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Systematic analysis of leucine-rich repeat disease resistance genes in maize

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Abstract

Leucine-rich repeat disease resistance (LRRDR) genes are important for defending plants from a range of pathogens. However, little information has been reported on the systematic analysis of LRRDR genes in maize. In this study, 235 LRRDR genes were identified in the complete genome sequence of maize (*Zea mays* cv. B73), classified as six different structural types, and then characterized based on conserved protein motifs, chromosomal locations and gene duplications. Subsequent phylogenetic comparisons indicated that ~20 pairs of maize LRRDR proteins possessed high similarities to LRRDR proteins with known functions. Analyses of the physical locations and duplications of LRRDR genes indicated that gene duplication events involving LRRDR genes were high in maize and 84% occurred between chromosomes, which may ensure the functional performance and enhancement of maize LRRDR genes. Meanwhile, the functions and expression patterns of the LRRDR genes were associated with their conserved protein secondary structures, suggesting that different conserved domains might distinguish their biological functions. Transcripts of 13 genes were regulated by two or more fungal pathogens, respectively, indicating that one LRRDR gene might mediate resistance to multiple fungal pathogens, suggesting that the signal networks of the maize-fungal pathogen interactions were partially crossed. Additionally, we screened five candidate LRRDR genes for ear rot resistance. The results reported in this study contribute to an improved understanding of the LRRDR gene family in maize.

Keywords: maize, leucine-rich repeat, disease resistance genes, genome, expression pattern

Introduction

One of the six prolific types of protein repeats, the leucine-rich repeat (LRR), is a widespread structural motif (Andrade et al, 2001). LRRs are protein interaction modules (Kajava, 1998; Kobe and Deisenhofer, 1995), and the LRR domain adopts a slender conformation that maximizes surface area, which is ideal for mediating protein-protein interactions (Bella et al, 2008; Padmanabhan et al, 2009). LRR proteins have been identified in thousands of protein sequences in all life forms, from viruses to eukaryotes (Bella et al, 2008). Proteins containing tandem repeats of two of more LRRs (LRR proteins) form the continuously expanding LRR superfamily (Buchanan and Gay, 1996). These LRR proteins possess diverse functions, such as extracellular matrix assembly (Hocking et al, 1998), cell adhesion and signaling (Hohenester et al, 2006; Kresse and Schonherr, 2001), neuronal development (Chen et al, 2006), adhesion and invasion of pathogenic bacteria to host cells (Bierne et al, 2007; Niemann et al, 2004), disease resistance response, and pathogen recognition in plants (Gay and Gangloff, 2007; Martinon and Tschopp, 2005; West et al, 2006). Thus, LRR proteins are important throughout the plant lifecycle.

In disease resistance, a recurrent theme shows the LRR proteins taking center stage (Jones and Jones, 1997; Padmanabhan et al, 2009). The innate immune system of both plants and animals uses immune receptors to detect pathogens and trigger defense responses (Padmanabhan et al, 2009). Despite having distinct evolutionary origins, just like animal immune receptors, most plant receptors contain LRR domains (Ausubel, 2005), such as FLS2 (Gomez-Gomez and Boller, 2000), EFR (Zipfel et al, 2006), or Xa21 (Song et al, 1995). Plant Pathogen Recognition Receptors (PRRs) resemble mammalian Toll-like receptors with both using extracellular LRRs to perceive mitogen-associated protein kinases and intracellular serine threonine kinases to activate downstream signaling (Palsson-McDermott and O'Neill, 2007). The LRR module serves as a highly adaptable structural platform into which diverse binding specificities can be incorporated, and it appears that plant nucleotide binding-leucine rich repeats (NB-LRRs) have put the LRR domain to good use (Padmanabhan et al, 2009). From using it as a recognition motif, to regulating protein activation and signal transduction, the LRR domain is an indispensable player in plant defense (Bella et al, 2008; Padmanabhan et al, 2009; Palsson-

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McDermott and O'Neill, 2007). Leucine-rich repeat receptor kinases are members of LRR superfamily in plant (Torii, 2004), and they regulate a wide variety of defense-related processes, including host-specific, as well as non-host-specific, defense responses, wounding responses and symbiosis (Bishop and Koncz, 2002; Gomez-Gomez and Boller, 2002; Jones and Jones, 1997; Kistner and Parniske, 2002; Torii, 2004). Therefore, analyzing the characteristics and organic expression patterns of LRR proteins is necessary for studying their biological functions, especially in disease resistance.

Maize (*Zea mays* L) is an important crop grown worldwide (Xiang et al, 2010a) that is environmentally adaptable (Reif et al, 2006; Yang et al, 2008). In this study, LRRDR proteins were identified from the complete maize genome. The identified LRRDR proteins were further characterized by their structural diversity, chromosomal distributions and gene duplications. Meanwhile, the normal expression patterns of LRRDR genes and their expression patterns after fungal inoculation were investigated using microarray databases. This study will provide a starting point for further experimental investigations.

