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Genome-Wide Identification and Bioinformatics Analysis of the ScNRAMP Gene Family in Sugarcane: Postprint

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Abstract

NRAMP proteins (natural resistance-associated macrophage proteins) play important roles in plant responses to heavy metal stress and can transport heavy metal ions such as Fe2+, Mn2+, Zn2+, and Cd2+. To investigate the characteristics of the sugarcane ScNRAMP gene family, this study identified the ScNRAMP gene family based on the Saccharum spontaneum genome and conducted analyses of physicochemical properties, gene structure, cis-acting elements, conserved motifs, domains, and evolutionary relationships. The results showed that the sugarcane ScNRAMP gene family contains 29 members, which are unevenly distributed across 19 chromosomes; the encoded proteins are all unstable, lack signal peptides, and are subcellularly localized to the plasma membrane; the number of conserved motifs ranges from 6 to 10, and the number of transmembrane domains ranges from 6 to 12 across members; the main components of the secondary structure are α-helices and random coils; cis-acting element analysis indicated that the sugarcane ScNRAMP gene family may participate in biological processes such as stress response and growth and development through hormone metabolism; tissue-specific analysis using RNA-seq transcriptome data from Saccharum spontaneum revealed that ScNRAMP exhibits spatiotemporal expression characteristics in leaves and stems at different developmental stages of sugarcane; phylogenetic analysis divided the sugarcane ScNRAMP family members into three subfamilies (I, II, and III). This study systematically identified the NRAMP gene family in Saccharum spontaneum, one of the ancestral species of modern cultivated sugarcane, at the whole-genome level, providing a foundation for further understanding of sugarcane NRAMP genes and important candidate genes for subsequent heavy metal research in sugarcane.

Full Text

Preamble

Genome-Wide Identification and Bioinformatics Analysis of the ScNRAMP Gene Family in Sugarcane

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Abstract

NRAMP (natural resistance-associated macrophage protein) family proteins play essential roles in plant responses to heavy metal stress by transporting divalent metal ions such as Fe^{2+}, Mn^{2+}, Zn^{2+}, and Cd^{2+}. To investigate the characteristics of the sugarcane ScNRAMP gene family, we identified ScNRAMP family members from the *Saccharum spontaneum* genome and conducted comprehensive analyses of their physicochemical properties, gene structure, cis-acting elements, conserved motifs, domains, and evolutionary relationships. The results revealed that the sugarcane ScNRAMP gene family comprises 29 members unevenly distributed across 19 chromosomes. The encoded proteins were all predicted to be unstable, signal peptide-free, and localized to the plasma membrane. Family members contained 6–10 conserved motifs and possessed 6–12 transmembrane domains, with α-helices and random coils as the predominant secondary structure elements. Cis-acting element analysis suggested that the ScNRAMP gene family may participate in stress responses and developmental processes through hormone metabolism. Using publicly available RNA-seq transcriptome data, tissue-specific expression analysis revealed spatiotemporal expression patterns of ScNRAMP genes in leaves and stems at different developmental stages. Phylogenetic analysis classified the 29 ScNRAMP family members into three subfamilies (I, II, and III). This study provides the first systematic genome-wide identification of the NRAMP gene family in *S. spontaneum*, an ancestor of modern cultivated sugarcane, establishing a foundation for further understanding of sugarcane NRAMP genes and offering important candidate genes for future research on heavy metal stress in sugarcane.

Keywords: sugarcane, *Saccharum spontaneum*, ScNRAMP, gene family, heavy metal stress

Introduction

Sugarcane (*Saccharum* spp.) is an important annual or perennial sugar crop belonging to the monocotyledonous family Poaceae (Irvine, 1999). As a C4 plant with high photosynthetic efficiency, sugarcane is characterized by large biomass, low CO_2 compensation point, drought tolerance, wide adaptability, and high yield (Fang et al., 2014). Sugarcane is an allopolyploid with ploidy levels ranging from 5× to 16× and a genome size of approximately 10 Gb (Chen et al., 2011). Modern cultivated sugarcane is the world' s most important sugar source, accounting for 80% of global sugar production (Liu et al., 2020).

