

New Insights into the Structure-Function Relationship of the Endosomal-Type Na⁺, K⁺/H⁺ Antiporter NHX6 from Mulberry (*Morus notabilis*)

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Supplemental data

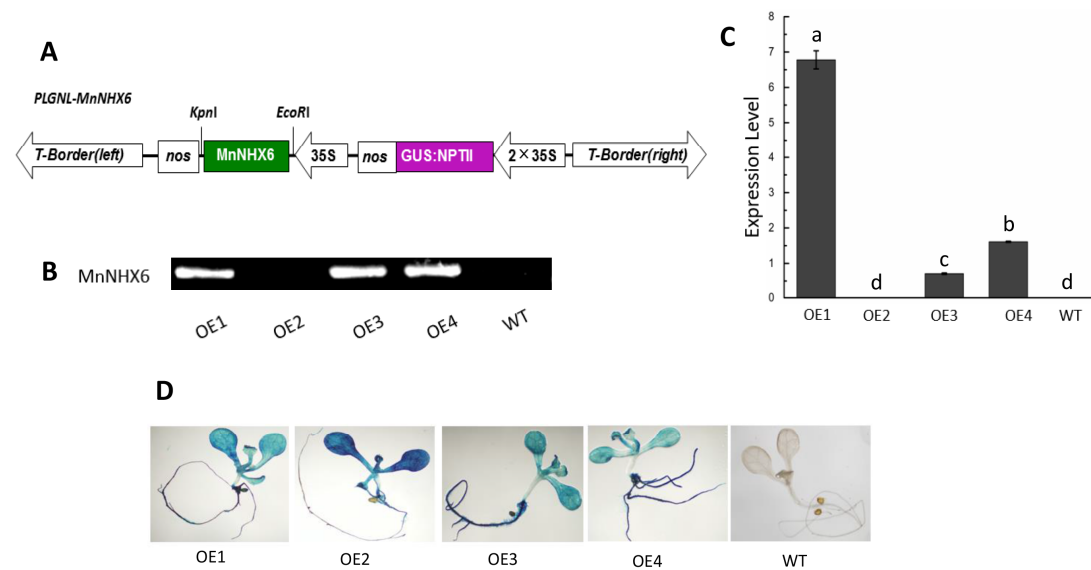


Figure S1. Confirmation of transgenic arabidopsis plants. (A) Diagram of the transgenic vector. (B) Genomic PCR analysis of transgenic lines. (C) Quantitative real-time PCR. Data are means $\pm$ SDs ($n = 3$), $p < 0.05$. Significant differences are indicated by different letters above the bar. (D) Histochemical GUS staining of transgenic lines.

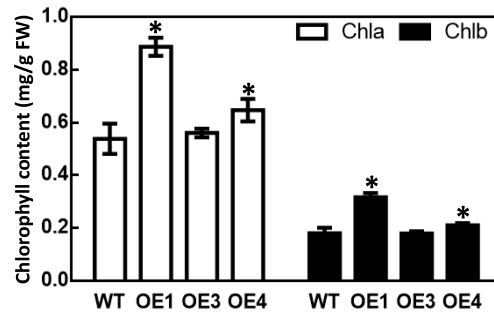


Figure S2. The chlorophyll a (Chla) and chlorophyll b (Chlb) contents in MnNHX6 transgenic Arabidopsis and WT plants under NaCl-stress conditions. Data are means $\pm$ SDs ($n = 6$), * $P < 0.05$.

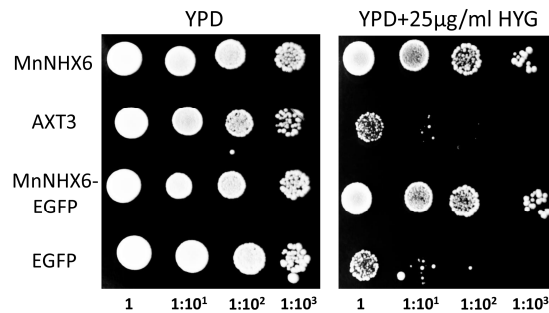


Figure S3. Growth of the strains on the YPD supplemented with or without hygromycin B. The MnNHX6 open-reading frames were cloned into the plasmid pYPGE15 and the plasmid pYPGE15-EGFP without the stop codon. These constructs were introduced into the yeast mutant strain AXT3. The AXT3 (empty pYPGE15) and EGFP (pYPGE15-EGFP) were used as negative controls. 4 μ L of 10-fold serial dilutions of these strains from saturated cultures were spotted onto the YPD plates with or without hygromycin B (25 μ g/mL).

