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Full Length Article

Genome-Wide Identification and Expression Profile of NINJA and AFP Genes in Rice

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Abstract

NINJA and AFP genes have shown important roles in plants growth and stress tolerance. However, little information is available in rice, whose yield is central to world food security. In this study, a systematic analysis has been conducted in rice. Three AFPs were identified from rice genome on chromosome 3 and chromosome 7, one NINJA was identified on chromosome 5. Phylogenetic trees showed NINJA and AFPs were not the same clade at all, although they were highly homologous. Domain and motif analysis revealed that C and N termini of OsNINJA and OsAFPs were highly conserved, but the middle region of them was highly variable. OsNINJA and OsAFPs gene structure presented less introns. Gene duplication analysis indicated segmental duplication or whole genome duplication might be the main way of gene expansion. Gene expression pattern suggested that OsNINJA and OsAFPs might play a role in floral organ and root development. Furthermore, Real-time fluorescence quantitative and *cis*-element analysis implied that they might be involved in various stresses resistance and signaling pathways, like abscisic acid and jasmonic acid. Variation analysis illustrated that obvious indica-japonica differences, namely Single nucleotide polymorphisms and indels, existed in these loci. Our study investigated the characteristics of NINJA and AFPs in rice and predicted their potential functions, which laid foundations for studying their biological functions.

Keywords: Rice; Novel INteractor of JAZ; ABI five binding protein; Expression analysis; Abiotic stress; Natural variation

Introduction

Biotic and abiotic stress have significant negative impacts on plant growth and development, eventually causing great crop losses. Furthermore, plants are always exposed to unfavorable environments throughout their life cycles (Mittler and Blumwald 2010; Wang *et al.* 2019). For adapting to challenging environments, plants have evolved a series of self-protecting strategies by changing morphological, physiological, molecular and biochemical characteristics (Sah *et al.* 2016). Many important genes involved in adaptation to stress are transcriptional regulators, suggesting that transcription factor (TFs) are essential for plant stress tolerance (Rabara *et al.* 2014).

ABI5, a member of bZIP gene family, is involved in abiotic stress through ABA pathway. Recently, it has reported that AFPs (ABI5 binding protein) regulate the activation and degradation of ABI5 (Tang *et al.* 2016). *AtAFP1* were firstly identified from *Arabidopsis*, and then many AFP proteins and NINJA (Novel Interactor of JAZ) adaptor proteins were continuously isolated. NINJA, which is homologous to AFPs (ABI5 binding protein) in *Arabidopsis*, is present in all land plants. NINJA and AFPs contain two highly conserved domains (A and C domain) and one variable domain (B) (Garcia *et al.* 2008; Lynch *et al.* 2016). A domain located at the N-terminal region overlaps with EAR (Ethylene-responsive binding factor-associated repression) motif. EAR motif shared recognition sequence LxLxL or DLNxxP and always functions as transcriptional repressor (Kagale and Rozwadowski 2011). Except AFPs and NINJA, there are many other EAR motif-containing proteins, such as AUX/IAA proteins, TFIIIA-type and SUPERMAN-type ZFP proteins, II-type ERF proteins, *etc*. (Yang *et al.* 2018). NINJA and AFPs directly bind TPL co-repressors by EAR domain, and EAR domain is essential (Pauwels *et al.* 2010). B domain of AFPs contains a putative nuclear localization signal (NLS) and could slightly enhance binding capacity of C domain

(Lynch *et al.* 2016). However, the function of B domain in NINJA is still unknown. C domain located at the C-terminal region possesses signature sequence: IxCxCx(12)HAG. C domain, which directly associated with repressive effects is sufficient and necessary for interaction with functional proteins, like the bZIP transcription factor ABI5 (ABSCISIC ACID INSENSITIVE 5) and ZIM domain proteins JAZs (Garcia *et al.* 2008; Pauwels *et al.* 2010).

Although NINJA and AFPs are highly homologous, they function as negative regulators via different signaling pathways, NINJAs involved in JA signaling pathway by interacting with ZIM domain proteins JAZs, but AFPs participates in ABA signaling pathway by interacting with bZIP transcription factors, like ABI5 (ABSCISIC ACID INSENSITIVE 5) (Garcia *et al.* 2008; Pauwels *et al.* 2010). In JA signaling pathway, NINJAs bind to JAZ-TPL (Groucho/Tup1-type co-repressor TOPLESS)/TPRs (TPL-related proteins) forming complexes and negatively regulate JA signaling pathway (Pauwels *et al.* 2010). In the ABA signaling pathway, AFPs directly target *ABI5* and promote ubiquitin-mediated degradation to negatively regulate ABA responses through both TOPLESS-dependent and -independent chromatin modification (Lopez-Molina *et al.* 2003; Lynch *et al.* 2016).

Interestingly, NINJAs always are related to plant growth, development and biotic stress, but AFPs tend to be associated with abiotic stress. It was firstly reported that *AtNINJA* maintained normal elongation of root by negatively regulating JA signaling pathway, but functions in root and aerial tissues are obviously different (Acosta *et al.* 2013; Gasperini *et al.* 2015). Furthermore, *AtNINJA* maintains root stem cell niche functions by interacting with Topoisomerase II-associated protein PAT1H1 (Yu *et al.* 2016). *AtNINJA* regulates leaf flatness by repression of CYCLIN D3 genes expression (Baekelandt *et al.* 2018). *AtNINJA* is involved in mitigate pattern-triggered immunity (PTI) against *Botrytis cinerea* and *Pseudomonas syringae* through the interaction with ERF19 (Huang *et al.* 2018). As a co-repressor, *OsNINJA* interacted with different proteins for different functions. *OsNINJA* association with *OsJAZ8* regulates bacterial blight resistance by repression of JA signaling pathway (Yamada *et al.* 2012). However, OsNINJA forms complexes with *OsJAZ9* and *OsbHLH062* to regulate expressions of salt-related genes (Wu *et al.* 2015). Recently, *OsNINJA1* has been shown to negatively affects rice bacterial blight resistance and JA-regulated leaf senescence through OsMYC2-mediated JA signaling (Kashihara *et al.* 2019). In cotton (*Gossypium hirsutum*), GhNINJA-silenced plants using virus-induced gene silencing technique (VIGS) showed inhibition of root growth and higher tolerance to *Verticillium dahliae* infection, suggesting *GhNINJA* negatively regulates resistance to V. dahliae and positively regulates root growth (Wang *et al.* 2017). In wild tobacco (*Nicotiana attenuata*), *NaNINJA-like*, an atypical NINJA protein, recruits TOPLESS to regulate flower defenses via flower-specific JA signaling pathway (Li *et al.* 2017).

