Genomic Organization and Evolutionary Insights on *GRP* and *NCR* Genes, Two Large Nodule-Specific Gene Families in *Medicago truncatula*

Benoit Alunni, Zoltan Kevei, Miguel Redondo-Nieto, Adam Kondorosi, Peter Mergaert, and Eva Kondorosi

Institut des Sciences du Végétal, Centre National de la Recherche Scientifique UPR2355, Bat 23, Avenue de la Terrasse, 91198 Gif-sur-Yvette cedex, France

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Deciphering the mechanisms leading to symbiotic nitrogen-fixing root nodule organogenesis in legumes resulted in the identification of numerous nodule-specific genes and gene families. Among them, *NCR* and *GRP* genes encode short secreted peptides with potential antimicrobial activity. These genes appear to form large multigenic families in *Medicago truncatula* and other closely related legume species, whereas no similar genes were found in databases of *Lotus japonicus* and *Glycine max*. We analyzed the genomic organization of these genes as well as their evolutionary dynamics in the *M. truncatula* genome. A total of 108 *NCR* and 23 *GRP* genes have been mapped that were often clustered in the genome. These included 29 new *NCR* and 17 new *GRP* genes. Reverse transcription-polymerase chain reaction analyses of the novel genes confirmed their exclusive nodule-specific expression similar to the previously identified members. Protein alignments and phylogenetic analyses revealed traces of several duplication events in the history of *GRP* and *NCR* genes. Moreover, microsyntenic evidences between *M. truncatula* and *L. japonicus* validated the hypothesis that these genes are specific for the inverted repeat-lacking clade of hologalegoid legumes, which allowed dating the appearance of these two gene families during the evolution of legume plants.

Nitrogen access in natural ecosystems is one of the key limiting factors for plant growth and development. To face and overcome this problem, most legume plants developed the ability to form symbiotic nitrogen-fixing nodules in association with rhizobia. The process of nodule development requires several recognition steps by both the bacteria and the plant. Nodule organogenesis is triggered by *Rhizobium* Nod factors in the host plant. Therefore, during recent years, surveys focused on the Nod factor signaling pathway, leading to the discovery of crucial genes playing roles in Nod factor recognition and signal transduction in the two model legumes *Medicago trun-*

catula and *Lotus japonicus* (Stacey et al. 2006). Moreover, several hundreds of genes have been identified during the last decade, which were activated at different stages of symbiotic nodule development (El Yahoui et al. 2004; Fedorova et al. 2002; Györgyey et al. 2000; Kouchi et al. 2004). Among them, two nodule-specific gene families encode secreted peptides. These are glycine-rich proteins (GRP; Kevei et al. 2002) and the cysteine-rich peptides (NCR; Mergaert et al. 2003). Beside their exclusive nodule-specific expression, *GRP* and *NCR* genes have also been shown to display a wide range of spatio-temporal patterns in the infected cells throughout nodule organogenesis.

GRP are known to be involved in many biological processes, including biotic and abiotic interactions between plants and their environment (Sachetto-Martins et al. 2000). Usually, the glycine content of GRP is 80% and glycine residues are ordered in typical motifs. Nevertheless, nodule-specific GRP are different; in general, they are shorter and contain fewer glycines (20 to 30%), with no clear motif structures. The studied nodGRP in *M. sativa* and *M. truncatula* formed three groups based on the divergent glycine-rich regions. Such GRP were found in *Vicia faba*, *Trifolium repens*, and *Medicago* species (Kevei et al. 2002), all belonging to the inverted repeat-lacking clade (IRLC) of legumes. This clade of legume species was previously often referred to as galegoids or hologalegoids, but recent phylogenetic studies supported a more accurate definition of the clade as IRLC (Wojciechowski et al. 2004).