NRAMP transporters play crucial roles in maintaining ion homeostasis, particularly in the transport of divalent metal ions, and this protein family is highly conserved during evolution (Nevo & Nelson, 2006). The amino acid sequence of mammalian NRAMP genes shows 46%, 58%, and 73% similarity with those of yeast, rice, and fruit flies, respectively (Belouchi et al., 1995). NRAMP proteins and other membrane transporters are essential for heavy metal absorption in plants (Cellier et al., 1995). Mäser et al. (2001) cloned and phylogenetically analyzed six NRAMP genes in *Arabidopsis thaliana*, dividing them into two subfamilies: AtNRAMP1 and AtNRAMP6 in the first subfamily, and AtNRAMP2-AtNRAMP5 in the second. AtNRAMP1, the primary high-affinity manganese transporter in *Arabidopsis*, is localized to the plasma membrane and functions in transporting heavy metals into the cytoplasm (Cailliatte et al., 2010). Pottier et al. (2015) found that induced expression of AtNRAMP4 in yeast mutants decreased cadmium and zinc uptake without affecting iron absorption, suggesting that engineered mutations in NRAMP transporters could reduce Cd uptake and transport in plants and animals. NRAMP family members exhibit tissue-specific expression patterns across different plants: AtNRAMP1 is highly expressed in roots (Castaings et al., 2016), AtNRAMP2 is primarily expressed in root epidermis and root tips (Gao et al., 2018), and OsNRAMP1 shows high expression in roots during vegetative growth but in leaves and stems during reproductive growth (Takahashi et al., 2011).

Like other plants, sugarcane faces heavy metal pollution challenges. Heavy metals play vital roles in plant growth—some divalent ions such as Cu^{2+}, Zn^{2+}, and Mn^{2+} are essential micronutrients—whereas excessive accumulation of others (e.g., Cd^{2+}, Pb^{2+}) causes heavy metal toxicity and reduces yield (Rosa-Santos et al., 2020). While NRAMP protein functions have been extensively studied in *Arabidopsis* (Thomine et al., 2000), rice (Luo et al., 2018), potato (Tian et al., 2021), soybean (Qin et al., 2017), and rapeseed (Meng et al., 2017), no studies on sugarcane NRAMP proteins have been reported to date.

This study identified ScNRAMP gene family members from the *S. spontaneum* genome using bioinformatics approaches, and performed phylogenetic tree construction, chromosomal localization, gene structure analysis, and protein characterization to provide a reference for future research on this gene family.

Materials and Methods

1.1 Data Sources

Protein sequences of the AtNRAMP gene family were obtained from the *Arabidopsis* TAIR database. The sugarcane whole-genome sequence and gene annotation files were downloaded from http://www.life.illinois.edu/ming/downloads/Spontaneum_{genome}/ (Zhang et al., 2018).

1.2.1 Identification of the Sugarcane ScNRAMP Gene Family

Using *Arabidopsis* NRAMP protein sequences as queries, we performed an initial BLASTp search in TBtools (v1.0971; Chen et al., 2020), followed by a second BLASTp search in NCBI. Candidate gene sequences were then screened using SMART (http://smart.embl-heidelberg.de/) to identify NRAMP conserved domains. Sequences lacking the NRAMP domain were manually removed, and the remaining sequences were designated as sugarcane ScNRAMP gene family members.

1.2.2 Physicochemical Properties of Encoded Proteins

Physicochemical properties including isoelectric point, molecular weight, sequence length, grand average of hydropathicity (GRAVY), instability index, and aliphatic index were predicted using the ExPASy online tool (https://us.expasy.org/tools/protparam.html/). Signal peptide prediction and subcellular localization analysis of the 29 protein sequences were performed using SignalP v4.1 (http://www.cbs.dtu.dk/services/SignalP-4.1/) and CELLO v2.5 (http://cello.life.nctu.edu.tw/), respectively.

1.2.3 Protein Secondary Structure and Transmembrane Domain Analysis

Secondary structure analysis of NRAMP proteins was conducted using SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_{automat}.pl?page=/NPSA/npsa_{sopma}.html). Transmembrane structures were predicted using the TMHMM Server v2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

1.2.4 Analysis of Conserved Motifs, Domains, and Gene Structure

MEME (https://meme-suite.org/meme/) and NCBI-CDD (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrps were used to identify conserved motifs and domains within the sugarcane ScNRAMP family. Intron and exon distribution patterns were obtained from genome annotation files, and all results were visualized using TBtools.