In Arabidopsis, AFPs have been described to be involved in ABA signaling pathway (Lopez-Molina *et al.* 2003; Garcia *et al.* 2008; Lynch *et al.* 2016). *AtTMAC2* (*AtAFP4*) overexpressing plants are insensitive to ABA and salinity during seed germination, which caused low survival rate, but TMAC2-RNAi transgenic plants show the opposite phenotype (Huang and Wu 2007). *AtAFP2* also helps seeds to break secondary dormancy induced by high temperature via altering expression of SOM, a CCCH-type zinc finger protein involving in light-dependent seed germination, and dynamically adjusting balance of GA/ABA (Chang *et al.* 2018). Additionally, *AtAFP2* negatively regulates flowering time through CO-AFP2-TPR2 complexes, whereas *AtAFP3* and *AtAFP4* also affect flowering (Huang and Wu 2007; Chang *et al.* 2019). Functions of AFPs in other species also have been identified. In rice, Mediator of *OsbZIP46* deactivation and degradation (*OsMODD*) suppresses the expression of *OsbZIP46 via* two complexes: MODD-OsTPR3-HDA702 and MODD-OsOTLD1 at recovery stage after drought, which alleviates secondary damages of excessive expression of drought-related genes (Tang *et al.* 2016; Ma *et al.* 2018). In sunflower (*Helianthus annus*), *AtAFP3* homologous gene *HaABRC5* responds to drought, salinity and ABA (Liu and Baird 2004). Heterologous overexpression of *GgAFP-like* in Arabidopsis, a NINJA homologous gene in Gladiolus (*Gladiolus gandavensis*), decreased ABA sensitivity during seed germination (Wu *et al.* 2016).

Rice (*Oryza sativa* L.) is one primary cereal crop, which feeds about half of the population all over the world. To meet demands for food of global populations by 2050, about additional 110 billion kilograms of rice should be produced in existing arable land, which requires higher stress tolerance rice varieties (Elert 2014). NINJAs and AFPs play an important role in stress tolerance. However, characterization and evolution of NINJA gene family have not been systematically analyzed in rice. In this study, one NINJA and three AFPs were identified in the rice genome. The intraspecific and interspecific evolution were systematically analyzed based on the results of multiple sequence alignment, phylogenetic relationships, gene structures, conserved domains, gene duplications and micro-collinearity. Additionally, functions of OsNINJA and OsAFPs were predicted according to outcomes of *cis*-element analysis, expression patterns and expression profiles under abiotic stress. Natural variation for these genes was also analyzed in different rice varieties. This research provides valuable information for functional characterization and resistance improvement.

Materials and Methods

Identification of NINJA genes in rice

Whole genome sequences of rice were retrieved from MSU Rice Genome Annotation Project Database (RGAP, http://rice.plantbiology.msu.edu/). *Arabidopsis* and

Brachypodium genome sequences were obtained from Phytozome Annotation Databases v12.0 (https://phytozome.jgi.doe.gov/pz/portal.html). NINJA and AFP family in rice were identified as follows: first, Hidden Markov Models (HMMs) of NINJA and AFP gene family (PF16136, PF07897 and PF16135) were obtained from Pfam Databases V32 (http://pfam.xfam.org/) and used to scan rice genome sequence with HMMER 3.0 (default parameters). Secondly, NINJA and AFP protein sequences in Arabidopsis, which came from PANTHER database V14.0 (http://www.pantherdb.org/), were used as queries to search rice genome by using Blastp (E-value<1e-20). Uniprot database (https://www.uniprot.org/) was used to search rice NINJA genes with keywords " NINJA and AFP family in rice " or " NINJA; AFP and rice ". Thirdly, non-redundant candidate proteins were checked for the presence of conserved domain by Pfam, SMART (http://smart.embl-heidelberg.de/) and NCBI Conserved Domain database (https://www.ncbi.nlm.nih.gov/cdd/). Finally, putative proteins without complete domains were removed, and the rest were renamed according to their locations on chromosome. The information about locus ID; CDS Coordinates; ORF length; exons and splice variants came from RGAP database, number of amino acids; molecular weight and isoelectric point were computed by ExPASy ProtParam tool (https://web.expasy.org/protparam/). Protein subcellular localizations were predicted by Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/) and then modified based on previous experimental results.

Multiple sequence alignment; domain organization and phylogenetic relationship

Domain sequences of NINJA and AFP genes in rice were extracted. Full sequences and domain sequences were used to conducted multiple sequence alignment using ClustalX 1.83 separately, with default parameters. Another multiple sequence alignment of 18 protein sequences was performed by MUSCLE program in MEGA7.0, and sequences came from four species: Arabidopsis (*Arabidopsis thaliana*); rice (*Oryza sativa*); maize (*Zea mays*) and common wheat (*Triticum aestivum*). Alignment results were used to build an unrooted phylogenetic tree using Neighbor-Joining method with the bootstrap test (1000 replicates) and evolutionary distances were computed using the Poisson correction model. Phylogenetic tree was modified by FigTree v1.4.30. Information about domains were source from SMART database.

Chromosomal distributions, gene duplication and microcollinearity analysis

Correspond relationships between NINJA members and rice genome were obtained by Circos based on physical locations in RGAP database. Gene duplications were performed by Multiple Collinearity Scan toolkit (MCScanX) with default parameters. Orthologous NINJA genes between rice and other species (*Arabidopsis*, *Brachypodium*) were visualized by TBtools (Chen *et al.* 2018). Non-synonymous substitution rate (Ka) and synonymous substitution rate (Ks) were computed by KaKs_Calculator 2.0 with GYN method.

Gene structure analysis and conserved motifs prediction

A phylogenetic tree between OsNINJA and AFPs was generated by MEGA 7.0 using Neighbor-Joining method (1,000 bootstrap). Coding DNA sequences and genome sequences were used to predict exon-intron structures by Display Server online website (GSDS, http://gsds.cbi.pku.edu.cn/) with default parameters. MEME Suite (http://meme-suite.org/) predicted conserved motifs with classic model, other parameters were as follows: Any number of repetitions; four motifs to find; the width of each motif was 6–100 aa.

Analysis of promoter sequences

1500bp genome sequences upstream of transcription initiation codon ATG were defined as promoter regions. PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to identify *cis*-elements.

Expression profiles of OsNINJA and AFPs

To analyse expression pattern of OsNINJA and AFPs, microarray data were downloaded from The Rice Expression Profile Database (RiceXPro, Version3, http://ricexpro.dna.affrc.go.jp/). Expression data of japonica rice (Nipponbare) were used to analyze expression pattern. Heatmaps were generated by HemI (Heatmap Illustrator, version 1.0, http://hemi.biocuckoo.org/) with normalized data (log2).