NCR genes form an exceptionally large family consisting of more than 300 genes in *M. truncatula* (Fedorova et al. 2002; Mergaert et al. 2003). *NCR* genes code for short secreted peptides with four or six cysteine residues in conserved positions along the 50 to 60 amino acids of the mature peptide. They are similar to scorpion toxins, *Conus* spp. toxins, plant and animal defensins, or determinants of self incompatibility (Mergaert et al. 2003), which also form multigenic families and mostly play roles in interactions between different organisms, in pathogenicity, or innate immunity processes (Klotman and Chang 2006; Olivera 2006; Rodriguez de la Vega and Possani 2005; Silverstein et al. 2005) but can be involved in regulation of reproduction as well (Schopfer et al. 1999; Watanabe et al. 2000). *NCR* are members of a large cysteine cluster protein (CCP) family (Graham et al. 2004). This family of cysteine-rich proteins comprises 10 subgroups, including the nodule-specific CCP (corresponding to *NCR*), the seed-specific CCP, and numerous ubiquitous CCP (harboring 4, 6, 8, or 10 conserved cysteines) (Graham et al. 2004; Silverstein et al. 2006). While *NCR* genes were found so far only in expressed sequence tag (EST) databases and genomic libraries of IRLC legumes

B. Alunni and Z. Kevei have contributed equally to this work.

Corresponding author: E. Kondorosi; E-mail: Eva.Kondorosi@isv.cnrs-gif.fr; Fax: +33(0)169823695.

Current address of M. Redondo-Nieto: Dpto. Biología. Facultad de Ciencias, Univ. Autónoma de Madrid, E-28049 Madrid, Spain.

All sequences corresponding to *NCR* and *GRP* family members mapped on the genome (mRNA and protein sequences) are submitted to GenBank and are available under accession numbers EF414284 to EF414410.

(Mergaert et al. 2003), other types of *CCP* were not specific for IRLC (e.g., *Glycine max*) and were identified also in non-legume plants, such as *Arabidopsis thaliana* (Graham et al. 2004; Silverstein et al. 2006). The large number of *NCR* genes and the significantly divergent amino-acid sequences within the family are reminiscent of other gene families, such as disease resistance (*R*) genes, which are also involved in interactions between organisms (Meyers et al. 2003).

The advanced sequencing projects of the two model legumes *M. truncatula* and *L. japonicus* allow comparative genomic studies. Though neither of them is entirely sequenced, a 54% general syntenic relation between the two plants has been shown recently (Cannon et al. 2006), and detailed microsyntenic analysis can give new insights in the evolution of larger gene families.

The aim of our study was to describe gene structure and organization of *GRP* and *NCR* families in the *M. truncatula* genome. Therefore, genomic databases were screened to identify formerly studied and new members of these gene families. Using this strategy, we mapped 23 *GRP* and 108 *NCR* genes, including, respectively, 17 and 29 novel genes and confirmed their nodule-specific expression. Many of the *GRP* and *NCR* genes were found in clusters as the consequence of numerous local duplication events. These data were supported by their phylogenetic analysis and also by the evolution study of their signal peptide and mature domain. Moreover, evidences for microsyntenic relations between *M. truncatula* and *L. japonicus* were found in the neighboring genomic regions of *NCR* and *GRP* genes, but the *NCR* and *GRP* clusters themselves were absent in the *Lotus* genome.

RESULTS

Nodule-specific *GRP* genes in *M. truncatula*.

The different protein sequences deduced from cDNAs of the *MtnodGRP* gene family (*MtnodGRP1A*, *MtnodGRP1B*, *MtnodGRP1C*, *MtnodGRP2A*, *MtnodGRP2B*, *MtnodGRP3*) (Kevei et al. 2002) were used for tBLASTn homology searches in the National Center for Biotechnology Information (NCBI) database. The searches resulted in three entirely sequenced *M. truncatula* genomic regions containing these genes. As the signal peptides were highly or well conserved in the three groups of *GRP*, these were used for tBLASTn searches to identify further potential *GRP* genes in the *M. truncatula* genome. This *nodGRP* gene hunting led to the discovery of additional members that were localized in six different genomic regions in total.

