

Note

Functional relationship heavy metal P-type ATPases (OsHMA 2 and OsHMA3) of rice (*Oryza sativa*) using RNAi

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Abstract *Oryza sativa* heavy metal P-type ATPase 3 (*OsHMA3*) plays a major role in the hyperaccumulation of cadmium (Cd) by the rice cultivars ‘Cho-Ko-Koku (CKK)’ and ‘Anjana Dhan.’ On the other hand, mutants that accumulate Cd in the shoots at a very low level have suggested the function of several genes such as *OsHMA2* (which is necessary to load Cd into xylem) or *O. sativa* natural resistance-associated macrophage protein 5 (*OsNRAMP5*, which regulates the uptake of Cd from soil into the roots). Although many studies have examined metal transporters, few have investigated the function of combination of multiple gene in plant. Herein, first, we used an RNA interference (RNAi) method to down regulate *OsHMA3* and recovered plants in which the expression of *OsHMA3* was strongly suppressed. Second, crossing of *OsHMA3* RNAi plants with *oshma2* mutants revealed a functional relationship between *OsHMA3* and *OsHMA2* in plant. We concluded that a complete loss of function of *OsHMA2* is crucial to minimize Cd translocation from the roots to the shoots. The current findings also implied that the activity of *OsHMA2* can be accelerated in plant in the roots without the C-terminal region of the protein.

Key words: Cadmium, *Oryza sativa*, *OsHMA2*, *OsHMA3*, RNAi.

Heavy metal pollution of soil is harmful to human health and agriculture (Clemens et al. 2012; Nriagu and Pacyna 1988; Wei and Zhou 2008). To breed new food crops that do not accumulate heavy metals, and to develop plants for phytoextraction that accumulate high levels of heavy metals, the molecular mechanisms of several key processes in heavy metal accumulation must be elucidated (Mendoza-Cozatl et al. 2011; Milner and Kochian 2008; Verbruggen et al. 2009).

Rice is a major staple crop for sustaining the world's population and a model system for monocot species, which explains why the elucidation of molecular knowledge regarding heavy metal accumulation, especially for cadmium (Cd), has recently accelerated (Clemens et al. 2012). Several studies showed that *Oryza sativa* natural resistance-associated macrophage protein 5 (*OsNRAMP5*) accounts for most of the Cd uptake from the soil by rice (Ishikawa et al. 2012; Ishimaru et al. 2012; Sasaki et al. 2012). The function of *O. sativa* heavy metal P-type ATPase 3 (*OsHMA3*) was revealed in the analysis of the rice cultivars ‘Cho-Ko-Koku (CKK)’ and ‘Anjana Dhan,’ whose shoots accumulate high levels of Cd.

Miyadate et al. (2011) and Ueno et al. (2010) reported that the phenotype of these two cultivars resulted from a defect in the structure of *OsHMA3* that transports Cd into vacuoles in rice roots and limits Cd translocation from roots to shoots. On the other hand, insertion mutants resulting in the loss of the function of another heavy metal P-type ATPase in rice, *OsHMA2* were used to demonstrate the gene function. *OsHMA2* transports Cd and zinc (Zn) into the xylem and is responsible for the translocation of Cd and Zn (Nocito et al. 2011; Satoh-Nagasawa et al. 2012; Takahashi et al. 2012).

Although studies on heavy metal transporters in rice have progressed markedly, most of them focused on a single transporter. However, more information about the function of transporters may be gained when combination of genes are investigated simultaneously through genetic engineering. For example, functional redundancy or an additive effect can be revealed by such studies (Hussain et al. 2004; Ishimaru et al. 2012). Herein, we used *OsHMA3* RNA interference (RNAi) plants to investigate the relationship between *OsHMA3* and *OsHMA2* in plant by analyzing the F₂ progeny of

Abbreviations: *OsHMA3*, *Oryza sativa* heavy metal P-type ATPase 3; Cd, cadmium; CKK, Cho-Ko-Koku; RNAi, RNA interference; Zn, zinc; *OsNRAMP5*, *Oryza sativa* natural resistance-associated macrophage protein 5; NPT, neomycin phosphotransferase; HPT, hygromycin phosphotransferase; LB, left border; RB, right border; UTR, untranslated region; RT-PCR, reverse transcription polymerase chain reactio.

