

# Comprehensive Genomic Analysis and Expression Profiling of the NOX Gene Families under Abiotic Stresses and Hormones in Plants

Yan-Li Chang<sup>1,†</sup>, Wen-Yan Li<sup>2,†</sup>, Hai Miao<sup>1</sup>, Shuai-Qi Yang<sup>1</sup>, Ri Li<sup>1</sup>, Xiang Wang<sup>1</sup>, Wen-Qiang Li<sup>1</sup>, and Kun-Ming Chen<sup>1,\*</sup>

<sup>1</sup>State Key Laboratory of Crop Stress Biology in Arid Areas, College of Life Sciences, Northwest A&F University, Yangling, Shaanxi, P. R. China

<sup>2</sup>Guangdong Academy of Agricultural Sciences, Argo-Biological Gene Research Center, Guangzhou, Guangdong, P. R. China

<sup>†</sup>These authors contributed equally to this work.

\*Corresponding author: E-mail: kunmingchen@nwsuaf.edu.cn, kunmingchen@163.com.

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## Abstract

Plasma membrane NADPH oxidases (NOXs) are key producers of reactive oxygen species under both normal and stress conditions in plants and they form functional subfamilies. Studies of these subfamilies indicated that they show considerable evolutionary selection. We performed a comparative genomic analysis that identified 50 ferric reduction oxidases (FRO) and 77 NOX gene homologs from 20 species representing the eight major plant lineages within the supergroup Plantae: glaucophytes, rhodophytes, chlorophytes, bryophytes, lycophytes, gymnosperms, monocots, and eudicots. Phylogenetic and structural analysis classified these FRO and NOX genes into four well-conserved groups represented as NOX, FRO I, FRO II, and FRO III. Further analysis of NOXs of phylogenetic and exon/intron structures showed that single intron loss and gain had occurred, yielding the diversified gene structures during the evolution of NOXs family genes and which were classified into four conserved subfamilies which are represented as Sub.I, Sub.II, Sub.III, and Sub.IV. Additionally, both available global microarray data analysis and quantitative real-time PCR experiments revealed that the NOX genes in *Arabidopsis* and rice (*Oryza sativa*) have different expression patterns in different developmental stages, various abiotic stresses and hormone treatments. Finally, coexpression network analysis of NOX genes in *Arabidopsis* and rice revealed that NOXs have significantly correlated expression profiles with genes which are involved in plants metabolic and resistance progresses. All these results suggest that NOX family underscores the functional diversity and divergence in plants. This finding will facilitate further studies of the NOX family and provide valuable information for functional validation of this family in plants.

**Key words:** NADPH oxidase (NOX), phylogenetic analysis, abiotic stress, hormone, coexpression.

## Introduction

Reactive oxygen species (ROS) have been shown to be toxic but also function as signaling molecules. This biological paradox of ROS and their underlie mechanism confer them vital roles in the integrity, fitness and aging of living organisms (D'Autréaux and Toledano 2007). NADPH oxidases (NOXs) are key enzymes of ROS generation and thus play crucial roles in a variety of biological processes in different kingdoms of life (Torres and Dang 2005; Bedard and Krause 2007; Bedard et al. 2007). While they are not found in prokaryotes and most unicellular eukaryotes, they are universally present in fungi, animals, and plants (Bedard et al. 2007). In fungi, only ancestral NOXs including one ferric reductase (FRE) and three

fungal NOXs were identified (Aguirre et al. 2005). In animals, total seven types of NOXs, namely NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2, were reported (Bedard and Krause 2007; Bedard et al. 2007). However, in plants, only NOX5-like type NOXs were found even although multiple members exist in different species (Sagi and Fluhr 2006; Bedard et al. 2007; Wang et al. 2013).

The plant NOXs, also named as respiratory burst oxidase homologs (RBOHs), are proposed to be major ROS producers of plants under normal and stress conditions (Foreman et al. 2003; Sagi and Fluhr 2006). The first plant NOX was isolated from rice as a gp91phox homolog of mammalian NOX (Groom et al. 1996). Thereafter, plant NOXs were identified

in *Lycopersicon esculentum* (Amicucci et al. 1999), *Nicotiana tabacum* (Yoshioka et al. 2001), *Solanum tuberosum* (Yoshioka et al. 2003), *Arabidopsis thaliana* (Sagi and Fluhr 2006), *Medicago truncatula* (Marino et al. 2011), *Phaseolus vulgaris* (Arthikala et al. 2014), and *Zea mays* (Nestler et al. 2014). In *Arabidopsis*, ten members of typical NOXs were reported with assigned to be AtRBOHA–J, respectively (Sagi and Fluhr 2006). In rice, at least nine typical NOXs were identified (Wong et al. 2007; Wang et al. 2013). Those rice NOXs were named as OsRBOHA–I or OsNOX1–9, respectively, and found to be diverse in protein domain composition and functioning (Wang et al. 2013). Besides the typical NOXs, some ferric reduction oxidases (FROs), which were considered as the isoforms of yeast ferric-chelate reductase (FRE), were also found in high plants (Sagi and Fluhr 2006). In *Arabidopsis* total eight FROs (AtFRO1–8) were identified (Wu et al. 2005) whereas in rice only two FROs were reported (OsFRO1 and OsFRO7) (Wang et al. 2013). These proteins are closely related but still different from the typical plant NOX. They contain six membrane-spanning domains, two hemes, and conserved motifs involved in NADPH and FAD binding but lack NADPH\_Ox domain and several calcium-binding EF-hand motifs that the typical NOX proteins have (Bedard et al. 2007; Wang et al. 2013).

Different NOX might have different functions both in animals and plants (Bedard and Krause 2007; Kaye et al. 2011). In *Arabidopsis*, two NOXs, AtRBOHD and AtRBOHF, were found to be essential for jasmonic acid-induced expression of genes regulated by the MYC2 transcription factor (Maruta et al. 2011) while AtRBOHD be participating in ABA-mediated ROS production and stomatal closure (Zhang et al. 2009). Although the activation mechanisms of AtRBOHD and AtRBOHF are similar in stress responses, AtRBOHD has a significantly greater ROS-producing activity than does AtRBOHF (Kimura et al. 2012). AtRBOHD and AtRBOHF also showed to be functioning in disease resistance (Chaouch et al. 2012) and salt stress tolerance (Xie et al. 2011). In addition, it was reported that AtRBOHD participates in endosperm development (Penfield et al. 2006), and AtRBOHB involves in seed after-ripening (Müller et al. 2009) whereas AtRBOHC functions in root-hair-tip growth (Takeda et al. 2008). Moreover, Evans et al. (2005) found that AtRBOHJ functions in salt stress tolerance and, more recently, it was reported that AtRBOHH and AtRBOHJ play essential roles in pollen tube tip growth via Ca<sup>2+</sup>-activated ROS production (Kaya et al. 2014, 2015). In maize, the expression of the four NOX genes could be induced by ABA treatment, implying that they also function in plant stress tolerance (Lin et al. 2009). In rice, the expression profiles of NOX-encoding genes showed unique stress-response characteristics (Wang et al. 2013). In wheat roots, NOXs ameliorate the oxidative stress induced by nickel (Hao et al. 2006). As a whole, NOXs participate in the plant immune response (Yoshioka et al. 2011), polar growth of root hairs (Nestler et al. 2014) and pollen tubes (Wudick and Feijó 2014; Kaya

et al. 2014, 2015), abscisic acid (ABA)-mediated stomatal closure (Zhang et al. 2009; Shi et al. 2012), apoptosis (Tewari et al. 2012), tapetal programmed cell death (PCD) and pollen development (Xie et al. 2014), control of cell differentiation and proliferation (Cano-Domínguez et al. 2008), and seed after ripening (Müller et al. 2009) in higher plants.

The ancient forms of NOXs also exhibit important roles. These ancient forms including fungal NOXs and yeast FREs are responsible either for ROS production or for iron uptake (Bedard et al. 2007). Fungal NOXs were found playing key roles in fungal cellular differentiation, pathogenicity, and fungal-plant symbiosis (Scott and Eaton 2008; Segmüller et al. 2008). Their counterparts in higher plants such as AtRFOs and OsRFOs, however, mainly function in iron acquisition, metabolism, and/or homeostasis (Wu et al. 2005; Sperotto et al. 2010).

As the major producer of ROS during cell growth, plant development, and stress responses, the increasing evidence also show that plant NOXs participate in a number of signaling pathways involving mitogen-activated protein kinases (Zhang et al. 2014), Ca<sup>2+</sup>-dependent protein kinases (Asano et al. 2012), receptor activated C-kinases (Nakashima et al. 2008), phosphatidylinositol (Kaye et al. 2011), phospholipase D $\alpha$ 1 and phosphatidic acid (Zhang et al. 2009), Ca<sup>2+</sup> (Wudick and Feijó 2014), NO (Delledonne et al. 2002), cGMP (Li et al. 2011), extracellular ATP (Song et al. 2006), and hormone-signaling transduction cascades (Zhang et al. 2014). Recently, it was suggested that a cyclin-dependent protein kinase/NOX activation circuit is required for rapid defense signal propagation in *Arabidopsis* (Dubiella et al. 2013). In addition, the clathrin- and microdomain-dependent endocytic pathways were found to cooperatively regulate AtRBOHD dynamics in *Arabidopsis* and the regulation of AtRBOHD activity by clustering and endocytosis could facilitate the activation of redox signaling pathways under salt stress (Hao et al. 2014). Thus, NOXs may serve as molecular ‘hubs’ during ROS-mediated signaling in plants (Marino et al. 2012), therefore, crucially involve in plant stress response and normal growth and development.

Although these studies in fungi, yeast, human, and *Arabidopsis* have led to an understanding of the biochemical properties and physiological functions of NOXs, there has been no systematic study of the evolution and functional divergence of the NOX gene family, especially in Plantae. Here, we performed a comprehensive analysis of the NOX gene family in 20 species, representing the eight major plant lineages within the supergroup Plantae. Phylogenetic analysis was performed to delineate the evolutionary history of the NOX family in Plantae, and exon/intron structure analysis was performed to gain insight into the possible mechanisms of the structural diversity of NOX gene family. Finally, the tissue-specificity, inducibility, and coexpression networks of NOX gene expression in *Arabidopsis* and rice (*Oryza sativa*) were characterized by examining publicly available microarray data sets and quantitative real-time PCR (qRT-PCR) experiments.

The results obtained here will broaden our understanding of the roles of plant NOXs and provide a framework for further functional investigations of these genes in plants.