Materials and Methods

Identification and classification of LRRDR proteins

The maize (*Zea mays* cv. B73) genome sequence was obtained from the MaizeGDB (http://www.maizegdb.org/; Lawrence et al, 2004) and NCBI (http://www.ncbi.nlm.nih.gov/guide/) databases. Plant LRRDR proteins are classified into four types based on protein secondary structure (Pan et al, 2000), Xa21 (Song et al, 1995), Cf-9 (Jones et al, 1994), RPP1 (Botella et al, 1998), and RPS2 (Mindrinos et al, 1994). The B73 reference genome used is assembly B73 RefGen_v2. The input parameters were as follows: E-value cutoff was $1e^{-4}$, and max hits were 500. The LRR domains of the four protein types were run as query using the BLASTP algorithm against the reference genomes. These obtained amino acid sequences were subsequently run as query using the BLASTP algorithm against the NCBI database. The amino acid sequences with 100% identity were downloaded, as were their GenBank and MaizeGDB IDs.

The SMART protein motif analysis (http://smart.embl-heidelberg.de/; Letunic et al, 2012; Schultz et al, 1998) and the NCBI putative conserved domains (http://www.ncbi.nlm.nih.gov/guide/) were used to determine whether the corresponding candidate LRRDR proteins encoded LRR motifs. Clustering of the LRRDR proteins was carried out using ClustalW, followed by the Neighbor-joining method in MEGA6 (Tamura et al, 2013). The operating parameters for the Neighbor-joining method was 1,000 bootstrap replications. The substitution model was the Poisson Model and pairwise deletion was used as the gap/missing data treatment. The ClustalW references proteins were the default.

Chromosomal locations of LRRDR genes and gene duplications

All LRRDR gene starting positions were confirmed based on the MaizeGDB database (http://www.maizegdb.org/; Andorf et al, 2010). MapChart 2.2 was subsequently used to graphically portray maize LRRDR genes.

Gene-duplication events involving LRRDR genes were also examined. The definition of gene duplication refers to that in the modified method of Cheng et al (2012). We defined gene duplications if the aligned proteins had an identities > 70%. LRRDR proteins

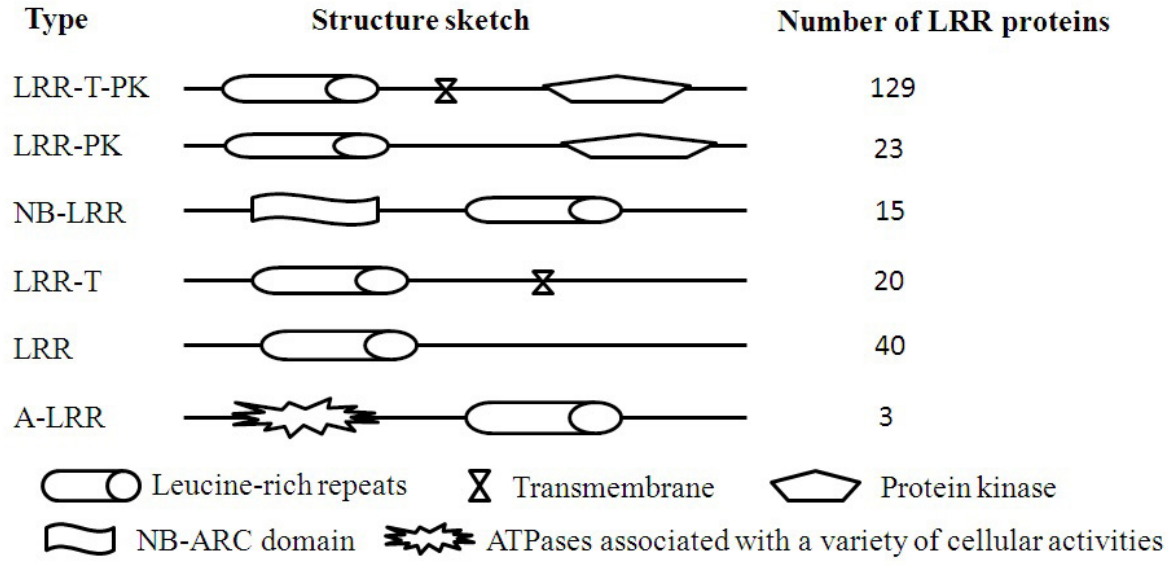

Figure 1 - Six structural types of leucine-rich repeat (LRR) proteins. The details include type, structure sketch and number of LRR proteins.

were aligned by MEGA6 for homology calculations (Cheng et al, 2012; Tamura et al, 2013).