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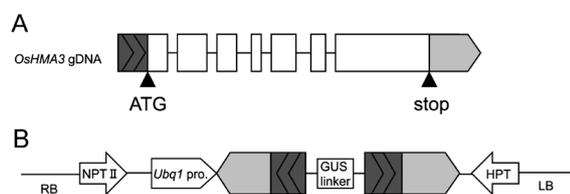


Figure 1. Structure of the RNAi construct of *OsHMA3*. (A) *OsHMA3* and the inverted repeat region in the pANDA vector. *OsHMA3* has seven exons (white boxes). Dark gray boxes are 5'-UTR and gray boxes are 3'-UTR. ATG is the translation start site. (B) The fusion between 180 bp of the 5'-UTR and 250 bp of the 3'-UTR of *OsHMA3* in the pANDA vector. RB, right border; NPTII, neomycin phosphotransferase II; Ubq1 pro., maize ubiquitin 1 promoter; HPT, hygromycin phosphotransferase; LB, left border.

OsHMA3 RNAi plants and *OsHMA2* mutants that were both in the 'Nipponbare' genetic background.

To downregulate *OsHMA3* exclusively without affecting the expression of other HMA-family genes, we used the 5'-untranslated region (UTR) and 3'-UTR regions of *OsHMA3* for targeting RNAi suppression (Figure 1). To create the construct, we performed three polymerase chain reactions (PCRs) with KOD Plus (Toyobo, Osaka, Japan). The first PCR was performed with primers HAF1 and P42 to amplify the 5'-UTR of *OsHMA3*, and primers P6 and P43 to amplify the 3'-UTR of *OsHMA3*. The sequences of all primers used in this study are listed on Table 1. PCR was carried out for 35 cycles of 30 s at 94°C, 30 s at 68°C, and 30 s at 68°C. For fusing the 5'- and 3'-UTRs, a second PCR was performed with primers P44 and P45 that contain sequences overlapping with the forward *attB1* and reverse *attB2* Gateway primers, respectively. After the third PCR with primers P50 and P51, which also include the forward *attB1* and reverse *attB2* sequences, Gateway cloning into the pANDA vector (Miki et al. 2005; Miki and Shimamoto 2004) was performed as described by Mohanty et al. (2009). The pANDA-*OsHMA3* RNAi construct and empty pANDA vector (control) were used for *Agrobacterium tumefaciens*-mediated transformation of 'Nipponbare' calli (Hiei et al. 1994). Sixteen lines containing the pANDA-*OsHMA3* RNAi construct and 14 lines containing the pANDA vector were regenerated on hygromycin medium, then transferred to soil in pots in a greenhouse with temperatures ranging from 20 to 28°C and natural light conditions. Part of the T₀ plants was used for a preliminary analysis, and the T₁ seeds were collected from plants in the greenhouse.

For analyzing the genotypes of T₀ and T₁ plants and selecting them for further analyses, DNA was extracted from seedlings using the method of Klimyuk et al. (1993), and PCR was performed using ExTaq (TaKaRa, Shiga, Japan) with primers P45 and P61 to select plants containing the pANDA-*OsHMA3* RNAi construct or pANDA vector (control). It was carried out for 35 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C.

Table 1. Primer sequences used in the study.

HAF1	5'-CTGCCATACACAACCATTGC-3'
P4	5'-CTGTTGAGCACGACGAGGAG-3'
P6	5'-GACACGCTTGTGGCTATTGAG-3'
P13	5'-ATTGGGAACAGAAGGACATTG-3'
P42	5'-CTTGCAACACGCCGTCGTCTCGTCCACCGGCCTC-3'
P43	5'-ACGACGGCGTGTGCAAGATTGACCAGTAATTCAC-3'
P44	5'-GCTCGATCCACCTAGGCTCTGCCATACACAACATTGC-3'
P45	5'-CGTAGCGAGACCACAGGAGACACGCTTGTGGCTATTGAG-3'
P48	5'-GGTATCGTCAGCAACTGGGATGATATGG-3'
P49	5'-GCTCCGTCAGGATCTTCATGAGGTAATC-3'
P50	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGCTCGATCCACCTAGGCT-3'
P51	5'-GGGACCACTTTGTACAAGAAAGCTGGGTCGTAGCGAGACCACAGGA-3'
P61	5'-GTATCAGTGTGCATGGCTGG-3'