	type		
AtNHX1		MLDSLVSK.....LPSLSTSDHRSVVALNLFVALCACVACVCHLLEN..RWNNSITALLLGGTVTLILISGKKS.S.HLIVSSEDLLEFLLPPIIFNAQFV	98
AtNHX2		MTWFSLSISK.....MLSVSTSDHRSVVALNLFVALCACVACVCHLLEN..RWNNSITALLLGGTVTLILISGKKS.HLIVSSEDLLEFLLPPIIFNAQFV	100
AtNHX3		MSIGLTFE.....VTNKLAAEHPQVPIQSVFIALCLCLVCHLLEN..RWNNSITALLLGGTVTLILISGKKS.HLIVSSEDLLEFLLPPIIFNAQFV	98
AtNHX4		MVIGLSMTLEK.....TEALFASDHRSVVALNLFVALCACVACVCHLLET..RWNNSITALLLGGTVTLILISGKKS.RLIVSSEDLLEFLLPPIIFNAQFV	101
MdNHX1		MAVPHLSMLISK.....LQNLSTSDHRSVVALNLFVALCACVACVCHLLEN..RWNNSITALLLGGTVTLILISGKKS.HLIVSSEDLLEFLLPPIIFNAQFV	102
AtNHX5		MEEVMISVEHD.....PQQQVQQAAGAGGILLQIMMVLSVSWGVRRHRHFYLLPQASASLLLGLIVGLANISNTETSIKTFWNGDEEPPFGLPPIIFOSSEFL	105
AtNHX6		MSSELQISPAIHD.....PQQQVQQAAGAGGILLQIMMVLSVSWGVRRHRHFYLLPQASASLLLGLIVGLANISNTETSIKTFWNGDEEPPFGLPPIIFOSSEFL	106
MnNHX6		MAVEDAQMLGLRISPSDSNGSSIPTTPGKEQQAAGGILLQIMMVLSVSWGVRRHRHFYLLPQASASLLLGLIVGLANISNTETSIKTFWNGDEEPPFGLPPIIFOSSEFL	117
LeNHX2		MEDHLQISPAIG.....AKAIPGKEQQAAGGILLQIMMVLSVSWGVRRHRHFYLLPQASASLLLGLIVGLANISNTETSIKTFWNGDEEPPFGLPPIIFOSSEFL	105
PeNHX6		MSTVVEELQMQISPAAGGGGRDSQSHPKKEQQAAGGILLQIMMVLSVSWGVRRHRHFYLLPQASASLLLGLIVGLANISNTETSIKTFWNGDEEPPFGLPPIIFOSSEFL	117
AtNHX1		KKKQPFNFVTIMLSGAVTIIISCTIISLGVTOFFKKLDIGTFDLGDYALGAIAPANTSSCTLOVLNQD.ETPLLVSDFVGEVNDATSVVFNATQSFDLTHLNHEAAFHLLGNSEY	217
AtNHX2		KKKQPFNFVTIMLSGAVTIIISCTIISLGVTOFFKKLDIGTFDLGDYALGAIAPANTSSCTLOVLNQD.ETPLLVSDFVGEVNDATSVVFNATQSFDLTHLNHEAAFHLLGNSEY	219
AtNHX3		KKKQPFNFVTIMLSGAVTIIISCTIISLGVTOFFKKLDIGTFDLGDYALGAIAPANTSSCTLOVLNQD.ETPLLVSDFVGEVNDATSVVFNATQSFDLTHLNHEAAFHLLGNSEY	217
AtNHX4		KKKQPFNFVTIMLSGAVTIIISCTIISLGVTOFFKKLDIGTFDLGDYALGAIAPANTSSCTLOVLNQD.ETPLLVSDFVGEVNDATSVVFNATQSFDLTHLNHEAAFHLLGNSEY	220
MdNHX1		KKKQPFNFVTIMLSGAVTIIISCTIISLGVTOFFKKLDIGTFDLGDYALGAIAPANTSSCTLOVLNQD.ETPLLVSDFVGEVNDATSVVFNATQSFDLTHLNHEAAFHLLGNSEY	221
AtNHX5		QRFQPFNFAGAVTIIISCTIISLGVTOFFKKLDIGTFDLGDYALGAIAPANTSSCTLOVLNQD.ETPLLVSDFVGEVNDATSVVFNATQSFDLTHLNHEAAFHLLGNSEY	225
AtNHX6		QRFQPFNFAGAVTIIISCTIISLGVTOFFKKLDIGTFDLGDYALGAIAPANTSSCTLOVLNQD.ETPLLVSDFVGEVNDATSVVFNATQSFDLTHLNHEAAFHLLGNSEY	225