Plant materials, growth conditions and stress treatment

HuaZhan (*Oryza sativa L. ssp indica cv*), a cultured variety, was used to perform all experiments. Seeds were treated with 75% ethanol for 5 min, 0.15% $HgCl_2$ for 2 min and subsequently rinsed several times with sterile water. Seeds were planted on 1/2 strength MS (Murashige-Skoog) solid medium for about four days in a greenhouse (dark, 26^{o}C). Then, seedlings with same growth status were transplanted into 1/2 MS liquid medium and grown in a greenhouse at 28^{o}C under long-day condition (16 h light /8 h dark Photoperiod). Four week-old seedlings were subjected to abiotic stress and phytohormone treatments. For drought stress, seedlings were put into air without water added. For Salt stress, seedlings were transferred into 1/2 MS liquid medium containing 200 m*M* NaCl. For high and low temperature treatment, seedlings were placed in an incubator at 42^{o}C or 4^{o}C. Hormone treatments were

conducted by spraying 1/2 MS liquid medium with 100 μM ABA (Abscisic acid treatment), GA_3 (Gibberellin treatment), IAA (Auxin treatment) and 1 m*M* MeJA (Jasmonic Acid treatment). Seedlings subjected to drought stress or treated with hormones were sampled at 0 h, 0.5 h, 1 h, 3 h, 6 h, 12 h and 24 h, separately. Samples were taken at 0 h, 1 h, 3 h, 6 h, 12 h and 24 h in other treatments. Samples were rapidly frozen using liquid nitrogen and stored at -80^{o}C. All experiments were conducted at least three times.

RNA extraction, cloning, qRT-PCR analysis and variation analysis

Total RNA was extracted using TRIzol™ Reagent (Invitrogen) according to procedural guidelines. DNase-treated RNA was reverse-transcribed into cDNA according to manufacturer's instructions using RevertAid First Strand cDNA Synthesis Kit (Thermo). qRT-PCR analysis was performed in LightCycler 480 (Roche) with TransStart Green qPCR SuperMix UDG (Transgen Biotech). Rice UBQ (LOC_Os03g13170) was used as an endogenous control. The relative expression was computed with $2^{-\Delta\Delta CT}$ method.

cDNA from leaves under normal condition were used as template to amplify target fragments using Phanta Max Super-Fidelity DNA Polymerase (Vazyme Biotech). RT-PCR program was as follows: 95^{o}C 3 min, 98^{o}C 30 s, 60^{o}C 30 s, 72^{o}C 1 min 30 s, 72^{o}C 5 min, 16^{o}C ∞, 35 cycles. PCR product was sequenced in TsingKe Biotech. Primers were designed with Primer 6.0 and specificities of them were checked by Primer-Blast (NCBI, https://www.ncbi.nlm.nih.gov/tools/primer-blast/). All primer pairs are listed in Table 1. RiceVarMap v2.0 (http://ricevarmap.ncpgr.cn/v2/) was used to analyse genomic variations of OsNINJA and AFPs with the following parameters: 1.5kb upstream of 5' end of mRNA and 0 kb down of 3' end (Zhao *et al.* 2015).

Table 1: Primer pairs used in this study

Primer name	Sequence (5'-3')	Function
AFP1-F	ATGGAGGGCTTCTCGAGGGACTTG	RT-PCR
AFP1-R	TCAGTATAAGGATGGCGGGATAGG	RT-PCR
AFP2-F	ATGGCGTCGAGGGACTTCTTG	RT-PCR
AFP2-R	TTACAAGAACACCGATGGCGAG	RT-PCR
NINJA-F	ATGGACGATGAGAATGGCCTTG	RT-PCR
NINJA-R	TTAGTTTTGGGCTGAGGCTGCTTG	RT-PCR
AFP3-F	ATGGCGGCGTCGAGGGATTTCTT	RT-PCR
AFP3-R	TCACAAGAACACCGACCGCGAG	RT-PCR
Q- AFP1-F	GGATGATCAAGAACAGGGAGTC	qRT-PCR
Q- AFP1-R	GATTCTCTCCATGACCTCATCATTTTG	qRT-PCR
Q- AFP2-F	CACTTCCTCCAACAGCATCAACT	qRT-PCR
Q- AFP2-R	GCTCGTCCGCATTGTCAGTGA	qRT-PCR
Q- NINJA-F	GCTCCACCTCAGGCTATGTCC	qRT-PCR
Q- NINJA-R	AGGAAGCACCAGCCTCTTGAGT	qRT-PCR
Q- AFP3-F	AGGTGAGGATCGTGTGCGTCTG	qRT-PCR
Q- AFP3-R	ATGTGCCTGAGCGGGTTGGT	qRT-PCR
Q-UBQ-F	AACCAGCTGAGGCCCAAGA	qRT-PCR
Q-UBQ-R	ACGATTGATTTAACCAGTCCATGA	qRT-PCR

Results

Identification of NINJA genes in rice

To accurately identify NINJA genes in rice, we used different databases and tools, such as NCBI; RGAP; BLAST; HMMER and PANTER, among others. Finally, one putative NINJA and three AFPs proteins in rice were identified (Table 2), including two previously published genes: *OsAFP1* (*OsMODD*) and *OsNINJA* (*OsNINJA*). AFPs were renamed from *OsAFP1* to *OsAFP3* according to physical positions on chromosome. Lengths of four coding DNA sequences ranged from 924 bp (*OsAFP2*) to 1356 bp (*OsNINJA*). NINJA and AFP proteins contained 307–451 amino acids (aa) with an average of 370 aa. Furthermore, AFPs proteins showed high isoelectric point (pI >9), *OsNINJA* was 5.35. Predictions of subcellular localization showed that, except *OsAFP1*, which was localized to a variety of organelles: cell membrane; chloroplast; cytoplasm; mitochondrion; nucleus and peroxisome, all the other proteins were localized to the nucleus. However, it has been demonstrated that *OsAFP1* was a nuclear protein, so we recognized that *OsAFP1* was also localized to the nucleus (Tang *et al.* 2016).

Multiple sequence alignment and phylogenetic relationship

To reveal conserved regions among NINJA and AFP gene family in rice, full and domain sequences were aligned respectively (Fig. 1). Results showed that all members were relatively conservative, especially in domain regions. Three domains were shared in NINJA and AFP family members. A and C domain were located at each N- and C-terminus respectively, Domain B of NINJA was located in the middle. Due to the fact that full-length sequence alignment might not accurately show conservation of domains, domain sequences predicted by pfam database were aligned. EAR domain had an LxLxL signature but not DLNxxP in NINJA and AFP family of rice. C domain was identified by IxCxCx(12)HAG signature. B domain of OsNINJA/AFP was highly variable.

To explain the evolutionary relationships and functional differentiations of NINJA and AFPs in plants, 16 NINJA proteins from four species were chosen to construct a phylogenetic tree (Fig. 2B). The phylogenetic tree presented two different clades: NINJAs and AFPs. AFPs group contained most of proteins, up to 13 proteins (13/16), NINJAs group only contained three proteins (*OsNINJA; AtNINJA* and *GRMZM2G043764A*). Interestingly, it happened that three proteins in NINJA group belonged to three plants respectively, but all proteins in wheat were distributed in AFPs group. Furthermore, AFPs proteins of each species had a tendency to cluster together. There was a defined boundary between monocots and dicots in AFPs group.

