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ARTICLE ADDENDUM

Plant endosomal NHX antiporters: Activity and function

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ABSTRACT

The Arabidopsis NHX antiporter family contains eight members that are divided into three subclasses: vacuolar, endosomal, and plasma membrane. While the plasma membrane and vacuolar NHXs have been studied extensively, the activity and function of the endosomal NHXs are beginning to be discovered. AtNHX5 and AtNHX6 are endosomal $Na^+,K^+/H^+$ antiporters that share high sequence similarity. They are localized in the Golgi, trans-Golgi network (TGN), and prevacuolear compartment (PVC). Studies have shown that AtNHX5 and AtNHX6 mediate K^+ and Na^+ transport, and regulate cellular pH homeostasis. Sequence alignment has found that AtNHX5 and AtNHX6 contain four conserved acidic amino acid residues in transmembrane domains that align with yeast and human NHXs. Three of these conserved acidic residues are critical for K^+ transport and seedling growth in *Arabidopsis*. Moreover, studies have shown that the precursors of the seed storage proteins are missorted to the apoplast in the *nhx5 nhx6* knockout mutant, suggesting that AtNHX5 and AtNHX6 regulate protein transport into the vacuole. Further analysis found that AtNHX5 and AtNHX6 regulated the binding of VSR to its cargoes. Taken together, AtNHX5 and AtNHX6 play an important role in cellular ion and pH homeostasis, and are essential for protein transport into the vacuole.

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$Na^+,K^+/H^+$ antiporters (NHX antiporters) are H^+-coupled cotransporters that transfer the Na^+ or K^+ across a membrane in exchange for protons (H^+).[1-3] Plant NHX antiporters are critical for cellular ion homeostasis and pH regulation. They play significant roles in Na^+ and K^+ movement, pH homeostasis, vesicle trafficking, protein transport, regulation of cell cycle and cell proliferation, salt tolerance, and growth and development.[4-10]

In *Arabidopsis*, the NHX antiporter family contains eight members. Based on their subcellular localizations, the AtNHX family is categorized into three subclasses: vacuolar (AtNHX1-AtNHX4), endosomal (AtNHX5 and AtNHX6), and plasma membrane (AtNHX7/SOS1and AtNHX8).[4-6,11] Studies have shown that the plasma membrane NHX AtNHX7/SOS1 is critical for cellular Na^+, K^+ and pH homeostasis, and play an important role in salt tolerance.[12-17] In addition, the vacuolar NHXs are involved in the regulation of cellular ion and pH homeostasis, and are critical for salt tolerance, K^+ homeostasis, and plant growth and development.[1,4,5,18]

The activity and function of the endosomal NHXs are beginning to be explored. AtNHX5 and AtNHX6 share high sequence similarity (78.7%).[4,19] They are localized in the Golgi, TGN, and PVC.[10,20,21] Recent studies have found that the endosomal NHXs play an important role in cellular ion and pH homeostasis, and are essential for plant growth and development.[10,20,21]

AtNHX5 and AtNHX6 regulate ion and pH homeostasis in Arabidopsis

Studies have shown that the *Arabidopsis* vacuolar NHXs[22-25] and plasma membrane NHXs[13,14,16] mediated K^+ and Na^+ transport in *Arabidopsis*. Do the endosomal NHXs share a common mold of action with the plasma membrane and vacuolar NHXs and are involved in K^+ and Na^+ transport in *Arabidopsis*?

Wang et al. recently show that the endosomal NHXs AtNHX5 and AtNHX6 mediate K^+ and Na^+ transport in *Arabidopsis*.[21] Using a yeast expression system, they found that AtNHX5 and AtNHX6 recovered yeast tolerance to high K^+ or Na^+. They show that AtNHX5 and AtNHX6 function at high K^+ at acidic pH while AtCHX17 at low K^+ under alkaline conditions, suggesting that AtNHXs and AtCHXs may have different modes of action in mediating K^+ homeostasis. Wang et al. further examined the role of AtNHX5 and AtNHX6 in K^+ homeostasis in plants.[21] They found that root growth of *nhx5 nhx6* was inhibited significantly at low K^+; overexpression of the *AtNHX5* or *AtNHX6* genes in *nhx5 nhx6* recovered root growth to the wild-type level. In addition, they found that the K^+ levels in *nhx5 nhx6* were dramatically reduced compared to the wild type seedlings.[21] On the other hand, Bassil et al. characterized the role of AtNHX5 and AtNHX6 in salt tolerance.[20] They found that *nhx5 nhx6* was sensitive to salt stress: the fresh

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weight of *nhx5 nhx6* was significantly reduced under the stress of 150 mM NaCl. They further show that seedling growth of *nhx5 nhx6* was arrested after cotyledon emergence under salt stress.[20] These results suggest that AtNHX5 and AtNHX6 are critical to K^+ and Na^+ homeostasis in *Arabidopsis*. These results also suggest that AtNHXs share a common mold of action with the plasma membrane NHXs and the vacuolar NHXs.