MnNHX6		QRFQPFNFAGAVTIIISCTIISLGVTOFFKKLDIGTFDLGDYALGAIAPANTSSCTLOVLNQD.ETPLLVSDFVGEVNDATSVVFNATQSFDLTHLNHEAAFHLLGNSEY	237
LeNHX2		SPRFQPFNFAGAVTIIISCTIISLGVTOFFKKLDIGTFDLGDYALGAIAPANTSSCTLOVLNQD.ETPLLVSDFVGEVNDATSVVFNATQSFDLTHLNHEAAFHLLGNSEY	225
PeNHX6		SPRFQPFNFAGAVTIIISCTIISLGVTOFFKKLDIGTFDLGDYALGAIAPANTSSCTLOVLNQD.ETPLLVSDFVGEVNDATSVVFNATQSFDLTHLNHEAAFHLLGNSEY	236
TM8 of AtNHX1			
* MdNHX1 Ser275			
AtNHX1		LDLSTLLAATGLISAVIKKLYFGRHST..EVALMMMAISYMLAEFLDSGLTVFEGGVVSHYTMHNTSSRITTKHTATLSAEFLSGLVSLDIDKWRVSVDTFG	336
AtNHX2		LDLSTLLAATGLISAVIKKLYFGRHST..EVALMMMAISYMLAEFLDSGLTVFEGGVVSHYTMHNTSSRITTKHTATLSAEFLSGLVSLDIDKWRVSVDTFG	338
AtNHX3		LDLSTLLAATGLISAVIKKLYFGRHST..EVALMMMAISYMLAEFLDSGLTVFEGGVVSHYTMHNTSSRITTKHTATLSAEFLSGLVSLDIDKWRVSVDTFG	336
AtNHX4		LDLSTLLAATGLISAVIKKLYFGRHST..EVALMMMAISYMLAEFLDSGLTVFEGGVVSHYTMHNTSSRITTKHTATLSAEFLSGLVSLDIDKWRVSVDTFG	339
MdNHX1		LDLSTLLAATGLISAVIKKLYFGRHST..EVALMMMAISYMLAEFLDSGLTVFEGGVVSHYTMHNTSSRITTKHTATLSAEFLSGLVSLDIDKWRVSVDTFG	340
AtNHX5		TVGQMSAAGVGVGTSAALLPKYAGLDIDNLQ..ECCLFVFPFVPSYMLAEFLDSGLTVFEGGVVSHYTMHNTSSRITTKHTATLSAEFLSGLVSLDIDKWRVSVDTFG	338
AtNHX6		TVGQMSAAGVGVGTSAALLPKYAGLDIDNLQ..ECCLFVFPFVPSYMLAEFLDSGLTVFEGGVVSHYTMHNTSSRITTKHTATLSAEFLSGLVSLDIDKWRVSVDTFG	338
MnNHX6		TVGQMSAAGVGVGTSAALLPKYAGLDIDNLQ..ECCLFVFPFVPSYMLAEFLDSGLTVFEGGVVSHYTMHNTSSRITTKHTATLSAEFLSGLVSLDIDKWRVSVDTFG	350
LeNHX2		TVGQMSAAGVGVGTSAALLPKYAGLDIDNLQ..ECCLFVFPFVPSYMLAEFLDSGLTVFEGGVVSHYTMHNTSSRITTKHTATLSAEFLSGLVSLDIDKWRVSVDTFG	338
PeNHX6		TVGQMSAAGVGVGTSAALLPKYAGLDIDNLQ..ECCLFVFPFVPSYMLAEFLDSGLTVFEGGVVSHYTMHNTSSRITTKHTATLSAEFLSGLVSLDIDKWRVSVDTFG	349
MnNHX6 Ser292*			
AtNHX1		TTSVAVSSILMGLVMVGRAPFVPLSPUSNAKKHQ..SEKISFNFMVVMWSGIMRGAVSMALAYNKEFRAGHTDVRGNAIMISGITVCLFSTVVFVLMKPLISYLLPHONATTS..	451
AtNHX2		TTSVAVSSILMGLVMVGRAPFVPLSPUSNAKKHQ..SEKISFNFMVVMWSGIMRGAVSMALAYNKEFRAGHTDVRGNAIMISGITVCLFSTVVFVLMKPLISYLLPHONATTS..	456
AtNHX3		GTIGVSGVITAVI.IISGAAPVPIPVGMNMRHTERNKSTTPKHQVIMWAGIMRGAVSMALAYNKEFRAGHTDVRGNAIMISGITVCLFSTVVFVLMKPLISYLLPHONATTS..	456