The previously described *MtnodGRP1A*, *MtnodGRP1B*, and *MtnodGRP1C* genes were on the same DNA contig, which contained, in addition, nine new *MtnodGRP1* genes

(*MtnodGRP1D* through *MtnodGRP1L*) (Fig. 1). On the other hand, the *MtnodGRP1B* mRNA turned out to be an alternatively spliced form of the *MtnodGRP1A* gene. The 11 *MtnodGRP1* genes were scattered in a 270-kb region, but eight of them were within a 70-kb distance. Six of the 11 *MtnodGRP1* genes possessed EST sequences in the database but not the *MtnodGRP1D*, *MtnodGRP1E*, *MtnodGRP1F*, *MtnodGRP1G*, or *MtnodGRP1I* genes.

The *MtnodGRP2* contig on chromosome 2 clustered four genes (*MtnodGRP2A* to *MtnodGRP2D*) that were in the same orientation (Table 1). *MtnodGRP2C* and *MtnodGRP2D* were novel genes, but EST were found only for *MtnodGRP2D*. The *MtnodGRP3* search revealed three homologous genes in the same region (*MtnodGRP3A*, *MtnodGRP3B*, *MtnodGRP3C*) and EST for *MtnodGRP3A* and *MtnodGRP3B*. The remaining *GRP*-related genes formed three new clusters located in three different genomic regions (Table 1); chromosome 2 carried two gene copies both for *MtnodGRP5* (*MtnodGRP5A* and *MtnodGRP5B*) and for *MtnodGRP6* (*MtnodGRP6A* and *MtnodGRP6B*), whereas the unique *MtnodGRP4* gene was on a region that has not yet been assigned to any chromosome of *M. truncatula* (designated as chromosome 0). EST were available for *MtnodGRP4* and *MtnodGRP5A*.

New *NCR* genes in the *M. truncatula* genome.

In the EST databases, sequences were available for 311 *NCR* genes (*NCR001* to *NCR00311*) (Mergaert et al. 2003). In order to localize these *NCR* genes in the genome, BLASTn homology searches were performed on *M. truncatula* bacterial artificial chromosome (BAC) sequences. A total of 51 different genomic regions carrying 79 of the known *NCR* genes were identified, and thus, the contigs often combined multiple *NCR* genes (Fig. 1; Table 1).

To verify the existence of further *NCR* on the *NCR*-containing BAC clones, tBLASTn searches were performed. The typical cysteine motifs and signal peptide parts helped to find neighboring *NCR* genes (Graham et al. 2004; Mergaert et al. 2003). These searches resulted in the identification of 29 new *NCR* genes (*NCR312* to *NCR340*) that were present in the vicinity of known *NCR* genes on 17 different contigs (Table 1). Of the 29 *NCR*, 19 peptides harbored the four-cysteine and nine the six-cysteine motif. Of the new *NCR* genes, 26 had no EST. There were EST for the *NCR314*, *NCR319*, and *NCR337* genes, which were, however, not annotated before as *NCR*. *NCR323*, *NCR324*, and *NCR335* corresponded to the formerly described *CCP1*, *CCP3*, and *CCP4*, respectively (Graham et al. 2004). In the whole genome, 27 *NCR* contigs contained one, nine contained two, six contained three, three contained four,

MtnodGRP1-contig (mth2-174i23, mth2-24k1, mth2-170k9) - chr5

NCR024/026/088/138/316/334-contig (mth2-12a23) - chr2

NCR053/054/056/057/317-contig (mth2-35n12) - chr1

NCR239/325/326/327/328-contig (mth2-192a14) - chr7

Fig. 1. Genomic organization of the *MtnodGRP1* contig and three *NCR* contigs in *Medicago truncatula*. The *MtnodGRP1* contig contains 11 *GRP1* genes (called *GRP1A* to *GRP1L*). The BAC clone numbers corresponding to the contigs are indicated between brackets and followed by the chromosome number. The location and orientation of genes are indicated by arrows. Genes and intergenic regions are not drawn at scale. Black arrows represent the *GRP* or *NCR* genes and their underlining indicates the absence of expressed sequence tags. Other genes are shown with white arrows.

four contained five, one contained six, and another one seven *NCR* genes, suggesting hot spots for gene duplications in certain regions (Table 1).