To analyze the expression of *OsHMA3* in the T₁ plants, total RNA was extracted according to the methods of Satoh-Nagasawa et al. (2012). Then, reverse transcription PCR (RT-PCR) was performed with KOD plus (Toyobo) and primers P4 and P13. *Actin* (LOC_OS03G50885.1) cDNA amplified with Ex Taq (TaKaRa) and primers P48 and P49 were used as the standard (Sasaki et al. 2012; Satoh-Nagasawa et al. 2012). PCR was performed for 28 and 29 cycles for *OsHMA3* and 23 and 24 cycles for *Actin*. The PCR program was the same as that above. Quantity One (Bio-Rad, Hercules, CA, USA) was used to measure the band volumes to ensure that cDNAs were exponentially amplified. The relative *OsHMA3* expression levels measured by RT-PCR were normalized to the *Actin* control.

Plants obtained by RNAi showed several patterns of downregulation of *OsHMA3*. In lines R3 and R6, the expression of *OsHMA3* was highly suppressed, whereas in line R8, the suppression was only partial; in line R13, *OsHMA3* expression was not suppressed (Figure 2A). For statistical analysis, JMP 5.1.2 was used (Tukey method; SAS Institute, Cary, NC, USA).

With the aim of investigating the effect of suppressing *OsHMA3* expression, selected T₁ plants containing the RNAi constructs and controls were grown hydroponically. Rice seeds were surface sterilized, incubated, and cultured for 27 days according to the method of Satoh-Nagasawa et al. (2012). For Cd treatment, all seedlings were grown hydroponically in a nutrient solution containing 5 μg l⁻¹ CdCl₂ for 3 weeks. The nutrient solution was renewed every 2 days, and CdCl₂ was added daily. All experiments were performed in a growth chamber (14 h of light at 26°C and 10 h of darkness at 22°C). Following the hydroponic culture period, the Cd content was measured according to the method of Satoh-Nagasawa et al. (2012). The

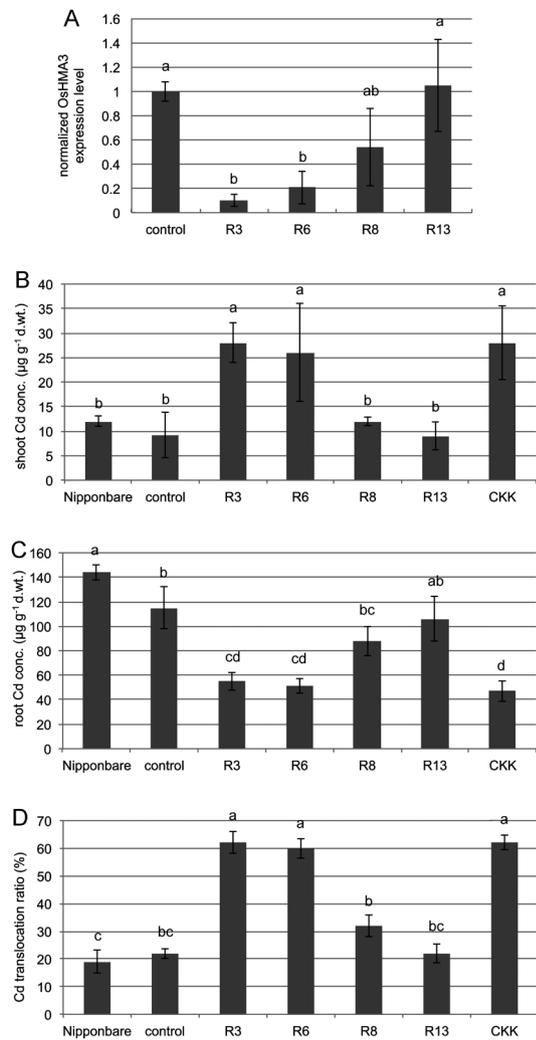


Figure 2. RNA silencing of *OsHMA3* estimated with semiquantitative RT-PCR and the phenotype of the RNAi T_1 lines for Cd accumulation. (A) Normalized *OsHMA3* expression level in four RNAi T_1 lines; (B) shoot Cd concentration; (C) root Cd concentration; (D) Cd translocation ratio. The data for untransformed Nipponbare, the empty pANDA vector (control), and CKK are included. Vertical bars represent the standard deviation of four plants. Letters above the bars indicate the level of significance at $p < 0.05$ using the Tukey method.

translocation ratio of metals from the root to the shoot was estimated as the percentage of the metals in the shoot compared to that in the whole plant.