AtNHX4		QSTIGVSSILGLLILGRAPFVPLSPUSNAKKHQ..DEKIDLKQCVIMWAGIMRGAVSMALAYNKEFRAGHTDVRGNAIMISGITVCLFSTVVFVLMKPLISYLLPHONATTS..	455
MdNHX1		TTSVAVSSILGLLILGRAPFVPLSPUSNAKKHQ..HEKISLRQCVIMWAGIMRGAVSMALAYNKEFRAGHTDVRGNAIMISGITVCLFSTVVFVLMKPLISYLLPHONATTS..	456
AtNHX5		SHVGFIFSLPIFIVARAVNVGCGYLDNARPAH..RQPLKHKOKALMYSGLR..GAMABALALQSHDLPEGHG...QTIFPATTAIVLVTLVLLISGSTMLEADVVGDSHDT..S	449
AtNHX6		SHLGFIFSLPIFIVARAVNVGCGYLDNARPAH..RQPLKHKOKALMYSGLR..GAMABALALQSHDLPEGHG...QTIFPATTAIVLVTLVLLISGSTMLEADVVGDSHDT..S	449
MnNHX6		SHVGFIFSLPIFIVARAVNVGCGYLDNARPAH..RQPLKHKOKALMYSGLR..GAMABALALQSHDLPEGHG...QTIFPATTAIVLVTLVLLISGSTMLEADVVGDSHDT..S	463
LeNHX2		SHVGFIFSLPIFIVARAVNVGCGYLDNARPAH..RQPLKHKOKALMYSGLR..GAMABALALQSHDLPEGHG...QTIFPATTAIVLVTLVLLISGSTMLEADVVGDSHDT..S	449
PeNHX6		SHVGFIFSLPIFIVARAVNVGCGYLDNARPAH..RQPLKHKOKALMYSGLR..GAMABALALQSHDLPEGHG...QTIFPATTAIVLVTLVLLISGSTMLEADVVGDSHDT..P	460
AtNHX1		MLSDD..STPKS.IHIFLLDQ...QDSFIEFGSNHNVFRPDSIRGFTIRPFR..TVHYIYWRQDQDAFMRFVGGRGVFPVFGSGPTERNPFDLSKA.....	538
AtNHX2		MLSDD..STPKS.IHIFLLDQGEGLDSFELPGSHQVFRPNSLGRFLMRPFR..TVHYIYWRQDQDAFMRFVGGRGVFPVFGSGPTERNPFDLSKA.....	546
AtNHX3		GRKTEPGSPKEDATLELLS...FDGASATFNFRKADISILMEPVY..TIHRYWKRQDQDAFMRFVGGRGVFPVFGSGPTERNPFDLSKA.....	529
AtNHX4		ALGVTPLKSS.FHDETLHEPLSTQD...SEYFPEQGVFRFMFKSPSR..AIHRYWKRQDQDAFMRFVGGRGVFPVFGSGPTERNPFDLSKA.....	552
MdNHX1		MLSEPTTPKS.IIIFLLG...QDSVDD..LVIDIRIRASIRDLITTPENRHTVHRWVKEDAFMRFVGGRGVFPVFGSGPTERNPFDLSKA.....	544
AtNHX5		NSEGFPEDSHQVYVPE...FSIGASDEDDSSSRFKMKLKEFHK...TTSTETALDNFLTPETNSGDDGDE..E.....	521
AtNHX6		LGDDGFVFNRSYMT.....SYDDDETTPPGSFRFTKLREFHK...SAASFTEDENYLTPETNSNGDDYDDEGNMROHHEERIPFTRRGNLNNRG..	535
MnNHX6		LGDNFE..GNGYLAPSTQVNGNHGYHAPPYDEPSSGSRKMKLKEFHK...STTSFSALDENYLTPETNSNGD.DVGDDEFPVPMSSRRSYG.....	555
LeNHX2		MDTEFE..GNGGYIAPS.....YSDESVDGSPSSGNFRMKLKEFHK...STTSFSALDENYLTPETNSNGD.EDE..DEPIMSSRRAGYDGH.....	531
PeNHX6		LSDESLD..GNGGYIAPS.....YNEATSSNRLKMKLKEFHK...STASTETALDENYLTPETNSNGD.DEE...EHGKIDYCNQA.....	533