## Materials and Methods

### Data Retrieval and Identification of FRO and NOX Genes

The protein sequences of 20 completely or partially sequenced plant genomes representing the eight major plant lineages (i.e., the glaucophyte *Cyanophora paradoxa*, the rhodophytes *Cyanidioschyzon merolae* and *Galdieria sulphuraria*, the chlorophytes *Ostreococcus tauri*, *Ostreococcus lucimarinus*, *Micromonas pusilla* strain RCC299 and strain CCMP1545, *Chlorella variabilis* NC64A, *Coccomyxa subellipsoidea* C-169, *Chlamydomonas reinhardtii* and *Volvox carteri*, the bryophyte *Physcomitrella patens*, the lycophyte *Selaginella moellendorffii*, the gymnosperm *Picea abies*, the monocots *Brachypodium distachyon*, *O. sativa* and *Z. mays* and the eudicots *A. thaliana*, *Populus trichocarpa*, and *Vitis vinifera*) were retrieved from public databases. All of the protein sequences were the most current nonredundant sequences and all data sources and versions were the same as in our previous study (Li et al. 2014), except for the gymnosperm *P. abies* version from ConGenIE 1.0 (<http://congenie.org/start>, last accessed February 29, 2016; Nystedt et al. 2013). Additionally, the most current and nonredundant protein sequences of *Homo sapiens* (e!Ensembl release 78, [http://asia.ensembl.org/Homo\\_sapiens/Info/Index](http://asia.ensembl.org/Homo_sapiens/Info/Index), last accessed February 29, 2016), *Saccharomyces cerevisiae* (SGD release R64.2, <http://www.yeastgenome.org/>, last accessed February 29, 2016) and *Escherichia coli* K-12 (EcoGene v3.0, <http://ecogene.org/>, last accessed February 29, 2016; Zhou et al. 2013) were also collected from public databases. All of the above protein sequences were integrated into a local protein database and the same methods in our previous study (Li et al. 2014) were used for the subsequent identification of the FRO and NOX homologs. The only difference was that the three family specific domain viz., Ferric\_reduct (PF01794), FAD\_binding\_8 (PF08022), NAD\_binding\_6 (PF08030) and four family specific domain viz., NADPH\_Ox (PF08414), Ferric\_reduct (PF01794), FAD\_binding\_8 (PF08022), NAD\_binding\_6 (PF08030) HMM profiles were used in HMM search against the local protein database for the identification of FRO and NOX homologous genes, respectively.

### Phylogenetic Relationships and Exon/Intron Structure Analysis

The amino acid sequences alignment of NADPH\_Ox, Ferric\_reduct, FAD\_binding\_8 and NAD\_binding\_6 domains of the candidate FRO and NOX proteins were used for the construction of phylogenetic trees, and the detailed methods were referenced to our previous study (Li et al. 2014). The exon/intron structures of individual FRO and NOX genes were obtained through the online Gene Structure Display Server

(GSDS, <http://gsds.cbi.pku.edu.cn>, last accessed February 29, 2016; Hu et al. 2015) by aligning the coding or cDNA sequences with their corresponding genomic DNA sequences from Phytozome v9.1 (<http://www.phytozome.net/>, last accessed February 29, 2016; Goodstein et al. 2012). To illustrate the evolution of introns and the predictions pertaining to the types of introns, we constructed a gene model for annotation of introns and the detailed methods were referenced to our previous study (Li et al. 2014).

### Cis-Regulatory Elements, In Silico Expression Profiles

The 1,500 bp upstream of the transcription start site of all NOX genes in *Arabidopsis* and rice were obtained from Phytozome v9.1 (<http://www.phytozome.net/>), and the cis-regulatory elements were identified using the PlantCARE program (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, last accessed February 29, 2016; Lescot et al. 2002).

### In Silico Expression Profiles and Coexpression Network Analysis

For the expression profile analysis of NOX genes in *Arabidopsis* and rice, ATH1 22k and Os 51k microarray data in the Genevestigator V3 database were used, and then the heat maps were constructed using the obtained gene expression data sets, respectively (Hruz et al. 2008).

Coexpression network analysis of NOX genes in *Arabidopsis* and rice were performed using Mutual Rank (MR) values calculated by using the online tools ATTED-II (version 7.1, <http://atted.jp/>, last accessed February 29, 2016; Obayashi et al. 2007) and RiceFRIEND (version 2.0, <http://ricefriend.dna.affrc.go.jp/>, last accessed February 29, 2016; Sato 2012) coexpression databases, respectively. The following criteria for interactome frameworks were performed at the cut-off MR value  $\leq 20$  between two genes and hierarchy=2 with the NOX gene as the center. The Cytoscape v3.2.1 software (<http://www.cytoscape.org/>, last accessed February 29, 2016; Shannon et al. 2003) was used to draw the coexpression networks.

### Plant Materials, Treatments, and qRT-PCR Analysis

Preparation of the *Arabidopsis* (Col-0) and rice (*O. sativa* ssp. *japonica* cv. Dongjin) seedlings were referenced to our previous study (Li et al. 2014). Meanwhile, the detailed methods for seedling under various abiotic stresses, that is, cold (4 °C), heat (30 °C for *Arabidopsis*/40 °C for rice), salt (200 mM NaCl), drought (20% PEG6000), and oxidation (30  $\mu$ M MV) and hormones, that is, ABA (100  $\mu$ M) and MeJA (100  $\mu$ M) were the same as in our previous study as well (Li et al. 2014). All the MV, MeJA, and ABA used for treatments were purchased from Sigma-Aldrich. All samples were collected following treatment and immediately frozen at  $-80$  °C.

Total RNA was extracted by using Trizol reagent (Takara, Japan) according to the manufacturer's instructions and the subsequent qRT-PCR analyses were referenced to our previous

study (Li et al. 2014). All gene-specific primers were designed to avoid the conserved region and span introns or cross an exon-exon junction. The detailed primer sequences are shown in [supplementary table S3, Supplementary Material](#) online. The *AtTub6* (AT5G12250) and *OsActin1* (Gene ID: KC140126) genes were chosen as the internal control in the analysis of *Arabidopsis* and rice, respectively.

## Results

### Identification of FRO and NOX Family Members in Plants

To comprehensively investigate and characterize the FRO and NOX gene families in plants, 20 species representing the eight major plant lineages within the supergroup Plantae, were selected for analysis (fig. 1). A Hidden Markov Model (HMM) search was performed with the obtained sequences and total 50 FRO and 77 NOX homologs were identified in Plantae (fig. 1; [supplementary table S1, Supplementary Material](#) online). One or more FRO genes and/or four or more NOX genes were identified in each genome of the selected species (fig. 1). Most of the aquatic algae, including glaucophytes, rhodophytes, and chlorophytes, contained none NOX genes per genome and only one FRO genes except one species in the chlorophytes, *C. reinhardtii*, carried three FRO genes per genome (fig. 1). In contrast, two or more FRO genes and four or more NOX genes were found in all land plants, including bryophytes, lycophytes, gymnosperms, monocots, and eudicots (fig. 1). In addition, none plant-type FRO gene was identified per genome in bacteria (*E. coli* K-12) and none plant-type NOX gene was identified per genome in animal (*H. sapiens*), fungi (*S. cerevisiae*), and bacteria (*E. coli* K-12; fig. 1).

The Pfam and SMART databases were used to analyze the functional domains of the identified FRO and NOX candidates. All of the putative FROs possess a transmembrane region with Ferric\_reduction domain (Pfam accession number PF01794), and two cytosol regions, FAD\_binding\_8 domain (PF08022) and NAD\_binding\_6 domain (PF08030). However, besides the domains as FROs have, all NOXs also possess a typical NADPH\_Ox domain (PF08414).

### Phylogenetic Analysis and Classification of the FRO and NOX Family

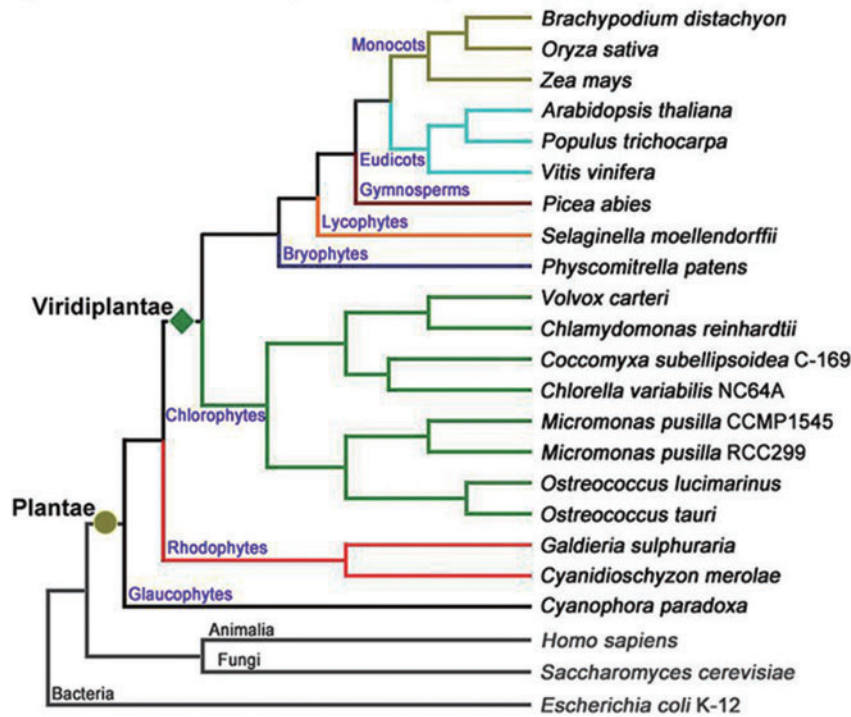
To explore the phylogenetic relationships among FRO and NOX family members in plants, we first generated an unrooted maximum-likelihood phylogenetic tree with the 50 FROs and 77 NOXs (fig. 2A), which were inferred from the amino acid sequences of the Ferric\_reduct, FAD\_binding\_8 and NAD\_binding\_6 domains ([supplementary fig. S1, Supplementary Material](#) online). FRO and NOX homologs in plants can be classified into four well-conserved subfamilies (NOX, FRO I, FRO II, FRO III; fig. 2) with high statistical support, according to the topology and the deep duplication nodes of FRO and NOX paralogues in the maximum-likelihood tree.

Interestingly, the topological relationship of members within subfamilies was highly consistent with the evolutionary relationships between species in Plantae (figs. 1B and 2A). The majority of aquatic algae contained none FRO and NOX genes per genome, except for chlorophytes *C. reinhardtii* carried three FROs, *V. carteri*, *Coccomyxa subeupsoides c-169*, and *Chlorella variabilis nc64A* carried one FRO and grouped into FRO II, whereas the majority of land plants, except for the gymnosperm *Picea sitchensis*, carried  $\geq 2$  FRO and 4 NOX genes per genome and were clustered into NOX and FRO II (fig. 2). Further analysis of the functional domains showed that domain organization of the proteins between FRO and NOX subfamilies varied considerably (fig. 2B). All of the subfamilies contained a transmembrane domain Ferric\_reduct, FRO I was considered as ancestor NOX, FRO II and FRO III contained FAD\_binding\_8 and NAD\_binding\_6 domains, but NOX also contained a typical NADPH\_Ox domain (fig. 2B).

