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Letter to the Editor

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Evolution of NBS-LRR Gene Copies among Dicot Plants and its Regulation by Members of the miR482/2118 Superfamily of miRNAs

Dear Editor,

A major function encoded in plant genome sequences is devoted to defense against pests and pathogens. Among the defense-related functions in the plant genomes sequenced, there is a group of genes coding for NBS-LRR proteins that have been associated with effector-triggered immunity (Jones and Jones, 1997). The majority of genes responsible for race-specific resistance in plants belong to this family.

The composition of the NBS-LRR family in the plant genomes sequenced to date varies from 600 members in apple to 65 in papaya (Supplemental Table 1). This variation is likely to reflect an adaptation of the plant immune systems to changes in the pathogen populations in different environments: plants subject to intensive infection pressure might benefit from more variation in NBS-LRR proteins than those that are exposed to a narrow range of pests and pathogens. However, the resistance genes may impose a fitness cost on host plants (Purrington, 2000), and thus their expression needs a high degree of control, particularly in plants with many NBS-LRR protein genes.

One of the potential control levels is through miRNA modulation of NBS-LRR mRNAs. Many plants produce a superfamily of miRNAs (miR482) that are complementary to a conserved region of NBS-LRR mRNAs. In infected tissue, these miRNAs participate in a feedback control of NBS-LRR protein expression whereby the switch is provided by pathogen-derived suppressors of RNA silencing (Shivaprasad et al., 2012; Li et al., 2012). The miR482 superfamily may reduce the cost of disease resistance by down-regulating the production of NBS-LRR proteins in the absence of pathogens.

A major purpose of this study was to investigate the relationship between miR482 and NBS-LRR diversity in the genomes of different plant species. Based on previous work, we proposed that the cost of multiple NBS-LRR protein genes would be compensated by the diversity of the miR482 superfamily. However, in plants with fewer NBS-LRR protein genes, the intrinsic cost of disease resistance would be relatively low and the advantage of a diverse miR482 superfamily would be reduced. Our prediction, therefore, was that diversity of the miR482 superfamily and NBS-LRR genes would be correlated.

Our approach to investigate this point was to exploit the genome sequences of diverse dicotyledonous plants including horticultural and fruit tree species. Some of these species have large and complex genomes while others have genomes of a relatively reduced size. We focused in particular on the Cucurbitaceae and on *Prunus* species in the Rosaceae. These two families have genomes of a similar size and are phylogenetically closely related. They have not undergone major genome duplication since they diverged from a common ancestor, and there is clear collinearity within each family at two major clusters of NBS-LRR genes (see below and Figure 1). Other species have complex genomes that have often been produced by more than one round of genome duplication since divergence from a common ancestor. Collinearity of genome sequences in these other genomes has been lost as a result of sequence divergence and genome rearrangement.

NBS-LRR GENES

A feature of sequenced plant genomes is the great variation in the number of NBS-LRR genes (Marone et al., 2013). Figure 1A shows an estimation of the NBS-LRR content per diploid genome of sequenced dicot species; Supplemental Figure 1 expands it to all available annotated plant genomes and further classifies NBS-LRR genes into Coiled-Coil (CC) and TIR-domain containing proteins. The difference in NBS-LRR gene number is particularly striking between closely related Cucurbit and Prunus species. Melon (Garcia-Mas et al., 2012) and peach (Verde et al., 2013) genome assemblies can be considered good-quality genomes in terms of base accuracy, scaffold sizes, short-range contiguity, or genome coverage and, therefore, amenable to a more precise examination. Notably, there are only 104 NBS-LRR in melon, contrasting with 408 in peach, although their genomes are of relatively similar size (450 and 265 Mb, respectively), and there is no evidence for genome duplication since the divergence of these genomes from a common ancestor.

In melon the NBS-LRR genes are present in different chromosomes, but there are two large clusters in linkage groups (LG) IX and V. Variation at these two clusters, especially that in LG V (González et al., 2013), accounts for much of the variation within the Cucurbitaceae (Huang et al., 2009; Guo et al., 2013) and some of the variation between the Cucurbitaceae and Rosaceae. In the peach genome, for example, there is a significant degree of collinearity of orthologous genes in the environment containing these NBS-LRR clusters. However, there are only two NBS-LRR genes in the homologous peach region (Figure 1B). Conversely, there is a large cluster of NBS-LRR genes in the peach chromosome II, but the corresponding syntenic regions in the genomes of cucurbits completely lack NBS-LRR genes (Figure 1C). Therefore, it can be concluded that the appearance and amplification of the NBS-LRR clusters are