Nodule specificity is a common feature for all *GRP* and *NCR* genes.

In order to confirm that the newly predicted *GRP* and *NCR* genes without EST are indeed expressed genes, we tested their

transcriptional activity by reverse transcription-polymerase chain reaction (RT-PCR) and defined their expression pattern. RNA was isolated from different *M. truncatula* organs, root, mature nodule, leaf, stem, and flower, and cDNA samples were synthesized. Specific oligonucleotides were designed for each of the 10 new *MtnodGRP* genes (*MtnodGRP1D*, *MtnodGRPE*, *MtnodGRPF*, *MtnodGRPG*, *MtnodGRPI*; *MtnodGRP2C*; *MtnodGRP3C*; *MtnodGRP5B*; *MtnodGRP6A*, and

Table 1. Genomic organization of *GRP* and *NCR* clusters

Contig name ^a	Genes	Orientations in contig	ORF coding exons	BAC clones	Chromosome
MtnodGRP1A/C/D/E/G/H/I/J/K/L	11	+/+/+/+/+/+/+/+/+	2/3/2/2/2/2/2/2/2/2/2	mth2-24k1 mth2-174i23 mth2-170k9	5
MtnodGRP2A/B/C/D	4	+/+/+/+	2/2/2/2	mth2-18j19	2
MtnodGRP3A/B/C	3	+/+/+	2/4/2	mth2-8c19	2
MtnodGRP4	1	+	2	mth2-28h7	0
MtnodGRP5A/B	2	+/+	2/2	mth2-28b21	2
MtnodGRP6A/B	2	+/+	2/2	mth2-168l8	2
NCR010/111/303	3	+/+/+	2/2/2	mth2-5j8	4
NCR012	1	+	2	mth2-101h21	1
NCR019	1	+	2	mth2-167a19	5
NCR020/147	2	+/-	1/1	mth2-67a20	7
NCR021	1	+	2	mth2-59j17	7
NCR024/026/088/138/316/334	6	+/+/+/+/+/+	2/2/2/2/2/2	mth2-12a23	2
NCR028	1	+	2	mth2-62p5	7
NCR043	1	+	1	mth2-39k21	4
NCR053/054/056/057/317	5	+/+/+/+/+	2/2/2/2/2	mth2-35n12	1
NCR062/074/076/128/145/312/313	7	+/-+/+/+/+/+	2/2/2/2/2/2/2	mth2-65c4	4
NCR065	1	+	2	mth2-10p14	2
NCR068	1	+	2	mth2-175n24	6
NCR077	1	+	2	mth2-68d12	3
NCR086/136/165/301/314	5	+/+/+/+/+	2/2/2/2/2	mth2-10a20	4
NCR090/217/290/304/315	5	+/-/-/-/-	2/2/2/2/2	mth2-15h3 mth4-42c9 mte1-10p15	5
NCR093	1	+	2	mth2-101o18	0
NCR094	1	+	2	mte1-26c9	5
NCR096	1	+	2	mth2-10c8	3
NCR101/336	2	+/-	2/2	mth2-16d16	3
NCR103/221	2	+/+	2/2	mth2-7h21	3
NCR118/318/319	3	+/+/-	2/2/2	mth2-116e22	4
NCR119/323/324/335	4	+/+/+/+	1/1/1/1	mth2-24k24	4
NCR121/320	2	+/+	2/2	mth2-34p9	4
NCR122	1	+	2	mth2-63h19	2
NCR127/187	2	+/-	2/2	mth2-17e17	6
NCR129	1	+	2	mth2-11o4	4
NCR140	1	+	2	mth2-9k5	4
NCR144/266	2	+/-	2/2	mth2-34b22	4
NCR146/321/322	3	+/+/+	2/2/2	mth2-6h11	5
NCR159/172/200/329	4	+/+/+/+	2/2/2/2	mth2-7m17	5
NCR169	1	+	2	mth2-130k11	6
NCR171	1	+	2	mth2-5n2	7
NCR173	1	+	1	mth2-74f16	8
NCR175/330/331	3	+/+/+	2/2/2	mth2-15l17	2
NCR176/192	2	+/-	2/2	mth2-32m22	5
NCR181	1	+	3	mth2-16l1g1	0
NCR189	1	+	2	mth2-50j5	3
NCR201/206/332/333	4	+/+/+/+	3/3/2/2	mth2-116m2	3
NCR209	1	+	2	mth2-15e11	4
NCR224/228/310	3	+/-/+	2/2/2	mth2-31b9	4
NCR237/337	2	+/+	1/1	mth2-58d22	6
NCR239/325/326/327/328	5	+/+/+/+/+	1/1/1/1/1	mth2-20k24	7
NCR244/338	2	+/+	2/2	mth2-192a14	7
NCR263	1	+	2	mth2-28p22	5
NCR265	1	+	2	mth2-59b18	3
NCR278	1	+	2	mth2-58c3	5
NCR284	1	+	2	mth2-9f10	4
NCR286	1	+	2	mth2-59k24	3
NCR289/339/340	3	+/+/+	2/2/2	mth2-8d23	5
NCR300	1	+	2	mth2-4g23	7
NCR309	1	+	2	mth1-64n13	0
				mth2-116h6	4