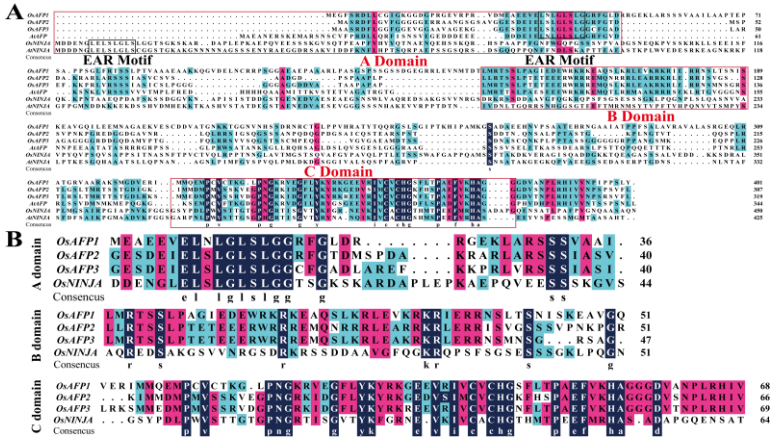

Fig. 1: Multiple sequences alignment of OsNINJA and OsAFP family members. (**A**) Full protein and AtNINJA sequences alignment by DNAMAN9. Red box shown domain location. (**B**) domain sequences alignment

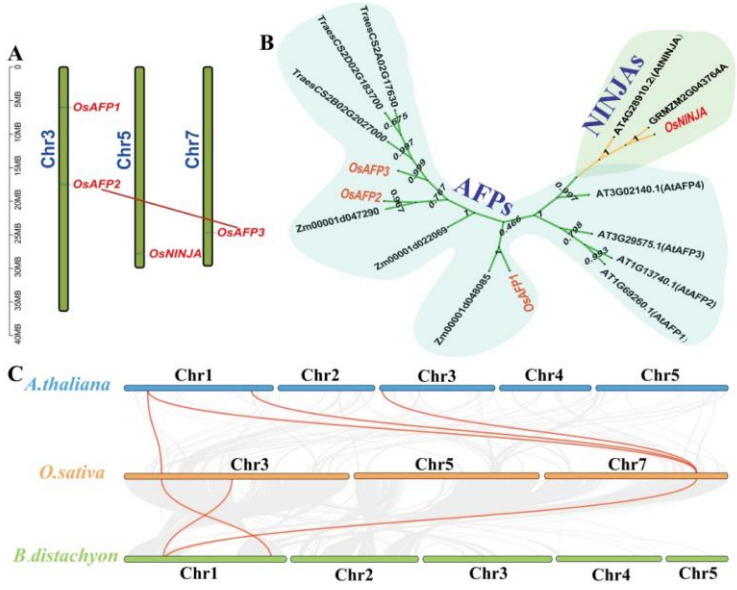

Fig. 2: The location and the evolutionary analysis. (**A**) Phylogenetic tree of NINJA proteins among rice, maize, common wheat and *Arabidopsis*. The different shading indicated different subgroups. Numbers represented the proportion of bootstrap test (1000 replicates). (**B**) Locations on chromosomes and gene duplication events of OsNINJA and OsAFPs. Four genes were localized on 3 chromosomes (Chr3; Chr5 and Chr7). The scale on the left shows length of chromosomes and position of genes (MB). The line in red indicates segmental duplication. (**C**) Synteny analysis of NINJA and AFPs between rice and 2 representative species (*Arabidopsis* and *Brachypodium*). Three chromosomes of rice with OsNINJA and AFPs were only displayed. Gray lines represented collinear blocks. lines highlight by red indicated the syntenic relationships

Chromosomal distribution, gene duplication and microcollinearity analysis

To understand the distribution of OsNINJA and AFPs on chromosomes, OsNINJA and AFPs were mapped to chromosomes in rice according to their physical locations. As shown in Fig. 2A, OsNINJA and AFPs were unevenly distributed on chromosomes: two were located on Chr3, others on Chr5 and Chr7 respectively. *OsAFP2* and *OsAFP3* were segmental duplications, but no tandem duplications were found among *OsNINJA and AFPs*.

To further explore evolutionary divergences of NINJA and AFPs in different species, microcollinearity analysis between rice and two model species: one dicotyledonous plant (*Arabidopsis*) and one monocotyledonous plant (*Brachypodium*), was conducted by BLASTP and MCScanX. 8 orthologous gene pairs were detected (Fig. 2C). Out of them, 5 pairs were from rice and *Arabidopsis*, 3 pairs were from rice and Brachypodium. Interestingly, *OsAFP1* and *OsAFP3* appeared in many orthologous gene pairs,

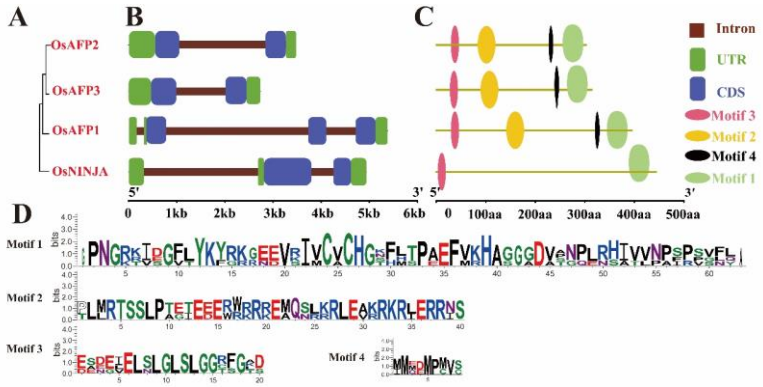

Fig. 3: Analysis of conserved relationships among OsNINJA and AFP family. the size of shapes represented corresponding structures size. (**A**) The phylogenetic tree of NINJA and AFPs proteins. (**B**) The distributions of Exons and introns. boxes in green and blue represented UTRs and Coding DNA Sequences (CDs), brown lines were introns. Bar showed the length of genomes. (**C**) Identification of motifs. Ellipses in various colors displayed four motifs, scale showed the length of proteins. (**D**) The conserved motifs sequences alignment in OsNINJA and OsAFPs

especially between rice and *Arabidopsis*. *OsAFP3* was associated with 4 gene pairs (50% of all orthologous gene pairs) alone. However, no gene pairs were associated with *OsNINJA*. Furthermore, *OsAFP2* had homology to genes in *Brachypodium* but not in *Arabidopsis*. Interestingly, orthologous genes in rice (*OsAFP2-OsAFP3*) just showed homology to one gene (Bradi1g22180.1.v3.1). To study selection pressure of gene pairs during evolution, values of Ka/Ks ratios were computed by KaKs_Calculator 2.0 (Table 3). Ka/Ks ratios of all orthologous AFP gene pairs were less than 1.

Gene structures analysis and conserved motifs predicted

To investigate distributions of OsNINJA and OsAFPs, a phylogenetic tree was generated by MEGA 7.0. The four genes were divided into two group, *OsNINJA* being the single member of one group (Fig. 3A). Functional diversification of gene family correlates with variation of gene structure, so exon-intron structures were studied with GSDS online tool. Results showed that (Fig. 3B and Table 2) OsNINJA and OsAFPs possessed similarly exon and intron distributions. Number of exons ranged from 2 to 3, and introns varied from 1 to 3. Interestingly, there was an intron inserted into 5' UTR of *OsAFP1*. Generally, gene structures were highly consisted with classification.