### Structure Analysis of FRO and NOX Family Genes

To better understand the evolution of the FRO and NOX gene families, we generated unrooted maximum-likelihood phylogenetic trees of the FRO and NOX family members (fig. 3A; [supplementary fig. S4, Supplementary Material](#) online), which were inferred from the amino acid sequences of the Ferric\_reduct, FAD\_binding\_8, NAD\_binding\_6, and/or NADPH\_Ox domains ([supplementary figs. S2 and S3, Supplementary Material](#) online). Numbers above the nodes represent bootstrap values from 1,000 replications. Intron position is generally very well-conserved in orthologous genes over long evolutionary time intervals, whereas exon/intron structure is slightly less, but sufficiently conserved in paralogous genes to reveal evolutionary relationships between introns (Rogozin et al. 2003; Li et al. 2009). To investigate the gene structural diversity and possible mechanisms for the structural evolution of FRO and NOX homologs in land plants, the rhodophytes (*Galdieria sulphuraria*), chlorophytes (*V. carteri*, *C. reinhardtii*, *Co. subeupsoides C-169*, *Chlorella variabilis NC64A*), bryophytes (*Ph. patens*), lycophytes (*Se. moellendorffii*), gymnosperms (*Picea abies*), eudicots (*A. thaliana*, *Po. trichocarpa*, and *Vi. vinifera*), and monocots (*B. distachyon*, *O. sativa*, and *Z. mays*), were considered to analyze the exon/intron organization in the coding sequence (fig. 3; [supplementary fig. S4, Supplementary Material](#) online). According to the exon/intron organization, the plant NOXs examined here can be divided into four conserved subfamilies which were represented as Sub. I, Sub. II, Sub. III, and Sub. IV, respectively (fig. 3); whereas, FRO II proteins can be grouped into three subfamilies named as FRO IIa, FRO IIb, and FRO IIc, respectively ([supplementary fig. S4, Supplementary Material](#) online). Overall, there was considerable diversity in the number and the length of introns in the FRO and NOX family genes, and NOX family members shared similar gene structure in terms of intron numbers and intron phases within

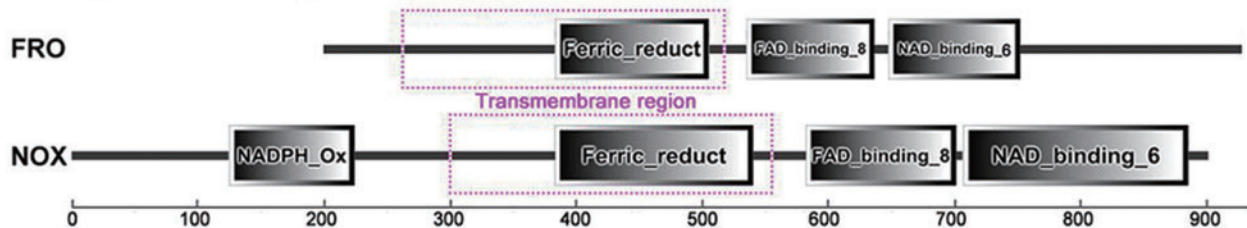
**A Systematic evolutionary relationships among species**



**B FRO NOX**

Species	FRO	NOX
<i>Brachypodium distachyon</i>	2	9
<i>Oryza sativa</i>	2	9
<i>Zea mays</i>	2	14
<i>Arabidopsis thaliana</i>	8	10
<i>Populus trichocarpa</i>	8	10
<i>Vitis vinifera</i>	6	7
<i>Picea abies</i>	1	6
<i>Selaginella moellendorffii</i>	9	8
<i>Physcomitrella patens</i>	5	4
<i>Volvox carteri</i>	1	0
<i>Chlamydomonas reinhardtii</i>	3	0
<i>Coccomyxa subellipsoidea</i> C-169	1	0
<i>Chlorella variabilis</i> NC64A	1	0
<i>Micromonas pusilla</i> CCMP1545	0	0
<i>Micromonas pusilla</i> RCC299	0	0
<i>Ostreococcus lucimarinus</i>	0	0
<i>Ostreococcus tauri</i>	0	0
<i>Galdieria sulphuraria</i>	1	0
<i>Cyanidioschyzon merolae</i>	0	0
<i>Cyanophora paradoxa</i>	0	0
<i>Homo sapiens</i>	7	0
<i>Saccharomyces cerevisiae</i>	8	0
<i>Escherichia coli</i> K-12	0	0

**C The typical domain organization of FRO and NOX proteins**



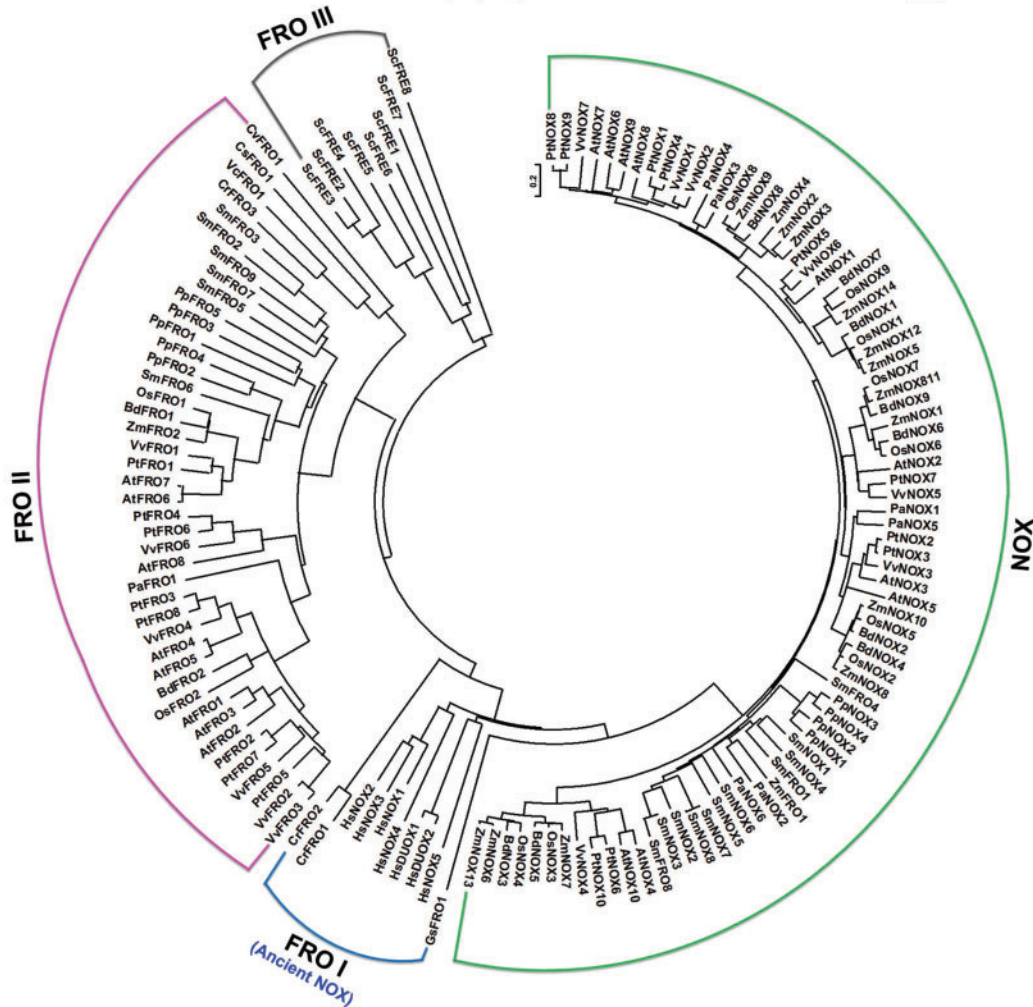
**Fig. 1.**—The FRO and NOX gene family in Plantae. (A) Systematic evolutionary relationships of 20 species among eight lineages within the supergroup Plantae. (B) The numbers of FRO and NOX homologs in each species. (C) The typical domain organization of FRO and NOX proteins. The transmembrane regions are shown with dotted pink box.

the same subfamily except Sub. IV (fig. 3; supplementary fig. S4, Supplementary Material online). For instance, NOX genes in Sub. I have 6–14 introns, 44.8% (13/29) genes have 11 introns, 48.3% (15/29) genes have 6–10 introns, and two genes have 12 and 14 introns, respectively. Whereas NOX genes in Sub. II, III, and IV have 11–13, 11–14, and 7–17 introns, respectively. We also investigated intron phases with respect to codons in the NOX and FRO genes. The intron phases were remarkably well conserved among subfamily members, whereas the intron arrangement and phases were strikingly distinct between subfamilies (fig. 3B; supplementary fig. S4, Supplementary Material online).

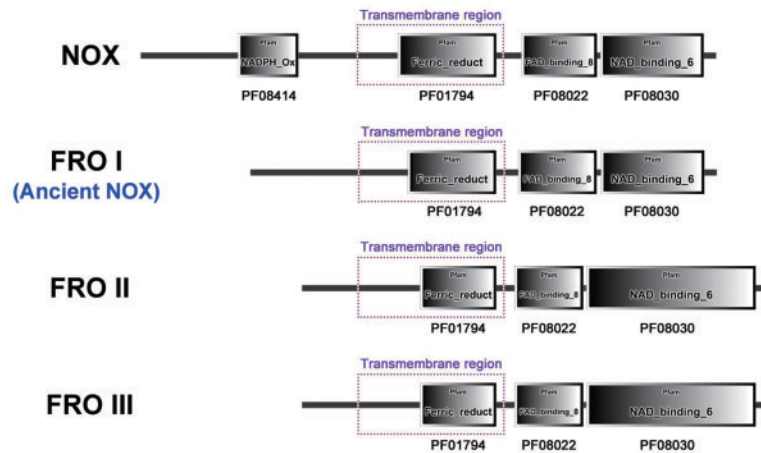
To further explore intron loss or gain within the NOX family genes, we examined the exon/intron organization of paralogous and orthologs in the land plants (fig. 4A). This analysis

revealed that single intron loss and gain likely occurred during the structural evolution of NOX family genes in land plants. For example, the paralogous genes *AtRBOHC/G* and *PtNOX8/9* showed conserved exon/intron structure in terms of the number of introns and exon length, whereas a single intron appears to have been lost during the evolution of the *SmNOX6/7/8* paralogs (figs. 3B and 4A). By contrast, a single intron gain occurred between the paralogs *ZmNOX3* and *ZmNOX2* (figs. 3B and 4A). Similar intron loss were seen with *BdNOX7/8* (subfamily I), *OsNOX5/6* (subfamily II), *BdNOX3/5* (subfamily III), and *PpNOX3/4* (subfamily IV; fig. 4A). Single intron gain was also observed in the NOX genes of land plants. For instance, *ZmNOX3* and *PaNOX4* in subfamily I and *AtRBOHE* and *PaNOX5* in subfamily II appear to have gained an intron, whereas *SmNOX2* and *PaNOX2* gain 2 and 3 introns,