^a Genes underlined possess no expressed sequence tags. The names of the contigs do not reflect their real gene order.

MtnodGRP6B) possessing no EST and for 12 new *NCR* genes without EST (*NCR312* to *NCR31318*; *NCR320* to *NCR324*) and were used for PCR experiments (Fig. 2). The *Mtc27* constitutive marker was used as control for cDNA quality. PCR products were then cloned and sequenced to distinguish the highly homologous genes and confirm their RNA splicing prediction. All investigated genes were transcribed, our splicing predictions were correct, and these genes exhibited exclusive nodule-specific expression (Fig. 2), as it was found for the previously identified *GRP* and *NCR* genes (Kevei et al. 2002; Mergaert et al. 2003).

Gene organization in *GRP* clusters.

Most *MtnodGRP* genes are composed of two exons where the first exon (approximately 80 bp) codes for the signal peptide and the second exon (between 200 and 700 bp) for the glycine-rich domain. Two exceptions were found: *MtnodGRP1C* had two introns, while the longer *MtnodGRP3A* was encoded by four exons. Intron sequences of the *GRP* genes were not homologous. In the different *GRP* contigs, all *GRP* genes were in the same orientation, except *MtnodGRP1H* and *K*, which were in opposite orientation to the others (Fig. 1).

Protein homology levels within the *GRP* gene clusters were investigated to elucidate the duplication events that occurred in the different contigs. Two examples are shown in Figure 3. In the *MtnodGRP2* contig, the homology among the four *MtnodGRP2* proteins was obvious; however, certain members were more similar to each other than to others, and divergence of the four proteins was predominant in the C-terminus (60 to 80% similarity). *MtnodGRP1A*, *MtnodGRP1D*, *MtnodGRP1E*, *MtnodGRP1F*, and *MtnodGRP1G* genes coded also for homologous proteins, but the identity was more apparent for the signal peptide domain (90% similarity). The homology and the same orientation of *MtnodGRP2* and these *MtnodGRP1* genes suggest that these genes had arisen from tandem duplication events within a restricted DNA region. In addition to the closely located *MtnodGRP1A*, *MtnodGRP1D*, *MtnodGRP1E*, *MtnodGRP1F*, and *MtnodGRP1G* genes, the *MtnodGRP1* contig contained six other *GRP* (*MtnodGRP1C*, *MtnodGRP1H*, *MtnodGRP1I*, *MtnodGRP1J*, *MtnodGRP1K*, *MtnodGRP1L*) that were more dispersed on the contig (Fig. 1). In these latter *MtnodGRP1* proteins, the signal peptides were similar but the glycine-rich domains were divergent. Thus, the duplication events resulting in the *MtnodGRP1C*, *MtnodGRP1H*, *MtnodGRP1I*, *MtnodGRP1J*, *MtnodGRP1K*, and *MtnodGRP1L* genes occurred earlier than the formation of the *MtnodGRP1A*, *MtnodGRP1D*, *MtnodGRP1E*, *MtnodGRP1F*, and *MtnodGRP1G* subgroup. This prediction is also supported by the insertions of numerous genes and by the larger distances between the presumably more ancient *MtnodGRP1* genes.