To further explore the evolutionary relationships among NINJA and AFPs proteins in rice, conserved motif analysis was conducted twice by MEME database (Fig. 3C). First, the searching number of motifs was set as 10, however last 6 motifs showed low confidence, so we performed the analysis again with 4 searching motifs. Motifs in proteins were highly conserved. Motif1 and motif3 had exactly similar distributions, but OsNINJA lacked motif 2 and motif 4. To speculate about the functions, motif sequences were extracted and confirmed by SMART. Results showed that motifs variated from 9 aa (Motif4) to 69 aa (Motif1), and they shared remarkably similar amino acid sequences (Fig. 3D). Motif1 and 4 were included in the C domain, motif2 was a part of B domain, and motif3 was EAR motif. Motif distributions and domain organizations were exceedingly identical (Fig. 3C).

***Cis*-element analysis of NINJA and AFPs in rice**

As a key component of genes, promoter plays an important role in controlling gene expression and in response to biotic and abiotic stresses. To predict functions of OsNINJA and OsAFPs and get insight into regulatory mechanisms, a *cis*-element analysis was performed with PlantCARE (Fig. 4). 283 *Cis*-elements were found in the OsNINJA and AFPs promoters, such as TATA-box; TC-rich repeats; ABRE and so on, with random distribution. *OsAFP3* had maximum elements (97), and *OsAFP2* was minimum (48) (Table 4). All elements were mostly divided into four types based on their functional annotations, except common *cis*-elements, for example CAAT-box and TATA-box. The first type (16, 5.65% of *cis*-elements) was stress response, including anaerobic stress and low-temperature. Anaerobic-responsive elements harboring ARE and GC-motifs were found across all members. However, LTR element, which is involved in response to low-temperature, was only contained in *OsAFP1*. TC-rich repeats appeared in *OsAFP3* promoter, and are related to defense and stress responsiveness. The second (62, 21.91% of *cis*-elements) type was hormone response, including abscisic acid response (ABRE), gibberellin response (GARE-motif), MeJA response (CGTCA-motif and TGACG-motif), salicylic acid response (TCA-element) and auxin response (TGA-element). *OsNINJA and AFPs* except *OsNINJA* had similar hormone-responsive *cis*-acting elements. They possessed many ABA-responsive elements (36, 58.06% of hormone-responsive elements), and a few MeJA response elements. Furthermore, *OsAFP2* lacked salicylic acid-responsive

Table 2: Identification and information of NINJA members in rice

Gene Name	Locus ID[a]	Previous name	CDS Coordinates (5'-3')[a]	ORF length(bp)[a]	Exons[a]	Splice variants[a]	Protein			subcellular localization[c]
							Amino acids[b]	MW(Da)[b]	PI[b]	
OsNINJA	*LOC_Os05g48500.3*	*OsNINJA*	Chr5: 27793442 - 27798381	1356	2	3	451	46973.41	5.35	Nucleus
OsAFP1	*LOC_Os03g11550.1*	*OsMODD*	Chr3: 5988828 - 5983457	1206	3	2	401	42644.31	9.25	Nucleus
OsAFP2	*LOC_Os03g30570.1*		Chr3: 17449206 - 17452682	924	2	2	307	32439.20	9.60	Nucleus
OsAFP3	*LOC_Os07g41160.1*		Chr7: 24636319 - 24633584	960	2	2	319	33396.57	9.33	Nucleus

[a] Information from Rice Genome Annotation Project database(http://rice.plantbiology.msu.edu/)
[b] Predicted by ProtParam tool(https://web.expasy.org/protparam/)
[c] Predicted by Plant-mPLoc(http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/) and verified by publication

Table 3: Orthologous gene pairs between rice and other 2 species

Orthologous Pairs		Ka	Ks	Ka/Ks	Selection pressure
OsAFP1	*AT1G13740.1*	1.06266	4.18151	0.254133	Purifying selection
OsAFP1	*Bradi1g69940.2.v3.1*	0.265095	4.80468	0.0551743	Purifying selection
OsAFP2	*Bradi1g22180.1.v3.1*	0.225737	2.45736	0.0918617	Purifying selection
OsAFP3	*Bradi1g22180.1.v3.1*	0.111861	1.72658	0.0647875	Purifying selection
OsAFP3	*AT1G13740.1*	0.655872	4.03242	0.16265	Purifying selection
OsAFP3	*AT1G69260.1*	0.88706	4.10381	0.216155	Purifying selection
OsAFP3	*AT3G02140.1*	1.21756	4.00016	0.304377	Purifying selection

The value of Ka/Ks were calculated by KaKs_Calculator 2.0 with GYN method

Table 4: The details of Cis-Elements in OsNINJA and OsAFPs

Cis-elements	Genes				Functional annotations	Categories
	OsAFP1	*OsAFP2*	*OsNINJA*	*OsAFP3*		
A-box	2				cis-acting regulatory element	
CAAT-box	4	21	9	21	common cis-acting element	
TATA-box	14	16	8	32	core promoter element	
ARE		1	5	2	anaerobic induction	Stress responsive
GC-motif	4	1		1	anoxic specific inducibility	Stress responsive
LTR	1				low-temperature responsiveness	Stress responsive
TC-rich repeats				1	defense and stress responsiveness	Stress responsive
ABRE	17	8		11	abscisic acid responsiveness	Hormone responsive
GARE-motif			6		gibberellin-responsive element	Hormone responsive
CGTCA-motif	3	1		3	MeJA-responsiveness	Hormone responsive
TGACG-motif	3	1		3	MeJA-responsiveness	Hormone responsive
TCA-element	1		2	1	salicylic acid responsiveness	Hormone responsive
TGA-element				2	auxin-responsive element	Hormone responsive
ACE		1			light responsiveness	Light responsive
ATCT-motif	1	1	1		a conserved DNA module involved in light responsiveness	Light responsive
Box 4			1		a conserved DNA module involved in light responsiveness	Light responsive
circadian			1		circadian control	Light responsive
G-Box	13	10		15	light responsiveness	Light responsive
chs-CMA1a	1				light responsive element	Light responsive
GATA-motif				1	light responsive element	Light responsive
I-box		1			light responsive element	Light responsive
TCCC-motif			1		light responsive element	Light responsive
TCT-motif			1		light responsive element	Light responsive
GT1-motif		1	8	3	light responsive element	Light responsive
Sp1	3	2		1	light responsive element	Light responsive
MRE			2		MYB binding site involved in light responsiveness	Light responsive
CCAAT-box	2				MYBHv1 binding site	binding site
CAT-box	3		2		meristem expression	meristem expression
O2-site		1	1		zein metabolism regulation	metabolism regulation
Total	72	66	48	97		