1.2.5 Cis-Acting Element Analysis of the ScNRAMP Gene Family

The 3,000 bp upstream region of each ScNRAMP gene was extracted as the promoter sequence. Cis-acting elements within these promoter regions were analyzed using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

1.2.6 Chromosomal Localization of ScNRAMP Genes

Chromosomal positions of ScNRAMP genes were visualized using TBtools based on the sugarcane genome annotation files and family member ID lists.

1.2.7 Phylogenetic Tree Construction

Multiple sequence alignment was performed using the local software MEGA X, and a phylogenetic tree was constructed using the Neighbor-Joining method with 1,000 bootstrap replicates (all other parameters set to default). The tree was edited and visualized using the online Evolview tool (https://evolgenius.info//evolview-v2/#login).

1.2.8 Expression Analysis of ScNRAMP Genes

Expression analysis was performed using publicly available RNA-seq transcriptome data from *S. spontaneum* (Li et al., 2020). Expression levels (FPKM values) of ScNRAMP family members across different tissues and developmental stages were extracted, clustered, and visualized as heatmaps using TBtools.

Results

2.1 Physicochemical Properties of Sugarcane NRAMP Proteins

A total of 29 ScNRAMP gene family members were identified from the *S. spontaneum* genome. Their primary structures and physicochemical properties are summarized in Table 1 . The results showed that the amino acid length of ScNRAMP proteins ranged from 334 to 1,272 residues, with an average of 563 amino acids. Molecular weights were approximately 55 kDa, and isoelectric points varied widely from 4.77 to 9.43. Most family members had instability indices below 40%, with only seven members ranging between 40% and 48%. The grand average of hydropathicity (GRAVY) ranged from 0.015 to 0.949. None of the 29 sugarcane ScNRAMP proteins contained signal peptides, and subcellular localization analysis predicted plasma membrane localization for all members.

2.2 Protein Secondary Structure and Transmembrane Domain Analysis

Secondary structure and transmembrane domain analyses of sugarcane NRAMP proteins are presented in Table 2 . The secondary structure of ScNRAMP proteins was primarily composed of α-helices, random coils, extended strands, and β-turns, with α-helices being the most abundant (36.71%-64.32%) and β-turns the least abundant (1.62%-5.06%). The secondary structures were relatively uniform across family members, with all but Sspon.03G0024310-2B and Sspon.03G0024310-1A following the pattern α-helix > random coil > extended strand > β-turn. All 29 sugarcane NRAMP family members contained transmembrane domains, with numbers ranging from 6 to 12, indicating that these proteins are transmembrane proteins likely adapted for heavy metal ion transport.

2.3 Conserved Motifs, Domains, and Gene Structure Analysis

Analysis of conserved motifs, domains, and gene structures of ScNRAMP genes is shown in Figure 1 [Figure 1: see original paper]. Motif analysis revealed that motif 1 was highly conserved and present in all 29 *S. spontaneum* NRAMP family members. Some members contained two copies of motif 9, including Sspon.02G002160-2C, Sspon.02G0057810-1D, Sspon.02G002160-1A, and Sspon.02G0021270-1A. Most family members possessed nine conserved motifs, while Sspon.02G0029680-1A, Sspon.05G0038800-1D, and Sspon.02G0057180-1D had the fewest with only six motifs (Figure 1 and Table 3). These differences in conserved motifs may imply functional divergence among family members.

Gene structure analysis revealed that all ScNRAMP genes contained introns, with numbers ranging from 2 to 11. Some members lacked untranslated regions (UTRs) at the 5 and 3 ends. Domain analysis showed that five members (Sspon.02G0057810-1D, Sspon.05G0038800-1D, Sspon.01G0025840-2B, Sspon.01G0061080-1D, and Sspon.01G0025840-1A) contained the Nramp superfamily domain, while the remaining 24 members possessed the Nramp domain.