The phylogenetic comparison of the 23 *MtnodGRP* proteins divided them in two major groups separating most *MtnodGRP1* proteins (*MtnodGRP1A*, *MtnodGRP1C*, *MtnodGRP1D*, *MtnodGRP1E*, *MtnodGRP1F*, *MtnodGRP1G*, *MtnodGRP1I*, *MtnodGRP1J*, *MtnodGRP1K*, and *MtnodGRP1L*) from the others (Fig. 4). On the other branch, *MtnodGRP2*, *MtnodGRP5*, and *MtnodGRP6* proteins were closely related. Despite the gene cluster structures, some *GRP* showed higher homology to *GRP* present in other contigs, as was the case for *MtnodGRP1J*, which was more related to *MtnodGRP3A*, *MtnodGRP3B*, and *MtnodGRP4* than to the other *MtnodGRP1* proteins. *MtnodGRP3C* also displayed a closer relation to *MtnodGRP6* proteins than to the other *MtnodGRP3*. *MtnodGRP1H*, *MtnodGRP1I*, and *MtnodGRP1K* were more distant from the other *GRP* as well as the previously identified nodulin MtN29 (Gamas et al. 1996), which also belongs to the nodule-specific *GRP* (Kevei et al. 2002) but represents a distant member. In conclusion, the evolutionary history of the *GRP*

family is complex with ancient and more recent duplications that occurred both locally and at a long range between chromosomes.

NCR genes and their cluster organization.

Similarly to *GRP*, most of the identified *NCR* genes were composed of two open reading frame (ORF) coding exons. The first coded for the signal peptide (approximately 70 bp) and the second for the mature peptide (approximately 150 bp). The average intron length was 100 to 150 bp. Nevertheless, in a few cases *NCR* contained only one ORF coding exon (Table 1). Another uncharacteristic *NCR* gene structure consisted in three exon-containing genes (Table 1). In *NCR201* and *NCR206*, the third exon coded for the last seven and six amino acids, respectively. In the *NCR181* gene, the second exon was small, coding for eight amino acids and the cysteine-rich domain derived from the third exon. In the *NCR* clusters, the genes were usually in the same orientation (Table 1). In total, 24 contigs contained from two to seven *NCR* genes, and they were clustered within distinct lengths of genomic regions from 7 up to 364 kb.

The homology levels of *NCR* within their clusters were variable, as shown for three of them in Figure 3. Members of the

Fig. 2. Expression pattern of new *GRP* and *NCR* genes determined by reverse transcription-polymerase chain reaction (RT-PCR) in different tissues of *Medicago truncatula*. RT-PCR reactions were performed on cDNA prepared from roots (R), nodules (N), leaves (L), stems (S), and flowers (F). In the case of *NCR321* to *NCR 322*, primers could amplify products of similar sizes for both genes and sequencing the products allowed us to distinguish them. Amplification of *MtC27* was used as a positive control for each condition.

NCR159/172/200/329 cluster were highly homologous (74% average similarity), both on the signal peptide and on the mature parts of the proteins. In the NCR239/325/326/327/328 cluster, NCR325 and NCR326 shared 90% similarity, having a practically identical signal peptide and a few differences in the mature domain. In contrast, NCR239, NCR327, and NCR328 were less homologous (64% average similarity) and likely arose from earlier duplications than the *NCR325* and *NCR326* genes. The divergence in the NCR090/219/290/304/315 cluster was even more pronounced (55% average similarity), affecting the entire peptide and particularly the mature part, in which only the position of the cysteines and a few neighboring amino acids were conserved. In respect to all investigated clusters of the NCR family (Fig. 5), the position of the cysteines was conserved and the N-terminal signal peptide part was significantly more homologous than the C-terminal region.