Line R3 in which *OsHMA3* expression was well suppressed had a high concentration of Cd in the shoots and a low concentration of Cd in the roots, resulting in a high Cd translocation ratio. In contrast, Cd accumulation in line R13 in which *OsHMA3* expression was not suppressed was exactly the opposite (Figure 2). Note that in both cases, the concentrations of Cd in shoots and the Cd translocation ratios corresponded quite well with each other, as observed in our previous study (Satoh-Nagasawa et al. 2012). The results showed that *OsHMA3* is responsible for translocating Cd into the shoots. To determine whether *OsHMA3* is involved in the translocation of other metals, we

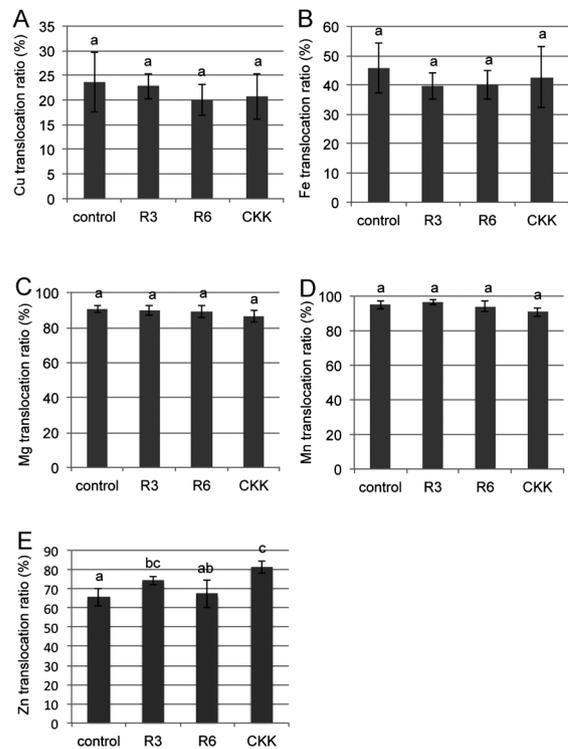


Figure 3. Translocation ratios of metals in *OsHMA3* RNAi T_1 lines, the control, and CKK. Translocation ratios of (A) Cu, (B) Fe, (C) Mg, (D) Mn, and (E) Zn when $5 \mu\text{g l}^{-1}$ CdCl_2 was added to the nutrient solution. Vertical bars represent the standard deviations of four plants. Letters above the bars indicate the level of significance at $p < 0.05$ using the Tukey method.

evaluated the translocation ratios of copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), and Zn. As shown in Figure 3, even in line R3 in which *OsHMA3* was strongly suppressed, no obvious differences were observed compared to the control. This result suggests that *OsHMA3* is a Cd-specific transporter, as reported in previous studies (Miyadate et al. 2011; Ueno et al. 2010).

After confirming the function of *OsHMA3* and providing evidence that a construct formed by the fusion of the 5'- and 3'-UTR of the *OsHMA3* gene downregulates its expression, we next used the *OsHMA3* RNAi plants acquired above to analyze the relationship between *OsHMA2* and *OsHMA3* in plant. Our original materials for the analysis were *oshma2* mutants and 'CKK'. As the *oshma2* mutants were in the 'Nipponbare' genetic background and 'CKK' is an *indica* cultivar, once we made crosses between *oshma2* mutants and 'CKK', the F_1 plants showed heterosis; vegetative growth continued for an extremely long time and the plants were nearly sterile. Although we harvested some F_2 seeds, the F_2 progeny showed phenotypic variation (e.g., variable plant height). Evaluating the function of *OsHMA3* and *OsHMA2* from such plants appeared to be difficult. In this experiment, *OsHMA3* RNAi plants were suitable materials for combining with *oshma2* mutants because all of the plants were in the 'Nipponbare' genetic