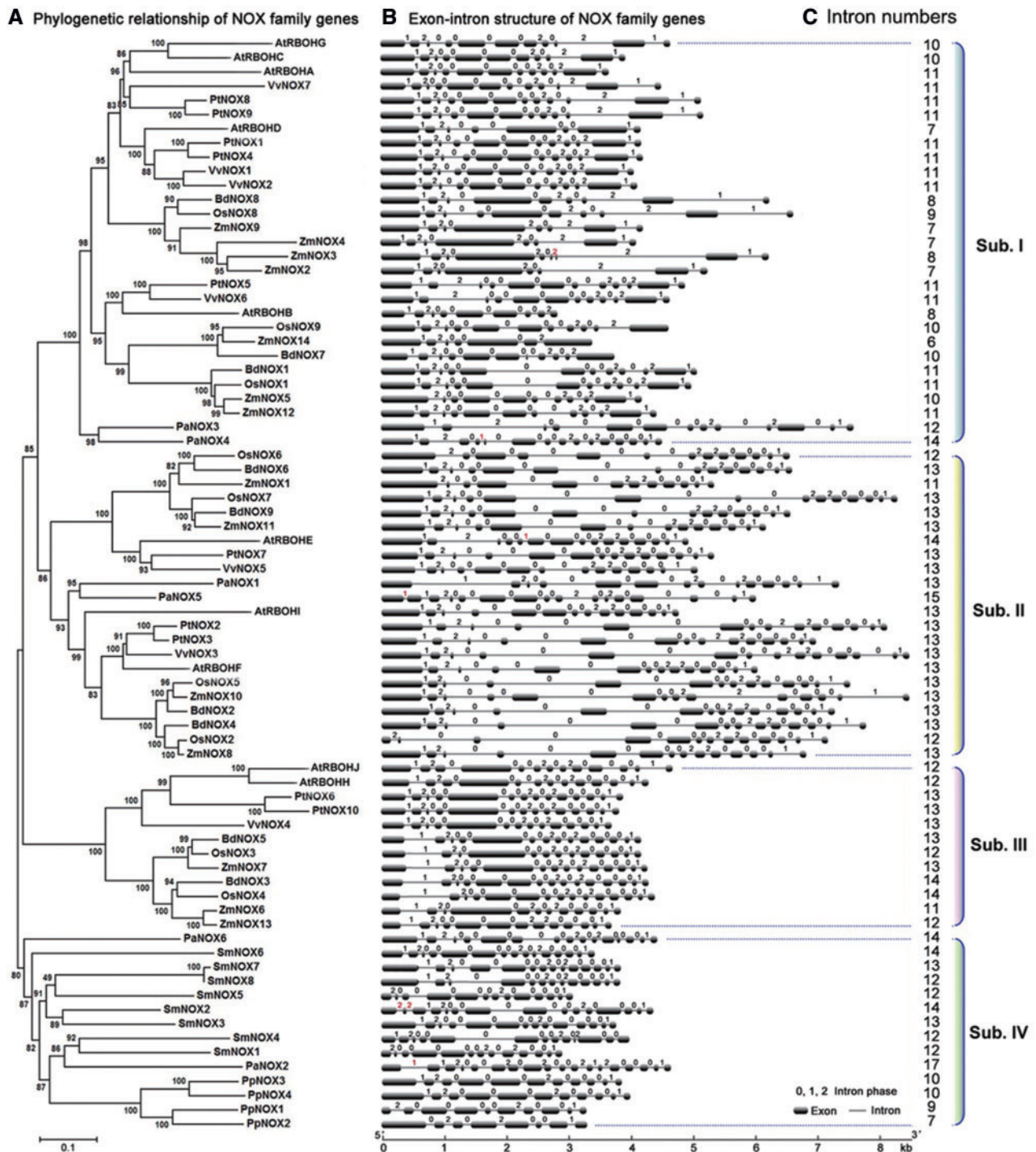
**A** The unrooted maximum-likelihood phylogenetic tree of NOX and FRO family genes



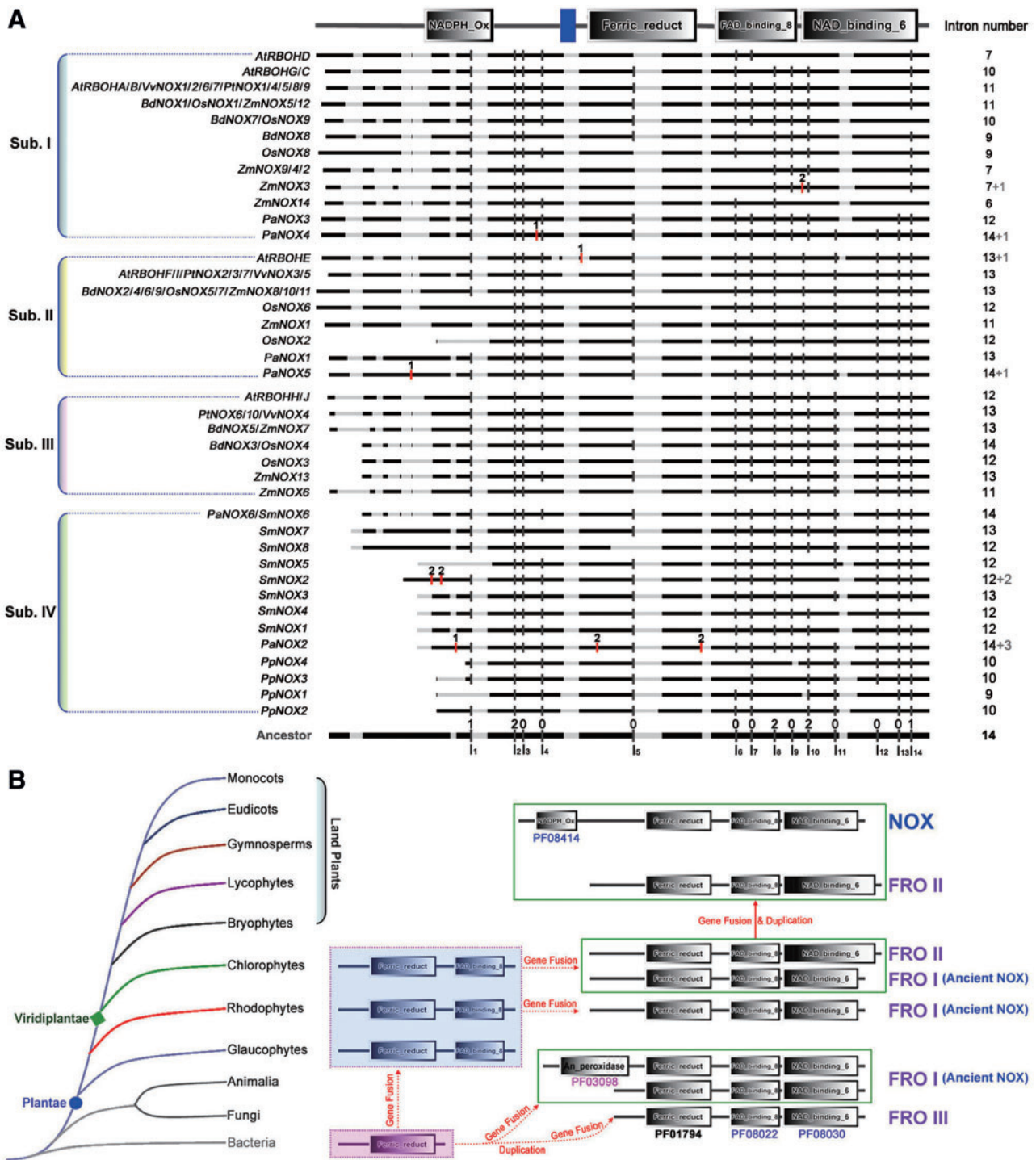
**B** The typical domain organization of NOX and FRO subfamily proteins



**Fig. 2.**—Phylogenetic relationships and domain organization of FRO and NOX genes in plants. (A) The unrooted maximum-likelihood phylogenetic tree of FRO and NOX family members was inferred from the amino acid sequence alignment of the Ferric\_reduct, FAD\_binding\_8 and NAD\_binding\_6 domains. The four conserved groups are marked by different colors and represented as NOX, FRO I, FRO II and FRO III. Scale bar represents 0.2 amino acid substitution per site. (B) Domain organizations of the four conserved groups of FRO and NOX proteins. The transmembrane regions are shown with dotted pink box.



**Fig. 3.**—Phylogenetic relationships and exon/intron structure of NOX family genes in land plants. (A) The unrooted maximum-likelihood phylogenetic tree of FRO and NOX family members was inferred from the amino acid sequence alignment of the NADPH\_Ox, Ferric\_reduct, FAD\_binding\_8 and NAD\_binding\_6 domains. Numbers above the nodes represent bootstrap values from 1,000 replications. (B) Exon/intron structure of NOX family genes in land plants. Black boxes represent exons; black lines represent introns; The length of the boxes and lines are scaled relative to the length of the gene, and longer introns are denoted by a double slash; numbers 0, 1, and 2 are intron phases, which indicates the positions of introns between or within codons. Phase 0 introns are located between two consecutive codons, phase 1 introns are located between the first and second nucleotides of a codon, and phase 2 introns are located between the second and third nucleotides of a codon. The introns labeled with red number, that is, “1” or “2” were gained introns. The four conserved subfamilies of NOXs are represented as Sub. I, Sub. II, Sub.III and Sub.IV at the left side of the gene structures.



**Fig. 4.**—The expansion and evolution of the FRO and NOX gene family in plants. (A) Schematic comparison of intron distribution in NOX orthologs of land plants generated with the CIWOG software. Black horizontal lines are aligned sequences; gray horizontal lines are gaps in the alignment; black vertical bars are conserved common introns and represented as I<sub>1</sub>?I<sub>14</sub>; red vertical bars are gained introns. The numbers 0, 1, and 2 are intron phases. The intron numbers of each NOX genes were showed on the right side and the gray numbers, that is, “+1,” “+2,” or “+3” are represented the number of gained introns. (B) A model for the expansion and evolution of the NOX gene family in Plantae. The evolutionary relationships among eight lineages (left) and a model for the expansion and evolution of the FRO and NOX gene family (right) within the supergroup Plantae.



respectively (fig. 4A). So the intron gain or loss may play a very important role in the structure evolution of NOX family.

Examination of the chromosomal locations of the NOX family genes in the genomes of bryophytes (*Ph. patens*), lycophytes (*Se. moellendorffii*), monocots (*B. distachyon*, *O. sativa*, and *Z. mays*), and eudicots (*Arabidopsis*, *Po. trichocarpa*, and *Vi. vinifera*) showed that NOX genes are randomly distributed (supplementary fig. S5, Supplementary Material online). Moreover, a search for NOX paralogs using the Plant Genome Duplication Database (PGDD; <http://chibba.agtec.uga.edu/duplication/>, last accessed February 29, 2016) (Lee et al. 2013) revealed twelve paralogous gene pairs in *A. thaliana*, *Po. trichocarpa*, *B. distachyon*, and *O. sativa* (supplementary fig. S6A, Supplementary Material online), but none in the other species.

To further explore association of positive selection with duplication and divergence of NOX family genes, the rate of nonsynonymous substitution ( $K_a$ ), synonymous substitution ( $K_s$ ), and the  $K_a/K_s$  ratios were calculated for the 12 paralogous gene pairs and used to estimate duplication and divergence times. The  $K_a/K_s$  ratios varied from 0.831 to 1.197 among the four different species (supplementary fig. S6B, Supplementary Material online). The fact that the  $K_a/K_s$  ratios were less than 1 suggests that the NOX family genes have undergone strong negative selection pressure and the duplication event was estimated to have occurred 6.217–39.499 Ma.

Based on all of these information, we construct a model of evolution of FRO and NOX gene families in plants (fig. 4B). In this model, it seems that all FRO family members originated from a common ancestor which contained only Ferric\_reduct domain. Ferric\_reduct domain then obtained FAD\_binding\_8 and NAD\_binding\_6 domains by first gene fusion and duplication and clustered into FRO I, FRO II, and FRO III subfamilies. FRO III mainly existed in fungi, FRO I perceived as ancient NOX mainly existed in animal which also gained an\_peroxidase domain (Pfam accession number PF03098) and in two kinds of algae, rhodophytes and chlorophytes. FRO I obtained another NADPH\_Ox domain (PF08414) which is represented as NOX family. For the evolutionary way in plants, FRO IIs existed both in chlorophytes and land plants but NOXs only in land plants (figs. 2 and 4B), implying their functional divergence.