The evolutionary relatedness of the 108 NCR by phylogenetic analysis is given in Figure 5. Generally the NCR that were present in the same cluster were the most homologous

and resulted from the most recent duplication events (Fig. 5, shaded boxes). However, others present in the same cluster, like NCR090/217/290/304/315, were distantly related and had arisen from more ancient duplication events permitting extensive diversifications during the evolution (Fig. 5, asterisks).

The GRP and NCR genes on *Medicago* chromosomes.

In addition to the cluster analysis, the GRP and NCR gene organizations were investigated on the whole-genome level. The chromosome visualization tool from the *Medicago truncatula* website was used to determine the gene or BAC positions on different chromosomes of *M. truncatula* (Fig. 6).

Of the six contigs containing the 23 GRP genes, four were on chromosome 2 and one on chromosome 5, while the *Mtnod-GRP4* gene was on chromosome 0. The three contigs containing the most related GRP, *MtnodGRP2*, *MtnodGRP5* and *Mtnod-GRP6* (Fig. 4), were also closely linked on chromosome 2.

Unlike the GRP, the 51 NCR loci were dispersed throughout the entire genome. Certain regions of chromosomes 3, 4, and 5

MtnodGRP1-cluster

MtnodGRP2-cluster

NCR159/172/200/329-cluster

NCR239/325/326/327/328-cluster

NCR090/217/290/304/315-cluster

Fig. 3. Peptide alignment of two representative GRP clusters and three representative NCR clusters. Not all the sequences from each cluster are included. Identical residues are shaded black, while similar residues are shaded gray. The big arrows delimit the signal peptide part of the proteins. The small arrows show the cysteines in conserved positions.

possessed numerous *NCR* genes, while a single gene was present on chromosome 8, and two loci were present on chromosome 1. Nevertheless, the *Medicago* sequencing project is still ongoing, and therefore, our results are not exhaustive and it is possible that additional *NCR* will be found. Interestingly, in a region on chromosome 2, the *NCR024/026/088/138/316/334* cluster and the *MtnodGRP3* genes were in the same contig. So far, we have no evidence for other genomic regions containing closely linked *GRP* and *NCR* genes, but in the absence of the complete genome sequence, we cannot exclude that either.

Gene evolution of *GRP* and *NCR*.

The comparison of *GRP* and *NCR* in Figure 3 indicates that the signal peptide and mature peptide part of the proteins might diversify to different extents. Their evolution patterns were separately analyzed by the ratios of synonymous (dS) and nonsynonymous (dnS) amino-acid substitution; a value for dS/dnS > 1 indicates purifying and dS/dnS < 1 indicates diversifying selection for the investigated domains. All 23 *GRP* in the six clusters and 37 *NCR* of the *NCR090*, *NCR159*, *NCR239*, *NCR053*, *NCR086*, *NCR024*, and *NCR062* clusters (Table 1) were used for the analysis. In the case of the *GRP*, 60% of the signal peptide pairwise comparisons (PC) fall into the dS/dnS > 1 category of purifying selection (Fig. 7A), while glycine-rich domains followed diversifying evolution (88% of PC with dS/dnS < 1) (Fig. 7B). In the 37 studied *NCR*, 55% of the signal peptide PC showed purifying selection (Fig. 7C), while 91% of the cysteine-rich domains PC (Fig. 7D) showed evidence for diversifying selection. Thus the diversification of the mature peptides was even more pronounced in the *NCR*. However, this statement concerns the overall comparison, and the dS/dnS values were variable depending on the *NCR* cluster and the time of the gene duplication events. Naturally, the more recently duplicated genes had less chance for diversification.

Fig. 4. Phylogenetic relationship between *GRP* proteins. All *GRP* except MtN29 have available genomic sequences.

Fig. 5. Phylogenetic relationship between *NCR* peptides. Only *NCR* for which the corresponding genomic sequence has been identified are included in this tree. Shaded boxes show homologous genes that are clustered in the genome. *NCR* marked with an asterisk form a cluster in the genome but are not homologous.