Gene ontology and digital expression analyses

Gene ontology (GO) was analyzed using Maize Cyc 2.2 (http://maizecyc.maizegdb.org/; Lawrence et al, 2004). The expression patterns of LRRDR genes under control conditions were investigated using Maize eFP Brower (http://bar.utoronto.ca/efp_maize/cgi-bin/efpWeb.cgi?dataSource=Sekhon_et_al; Sekhon et al, 2011; Winter et al, 2007). The expression patterns of LRRDR genes after fungal inoculation were based on the PLEXdb database (http://www.plexdb.org/plex.php?database=Corn&tmva=0|20|33|63|65|). Hierarchical clustering was performed using the Cluster V3.0 with the average linkage clustering method, and illustrated by the TreeView software (Saldanha, 2004).

Results

A total of 235 LRRDR proteins were identified within the whole maize genome (Supplementary Table 1). The amino acid lengths of the LRRDR proteins ranged from 139 to 1,693, with an average of 854. The amino acid length of most LRRDR proteins (191/235) was greater than 600. Based on the protein secondary structures, the LRRDR proteins of maize were classified into six types: LRR-Transmembrane (T)-Protein kinase (PK) (LRR-T-PK), LRR-PK, NB-LRR, LRR-T, LRR and ATPases (A)-LRR (A-LRR). Their numbers were 134, 23, 15, 20, 40, and 3, respectively. Most LRRDR proteins (57%) belonged to the type LRR-T-PK (Figure 1).

Chromosomal locations of LRRDR genes and duplication events

The 235 LRRDR genes were located across all of the chromosomes, but most LRRDR genes were found in clusters, with clusters occurring at both ends of every chromosome (Figure 2). The number of LRRDR genes distributed on chromosomes 1 to 10 was 35, 27, 31, 34, 22, 20, 20, 17, 14, and 15, respectively. The distribution of LRRDR genes belonging to the six types, except for the NB-LRR type, was random. The LRRDR genes belonging to the NB-LRR type were mainly distributed on chromosomes 1, 3,