Moreover, Reguera et al. investigated the role of AtNHX5 and AtNHX6 in regulating the pH of endomembrane compartments.[10] They used organelle specific pHluorin-based pH sensors to measure the luminal pH of Golgi, TGN, and the late prevacuolar compartment (LPVC). They found that *nhx5 nhx6* had a reduced pH in these endosomal compartments relative to the wild type.[10] This observation was supported by Wang et al.[21] They showed that *nhx5 nhx6* had a reduced pH in the mature roots and the cell sap extracted from rosette leaves, as measured by the fluorescein-based ratiometric pH indicator BCECF (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein) and a semimicroelectrode, respectively. These results suggest that AtNHX5 and AtNHX6 regulate cellular pH homeostasis.

Three conserved acidic residues in AtNHX5 and AtNHX6 are critical for K^+ transport and growth in Arabidopsis

Sequence alignment has found that there are four conserved acidic residues in the transmembrane domains of the NHX antiporters from bacteria, yeast and mammals.[26] Mutation of three of these conserved acidic residues in yeast ScNhx1p blocked protein trafficking in yeast, suggesting that these conserved amino acids are crucial for vacuolar trafficking in yeast. In addition, mutation of E262 in human NHE1 (E262 is equivalent to E225 of yeast ScNhx1p) abolished ion exchange activity, suggesting that these conserved residues are essential for ion transport activity.[27] Homology modeling shows that two of the residues (Asn262 and Asp267) of human NHE1are localized within TM5.[28] TM5 is located close to TM4 and TM11, which form an assembly structure and involve in conformation change at the cation-binding site following pH activation.[28,29] Thus, localization of these acidic residues in the proximity of the core structure suggests that they may function in binding and translocating cations in the process of ion exchange.[28,29]

Wang et al. reported that AtNHX5 and AtNHX6 contain ur conserved acidic amino acids in transmembrane domains that align with the ScNhx1p and human NHE1 sequences.[21] The four acidic residues of AtNHX5, D164, E188, D193 and E320, align with the D201, E225, D230 and E355 of ScNhx1p, respectively; similarly, the D165, E189, D194 and E320 of AtNHX6 line up with the D201, E225, D230 and E355 of ScNhx1p, respectively.[21] They further showed that mutation of three of the conserved residues in both AtNHX5 and AtNHX6 failed to recover yeast growth in high K^+ and hygromicin B. In addition, they expressed these mutated genes of AtNHX5 and AtNHX6 in *nhx5 nhx6*, and found that the mutants failed to complement the growth of the *nhx5 nhx6* seedlings. These results suggest that the conserved acidic residues play critical roles in K^+ transport and growth in *Arabidopsis*.[21] In addition, these results suggest that AtNHX5 and AtNHX6 may share similar core structure and transport mode to their yeast and human counterparts, and these conserved acidic residues may involve in binding and translocating cations in ion exchange.[21]

AtNHX5 and AtNHX6 regulate protein transport toward the vacuole

In plants, seed storage proteins are synthesized as precursors in the endoplasmic reticulum (ER). Then they are transported into the protein storage vacuoles (PSVs) and are converted to mature forms.[30-32] Studies have shown that proteins are transported to the vacuole through a vesicle-mediated trafficking pathway that includes the ER, Golgi, TGN, and MVB/PVC.[33] Hence, the Golgi, TGN and MVB/PVC are major protein sorting stations in vacuolar transport.[34,35] Since AtNHX5 and AtNHX6 are localized to the Golgi, TGN and PVC, where they overlap with the protein trafficking pathway, it is interesting to ask whether these two endosomal NHXs function in protein trafficking toward vacuole? If yes, how?

Reguera et al. recently reported that AtNHX5 and AtNHX6 were involved in protein trafficking toward vacuole.[10] They found that the precursors of the seed storage proteins 2S albumin and 12S globulin were missorted to the apoplast in the *nhx5 nhx6*. They further showed that the interaction between VSR2;1and its cargoes aleurain and 12S globulin was reduced in *nhx5 nhx6*. Moreover, they found that the luminal pH of VSR compartments as well as the trans-Golgi network and prevacuolar compartments was more acidic in *nhx5 nhx6* than the wild type.[10] These results suggest that AtNHX5 and AtNHX6 regulate the binding of VSR to its cargoes; endosomal pH homeostasis is critical for vacuolar trafficking of the seed storage proteins. How pH regulates the interaction between VSR and its cargoes, however, remains to be studied.

Disclosure of potential conflicts of interest

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