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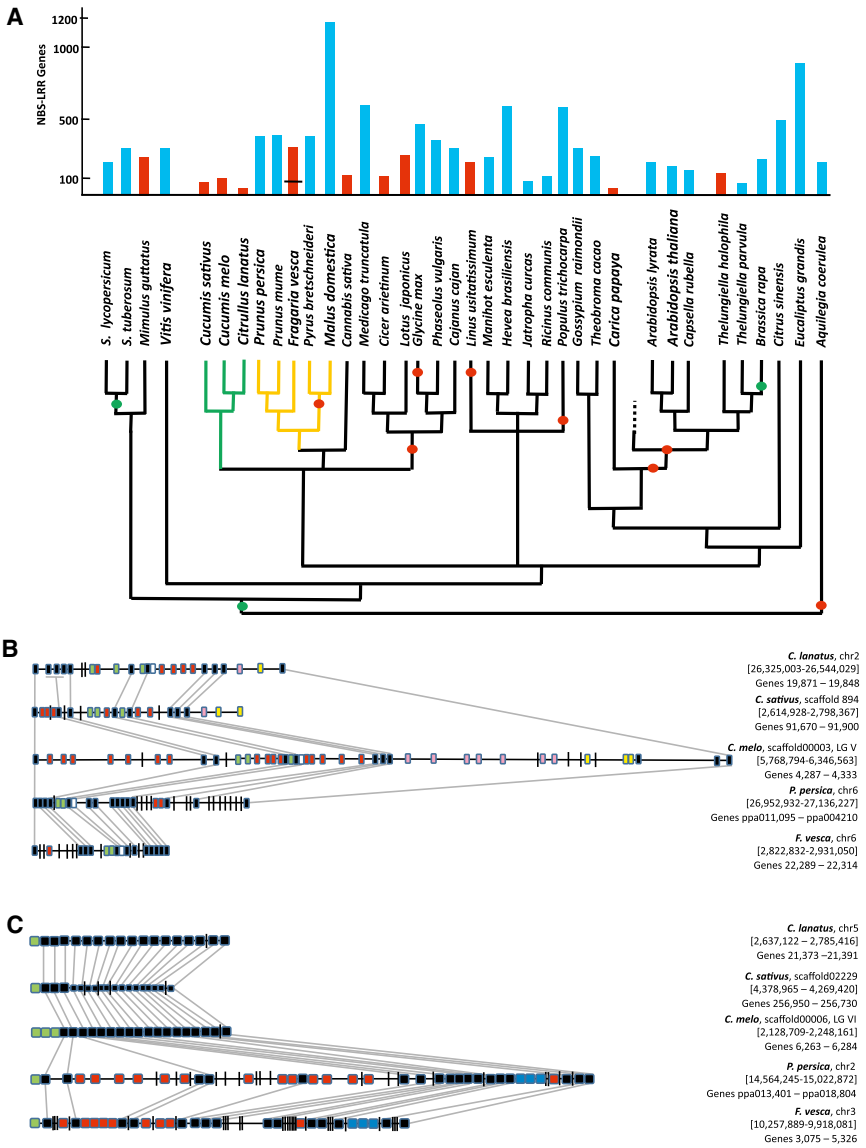

Figure 1. Variation in the Number of NBS-LRR Genes.

(A) Number of NBS-LRR genes and the presence/absence of miR482 genes in dicot plant species. Blue bars indicate that miR482 genes have been found while red bars indicate the absence of such genes. Bar lengths represent the number of NBS-LRR genes per diploid genome found in each species as described in Supplemental File 1. The horizontal black line in the *Fragaria vesca* bar marks the number of NBS-LRR reported in the bibliography. Dots in the phylogeny tree indicate whole-genome duplication events: green dots, genome tripling; red dots, genome doubling. Green and orange tree branches mark the position of, respectively, Cucurbitaceae and Rosaceae. Phylogeny tree based on an image found in http://genomevolution.org/wiki/index.php/Whole_genome_duplication.

(B and C) Overview of microsynteny between an NBS-LRR gene-rich 580 kb melon genome fragment **(B)** or an NBS-LRR gene-rich 460 kb peach genome fragment **(C)** and its homologous sequences in melon (*Cucumis melo*), cucumber (*Cucumis sativus*), watermelon (*Citrullus lanatus*), peach (*Prunus persica*), and strawberry (*Fragaria vesca*). Predicted genes with no apparent syntenic counterparts are marked by vertical bars. Syntenic genes are represented by boxes. Gray lines represent one-to-one syntenic relationships. Synteny between clusters of multi-copy genes is represented using equally colored boxes. Red boxes represent TIR-NBS-LRR genes and pink boxes, CC-NBS-LRR genes; other colors are used to mark clusters of genes other than R-genes. The source of the genome sequences is presented in Supplemental Table 1. Figure drawn to scale.

produced by very active mechanisms that occur during the process of speciation.