### Tissue-Specific Expression Patterns of NOX Genes in *Arabidopsis* and Rice

To investigate differences in the expression of NOX genes in *Arabidopsis* and rice during plant development, we analyzed the expression profiles of *AtNOXs* (i.e., *AtRBOHA–J*) and *OsNOXs* (i.e., *OsNOX1–9*) in ten and nine developmental stages/tissues, respectively, based on the microarray data reported in Genevestigator (fig. 5). Essentially identical developmental expression profiles for the *AtNOX* and *OsNOX* genes were also obtained with data from the *Arabidopsis* and rice eFP browsers in the Bio-Analytic Resource ([http://bar.utoronto.](http://bar.utoronto.ca/welcome.htm)

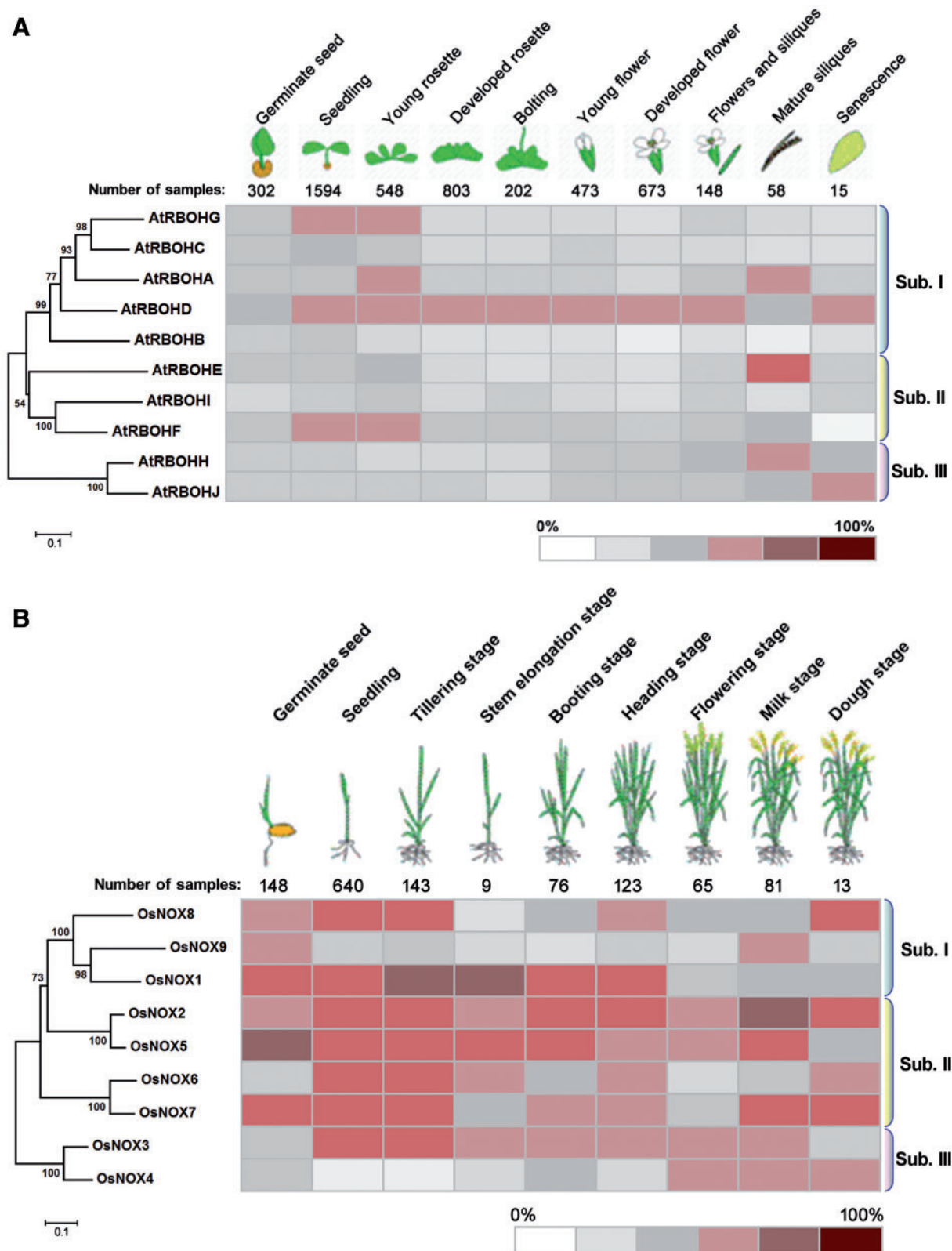
<http://bar.utoronto.ca/welcome.htm>, last accessed February 29, 2016) database (Winter et al. 2007). The expression patterns of *AtNOXs* and *OsNOXs* from the microarray data analyses are shown as heat maps in white/gray/red (low to high) that reflect the percent of expression (fig. 5).

Overall, all of the *AtNOX* and *OsNOX* genes are expressed during the vegetative and reproductive development stages, and they display strong tissue specificity. In *Arabidopsis*, *AtRBOHD* shows higher expression in most tissues and developmental stages than other *AtNOXs* (fig. 5A). The *AtRBOHD* expression is almost seen in all the tissues except germinate seed and mature siliques, whereas *AtRBOHG* and *AtRBOHF* are the highest expressed in the seedling and young rosette, *AtRBOHA* is mainly expressed in the young rosette and mature siliques (fig. 5A), the *AtRBOHE* expression is the highest in the mature siliques, whereas *AtRBOHH* and *AtRBOHJ* are mainly expressed in mature siliques and senescence leaves, respectively. In rice, the *OsNOX1*, *OsNOX2*, *OsNOX3*, *OsNOX5*, *OsNOX6*, *OsNOX7*, and *OsNOX8* transcripts tend to accumulate to higher levels than the *OsNOX4* and *OsNOX9* transcripts in most tissues/developmental stages, but their individual expression patterns differ (fig. 5B). Apparently, most *OsNOX* genes have higher expression at the vegetative development stages, but *OsNOX2*, *OsNOX5*, *OsNOX7*, and *OsNOX8* genes are also higher expressed at the reproductive development stages. It seems that *OsNOX4* is only highly expressed at the reproductive stages (fig. 5B).

### Response Profiles of NOX Genes under Abiotic Stresses and Hormone Treatments in *Arabidopsis* and Rice

To understand the molecular mechanism of *AtNOXs* and *OsNOXs* transcriptional regulation under abiotic stresses and hormone treatments, we first identified potential *cis*-elements in the promoter regions of each *NOX* gene using the PlantCARE program. The results obtained show that the promoter regions of *AtNOX* and *OsNOX* genes contain many response elements for several abiotic/biotic stresses, such as low temperature, heat, drought (MYB binding sites), anaerobic conditions, and pathogens (TC-rich repeats, W box, GCC box, Box S, Box-W1, and EIRE) (supplementary tables S4 and S5, Supplementary Material online). In addition, the *AtNOX* and *OsNOX* gene promoters contain *cis*-elements for responding to several hormones, such as auxin, gibberellin, ABA, ethylene, salicylic acid (SA), and methyl jasmonic acid (MeJA) (supplementary tables S4 and S5, Supplementary Material online). Further analysis showed that the majority of the *AtNOX* and *OsNOX* promoters contain anaerobic-, ABA-responsive elements, heat stress, drought (MYB binding sites), and MeJA response elements (supplementary tables S4 and S5, Supplementary Material online).

To further demonstrate the expression profiles of *AtNOX* and *OsNOX* genes induced by abiotic stresses, we again examined *Arabidopsis* and rice microarray data in the



**Fig. 5.**—Developmental expression patterns of NOX family genes in *Arabidopsis* and rice. Expression profiles of (A) AtNOXs (i.e., *AtRBOHA–J*) and (B) OsNOXs (i.e., *OsNOX1–9*) in different developmental stages obtained from microarray data reported in Genevestigator. Results are shown as heat maps in white/gray/red (low to high) that reflect the percent of expression.

Genevestigator database. As shown in [supplementary figures S7 and S8, Supplementary Material](#) online, the expression of *AtNOX* and *OsNOX* genes is induced to varying degrees by cold, heat, drought (PEG), salt (NaCl), and oxidative (methyl viologen, MV) stresses. As can be seen, *AtRBOHB* and *AtRBOHD*, both belong to Sub. I, exhibit strongly upregulations to hypoxia treatments both in seedlings and roots ([supplementary fig. S7A, Supplementary Material](#) online). *AtRBOHD* is also upregulated by drought, osmotic stress and salt treatments ([supplementary fig. S7A, Supplementary Material](#) online). In contrast, the two Sub. III genes (*AtRBOHH* and *AtRBOHJ*) have no much changes in expression under most abiotic stresses ([supplementary fig. S7, Supplementary Material](#) online). The *OsNOX* genes exhibit more complicated expression profiles in response to environmental stress treatments and no marked distinct orderliness in expression was observed ([supplementary fig. S8, Supplementary Material](#) online). It should be noticed that *OsNOX2* and *OsNOX9* are greatly upregulated during seed germination of rice under either anaerobic or aerobic conditions, whereas *OsNOX1*, *OsNOX3*, *OsNOX4*, and *OsNOX8* are downregulated at early stages during the seed germination process ([supplementary fig. S8A, Supplementary Material](#) online).

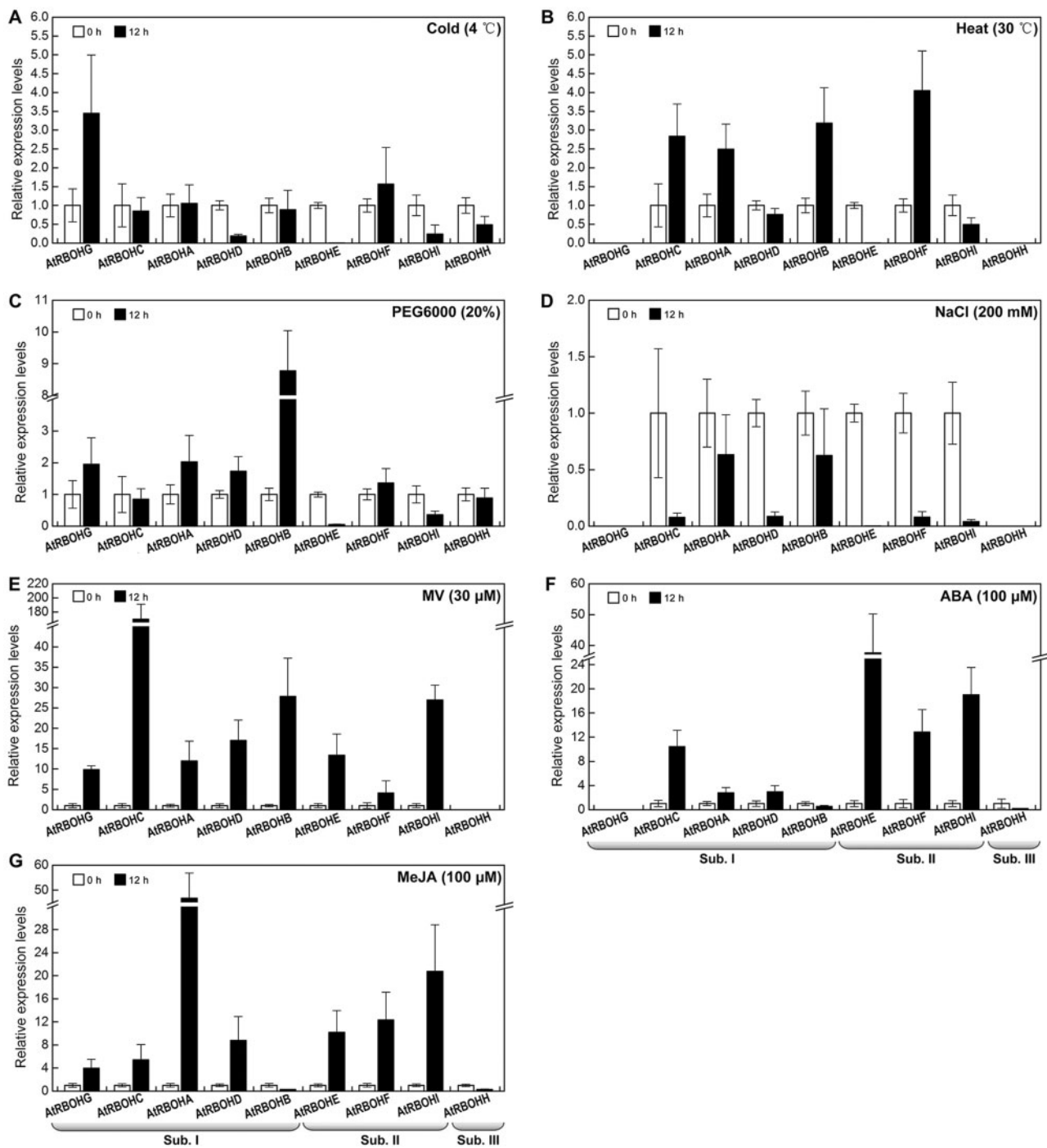
The expression profiles of *AtNOXs* and *OsNOXs* under different abiotic stresses were also examined by qRT-PCR experiments. In brief, cold greatly upregulates the transcripts of *AtRBOHA* but downregulates those of *AtRBOHD*, *E*, *I*, and *H*; heat markedly upregulates the transcripts of *AtRBOHA*, *B*, *C*, and *F* but downregulates those of *AtRBOHE* and *I*; dehydrate stress (PEG treatment) significantly upregulates the transcripts of *AtRBOHB* but downregulates those of *AtRBOHE* and *I*; in contrast, salt stress (NaCl treatment) greatly downregulates almost all the *AtNOX* genes, especially *AtRBOHE* and *I*; for oxidative stress (MV treatment), except *AtRBOHH*, all the other eight genes are significantly upregulated (fig. 6). More complicated expression profiles of NOX genes are presented in rice. As can be seen, the expression profiles of *OsNOX* genes exhibit obvious temporal and spatial variations (fig. 7). Under cold stress, almost all the *OsNOX* genes are upregulated in both shoots and roots at 12 h timepoint except *OsNOX9* is slightly downregulated at this timepoint. However, the transcripts of most *OsNOX* genes are fallen back at 24 h timepoint by cold stress but some are still upregulated at this timepoint, for example *OsNOX3* is upregulated both in shoots and roots whereas *OsNOX2*, *4*, *5*, *7*, and *9* are upregulated only in roots (fig. 7A). For heat treatment, almost all of the *OsNOX* genes are upregulated at 12 h timepoint in shoots and roots except *OsNOX1* and *3* are downregulated at this timepoint in roots, however, almost all the genes are markedly downregulated at 24 h timepoint in roots by this stress treatment (fig. 7B). In dehydrate stress experiment (PEG treatment), all *OsNOXs* are significantly upregulated at both 12 and 24 h timepoints in shoots but significant upregulations in roots were only observed from *OsNOX1*, *3*,