Different color background regions showed different categories according their functional annotations

elements and *OsAFP3* had 2 auxin-responsive elements alone. *OsNINJA* exhibited 6 gibberellin-responsive elements and 2 salicylic acid-responsive elements. Light response was the third type (6924.38% of *cis*-elements) with 13 kinds of elements. The composition of *OsNINJA* was not the same with the other members. GT1-motifs were the most abundant elements (8) among 13 kinds in *OsNINJA*, but G-Box were most abundant in others. OsNINJA and AFPs expression might be regulated by light, but the underlying mechanism might be different. The last type was other functional elements related to meristem expression and metabolism regulation. This type was the least abundant and only contained three elements: CCAAT-box, CAT-box and O2-site. CAT-box motifs, related to meristem expression, were present in *OsAFP1* and *OsNINJA*. Generally speaking, OsNINJA and AFPs

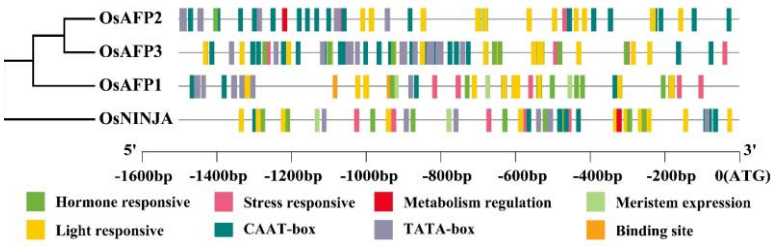

Fig. 4: Distribution diagram in promoter regions of OsNINJA and AFPs. Boxes in different colors presented different functions of elements. The bottom scale showed the distance between positions of elements and the initial codon (ATG)

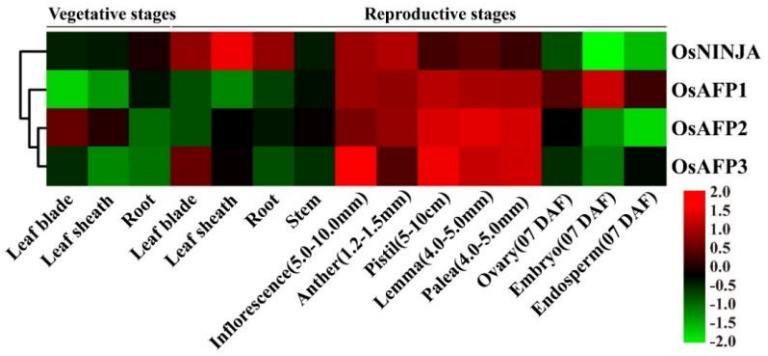

Fig. 5: Spatial and temporal expression analysis of OsNINJA and AFPs. The colorful scale on the lower right showed normalized (log2) signal intensity. red indicated high expressions and green represented low expressions. Heatmap was created by HemI with the mean value of 3 replicates from RiceXPro database. The below labels were different organizations, including vegetative stages (leaf blade, leaf sheath and root) and reproductive stages (leaf blade, leaf sheath, root, stem and so on). DAF, Days After Flowering

might play an important role in regulating plant development and stress tolerance.

Spatial and temporal expression profiles of OsNINJA and AFPs

Gene expression patterns are directly associated with gene functions. Hence, unknown functions might be inferred from similar expression profiles. To get insight into functions of OsNINJA and OsAFPs, gene expression pattern was analyzed. As shown in the heatmap (Fig. 5), OsNINJA and AFPs had significantly different expression profiles according to the hierarchical clustering. Two clusters showed high expression in some tissues during reproductive stages and lower expression during vegetative stages. Notably, *OsAFP2* was highly expressed in leaf blade during the vegetative stage. Genes from AFPs were highly expressed in different organs like inflorescence, anther, pistil, lemma and palea. *OsNINJA* was also abundant in root, leaf blade and leaf sheath at vegetative stage, indicating that *OsNINJA* might have specific functions.

Expression profiles under abiotic stress; cloning and genetic variation analysis

OsNINJA and AFPs might play a role in response to abiotic stresses and hormones according to the outcome of the above analysis. To study whether OsNINJA and AFPs have potential functions in abiotic stress tolerance, four-week-old seedlings experienced various treatments including four abiotic stresses (drought, high salinity, heat and cold) and four hormones (ABA, GA_3, IAA and MeJA). Then, expression profiles were examined by qRT-PCR analysis. As was shown in Fig. 6, OsNINJA and AFPs responded to various abiotic stresses and hormones, but showed different expression profiles under different treatments. OsNINJA and AFPs were strongly induced by drought. OsAFPs and *OsNINJA* were continuously up-regulated and reached the peak at 3 h. Interestingly, expression profiles of OsAFPs under ABA treatment showed similar expression patterns, but they reached peaks at different time. For salt stress, OsAFPs showed up-regulation at some points, especially at 2 h, 3 h and 24 h. However, *OsNINJA* were not induced by salt treatment. OsNINJA and OsAFPs showed no obvious changes in response to heat stress. When rice seedlings were exposed to cold treatment, the expression of *OsAFP1*, *OsAFP3* and *OsNINJA* were increased, *OsAFP1* was up-regulated at 24 h (1.7-fold), while the expression level of *OsAFP3* and *OsNINJA* were induced at 1h. Interestingly, all OsAFPs responded to ABA treatment at different response time. *OsAFP1* responded to ABA at late stage, but *OsAFP2* and *OsAFP3* were earlier. Furthermore, *OsAFP3* was

significantly up-regulated at 0.5 h (4.2-fold) under GA_3 treatment and *OsNINJA* was highly expressed at 0.5 h (2.5-fold) under IAA treatment. For MeJA treatment, only *OsNINJA* was significantly induced after 0.5 h and reached peak at 3 h. In conclusion, the four *OsNINJA and AFPs* were induced by multiple stresses and hormones.

To clone the *OsNINJA* family members, RT-PCR analysis was carried out using cDNA of indica rice variety (ZhongHua11) and japonica rice variety (HuaZhan) under normal condition. Bands of four OsNINJA and AFPs showed the expected size. Sequencing results showed that the sequence homology between Nipponbare and ZhongHua11 was significantly higher than that between Nipponbare and HuaZhan. Only two Single Nucleotide Polymorphisms (SNPs) between Nipponbare and ZhongHua11 were found in *OsAFP3* locus. On the contrary, there were many SNPs and even some indels between Nipponbare (ZhongHua11) and HuaZhan in *OsNINJA and AFPs*. To further learn about variations, SNPs and indels were scanned using RiceVarMap v2.0 database. A large number of SNPs and indels, ranging from 73 to 123, were found in *OsNINJA and AFPs*. In all variations, about 65–80% of variations were SNPs and only approximately 20–35% were indels (Fig. 7B). As was shown in Fig. 7A, SNPs and indels were unevenly distributed in *OsNINJA and AFPs*. The vast majority of them were in promoter and intron regions and only very few were in exon. Generally, mutation of SNPs and indels in exon region had great possibility to change the structure and function of proteins. Variations in exon were listed as follows: 4 SNPs (vg0305988313, vg0305984986, vg0317450790 and vg0527792378) and 4 indels (vg0724635438, vg0724635439, vg0724635447 and vg0724635451). The primary allele frequency varied from 0.37 to 0.998. Surprisingly, all indels occurred in *OsAFP3* and indel vg0724635439 in *OsAFP3* had 4 kinds of variations. To further investigate the variation between indica and japonica rice varieties, distributions of SNPs and indels in 4726 rice varieties, which might cause big effects, were analyzed. Interestingly, there were obvious divergences between japonica and indica in one SNP site (vg0317450790) and two indels sites (vg0724635438 and vg0724635439) (Fig. 7C). Vg0317450790 and vg0724635438 only had two alleles, but for vg0724635439 there were five, and other alleles accounted for 42.3%. Variations in indica varieties were main component of the primary allele in vg0317450790, which accounted for up to 84.68%. However, the main part of secondary allele was in japonica rice (94.08%). Vg0317450790 was similar. Interestingly, almost all variations of vg0724635439 in all japonica varieties were distributed in primary allele, whereas variations in indica were in the rest of the alleles.