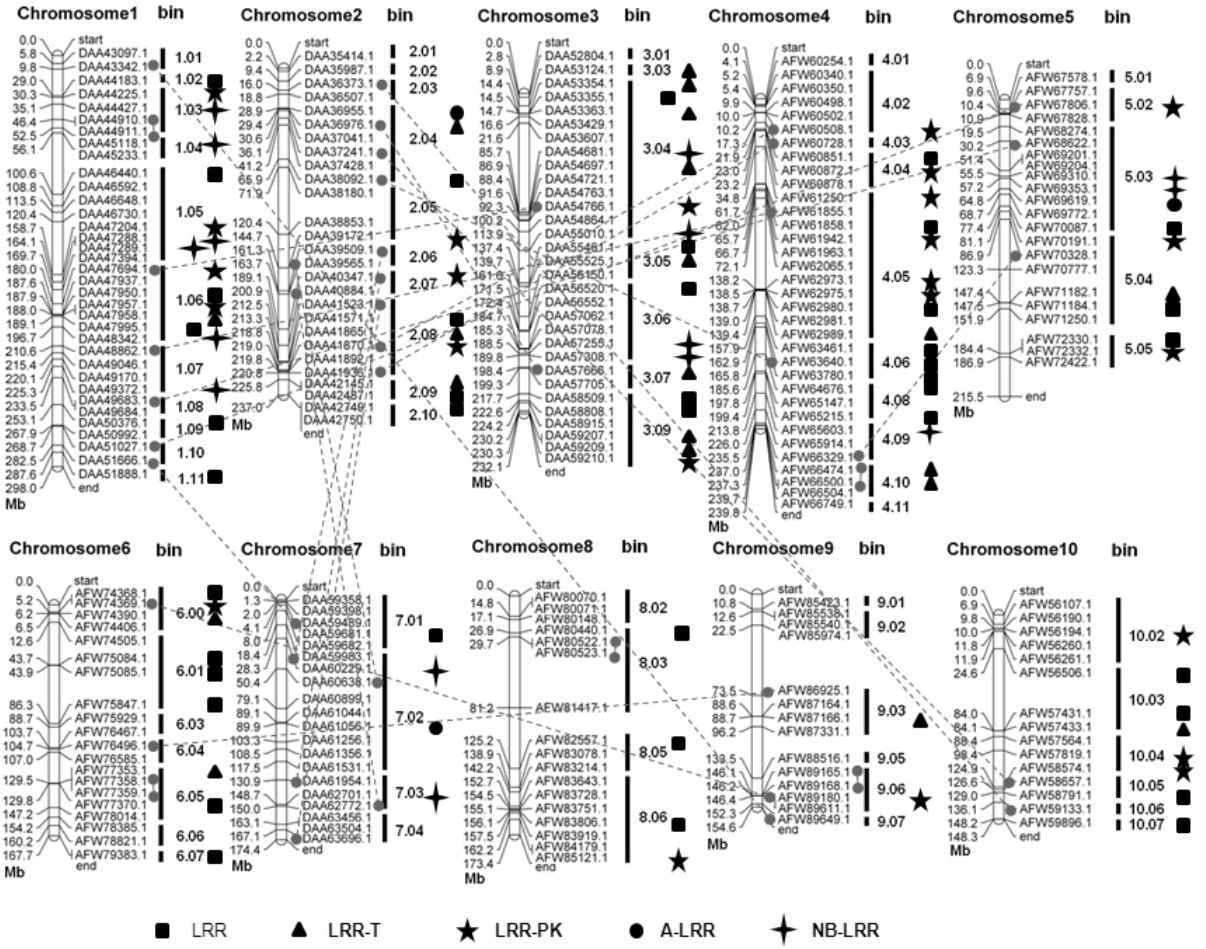

Figure 2 - Distribution of LRRDR genes on the maize chromosomes. Red dotted lines connect the LRRDR genes present on duplicated chromosomal segments. Markers beside the LRRDR genes indicate the structural type to which each protein belongs. The key of the protein types is given at the bottom. No marker beside the LRRDR gene indicates the LRR-T-PK type.

