSAF –ASLD(PSS) SVNSLRSAFL-ASLD(PSS) ASLD	5	121-141	146-170	189-208	230-249	284-302
ZnT1	*Rattus orvegicus*	Q62720	507	SLAIMLSDSPHMLSD(CSS)	6	11-28	42-61	79-101	114 -134	246-270
Cot1	*S. cerevisiae*	NP014961	439	SLALIADSPHMLND(CSS)	6	10 -29	43-60	78-100	114-133	243-267
ZRC1	*S. cerevisiae*	S56057	442	SLALIADSPHMLND(CSS)	6	9 -30	34-59	77-99	113-132	234-263
ZiTB	*Yersinia pestis*	Q8ZGY6	312	SLALLADAGHMLTD(CSS)	6	16 -35	48-66	84 -102	114-137	159-177
czcD	*Alcaligenes sp. CT14*	P94178	316	SLALISDAAHMLTD(CSS)	6	19 -37	47-67	84 -103	119-135	156-174

TM6		C-terminal Cation-efflux domain	His-rich domain	Molecular weight (D)	Isoelectric point	Solubility (soluble %)	Hydrophobic (%)	Hydrophilic (%)	Basic (%)	Acidic (%)
275-293	N-terminus inside	59-382	TM4 -TM5	42,074	6.32	71	46.1	28.5	14.1	11.3
280-298	N-terminus inside	59-388	TM4 -TM5	42,297	6.42	69	46.4	28.6	14.2	10.8
278-296	N-terminus inside	59-385	TM4 -TM5	42,426	6.47	69	46.8	27.0	14.8	11.4
269-287	N-terminus inside	58-376	TM4 -TM5	41,487	6.05	79	46.3	28.2	13.6	12.0
275-293	N-terminus inside	55-382	TM4 -TM5	41,728	6.39	68	45.0	29.6	14.4	11.0
291-309	N-terminus inside	58-398	TM4 -TM5	44,064	6.53	68	46.2	27.1	15.3	11.3
no	N-terminus inside	77-371	no	52,311	7.44	76	44.7	32.7	13.9	8.7
282-300	N-terminus inside	54-389	TM4 -TM5	42,920	6.09	76	45.5	27.5	14.4	12.6
283-301	N-terminus inside	54-390	TM4 -TM5	42,963	6.03	77	44.6	27.6	14.9	13.3
no	N-terminus outside	122-401	no	46,701	5.87	56	47.2	28.0	12.5	12.3
312-333	N-terminus inside	11-425	TM4 -TM5	55,142	6.43	55	45.2	30.8	13.0	11.0
278-297	N-terminus inside	10-386	TM4 -TM5	48,155	6.27	54	43.5	34.4	12.1	10.0
269-288	N-terminus inside	9-378	TM4 -TM5	48,345	6.34	62	44.8	32.6	12.4	10.2
179-197	N-terminus inside	16-291	no	34,828	6.94	64	52.6	24.7	14.1	8.7
174 -194	N-terminus outside	17-292	no	33,741	6.24	52	54.4	26.9	9.8	8.9

Table S2 Gene name, GenBank accession number and species

Gene Name	**Accession No.**	**Species**
BjCET1	AAO64482	*Brassica juncea*
BjCET2	AAO83658	*Brassica juncea*
BjCET3	AAO83659	*Brassica juncea*
BjCET4	AAO83660	*Brassica juncea*
BjMTP1	AAR83910	*Brassica juncea*
AtMTP1(ZAT)	NP_182203	*Arabidosis thaliana*
AtMTP2	NP_191753	*Arabidosis thaliana*
AtMTP3	NP_191440	*Arabidosis thaliana*
tMTP4	NP_180502	*Arabidosis thaliana*
AtMTP5	NP_187817	*Arabidosis thaliana*
AtMTP6	NP_182304	*Arabidosis thaliana*
AtMTP7	NP_564594	*Arabidosis thaliana*
AtMTP8	NP_191365	*Arabidosis thaliana*
AtMTP9	NP_178070	*Arabidosis thaliana*
AtMTP10	NP_173081	*Arabidosis thaliana*
AtMTP11	NP_181477	*Arabidosis thaliana*
AtMTP12	NP_178539	*Arabidosis thaliana*
TgMTP1	AAR83910	*Thlaspi goesingense*
TcMTP1	AAR83907	*Thlaspi caerulescens*
TaMTP1	AAR83906	*Thlaspi arvense*
AsMTP1	AAR83908	*Arabidopsis lyrata*
AsMTP2	AAR83909	*Arabidopsis lyrata*
AhCDF1-3	CAD89013	*Arabidopsis halleri*
AhMTP1-1	AJ704801	*Arabidopsis halleri*
AhMTP1-2	CAG28977	*Arabidopsis halleri*
ShMTP1	AY181256	*Stylosanthes hamata*
ZnT-1	AAA79234	*Rattus norvegicus*
ZnT-2	AAB02775	*Rattus norvegicus*
ZnT-3	AAB39732	Homo sapiens
ZnT-4	NP_742063	*Rattus norvegicus*
COT1	CAA99636	*Saccharomyces cerevisiae*
ZRC1	CAA88653	*Saccharomyces cerevisiae*
ZiTB	NP_670349	*Yersinia pestis*
czcD	BAA11062	*Alcaligenes sp.* CT14