Discussion

Rice is one of the major staple foods in the world, and high-stable yield varieties are crucial to world food security (Huo *et al.* 2017). However, biotic and abiotic stresses are serious threats for plant growth, and eventually they cause huge decreases of rice yields (Zhu 2016). NINJA and AFP gene family has been proven to be involved in regulating tolerance stresses in rice, like drought, salinity and bacterial blight resistance (Yamada *et al.* 2012; Wu *et al.* 2015; Tang *et al.* 2016; Ma *et al.* 2018). However, NINJA and AFP gene family has not been comprehensively studied in rice.

In this study, one NINJA and three AFPs were identified using different methods, which was in great agreement with previous researches (Table 2) (Wu *et al.* 2015; Tang *et al.* 2016). *Arabidopsis* contains at least one NINJA and four AFPs although *Arabidopsis* genome size and gene number were much smaller than those of rice (Garcia *et al.* 2008; Pauwels *et al.* 2010). This result might be caused by variabilities of gene duplications and uneven distributions on chromosomes of four OsNINJA and AFPs, which might locate in inactive regions of duplications (Fig. 2A). Analysis of phylogenetic relationships among OsNINJA and OsAFPs indicated that OsNINJA and AFPs were grouped into two categories (Fig. 3A). OsNINJA was divergent, and possessed the highest molecular weight (46973.41 Da) and lowest isoelectric point (5.35) comparing to OsAFPs (Table 2). These indicated that OsNINJA consisted of many acidic amino acids, like aspartic acid and glutamic acid. Additionally, all OsNINJA and AFPs, contained the 3 classic domains (A, B, C domain), but B domain in OsNINJA was highly varied. (Lynch *et al.* 2016). Interestingly, A domain was frequently found in transcriptional repressor proteins.

To further investigate expansions of NINJA and AFPs, an unrooted phylogenetic tree was built among four species, including dicotyledons (*Arabidopsis*) and monocotyledons (rice, wheat and maize) (Fig. 2B). NINJA proteins of dicotyledons and monocotyledons could be clustered in one group, which suggests divergences of NINJA and AFPs might appear early than that of dicotyledons and monocotyledons. NINJA and AFPs of rice and maize might have closer evolutionary relationships (Fig. 2B). Gene duplications played an important role in evolution and are widely present in gene families. In rice, only one segmental duplication and no tandem duplication were found (Fig. 2A). Eight orthologous gene pairs between rice and representative species (monocots: *Brachypodium*, dicots: *Arabidopsis*) were detected (Fig. 2C). These orthologous genes were selected by purifying selection (Table 3) during evolution. Collinearity analysis showed that NINJAs and AFPs likely originated in a common ancestor. Altogether, segmental duplication or whole genome duplication (WGD) has contributed to NINJA and AFPs expansions within specie or between species. Duplicated genes provided a possibility for forming new genes or new functions and assisted plants in adapting to complex environments.

Changes of exon-intron structure might cause divergences of gene function, and less introns were easier to

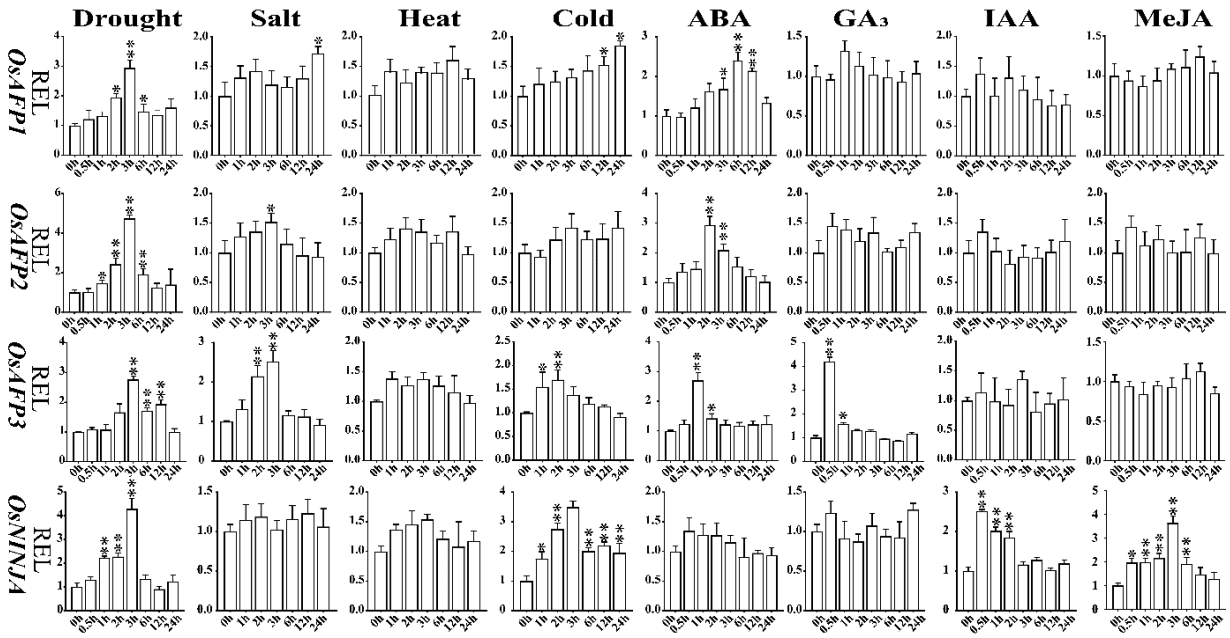

Fig. 6: qRT-PCR analysis of OsNINJA and AFPs under multiple abiotic stress and hormone treatments. REL, Relative expression level. X-axis represents designed points, Y-axis is the expression level of OsNINJA and AFPs under treatments compared to the value of that in normal environment (0 h), all gene expressions are normalized to reference gene UBQ (LOC_Os03g13170). Error bars, mean ± SD (n=3). * $P < 0.05$, ** $P < 0.01$