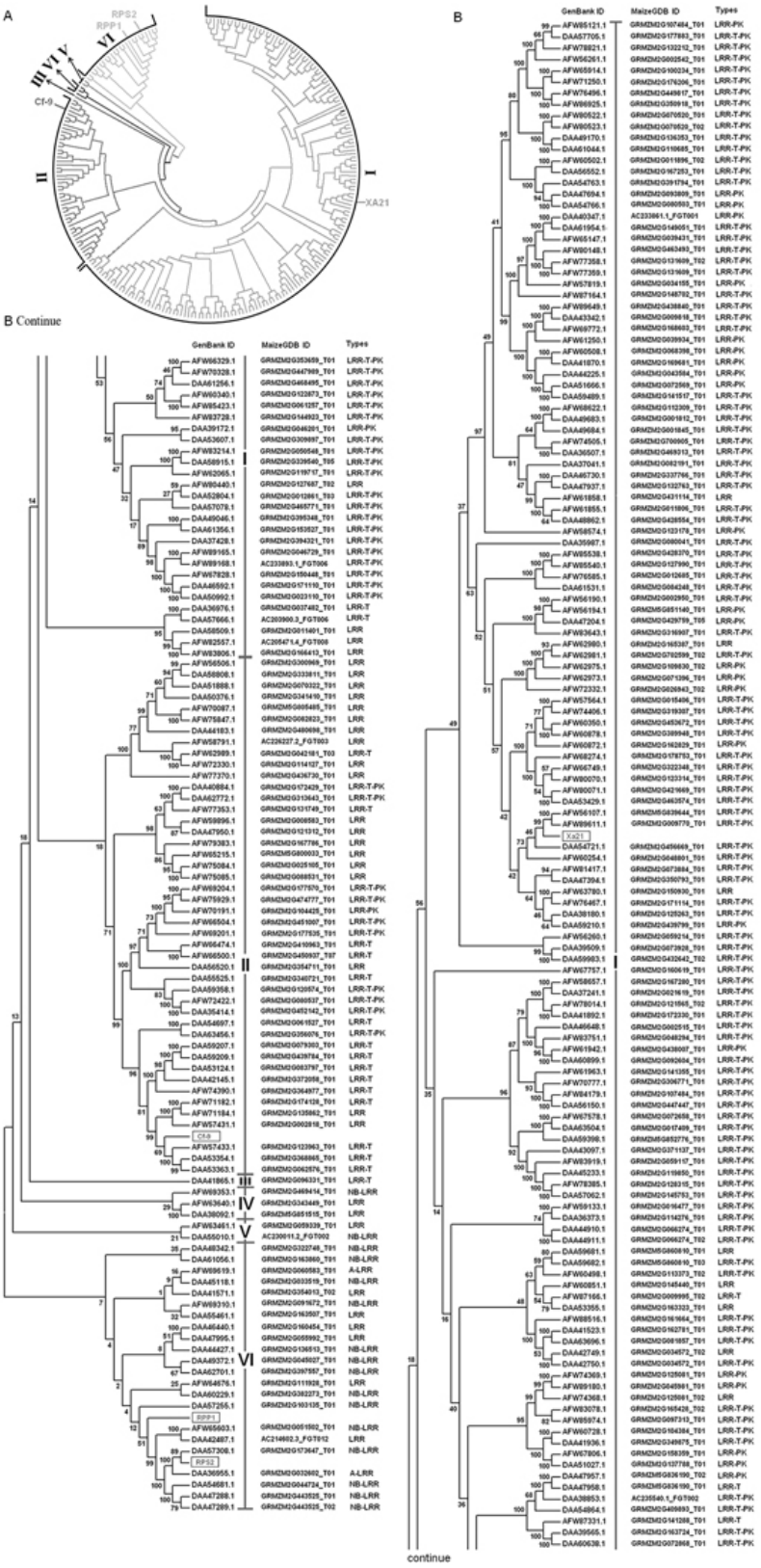

Figure 3 - Radiation chart and phylogenetic tree derived from LRRDR genes in maize. A. Radiation chart derived from LRRDR genes in maize. It was generated using the Neighbor-joining method with 1,000 bootstrap replications. A total of 235 LRRDR genes combined with 4 known plant disease resistance genes were analyzed. Different colors indicated different groups. B. Phylogenetic tree derived from LRRDR genes in maize. The information includes GenBank ID, MaizeGDB ID and type.

5, and 7, with the heaviest concentration on chromosome 1 (Figure 2).

Fifty LRRDR genes (25 pairs) with duplication events were detected in maize (Figure 2). There were 1, 2, 4, and 18 pairs belonging to the types LRR, LRR-T, LRR-PK, and LRR-T-RK, respectively, whereas there were none belonging to the types NB-LRR and A-LRR. Duplication events occurred on all chromosomes, and most of these were on chromosome 2. These duplication events were mainly present between chromosomes and not on the same chromosome. They were present between chromosomes 1 and 3, 1 and 4, 1 and 5, 1 and 7, 1 and 9, 2 and 3, 2 and 4, 2 and 7, 2 and 10, 4 and 5, and 6 and 9. There were none between the other chromosomes, suggesting that the duplication events of the LRRDR genes were nonrandom.

Phylogenetic tree of LRRDR genes

The phylogenetic relationships among LRRDR genes were inferred by constructing a combined phylogenetic tree with aligned LRRDR protein sequences. The 235 maize LRRDR protein sequences were aligned, and a phylogenetic tree was generated by the Neighbor-joining method. The phylogenetic tree constructed using maize LRRDR genes showed six groups (Figure 3). The types LRR-T-PK and LRR-PK were mainly clustered in Group I along with Xa21. The types LRR-T and LRR were mainly clustered in Group II along with Cf-9. The types NB-LRR and A-LRR were mainly clustered in Group VI along with RPP1 and RPS2. Only a few LRRDR proteins were found in Groups III, IV, and V. The above results indicated that the clustering was based on the protein secondary structures. As for the alignment of references sequences, the amino acid sequences and protein secondary structures of AFW56107.1 and AFW89611.1 had high identities (Statistical frequency > 60) with those of Xa21, whereas the amino acid sequences and protein secondary structures of AFW57433.1, DAA53354.1 and DAA53363.1 had high identities with those of Cf-9. Meanwhile, the amino acid sequences of DAA57308.1 and DAA36955.1 had high identities with that of RPS2, but the protein secondary structure of DAA57308.1 was not similar to that of RPS2. Additionally, none of the LRRDR proteins had a high identity with RPP1.

GO analysis

GOs of LRRDR proteins were closely associated with their structural types (Supplementary Table 1). The types LRR-T-PK and LRR-PK were related to protein phosphorylation (GO: 0006468). The type LRR was mainly related to protein binding (GO: 0005515). The types NBS-LRR and A-LRR were mostly related to defense response (GO: 0006952) and apoptotic process (GO: 0006915). The types LRR and LRR-T were largely related to protein binding (GO: 0005515). The results suggest that the functions of LRRDR proteins were associated with their protein secondary structures.

Expression pattern analysis of maize LRRDR genes

The expression patterns of the maize LRRDR genes were studies using a microarray database, resulting in the assignment of maize LRRDR genes to multiple groups based on their expression levels in anthers, leaves, embryos, endosperm and multiple tissues (Figure 4). No LRRDR gene was highly expressed in all of the organs. We found that most LRRDR genes in the same clustering groups possessed similar expression patterns and that most LRRDR genes within the same duplication event also possessed similar expression patterns. Meanwhile, most LRRDR genes related to defense response (GO: 0006952) also possessed similar expression patterns. The results suggest that their functions and organic expression patterns were associated with the protein secondary structures of LRRDR genes, and the duplicated LRRDR genes might have functions similar to those of the original genes.