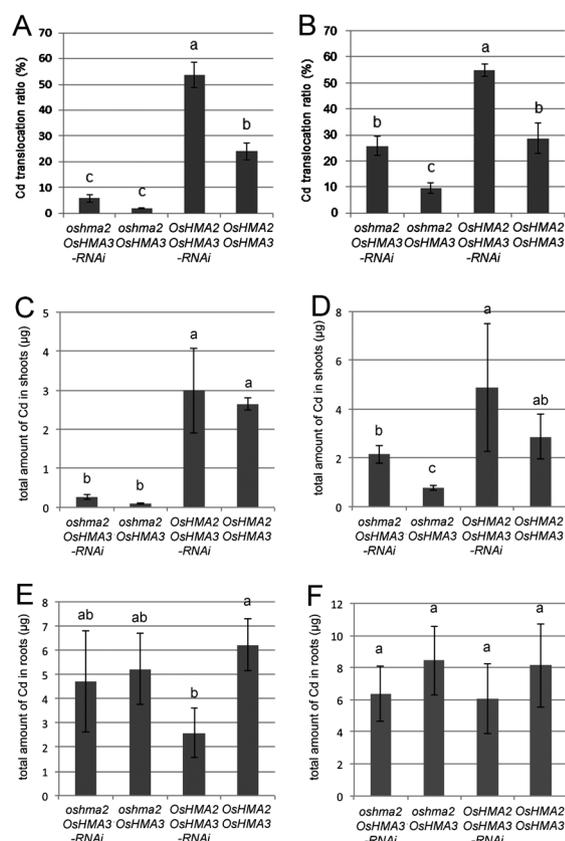


Figure 4. Cd translocation ratio and total amount of Cd in shoots and roots in *oshma2-1 OsHMA3-RNAi*, *oshma2-1 OsHMA3*, *OsHMA2 OsHMA3-RNAi*, and *OsHMA2 OsHMA3*. The *oshma2-1* allele was used for the analysis, respectively, in (A), (C) and (E) and the *oshma2-2* allele was used for the analysis, respectively, in (B), (D) and (F). Vertical bars represent the standard deviations of analyzed plants. Letters above the bars indicate the level of significance at $p < 0.05$ using the Tukey method.

background.

Prior to making the crosses, the lines were genotyped to verify the presence of *oshma2* mutations or *OsHMA3* RNAi constructs. Genotyping for the *Tos17* insertions in *oshma2* mutants was performed according to the method of Satoh-Nagasawa et al. (2012). Genotyping for *OsHMA3* RNAi constructs was conducted as described above. For the *oshma2* mutants, we used *oshma2-1* and *oshma2-2*. In *oshma2-1*, more than half of the *OsHMA2* protein was lost, with very little activity remaining; in *oshma2-2*, the C-terminal region of the *OsHMA2* protein was missing, but some function still remained (Satoh-Nagasawa et al. 2012). The R3 line of *OsHMA3* RNAi plants was used because all plants carrying the construct showed suppressed expression of *OsHMA3* in the T_1 generation, and the suppression was strongest among the lines we created (Figure 2). Heterozygous *oshma2* mutants were fertilized with pollen from the *OsHMA3* RNAi line R3 plants. The Cd translocation ratios were analyzed in the *oshma2* homozygote mutants with the *OsHMA3* RNAi construct (*oshma2 oshma3*), the *oshma2* homozygote mutants without the *OsHMA3*

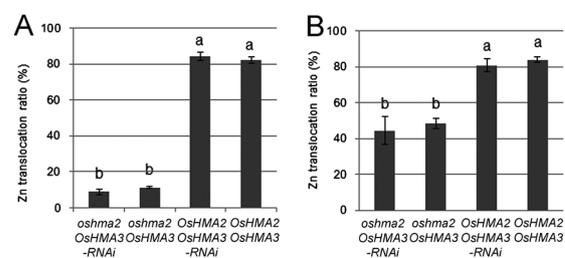


Figure 5. Zn translocation ratio in *oshma2-1 OsHMA3-RNAi*, *oshma2-1 OsHMA3*, *OsHMA2 OsHMA3-RNAi*, and *OsHMA2 OsHMA3*. The *oshma2-1* allele was used for the analysis in (A) and the *oshma2-2* allele was used for the analysis in (B). Vertical bars represent the standard deviations of analyzed plants. Letters above the bars indicate the level of significance at $p < 0.05$ using the Tukey method.