Figure S4. The alignment of the amino acid sequences of plant vacuolar-type NHX and endosomal-type NHX. The TM8 segment of AtNHX1 is marked with red box, which had been to determine by experiment [1]. The Ser275 (MdNHX1) [2] and Ser292 (MnNHX6) are marked with red asterisk.

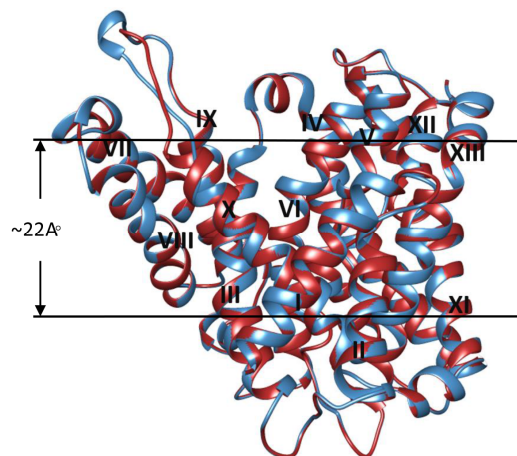


Figure S5. Comparison of the transmembrane domains in 3-D models of MnNHX6 (Wild-type, brown) and R402C (mutant, blue), the TM segments are marked with the black Roman numerals.

Table S1. Site-directed mutagenesis of all the highly conserved charged residues in the model of MnNHX6 were shown, including the conserved negatively charged residues Glu66, Glu98, Glu99, Asp176, Glu186, Asp190, Glu200, Asp205, Glu271, Glu287, Glu332, Asp342, Glu346, the conserved positively charged residues Lys120, Lys258, Arg367, Arg402, and the conserved structure-related residues Pro107, Pro107 and Ser292.

MnNHX6	Location	Conservation score
E66Q	TM2	8
E98Q	TM3	6
E99Q	TM3	6
D176N	TM5	9
E186Q	TM5-TM6	7
D190N	TM5-TM6	8
E200Q	TM6	9
D205N	TM6	9
E271Q	TM7-TM8	9
E287Q	TM8-TM9	8
E332Q	TM10	9
D342N	TM10-TM11	7
E346Q	TM10-TM11	6
K120C	TM3-TM4	5
K258C	TM7-TM8	9
R367C	TM11	9
R402C	TM12	9
P107C	TM3	9
P108C	TM3	9
S292C S292D S292K	TM8-TM9	9

Table S2. Primers used in this work.