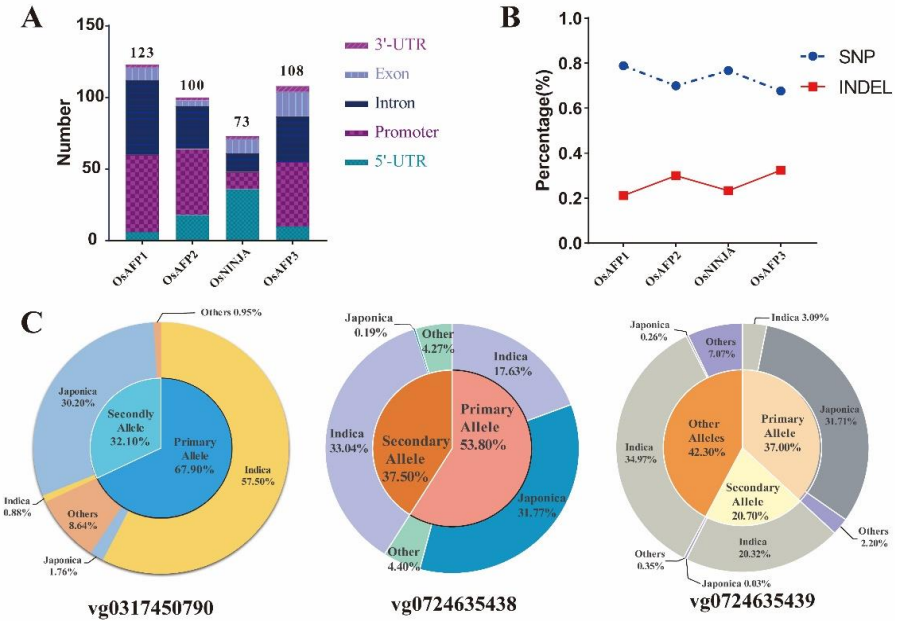

Fig. 7: Genetic variations in OsNINJA and AFPs. (**A**) The distributions of SNPs and INDELs. Promoter indicated the intergenic region, which was 1.5 kb upstream of gene. (**B**) the percentage of SNPs and INDELs. (**C**) three divergent variations between indica and japonica, the DEL Frequency were not shown

transcribe (Xu *et al.* 2012). OsNINJA and AFPs only possessed one to three introns, which might be favorable to respond to stresses (Fig. 5B). Surprisingly, intron region in 5'-UTR of *OsNINJA* happened to contain a splice site, which resulted in diverse splice variants, as happens with *AtNINJA* (*AT4G28910*). Notably, number of introns in Arabidopsis also ranged from one to three, suggesting that gene structure was conserved during evolution. Domain

structure (Fig. 1) and motif analysis (Fig. 3C) showed that OsNINJA and AFPs contained a classic EAR domain, which indicates that NINJA and AFPs might be transcriptional repressors (Pauwels *et al.* 2010; Kagale and Rozwadowski 2011).

Spatial and temporal expression profiles are related to gene functions. Hence, we conducted expression pattern analysis (Fig. 5). Particularly, OsNINJA and AFPs were abundant expression in inflorescence, which indicated OsNINJA and AFPs might play a role in floral organ development and suggests that there might be some functional redundancy. This also occurred in NINJA and AFPs in Arabidopsis (Huang and Wu 2007). *OsNINJA* was highly expressed in leaf and root, whereas *OsAFP2* and *OsAFP3* were slightly expressed in leaf blade, implying they might be induced by different stresses. *Cis*-elements were necessary for gene responses to external environment. Many *cis*-elements related to light, stress and hormone responses were found in promoters of OsNINJA and AFPs (Fig. 4), which indicated OsNINJA and AFPs might be involved in development, stress response and signal transmission. Coincidentally, *AFP2*, *AFP3* and *AFP4* could adjust flowering time in Arabidopsis, which might relate to light response (Chang *et al.* 2019). *OsNINJA* has been proved to relate to JA signaling pathway, but we did not find any MeJA-responsive elements, which including G-box; GCCbox; JASE1; JASE2; W-box; E-box; GAGTA and so on, in *OsNINJA* promoter (1500 bp upstream of ATG). We speculated that 1500 bp could not cover the *OsNINJA* promoter. We then research *cis*-elements in 2000 bp upstream of ATG, but only one CGTCA-motif and four G-box were found. *OsNINJA* was significantly induced by MeJA treatment, but not induced by ABA treatment, this is consistent with Pauwels' study (Pauwels *et al.* 2010). OsAFPs exhibited some MeJA response elements CGTCA-motif and TGACG-motif, but they were not induced by MeJA treatment (Fig. 6). AFP members are a pivotal component of ABA signaling pathway. Thus, OsAFPs are putatively involved in ABA response. As expected, OsAFPs were rich in ABRE elements, which are ABA response elements. ABA treatment could significantly induce the expression of *OsAFPs*, indicating they negatively regulate ABA signaling. These results were in accordance with former studies (Fig. 6) (Lopez-Molina *et al.* 2003; Lynch *et al.* 2016). ABA signaling pathway is activated upon many abiotic stresses. For example, *OsAFP1* negatively regulated drought through ABA signaling pathway (Tang *et al.* 2016), whereas *AtAFP4* negatively regulates salt stress (Huang and Wu. 2007). In this study, *OsAFP1* was also induced by drought, *OsAFP3*, homologous to *AtAFP4*, was also down-regulated by salt treatment. Recently, it was reported that *AtAFP2* altered balances of ABA and GA_3 to break seed dormancy (Chang *et al.* 2018). *OsAFP3* also responded to GA_3 treatment. Taken together, our study indicates that OsNINJA and AFPs are induced by various stresses and might have redundant functions. Besides, we cloned four OsNINJA genes in two rice varieties (ZhongHua11 and HuaZhan). The sequencing results indicated that there were some genetic variations of NINJA members among different rice varieties. To further study the variations in OsNINJA and AFPs, genetic variation analysis was performed using RiceVarMap v2.0 database (Fig. 7A). Details of potential "big effects" variations implied that OsNINJA and AFPs were divergent in different varieties, especially between indica and japonica subspecies. We speculated that some OsNINJA and AFPs, like OsAFP3, might have different functions in different subspecies. However, it remained to be verified by molecular biotechnology, like gene editing. Further investigations should first confirm the specific functions of OsNINJA and AFPs by generating overexpression and knockout (knockdown) transgenic plants, then exploring the underlying mechanism of functions and finally applying these gene resources to rice variety improvement

Conclusion

In summary, one OsNINJA and three OsAFPs were identified in rice genome through genome-wide identification, then their phylogenetic relationship, conserved domain, gene structure, gene duplication, *cis*-element in promoter and expression profile were systematically analyzed. We also cloned OsNINJA and AFPs by RT-PCR and analyzed SNPs and indels between indica and japonica rice. Results showed that OsNINJA and AFPs were highly conserved in gene structure and regulation. Segmental duplication or whole genome duplication (WGD) might be the main reason for the expansion of NINJA and AFPs. OsNINJA and AFPs might play a vital role in floral organ and root development, ABA and JA signal transduction and stress adaptation, especially in drought stress. Additionally, there was a clear indica-japonica differentiation in some SNPs and indels sites, which might cause the functional differentiation. This study provided a wealth of valuable information for further investigating the underlying mechanism of OsNINJA and AFPs, which accelerates the genetic improvement of rice.

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