2.4 Cis-Acting Element Analysis of the ScNRAMP Gene Family

Analysis of the 3,000 bp promoter region upstream of the start codon identified several cis-acting elements in ScNRAMP gene promoters (Figure 2 [Figure 2: see original paper]). These included: (1) growth and development-related elements such as CAT-box (meristem regulatory element) and RY-element (seed-specific regulatory element); (2) hormone-responsive elements including ABRE (abscisic acid-responsive element), TGACG-motif (jasmonic acid-responsive element), TCA-element (salicylic acid-responsive element), TGA-element (auxin-responsive element), and TATC-box/P-box (gibberellin-responsive element); (3) abiotic stress-related elements such as TC-rich (defense and stress-responsive element), MBS (drought-responsive element), LTR (low-temperature responsive element), GT1-motif/G-box, MRE, I-box (light-responsive elements), and ARE (anaerobic response element); and (4) protein-related elements including MBSI and MYBHv1 (MYB transcription factor binding sites) and O2-site (zein metabolism regulation). Notably, elements such as G-box, MRE, TGACG-motif, and ABRE were present in all members. These results suggest that the ScNRAMP gene family likely participates in sugarcane responses to heavy metal stress through hormone metabolism via elements like TC-rich motif and MRE motif.

2.5 Chromosomal Localization of ScNRAMP Genes

Chromosomal localization results are shown in Figure 3 [Figure 3: see original paper]. The 29 ScNRAMP family members were unevenly distributed across 19 chromosomes. Chromosome Chr2A contained four genes; chromosomes Chr1B, Chr2C, and Chr2D each harbored three genes; chromosome Chr1A had two genes; and the remaining 14 chromosomes each contained a single gene.

2.6 Phylogenetic Analysis of the ScNRAMP Family

To understand the evolutionary relationships of NRAMP genes between sugarcane and other Poaceae species, we constructed a phylogenetic tree using NRAMP family members from rice, *Arabidopsis*, *S. spontaneum*, maize, and sorghum (Figure 4 [Figure 4: see original paper]). The tree formed three major branches corresponding to three subfamilies. Subfamily I contained 14 sugarcane ScNRAMP members clustered with 14 sorghum, 15 maize, 2 *Arabidopsis*, and 4 rice members. Subfamily II included 9 sugarcane ScNRAMP members grouped with 3 sorghum, 5 maize, 4 *Arabidopsis*, and 2 rice members. Subfamily III comprised 6 sugarcane ScNRAMP members clustered with 5 sorghum and 7 maize members, but lacked *Arabidopsis* and rice representatives. Homologous genes were identified among NRAMP family members across all five species, with maize and sugarcane showing the closest phylogenetic relationship.

2.7 Tissue-Specific Expression of ScNRAMP Genes

To investigate the functional and spatiotemporal expression patterns of the ScNRAMP gene family, we analyzed expression profiles across different tissues and developmental stages using publicly available *S. spontaneum* RNA-seq data (Figure 5 [Figure 5: see original paper]). The results revealed tissue-specific expression patterns: genes such as Sspon.04G0018970-2B, Sspon.04G0018970-4D, Sspon.04G0018970-1A, and Sspon.04G0018970-3C showed high expression in mature stems (mature-stem-3, 6, 9) but low expression in pre-mature and germination stages. In contrast, Sspon.03G0024310-3C, Sspon.03G0024310-2B, Sspon.03G0024310-1A, Sspon.01G0025840-2B, and Sspon.01G0061080-1D exhibited higher expression in developing leaves compared to pre-mature and mature stages. Notably, only one member, Sspon.05G0038800-1D, showed an FPKM value of 0 across all developmental stages and tissues, indicating no expression.

Discussion and Conclusion

Physicochemical analysis revealed considerable variation among different NRAMP proteins, with amino acid lengths ranging from 334 to 1,272 residues and isoelectric points spanning 4.77-9.43, suggesting that these proteins can adapt to different pH environments. All sugarcane NRAMP proteins had instability indices below 40%, indicating good stability, and GRAVY values of 0.015-0.949, classifying them as relatively hydrophobic proteins. The 29 family members were distributed across 19 chromosomes, with chromosome Chr2A containing the maximum of four members. All *S. spontaneum* NRAMP proteins possessed 6–12 transmembrane domains and were localized to the plasma membrane, likely adapting them for heavy metal ion transport. Gene structure analysis confirmed that all family members contained introns.