Expression patterns of LRRDR genes after fungal pathogen inoculation

Among the LRRDR genes, only 26 genes had defined probes. Therefore, we only analyzed the expression patterns of those 26 LRRDR genes after fungal pathogen inoculation (Figure 5A). Infection with *Ustilago maydis*, *Fusarium moniliforme*, and *Colletorichum graminicola* were used for analyzing the expression patterns of the LRRDR genes. Among the 26 LRRDR genes, 21 were regulated by *U. maydis*, whereas 11 and 10 genes were regulated by *F. moniliforme* and *C. graminicola*, respectively. We inferred that the maize - *U. maydis* interaction is more complicated than the other two interactions. Additionally, we found that AFW75847.1 was induced by *U. maydis* and *F. moniliforme* and AFW78821.1 was induced by *U. maydis* and *C. graminicola*. Meanwhile, AFW82989.1 was simultaneously induced by *U. maydis*, *F. moniliforme*, and *C. graminicola*. This indicated that these maize LRRDR genes overlapped in the different maize-fungal pathogen interactions, suggesting that the signal networks of the three maize-fungal pathogens' interactions might partially cross.

Discussion

The LRRDR genes, such as FLS2 (Gomez-Gomez and Boller, 2000), EFR (Zipfel et al, 2006), Xa21 (Song et al, 1995), Cf-9 (Jones et al, 1994), RPP1 (Botella et al, 1998) and RPS2 (Mindrinos et al, 1994), have been studied extensively in plants. However, the LRRDR disease resistance gene family has not been completely described in plant species. The present study not only confirmed locational patterns for the LRRDR genes of maize, but also clarified expression patterns and elucidated features. The most recent maize genome sequence data, NCBI and MaizeGDB, were employed in the present study, which enabled us to avoid overestimating the actual number of LRRDR genes. In the maize genome, we detected 235 genes containing the LRR domain. The LRRDR proteins

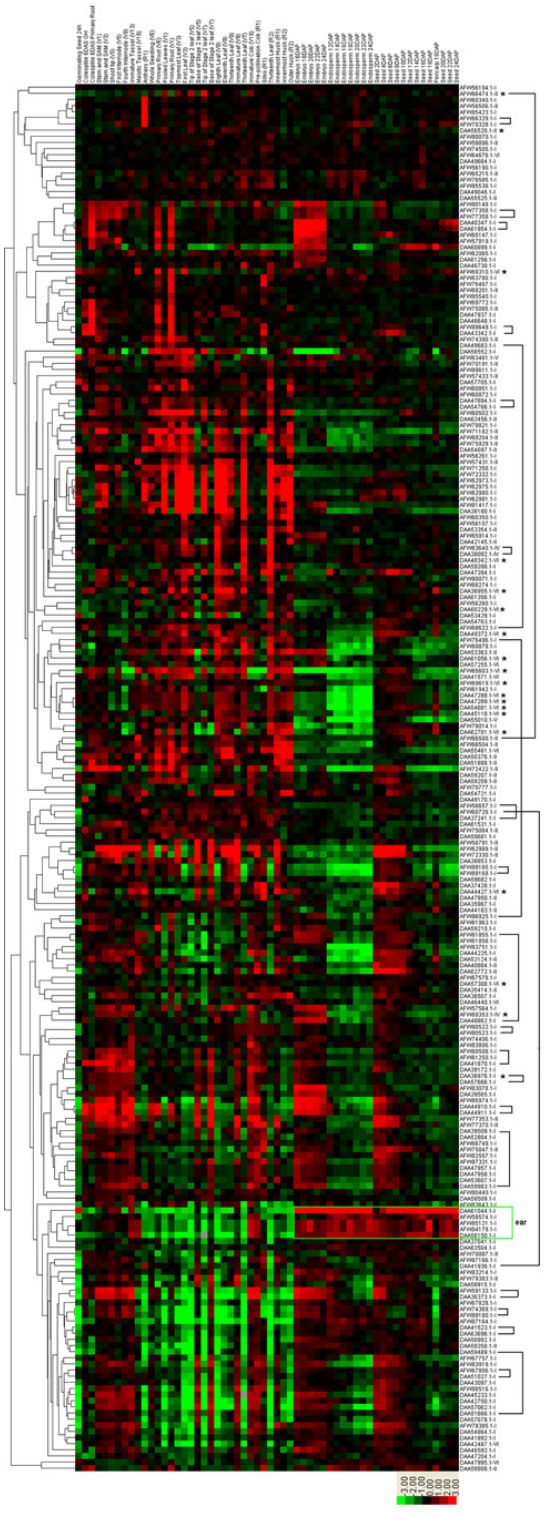

Figure 4 - Expression patterns of LRRDR genes in different tissues and organs. An asterisk beside the LRRDR genes indicates that their gene ontology (GO) terms included «defense response» (GO: 0006952). Folding lines connect the LRRDR genes present on duplicated genes. The LRRDR genes in green rectangle have ear-specific expression.

were classified into six types based on the protein secondary structure, and LRR-T-PK was found in the greatest abundance (134/235; 57%) (Figure 1).