OsHMA3 RNAi construct (*oshma2 OsHMA3*), plants without the *oshma2* mutations carrying the *OsHMA3* RNAi construct (*OsHMA2 OsHMA3-RNAi*), and plants with neither the *oshma2* mutation nor the *OsHMA3* RNAi construct (*OsHMA2 OsHMA3*) in the F_2 progeny.

Among 47 plants of the F_2 population in which the presence of the *OsHMA3* RNAi construct and the *oshma2-1* mutation were confirmed, six plants of *oshma2 OsHMA3-RNAi*, four plants of *OsHMA2 OsHMA3-RNAi*, six plants of *oshma2 OsHMA3*, and four plants of *OsHMA2 OsHMA3* were selected and treated with Cd. The Cd translocation ratio and total amount of Cd in shoots between *oshma2-1 OsHMA3-RNAi* and *oshma2-1 OsHMA3* plants was not statistically different, whereas those of *OsHMA2 OsHMA3-RNAi* were higher than those of *OsHMA2 OsHMA3* (Figures 4A, C). We speculate that loss of *OsHMA3* function leads to accumulation of Cd outside of vacuole in roots temporary. If *OsHMA2* activity was completely lost, Cd was not translocated from roots to shoots. On the other hand, if *OsHMA2* functioned normally, Cd translocated. As for total amount of Cd in roots, there were no obvious difference between four genotypes (Figure 4E). Moreover, the result also implied that the activities of genes other than *OsHMA2* are minor in loading Cd into xylem.

The same analysis of 49 plants of the F_2 population in which the existence of the *OsHMA3* RNAi construct and the *oshma2-2* mutation were confirmed revealed that genotype *oshma2-2 OsHMA3-RNAi* had a similar Cd translocation ratio and total amount of Cd to *OsHMA2 OsHMA3* rather than *oshma2-2 OsHMA3* (Figures 4B, D). This means that the activity of mutated *OsHMA2* is accelerated under high Cd concentrations in the roots even when the protein has lost its C-terminal region, which results in partial *OsHMA2* activity. *AtHMA2* ATPase activity is dependent on the metal concentration in vitro (Eren and Arguello 2004), whereas our findings demonstrated the accelerated activity of *OsHMA2* in plant. In addition, we acquired information about the region that is important for regulating protein activity.

Because *OsHMA2* also transports Zn, we analyzed the

Zn translocation ratio in the plants described above. The Zn translocation ratios of *oshma2 OsHMA3-RNAi* and *oshma2 OsHMA3* were similar, and those of *OsHMA2 OsHMA3-RNAi* and *OsHMA2 OsHMA3* were also similar (Figures 5A, B). That is, the genotype of *OsHMA2* is crucial for Zn translocation. If *OsHMA3* involve Zn transport into vacuoles, the genotype of *OsHMA3* should also affect Zn translocation. Thus, here, we confirmed that *OsHMA3* does not transport Zn.

As a whole, the phenotypes of *oshma2 OsHMA3-RNAi* plants in this study verified the sequential function of *OsHMA3* and *OsHMA2* in plant; *OsHMA3* transports only Cd into vacuoles (Miyadate et al. 2011; Ueno et al. 2010) and *OsHMA2* transports Cd and Zn into the xylem of rice roots (Nocito et al. 2011; Satoh-Nagasawa et al. 2012; Takahashi et al. 2012). Moreover, our results suggest the importance of the complete loss of function of *OsHMA2* to minimize Cd translocation from the roots to shoots. Recently, much attention has been paid to *OsNRAMP5* and *OsHMA3* for creating low and high heavy metal-accumulating rice for food and phytoextraction, respectively (Ishikawa et al. 2012; Miyadate et al. 2011; Ueno et al. 2010). Besides these genes about Cd accumulation in rice breeding, we propose that much remains to be learned about the functional relationships of other genes in plant for the practical application of such research, and that the combination of RNAi plants and mutants is a valuable way to analyze these relationships.

Acknowledgements

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