MIR482 SUPERFAMILY GENES

The Solanaceae miR482 superfamily has three genes in the case of tomato, and could target the P loop motif of at least 30% of the NBS-LRR mRNAs in some members of this family (Shivaprasad et al., 2012). To explore the relationship of the miR482 family with NBS-LRR gene diversity, we analyzed the genomes of several sequenced dicot plants. We searched for sequences with the potential to produce miR482 from a transcript with an extended fold-back structure like those of pre-miRNAs in plants. The results are shown in Figure 1A, where blue bars represent the presence of putative miR482 genes while those species for which we failed to detect miR482 sequences are marked with red bars. Negative results were confirmed by performing additional, less stringent searches using the mature miR482 sequences as template (see Supplemental File 1) in the case of cucurbits, strawberry, and papaya, and are consistent with published studies on the characterization of the small RNA component in several cucurbit species (Jagadeeswaran et al., 2012). In addition, a systematic target prediction was done to show that miR482 superfamily members are actually targeting NBS-LRR mRNAs of four selected plant species: *Glycine max* (as representative of legumes), *Malus domestica* (which has the higher number of NBS-LRR genes among the analyzed species), *Prunus persica* (as representative of Rosaceae), and *Vitis vinivera* (outgroup for species that have not undergone recent genome duplications). Detailed information on this subject is available in Supplemental File 2.

There is a good correlation between species with large numbers of NBS-LRR genes and those having miR482 superfamily members. This correlation is especially pronounced in cucurbits or papaya with few NBS-LRR genes and lacking miR482, and in the closely related peach and apple genomes with abundant miR482 and many NBS-LRR genes. Species with miR482 family genes have an average of 2.2 times more NBS-LRR genes than those with no miR482 genes (difference in means significant at $P < 0.05$ using a two-tailed *t*-test). Apparent exceptions might be due to particular strategies for

protection of the plants against pathogens, but in other cases the quality of the genome sequences may have precluded the detection of the miR482 or NBS-LRR genes. This may be the case for *Fragaria vesca*, in which our comparison with melon identifies 320 NBS-LRR genes but no miR482. However, the analysis of the unpublished genome of *Fragaria* × *ananassa*, another strawberry species, reveals the presence of miR2118 family genes which, due to its sequence similarity with miR482, can be considered as belonging to the miR482 superfamily (Shivaprasad et al., 2012) and, therefore, putatively involved in NBS-LRR regulation. Interestingly, all species in which miR2118 was detected also have miR482 genes, strawberry being the exception, while several species were found to have miR482 but no miR2118. A detailed representation of miR482/2118 presence/absence in sequenced plant genomes is given in Supplemental Figure 1.

To investigate the variation of miR482 superfamily, we compared the genomic sequence of the peach genomes with the syntenic regions of Cucurbitaceae. The genome of peach contains at least six miR482 genes, of which four are found in a cluster in chromosome 1. The region surrounding this cluster shows a high degree of gene synteny with a melon region in LG X. However, the 80 kb fragment containing the cluster of miR482 genes and 14 predicted protein-coding genes is absent from the melon syntenic region; in fact, no sequence showing homology with the analyzed peach fragment could be found in the melon genome (Supplemental Figure 2). This result also supports the hypothesis that the presence/absence of miR482 genes corresponds to the number of NBS-LRR genes in a species-specific manner.

In conclusion, two types of genomic variability have been observed in the genome of plants that may be the result of their adaptation to biotic stress: first, the formation of large clusters of NBS-LRR genes that are specific hot spots for genomic variability between families and between species in specific plant families; and second, the appearance of miRNA that modulates the expression of the NBS-LRR in the cases where this family of genes is highly amplified. These two factors have to be taken into account when explaining the ways plants adapt themselves to changing environments, and may provide ideas on directed mutations useful for breeding.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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Víctor M. González[1], Sebastian Müller[2], David Baulcombe[2] and Pere Puigdomènech[1,2,]*

[1]Centre for Research in Agricultural Genomics (CRAG), CSIC-IRTA-UAB-UB, Edifici CRAG, Campus UAB, 08193 Bellaterra, Barcelona, Spain
[2]Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, UK
***Correspondence: Pere Puigdomènech (pere.puigdomenech@cragenomica.es)**
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