The identification of 29 ScNRAMP genes in the *S. spontaneum* genome represents a significant expansion compared to rice (6 members) and *Arabidopsis* (6 members). This expansion likely reflects the polyploid nature and large genome size of sugarcane. As a polyploid plant, whole-genome duplication during polyploidization has promoted genome content expansion and gene family enlargement in sugarcane, consistent with findings that highly variable gene families in Solanaceae species increased in size through whole-genome duplication and tandem repeats (Mäser et al., 2001; Zhang et al., 2018; Tian et al., 2021; Wang et al., 2021). The genome sizes of *Arabidopsis* and rice are 125 Mb and 466 Mb, respectively, whereas the *S. spontaneum* genome reaches 3.36 Gb. Moreover, while *Arabidopsis* and rice are diploid, sugarcane is octoploid, and the sequenced *S. spontaneum* variety AP85-441 is an euploid (tetraploid) derived from octoploid SES208 through single pollen culture. Therefore, modern sugarcane cultivars (mostly allo-octoploid and aneuploid) would theoretically contain more than 29 ScNRAMP family members (Chen et al., 2011; Zhang et al., 2018).

Analysis of gene structure and motif composition provides important insights into gene family evolution (Boudet et al., 2001). Our analysis revealed that most genes within the same subfamily shared similar structural features in exons, motifs, or UTRs, a pattern observed in NRAMP families of other species (Belouchi et al., 1997; Lanquar et al., 2005). For example, AtNRAMP3 and AtNRAMP4 share similar gene structures and both exhibit high Fe^{2+} transport capacity (Lanquar et al., 2005), while OsNramp1, OsNramp2, and OsNramp3 share 64%–75% sequence similarity (Belouchi et al., 1997), suggesting that members within the same sugarcane NRAMP subfamily may have similar functions.

Phylogenetic analysis of NRAMP families from four monocot species (*S. spontaneum*, rice, sorghum, and maize) and one dicot species (*Arabidopsis*) classified the 29 ScNRAMP members into three subfamilies. Notably, subfamily III contained only members from sorghum (5), maize (7), and *S. spontaneum* (6), but none from rice or *Arabidopsis*, suggesting that these six sugarcane ScNRAMP members may possess specialized functions distinct from those in rice and *Arabidopsis* NRAMP proteins, with similar specialized functions likely present in sorghum and maize.

Plant promoters are among the most important cis-elements regulating functional gene expression, and promoter analysis helps elucidate gene expression regulation and response mechanisms. Analysis of cis-acting elements in promoter regions can identify important components for responding to specific biotic or abiotic stresses. Our promoter analysis suggests that *S. spontaneum* may respond to heavy metal stress through complex metabolic regulatory networks involving light-responsive factors, hormone-responsive elements, and stress-responsive factors (e.g., TC-rich motif). This is consistent with reports that rice NRAMP genes are regulated by JA and ABA to cope with pathogen infection, defense signals, and metal ions, thereby participating in plant defense responses (Zhou et al., 2004). The presence of jasmonic acid-responsive elements (TGACG-motif) and auxin-responsive elements (TGA-element) in most ScNRAMP members suggests that this gene family may have evolved functions related to plant growth and stress responses. The tissue-specific expression of ScNRAMP genes indicates predominant expression in specific tissues and organs. Notably, Sspon.01G0025840-2B and Sspon.01G0061080-1D showed significantly higher expression in leaves and stems during the seed germination stage compared to other stages. Analysis of highly expressed genes across developmental stages and tissues revealed the presence of hormone-responsive elements such as MBS, ABRE, and TCA-element, suggesting their involvement in hormone metabolism and subsequent influence on sugarcane responses to heavy metal stress.

The NRAMP gene family has been extensively studied in economic crops such as common bean (Ishida et al., 2018), soybean (Qin et al., 2017), and rapeseed (Meng et al., 2017). As a crucial sugar and energy crop, sugarcane is vital for global food security. Understanding the molecular mechanisms of heavy metal ion transport by gene families such as ScNRAMP is essential for mitigating the impact of heavy metal stress on sugarcane yield and sugar content. This comprehensive genome-wide analysis of 29 ScNRAMP gene family members in *S. spontaneum* facilitates functional characterization of the sugarcane ScNRAMP gene family and provides important candidate genes for molecular breeding of sugarcane, particularly for improving heavy metal stress tolerance.

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