and *5* genes at these two timepoints (fig. 7C). For NaCl treatment, great upregulation of gene transcripts were found from *OsNOX2*, *4*, *5*, *6*, and *9* at both 12 and 24 h timepoints in shoots whereas most of the genes keep no big changes in roots at these timepoints (fig. 7D). In contrast, oxidative stress (MV treatment) significantly upregulates the transcripts of all the genes detected in both shoots and roots, especially *OsNOX2*, *4*, *6*, and *9* in shoots (fig. 7E).

As for several hormone response elements were found in the promoter regions of *AtNOX* and *OsNOX* genes, we also examined *Arabidopsis* and rice microarray data in the Genevestigator database ([supplementary figs. S7 and S8, Supplementary Material](#) online), as well as by qRT-PCR experiments. The expression of *AtNOX* and *OsNOX* genes is induced by hormones such as ABA, SA, MeJA, Zeatin, GA, IAA, KT, 6-BA, JA, and ACC. As can be seen, *AtRBOHB* and *AtRBOHD* are markedly downregulated by ABA, Zeatin, and IAA treatments but upregulated by MeJA ([supplementary fig. S7B, Supplementary Material](#) online). Three others in this subfamily genes (*AtRBOHA*, *AtRBOHC*, and *AtRBOHG*) show similar expression profiles under these hormone treatments except they are not obviously induced by MeJA ([supplementary fig. S7B, Supplementary Material](#) online). Interestingly, the genes in Sub. II (*AtRBOHE*, *AtRBOHF*, and *AtRBOHI*) exhibit almost totally opposite expression profiles as the Sub. I genes have under ABA and IAA treatments ([supplementary fig. S7B, Supplementary Material](#) online). The *OsNOX* genes exhibit more complicated and irregular expression profiles ([supplementary fig. S8, Supplementary Material](#) online). As for the results of qRT-PCR, ABA treatment pronounced upregulates the transcripts of most *AtNOX* genes including *AtRBOHA*, *C*, *D*, *E*, *F*, and *I*, but downregulates those of *AtRBOHB* and *H*; similar expression profiles of the *AtNOX* genes were also found by treatment with MeJA (fig. 6). In rice, ABA treatment markedly upregulates the transcripts of *OsNOX3* and *7* at 12 h timepoint and the transcripts of *OsNOX2* and *4* at 24 h timepoint in shoots, but only the transcripts of *OsNOX1* are obviously upregulated in roots at 12 h timepoint. However, ABA treatment markedly downregulates the transcripts of *OsNOX5*, *6*, *7*, and *8* in roots at the two timepoints (fig. 7F). MeJA treatment significantly upregulates the transcripts of *OsNOX1*, *2*, *3*, *5*, *6*, *7*, *8*, and *9* at both 12 and 24 h timepoints in shoots. In roots, however, MeJA treatment only greatly upregulates the transcripts of *OsNOX3*, *4*, and *7* at both 12 and 24 h timepoints (fig. 7G). It should be noticed that the expression profiles of the members of both *AtNOX* and *OsNOX* genes have no distinct subfamily characteristics in response to these environmental stresses and hormone treatments.

#### Coexpression Analysis of NOX Genes in *Arabidopsis* and Rice

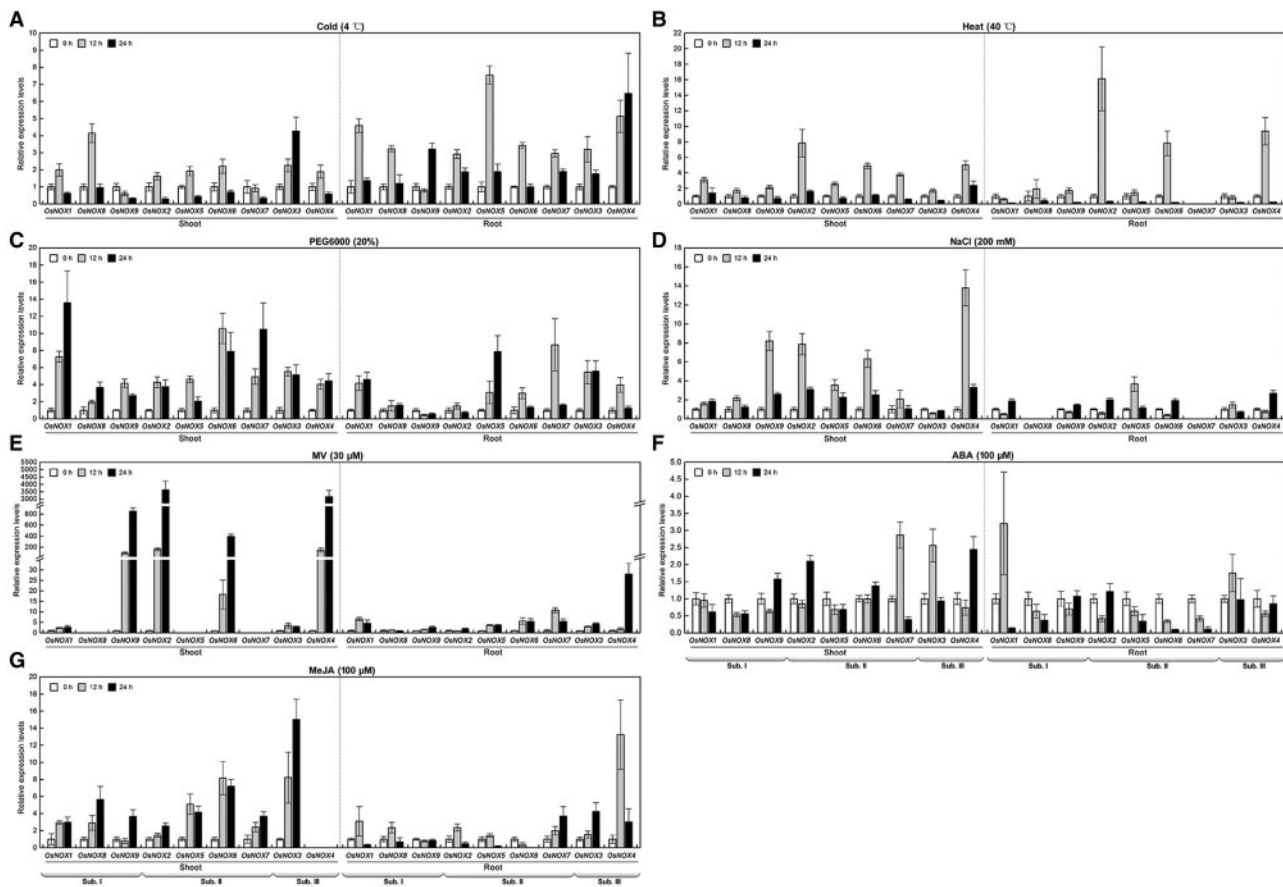
To further explore the system-level functionality of *OsNOXs* and *AtNOXs*, we constructed the framework for coexpression



**FIG. 6.**—Inducible expression patterns of NOX family genes in *Arabidopsis* under abiotic stresses and hormone treatments. Expression levels of *AtRBOHA–I* assayed by qRT-PCR under (A) cold (4 °C), (B) heat (30 °C), (C) drought (20% PEG6000), (D) salt (200 mM NaCl), (E) oxidative (30 μM MV) stresses and (F) ABA (100 μM), (G) MeJA (100 μM) hormone treatments. Data are means ±SD (*n* = 3) and are representative of similar results from three independent experiments.

analysis of NOX genes using the Cytoscapev3.2.1 software (supplementary fig. S9, Supplementary Material online). MR values were labeled on the connecting lines between the two coexpression genes. The coexpression of directly linked genes

with OsNOXs and AtNOXs were predicted by RiceFREND version 2.0 and ATTED-II version 7.1, respectively (supplementary table. S2, Supplementary Material online). In the frameworks of the coexpression networks, we observed that the patterns