Primer	Sequence	Use
MnNHX6-F1	ATGGCGGTTGAGGACG	Cloning of MnNHX6 (without the stop codon were fused the EGFP)
MnNHX6-R1	CTAACCGCGGTAACCTCTTAGAA	
E66Q F	TTCTATTATCTTCCAAGGCCAGTGCTTCC	
E66Q R	CTGTGGAAGATAATAGAATTGTGACGGCG	
E98Q F	TGGTTCAATTTTCACAGAGTTTTTCTTC	
E98Q R	CTGTGAAAATTGAACCATGATCTGATGCT	

E99Q F	TTCAATTTTCACGAGCAGTTTTTCTTCCTG	Point mutations of the conserved negatively charged residues of MnNHX6
E99Q R	CTGCTCGTGAAAATTGAACCATGATCTGAT	
D176N F	CTTATATCAGCGACTAATCCGGTCACTGTTTTG	
D176N R	ATTAGTCGCTGATATAAGAGCACCAAACATTAG	
E186Q F	TTGTCTATATTTTCAGCAACTCGGCACGGAT	
E186Q R	TTGCTGAAATATAGACAAAACAGTGACCGG	
D190N F	CAGGAACTCGGCACGAATATGAACCTATAT	
D190N R	ATTCGTGCCGAGTTCCTGAAATATAGACAA	
E200Q F	GCCTTGGTTTTTGGGCAATCCGTCTTGAAT	
E200Q R	TTGCCCAAAAACCAAGGCATATAGGTTTCAT	
D205N F	GAATCCGTCTTGAATAATGCTATGGCAATT	
D205N R	ATTATTCAAGACGGATTCCCCAAAACCAA	
E271Q F	AACCTGCAGAACTTGCAAGTGTGTCTATTT	
E271Q R	CTGCAAGTTCTGCAGGTTGTCAATGTCTAA	
E287Q F	TCGTACATGCTTGCAAGGTCTTAGCCTC	
E287Q R	TTGTGCAAGCATGTACGAGAAATATGGAAA	
E332Q F	ATATCATCACTCGCACAGACATTTGTTTTT	
E332Q R	CTGTGCGAGTGATGATATCAAATGAAAAAA	
D342N F	ATATACATGGGCTTCAATATCGCCATGGAA	
D342N R	ATTGAAGCCCATGTATATAAAAACAAATGT	
E346Q F	TTCGATATCGCCATGCAACAGCATAGCTGG	
E346Q R	TTGCATGGCGATATCGAAGCCCATGTATAT	
K120C F	TTCAGTCTTCCACCTTGTCTTCTTCTCA	Point mutations of the conserved positively charged residues of MnNHX6
K120C R	ACAAGGTGGAAGACTGAATCCTGACTGAAA	
K258C F	TCTGCTTTGCTTTTTTGCTATGCAGGGTTA	
K258C R	GCAAAAAAGCAAAGCAGAAATAAATGCAAC	
R367C F	TTATCATAGTTGCATGTGCAGCAAATGTC	
R367C R	ACATGCAACTATGATAAATATAATTGAGAA	
R402C F	TGGTACAGTGGACTCTGTGGGGCTATGGCT	
R402C R	ACAGAGTCCACTGTACCAAAGGGCTTTCTG	
P107C-F	TTCTGTCTTCTCTTATGTCCAATCATATTT	Point mutations of the conserved structure-related residues of MnNHX6
P107C-R	ACATAAGAGAAACAGGAAGAAAAACTCCTC	
P108C-F	CTGTTTCTCTTACCTGTATCATATTTTCA	
P108C-R	ACAAGGTAAGAGAAACAGGAAGAAAAACTC	
S292C-F	GAAGGTCTTAGCCTCTGTGGTATTGTGTCA	
S292C-R	ACAGAGGCTAAGACCTTCTGCAAGCATGTA	
S292D-F	GAAGGTCTTAGCCTCGATGGTATTGTGTCA	
S292D-R	ATCGAGGCTAAGACCTTCTGCAAGCATGTA	
S292K-F	GAAGGTCTTAGCCTCAAAGGTATTGTGTCA	
S292K-R	TTTGAGGCTAAGACCTTCTGCAAGCATGTA	

References

1. Yamaguchi, T.; Apse, M.P.; Shi, H.; Blumwald, E. Topological analysis of a plant vacuolar Na⁺/H⁺ antiporter reveals a luminal C terminus that regulates antiporter cation selectivity. *P. Natl. Acad. Sci. USA* **2003**, *100*, 12510-12515.
2. Sun, M.H.; Ma, Q.J.; Hu, D.G.; Zhu, X. P., You, C.X.; Shu, H.R. The glucose sensor mdhxl1 phosphorylates a tonoplast Na⁺/H⁺ exchanger to improve salt tolerance. *Plant Physiol.* **2018**, *176*, pp.01472.