Xa21, Cf-9, RPP1, RPS2 and Pto are references of plant disease resistance genes. Among them, only Pto's secondary structure revealed no LRR domains (Pan et al, 2000). Xa21 and RPS2 mediate bacterial disease resistance (Mindrinos et al, 1994; Song et al, 1995), whereas Cf-9 and RPP1 mediate fungal disease resistance (Botella et al, 1998; Jones et al, 1994). Queries using the amino acid sequences of the LRR domains from Xa21, Cf-9, RPP1 and RPS2, ensured the comprehensive identification of LRRDR proteins. The LRRDR proteins in Group VI lacked the PK domain (Figure 2). They were clustered with RPP1 and RPS2, possibly due to the absence of the PK domain. However, RPP1-like LRRDR proteins were not clearly distinguished from RPS-like LRRDR proteins. The LRRDR proteins belonging to the types LRR-T-PK and LRR-PK were mainly clustered into Group I, whereas those belonging to the types LRR and LRR-T were mainly clustered into Group II. Those clustering together with Xa21 in Group I might be related to bacterial disease resistance, whereas those clustering together with Cf-9 in Group II might be related to fungal disease resistance.

LRRDR proteins play important roles in disease resistance and pathogen recognition in plants (Gay and Gangloff, 2007; Martinon and Tschopp, 2005; West et al, 2006). In spite of their large numbers and significance, very few of these proteins have been functionally characterized in monocots, such as maize. In general, the orthologs clustered into a group shared similar protein architectural structures and functions (Du et al, 2012). A phylogenetic analysis of maize LRRDR proteins revealed LRRDR proteins in the same group have similar protein secondary structures. The major groups contained members of orthologous genes belonging to the types LRR-T-PK, LRR-T, and NB-LRR. The LRRDR proteins belonging to the same structural types were involved in similar GO terms. Meanwhile, by analyzing the expression patterns of the LRRDR genes, we found that LRRDR genes in the same structure-based groups had similar expression patterns. This was similar to the R2R3-MYB and chitinase gene families in maize (Du et al, 2012; Xiang et al, 2013). Although the roles of these maize LRRDR proteins remain to be elucidated, it is likely that members of a given group may have recent common evolutionary origins and also conserved functions.

LRRDR genes were distributed on all of the chromosomes of maize, and most of them clustered together. This is similar to other large gene families, such as HD-zip and R2R3-MYB (Du et al, 2012; Zhao et al, 2011). Gene duplication events occurred in ~20% of LRRDR genes. Gene duplications, including tandem and segmental duplications, play primary roles in the expansion of gene families (Leister, 2004).

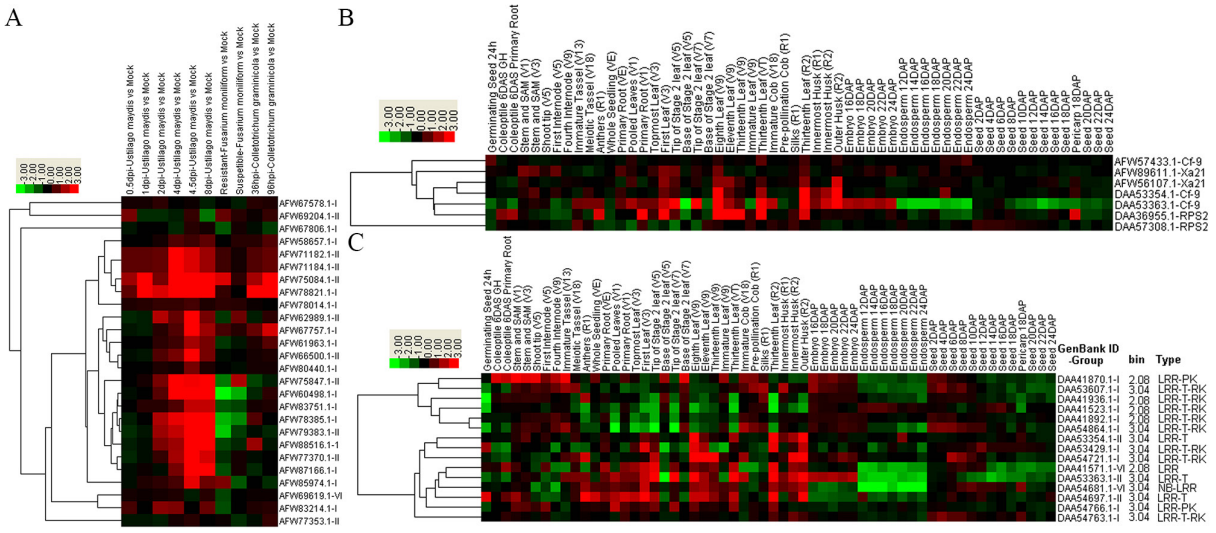

Figure 5 - Expression patterns of maize LRRDR genes. A) Expression patterns of 26 LRRDR genes after *Ustilago maydis*, *Fusarium moniliforme*, and *Colletorichum graminicola* inoculation. B) Expression patterns of maize genes homologous to Xa21, Cf-9, RPP1, and RPS2. No maize LRRDR gene was homologous to Arabidopsis RPP1. C) Expression patterns of maize LRRDR genes in «hot» chromosomal regions associated with ear rot resistance, including GenBank ID, bin and type.