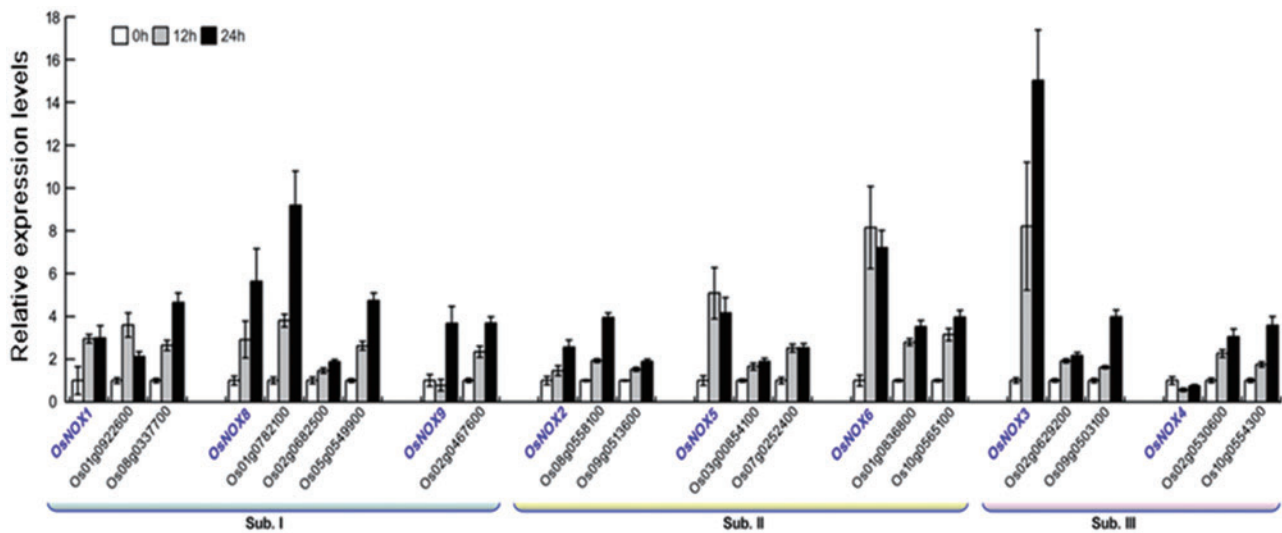


**Fig. 7.**—Inducible expression patterns of NOX family genes in rice under abiotic stresses and hormone treatments. Expression levels of *OsNOX1–9* assayed by qRT-PCR under (A) cold (4 °C), (B) heat (40 °C), (C) drought (20% PEG6000), (D) salt (200 mM NaCl), (E) oxidative (30 μM MV) stresses and (F) ABA (100 μM), (G) MeJA (100 μM) hormone treatments. Data are means ± SD (*n* = 3) and are representative of similar results from three independent experiments.

of correlation of expression profiles among NOXs and related genes which are involved in plant resistance and many metabolism progresses. For *AtNOXs*, *AtRBOHA/C/D/F* are mainly involved in plant-pathogen interaction and they are related to genes which are involved in biosynthesis of secondary metabolites, plant hormone signal transduction, phenylalanine metabolism, alpha-linolenic acid metabolism, amino sugar, and nucleotide sugar metabolism, phenylpropanoid biosynthesis (supplementary fig. S9A, Supplementary Material online). For example, *AtRBOHA* is coexpressed with a bZIP transcription factor family protein TGA10 (At5g06839), *AtRBOHB* is related with C2H2 like zinc finger protein (At5g44160), whereas *AtRBOHC* has relatedness between At3g01190 which belongs to peroxidase superfamily protein, and *AtRBOHD* shows a correlation with lipoxygenase protein LOX4 (At1g72520). Additionally, *AtRBOHE* is coexpressed with pollen Ole e 1 allergen and extensin family protein (At5g47635), *AtRBOHG/H/I* shows a correlation with Leucine-rich repeat protein kinase family protein, and

*AtRBOH/H/I* are involved in FRE-like transmembrane component family protein (supplementary fig. S9A and table S2, Supplementary Material online).

More complicated coexpression networks were found in rice NOXs. In brief, the *OsNOXs* are mainly coexpressed with the genes functioning in plant-pathogen interaction, metabolic pathways, N-glycan biosynthesis, endocytosis, and fatty acid and unsaturated fatty acid biosynthesis (supplementary fig. S9B and table S2, Supplementary Material online). For example, *OsNOX1* shows relatedness with RabGAP/TBC domain containing protein and SBP-domain protein 4 (SBP, SPL2), *OsNOX2* is coexpressed with serine/threonine-protein kinase PBS1, and *OsNOX3* has correlation with proteins which are similar to quinone-oxidoreductase QR1, HvPIP2;1 protein (PIP2;2) and a Nodulin-like domain containing protein. *OsNOX4*, *OsNOX5*, *OsNOX6*, *OsNOX7*, *OsNOX8*, and *OsNOX9* were found having relatedness with genes which encode an flavin-containing monooxygenase FMO family protein, cellulose synthase-8 (CESA6), metacaspase1, an protein



**Fig. 8.**—Expression profiles analysis of coexpression genes with *OsNOXs* by qRT-PCR under MeJA hormone treatment on the shoot of rice seedling. Data are means  $\pm$  SD ( $n = 3$ ) and are representative of similar results from three independent experiments. Abbreviations and locus names of genes used are available in [supplementary table S2, Supplementary Material](#) online.

kinase domain containing protein, *TRANSPARENT TESTA GLABRA 1* protein (TTG1 protein), and an AP2 domain containing protein RAP2.2 (AP2-EREBP) and cinnamate 4-hydroxylase CYP73, respectively ([supplementary fig. S9B and table S2, Supplementary Material](#) online).

For verifying the results of coexpression analysis, we choose some interesting genes to examine their expression profiles under MeJA treatment by qRT-PCR method. As can be seen, the selected genes exhibit very well coexpression patterns with their linked *OsNOX* genes (fig. 8). Among the examined eight genes, the transcripts of seven are up-regulated by MeJA treatment and also for their predicted coexpression genes. Although the transcripts of *OsNOX4* shows no match with those of its two predicted coexpression genes in this experiment (fig. 8), the coexpression could also be observed since its transcription is greatly upregulated in roots under MeJA treatment (fig. 7H).

## Discussion

### Expansion, Diversity, and Evolution of the FROs and NOXs in Plants

Gene duplication is a common phenomenon in eukaryotes and contributes to biological diversity during evolution (Van de Peer et al. 2009; Magadum et al. 2013). The FROs and NOXs are represented by at least one gene in land plants. Although some previous studies suggested that FRO proteins are the ancestral NOXs (Bedard et al. 2007; Wong et al. 2007), but the evolution history did not well examined, especially that in Plantae. In this study, we found that the three ancestors (cyanophytes, phodophytes, and chlorophytes) of Plantae,

contain very few FRO homologs and many species have zero or only one FROs per genome and no NOX homologs were found in these species (fig. 1). Based on their domain composition and gene structure characters of the two family proteins (figs. 1 and 2; [supplementary fig. S1, Supplementary Material](#) online), FROs in Plantae should be truly considered to be ancient forms of NOXs. A single gene duplication might lead to the expansion of the FRO and NOX gene families in plants, which occurred during the divergence of ancestral cyanophytes from Plantae. The scattered distribution of NOX family genes on chromosomes ([supplementary fig. S5, Supplementary Material](#) online) and the 12 paralogous NOX gene pairs found in seven land plants ([supplementary fig. S6, Supplementary Material](#) online) suggest that segmental duplications may have been involved in the expansion of the NOX gene family and caused differences in the number of NOX genes within subfamilies and species of land plants.

Gene fusion and exon shuffling after gene duplication are mechanisms that can enhance the functional divergence of duplicated genes by creating additional domains or rearranging the original functional domains (Morgante et al. 2005; Kaessmann 2010). In this study, phylogenetic analysis, together with the domain organizations and gene structures of FRO and NOX family genes, showed distinct evolutionary differences among subfamilies (figs. 2 and 4; [supplementary figs. S2 and S3, Supplementary Material](#) online). As suggested in our proposed model for FRO and NOX gene family evolution in plants (fig. 4B), there are two distinct evolutionary ways for the families. One leads to the emergence of fungi and animal FROs and another leads to evolution of plant FROs and NOXs. FRO III subfamily proteins, also named as FREs,

are more ancient forms of FROs, which were only identified in fungi. FRO II subfamily proteins are dominantly identified in algae and most land plants. Based on domain characteristics, FRO I subfamily proteins might be considered to be ancient NOXs, which were mainly identified in *C. reinhardtii* and *H. sapiens* (fig. 2). Apparently, the ancestral FRO I protein gained a NADPH\_Ox domain is an important event for the evolution of both plant and animal NOX proteins. Therefore, it is reasonable to conclude that the gene fusion and duplication might contribute to the expansion and evolution of the two family genes in plants. All FRO family members originated from a common ancestor, which contained only Ferric\_reduct domain and existed in all living organisms from prokaryotic bacteria to eukaryotic angiosperms. Then at least two gene fusion events occurred to yield the FROs of rhodophytes and green algae by obtained the FAD\_binding\_8 and a NAD\_binding\_6 domains. And then at least one gene duplication occurred to yield three FRO genes (one FRO II and two FRO I) in green algae and one FRO II gene duplication bring 2–9 FRO II proteins in land plants (figs. 1, 2, and 4B). Finally, another gene fusion event occurred in red algae (contains one FRO I protein) or green algae (contains two FRO I proteins) to yield plant NOXs and then one or two gene duplication bring four NOX genes in bryophytes (figs. 1 and 4B).

Gene structural diversity within gene families is another evolutionary mechanism that promotes variability, and intron loss or gain is important in generating structural diversity and complexity (Zhang and Kishino 2004; Li et al. 2009). Analyzing the exon/intron structures of FRO and NOX genes (fig. 3; [supplementary fig. S4, Supplementary Material](#) online), we found that the numbers of introns and intron phases among subfamily members are remarkably conserved, whereas the intron arrangement and intron phases are strikingly distinct between subfamilies. Further analysis of the exon/intron organization of orthologous and paralogous genes in land plants within the NOX family (fig. 4A) suggests that single intron loss and gain likely occurred and led to the diversification of gene structure. This consequently contributes to the functional diversity and divergence of the FRO and NOX family proteins during the evolution process in plants. This evolutionary mechanism was also found in our previous study (Li et al. 2014).

The NADPH\_Ox domain only occurred in land plants, implying this domain might specifically contribute to adaptation of the plants to terrestrial habitats. The increasing literatures have showed that NOXs are mainly responsible for the stress tolerance and development by producing ROS (Kaya et al. 2014; Wang et al. 2015) whereas FROs are mainly responsible for iron uptake and iron homeostasis in higher plants (Gross et al. 2003; Sperotto et al. 2010). However, the most homologs of FROs in fungi show dual functions of both ROS-generating and metal ion-reducing (Aguirre et al. 2005). Previously, several NOX homologs were identified from green algae and red algae but considering their lack of NADPH\_Ox domain in amino acid sequences (Hervé et al.

2006; Anderson et al. 2011), they therefore, should be assigned to FROs as suggested in our current study (figs. 1, 2, and 4). These algae FROs seem only functioning in ROS production but not iron homeostasis (Hervé et al. 2006; Anderson et al. 2011). These results clearly show the functional divergence of FROs and NOXs in Plantae during their long-term evolution history.

### Plant NOXs Respond to Abiotic Stresses and Hormone Treatments

Many studies have shown that ROS production and NOX activity were stimulated in plants under various environmental stress conditions, such as cold, heat, drought, salt, oxidative stress, and hormone treatments. In *Arabidopsis*, AtRBOHD and AtRBOHF were found participating in the ROS-involved response of the plants to pathogen attacks (Torres et al. 2002) and the ABA-mediated regulation of stomatal closure (Kwak et al. 2003). In rice, OsRbohB (OsNOX1) was reported to have a ROS-producing activity (TakahaShi et al. 2012) and using suspension cells, Yoshie et al. (2005) found that the two others, OsRbohA (OsNOX2) and OsRbohE (OsNOX3), participated in ROS-dependent immune responses. Recently, we examined the expression profiles of nine NOX and two FRO genes in rice under various environmental conditions and found unique stress response characteristics, indicating their related but distinct functions in response to different environmental stresses including drought, heat and salt (Wang et al. 2013). Among these NOX genes, OsRbohA (OsNOX2) was proved to be essential to rice normal developmental regulation and drought stress tolerance (Wang et al. 2015).