Segmental and tandem duplications contribute to the expansion of the maize LRRDR gene family, which was beneficial to the coevolution of maize and pathogens, because the LRR domain is ideal for mediating protein-protein interactions (Padmanabhan et al, 2009). For example, the Arabidopsis FLS2 LRR contributes to flagellin perception (Dunning et al, 2007). Cf-9 and Xa21 are expected to be involved in specific interactions between proteins and pathogens, possibly through the solvent-exposed amino acid residues in the LRR domain (Van der Hoorn et al, 2001; Wang et al, 1998). In addition to the interactions of host LRR domains and pathogen-specific proteins, the LRR domains of LRRDR proteins from plants are able to interact with other proteins from the plant itself that mediate disease resistance, such as RPP1 (Krasileva et al, 2010) and RPS2 (Banerjee et al, 2001). Thus, LRRDR gene duplication is necessary for defending plants against diseases.

Analyzing the expression of maize LRRDR genes facilitates the identification of gene functions and future genomic functional studies of plant growth and development, and responses to stress (Cheng et al, 2012). In total, 226 out of 235 maize LRRDR genes that were supported by expression evidence exhibited distinct expression patterns in different tissues and organs. Thus, maize LRRDR genes may have temporal and spatial expression patterns that vary by tissue type or developmental stage. For example, DAA61044.1, AFW58574.1, AFW85121.1, AFW84179.1, and DAA56150.1 exhibited tissue-specific expression patterns in the ear relative to other tissues (Figure 4), which suggests that they might function only in resistance to an ear disease or ear development. Conversely, LRRDR genes are also expressed in multiple tissues and organs, which indicates that they might play important roles during plant growth and development, because the LRR protein can affect plant development (Chen et al, 2013). Furthermore, the expression data revealed that most duplicated LRRDR gene groups exhibited similar expression patterns among members, which is inconsistent with the deduction of Blanc and Wolfe (2004). The function might be related to a special protein structure of the LRRDRs.

Xa21, Cf-9, RPP1, and RPS2 mediate *Xanthomonas oryzae* pv. *oryzae*, *Cladosporium fulvum*, *Peronospora parasitica*, and *Pseudomonas syringae* disease resistance, respectively (Botella et al, 1998; Jones et al, 1994; Mindrinos et al, 1994; Song et al, 1995). The four pathogens mainly infect plant leaves. On the basis of clustering results and organic expression patterns of the LRRDR genes (Figure 5B), the maize LRRDR genes, AFW56107, DAA55354, and DAA36955, might be homologs of rice Xa21, tomato Cf-9, and Arabidopsis RPS2, respectively.

Colletotrichum spp. and *U. maydis* are in the top 10 fungal plant pathogens (Dean et al, 2012). *F. moniliforme* is also a devastating maize pathogen (Reid et al, 1999; Zhang et al, 2006). The results of the present study revealed diverse expression patterns for maize LRRDR genes exposed to these three pathogens. Relative to the control, expression levels increased for most candidate genes after *U. maydis* or *C. graminicola* infection, whereas only particular genes were induced by *F. moniliforme*, suggesting that these LRRDR genes might be necessary for the maize-fungal pathogen interaction. Meanwhile, gene expression levels were usually higher under *U. maydis* inoculation compared to *C. graminicola* or *F. moniliforme* inoculation, suggesting that the maize-*U. maydis* interaction is stronger than the maize-*F.*

moniliforme or maize-*C. graminicola* interaction. Additionally, a MetaQTL analysis showed that bins 2.08 and 3.04 are very important chromosome regions associating with ear rot resistance (Xiang et al, 2010b). There were 6 and 12 LRRDR genes in the two chromosomal regions, respectively. Based on the combination of their structural types and expression patterns (Figure 5C), DAA41523.1 on bin 2.08 and DAA54864.1, DAA53607.1, DAA54681.1, and DAA54763.1 on bin 3.04 might be candidate genes for ear rot resistance.

In conclusion, we classified and characterized the LRRDR genes in maize by analyzing their structural diversity, chromosomal distribution, duplication events and phylogenetic relationships, and investigated their expression patterns under normal environmental conditions and after fungal pathogen inoculation using microarray databases. The results reported in the present study contribute to a better understanding of the maize LRRDR gene family, and also provide a potential opportunity to select for maize disease resistance-related LRRDR genes.

Acknowledgements

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