Although many studies have found that NOX family genes functionally in variety abiotic stresses, there has been no systematic studies of the functions of NOX genes response to various abiotic stresses. According to identify potential *cis*-elements in the promoter regions of *Arabidopsis* and rice NOXs, we found that the promoter regions of NOXs contain multiple response elements for various abiotic stresses ([supplementary tables S4 and S5, Supplementary Material](#) online). Further exploration of the expression profiles of NOXs in *Arabidopsis* using silico data and qRT-PCR results demonstrated that AtNOXs belong to different subfamilies might possess different expression profiles under abiotic stresses, although the results obtained from the two experiment ways were somewhat inconsistent (fig. 6; [supplementary fig. S7, Supplementary Material](#) online). For example, the two Sub I genes, *AtRBOHB* and *AtRBOHD*, are strongly upregulated by hypoxia, MV, and *AtRBOHD* is also upregulated by drought and osmotic stresses ([supplementary fig. S7A, Supplementary Material](#) online). Considering its strongly expression in various tissues and organs (fig. 5A; Suzuki et al. 2011), these results suggest a putative housekeeping role of this gene in various plant developmental progresses. However, the genes in Sub. II (*AtRBOHE*, *AtRBOHF*, and *AtRBOHI*) exhibit almost totally

opposite expression profiles as the Sub. I genes have according to the silico analysis (supplementary fig. S7B, Supplementary Material online). The qRT-PCR data also show somewhat differences in expression profiles between the two subfamily genes, especially under PEG (fig. 6). It was reported that AtRBOHF plays a crucial role in the interplay between intracellular oxidative stress and pathogenesis responses (Chaouch et al. 2012). In contrast, the Sub. III genes (*AtRBOHH* and *AtRBOHJ*) have no much changes in expression under most abiotic stresses and hormone treatments (fig. 6; supplementary fig. S7, Supplementary Material online) but they were found to be specifically expressed in stamens and pollen (Sagi and Fluhr 2006) and play crucial roles in pollen tube tip growth via Ca<sup>2+</sup>-activated ROS production (Kaya et al. 2014, 2015). The rice OsNOX genes also show great differential expression under various stresses and hormone treatments but their expression profiles exhibit no markedly distinct orderliness between the subfamilies (fig. 7; supplementary fig. S8, Supplementary Material online), implying their more complicated regulatory mechanisms and functions during the response to environmental stresses. Apparently, each NOX gene in rice has its unique response characteristics to different stresses but complicated cross-talks existing among the members of the gene family. OsNOX1 (OsRbohB) was found to modulate cytosolic Ca<sup>2+</sup> concentration by directly interaction with OsRac1, whereas OsNOX2 (OsRbohA) and OsNOX3 (OsRbohE) were proved to participate in the plant immune response and/or drought tolerance (Yoshie et al. 2005; Wong et al. 2007; Wang et al. 2015). All the functions of these NOXs were found to be related their activity in ROS production (Yoshie et al. 2005; Wong et al. 2007; Takahashi et al. 2012; Wang et al. 2015).

Hormone response elements were also found in the promoter regions of *Arabidopsis* and rice NOXs (supplementary tables S4 and S5, Supplementary Material online). *AtRBOHB* and *AtRBOHD*, are strongly upregulated by MeJA treatments but markedly downregulated by ABA, Zaelin, and IAA treatments (supplementary fig. S7A, Supplementary Material online), *AtRBOHE*, *AtRBOHF*, and *AtRBOHI* exhibit different expression profiles under ABA and IAA treatments (fig. 6 and supplementary fig. S7B, Supplementary Material online). In rice, almost all the rice NOX genes exhibit great upregulation under oxidative stress (MV) and the hormone (ABA, SA, and MeJA) treatments (fig. 7), indicating that their functions in stress responses might be involved in hormone signaling since ROS are important components of the cross-talk among ABA, JA, and SA signaling pathways during plant stress responses (Atkinson and Urwin 2012). It has been reported that the cross-talk between NOXs (mainly OsNOX1 and OsNOX3), H<sub>2</sub>O<sub>2</sub> and MAPK in ABA signaling plays crucial roles in the functioning of a novel C2H2-type zinc finger protein, ZFP36, in rice stress tolerance (Zhang et al. 2014). However, as reported recently, OsNOX2 might function with an ABA-independent defense pathway and mediate very complicated

signaling pathways might be involved in rice normal growth processes and drought stress response (Wang et al. 2015). All these results suggest the crucial roles of NOXs in the plant defense system.

### Coexpression Analysis of NOXs in *Arabidopsis* and Rice Reflects Their Multiple Functions Both in Normal Growth Regulation and Stress Response

As the key producers of ROS, NOXs play a crucial role in the development of plants, particularly in tip growing systems (Kaur et al. 2014). It was reported that loss function of AtRBOHF or AtRBOHD/F reduced the plant size and root length whereas knockout of AtRBOHC suppressed the root hair elongation (Torres et al. 2002; Foreman et al. 2003; Kwak et al. 2003). In addition, AtRBOHD was found participating in endosperm development (Penfield et al. 2006) and be closely related with vesicle cycle (Hao et al. 2014), AtRBOHB involving in seed after-ripening (Müller et al. 2009) whereas AtRBOHH and AtRBOHJ functioning in pollen tube tip growth (Kaya et al. 2014). In *N. tabacum*, Potocký et al. (2007) found ROS-generated from NOX in polarized growth of pollen tubes and lipid microdomain polarization was found playing a role in pollen tube tip growth in *Picea meyeri* (Liu et al. 2009). Kaur et al. (2014) conclude that the NOX may associate with polarized lipid microdomain in targeting apical plasma membrane region. MtrBOHA from *M. truncatula* and PvRBOHB from *P. vulgaris* were reported to be involved in plant-rhizobial symbiosis (Marino et al. 2011; Montiel et al. 2012). ROS production is also involved in various stages of seed, seedling development, and blue light responses of seedlings in plants (Kaur et al. 2014). Our coexpression analysis shows that both AtNOXs and OsNOXs are significantly correlated with many genes which are involved in metabolism and development progresses in plants (supplementary table S2 and fig. S9, Supplementary Material online). Such like, *AtRBOHC* was observed relatedness to peroxidase superfamily protein (supplementary table S2, Supplementary Material online), and which are involved in biosynthesis of secondary metabolism, phenylpropanoid biosynthesis, and plant-pathogen interaction (supplementary table S2, Supplementary Material online). In rice, *OsNOX1* shows a significantly correlated expression profiles with RabGAP/TBC domain containing protein and a protein similar to SBP-domain protein 4, implying it might participate in endocytic trafficking process and floral development since the RabGAP/TBC Armus integrates autophagy with signaling and endocytic trafficking by coordinates Rac1 and Rab7 functions (Carroll et al. 2013) and many SBP-box genes highly expressed in inflorescence apex, floral meristem, leaf, and floral primordial (Birkenbihl et al. 2005). In addition, one of the largest SBP-box genes, *SPL14*, was characterized as conferring resistance to the PCD-inducing fungal toxin fumonisin B1 (FB1) (Stone et al. 2005). *OsNOX2* was found to be coexpressed with a serine/threonine-protein



kinase PBS1 (supplementary table S2, Supplementary Material online). PBS1 and Pto were found to fulfill different functions in the recognition of pathogen avirulence proteins in *Arabidopsis* (Swiderski and Innes 2001). *OsNOX8* has a correlation with TTG1 protein (supplementary table S2, Supplementary Material online) which was found to regulate several developmental and biochemical pathways such as trichome differentiation and anthocyanin biosynthesis in *Arabidopsis* (Zhang et al. 2003).

As mentioned above, plant NOXs are mainly ROS generated enzymes, and ROS in turn mediate various abiotic responses and biotic interactions (Kaur et al. 2014). In early reports, various abiotic stresses and the role of phytohormones such as ABA, JA, brassinosteroid (BR), auxins, and SA in plants have achieved important progress. *AtRBOHD* and *AtRBOHF* are involved in pathogenic response (Torres et al. 2002) and *AtRBOHD* is upregulated by auxin (Peer et al. 2013) and responsible for negative regulation of cell death by triggering SA accumulation under *Botrytis cinerea* treatment when it was overexpressed (Torres and Dang 2005). A recent study found that *AtSRC2* can regulate the activation of *AtRBOHF* under cold treatment (Kawarazaki et al. 2013). Furthermore, ROS production was decreased in maize apoplast under salinity stress, suggesting that NaCl play a negative effect on NOX activity (Rodriguez et al. 2007). Comparing the role of NOXs (*RbohD* and *RbohF*) and peroxidases (*Prx103*, *Prx107*, *Prx112*, and *Prx118*) in *Triticum aestivum* which were infected by *Puccinia triticina*, Dmochowska-Boguta et al. (2012) concluded that NOXs are more complex and might be playing diverse roles in different kinds of interactions in dicots and monocots. In our result, *AtRBOHA* is coexpressed with a bZIP transcription factor family protein TGA10 (supplementary table S2, Supplementary Material online), TGA transcription factors have been identified overlapping roles in plant disease resistance and stress responses (Despres et al. 2003; Mueller et al. 2008). *AtRBOHB* shows a relatedness with C2H2-type zinc finger protein which has found playing a role in rice stress tolerance (Zhang et al. 2014). *AtRBOHD* shows a correlation with lipoxygenase protein LOX4, which play a role in plant-pathogen reaction, while researches have shown that *OsRBOHE* (*OsNOX3*) is involved in the regulation of defense-related genes *EI2* and *LOX* (Yoshie et al. 2005). It was also reported that LOX3 and LOX4 interfere with distinct metabolic and that LOX4 plays a major role in controlling plant defence against nematode infection (Ozalvo et al. 2014). *OsNOX3* also shows a correlation with a protein similar to Quinone-oxidoreductase QR1 (supplementary table S2, Supplementary Material online). QR1 is one of the earliest genes on the haustorium signal transduction pathway and is necessary for the redox bioactivation of haustorial inducing factors (Bandaranayake et al. 2010). *OsNOX6* exhibits to be highly coexpressed with a protein similar to Metacaspase 1 (supplementary table S2, Supplementary Material online), implying it might be involved in plant hypersensitive response

and cell death. Metacaspase proteins belong to caspase-like proteins which may contain zinc finger motifs resembling those in the plant hypersensitive response and cell death protein *Lsd-1* (Uren et al. 2000). *OsNOX9* has a correlation with proteins similar to AP2 domain containing protein RAP2.2 (AP2-EREBP) and two other AP2-EREBP proteins (supplementary table S2, Supplementary Material online), indicating its potential functions in redox balance and stress tolerance of the plant since AP2/EREBP transcription factors actively participate in hormone and redox signalings and gene regulatory networks under abiotic stresses (Shaikhali et al. 2008; Karim et al. 2009). *OsNOX9* is also highly coexpressed with cinnamate 4-hydroxylase CYP73. In tobacco, a class II cinnamate 4-hydroxylase, CYP73A15, was found participating in the production of lignin (Blee et al. 2000).

All these results suggest that NOXs in plants play a complicated role in many metabolic and resistance progresses. Different NOXs can be involved in similar stimuli but also show a complex and cross-talk signaling networks in response to a number of environmental stresses. However, the function of each NOX and the molecular mechanism to resistance are still unclear, and the interactions among NOXs are still barely reported. Further studies will be committed to explore the functions of single or multiple NOX genes in plants.

## Supplementary Material

Supplementary figures S1–S9 and tables S1–S5 are available at *Genome Biology and Evolution* online (<http://www.gbe.oxfordjournals.org/>).

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