Overall, these results show, for the GRP and NCR, a tendency of purifying selection for their signal peptides contrasting with the diversifying selection trend observed for their respective glycine-rich or cysteine-rich domains.

Microsyntenic analysis of *GRP* and *NCR* loci.

In the *L. japonicus* databases, there are no ESTs or genomic sequences corresponding to *M. truncatula* *GRP* and *NCR*. Therefore, we carried out microsynteny analysis in the two model legumes to verify whether the *GRP* and *NCR* genes are unique to *M. truncatula*. Annotated *M. truncatula* protein sequences corresponding to the flanking genomic regions of different *GRP* and *NCR* gene clusters were used for tBLASTn searches against *L. japonicus* genome databases. The corresponding *L. japonicus* regions were found for the *M. truncatula* loci carrying the *MtnodGRP2* and the *NCR062/074/076/128/145/312/313* clusters (Fig. 8). Analysis of gene occurrence and order in the two genomes demonstrated a syntenic relation between *M. truncatula* and *L. japonicus* in these regions.

In the *MtnodGRP2* region on chromosome 2, nine of 17 genes showed synteny to eight of nine genes of the *L. japonicus* chromosome 6 (Fig. 8). However, the four tandem-duplicated *MtnodGRP2A*, *MtnodGRP2B*, *MtnodGRP2C*, and *MtnodGRP2D* genes were absent in *L. japonicus*. In the syntenic region, all genes except one were in the same orientation in the two genomes and the syntenic proteins displayed, on average, 75% identity and 84% similarity. In the case of the *NCR062/074/076/128/145/312/313* contig on *M. truncatula* chromosome 4, 10 of 38 genes displayed syntenic relationship with 10 of 19 *L. japonicus* genes on chromosome 3. All the syntenic genes were in the same orientation in the two genomes, with one exception, and the encoded proteins shared an average of 81% identity and 92% similarity. Similarly to the *MtnodGRP2* contig, no *NCR*-like genes were present in the corresponding region on *L. japonicus* chromosome 3. However, unlike the *MtnodGRP2* region, many other genes were inserted between the syntenic genes and the *M. truncatula* *NCR* genes. In addition, the duplication events resulting in *NCR312* and *NCR074* genes were associated with gene inversion. These data suggest that the *MtnodGRP2* gene duplications occurred later than the amplifications of these *NCR*.

In the absence of complete genome sequences, our analysis was restricted to the available two examples. Nevertheless, this set of data, as well as the absence of *GRP* and *NCR* EST in *L. japonicus* and *Glycine max* but the presence of *GRP* and *NCR* transcripts in other IRLC legumes (Kevei et al. 2002; Mergaert et al. 2003) reinforce the idea that these gene families are indeed specific for IRLC legumes.

DISCUSSION

NCR and *GRP* gene families in *M. truncatula*.

NCR genes likely represent one of the largest multigenic families in plants with extreme natural diversity in the mature peptides. From the 311 *NCR* transcripts described by Mergaert and associates (2003), in this study, we localized the genomic loci for 79 genes, representing 25% of the known set in *M. truncatula*. Moreover, we identified 29 new *NCR* in the vicinity of known *NCR*, suggesting that the family is much larger than expected. One has to note that all verified new *NCR* were expressed genes and displayed nodule specificity as shown by RT-PCR experiments. Extrapolating our findings to the whole-genome level would predict a family size of about 430 nodule-specific *NCR* genes. Thus, our strategy to find new *NCR* family members was fruitful despite the fact that bioinformatics tools are not optimized to detect short and divergent genes, as discussed by Silverstein and associates (2005).

Until this study, many *GRP* remained hidden and were not annotated in the genome due to their high variability and lack of apparent glycine-rich motifs in the mature peptide. As in the case of *NCR*, we were able to identify 17 novel genes, mainly on the basis of the highly conserved signal peptide. At present, 23 *GRP* belonging to six subgroups are known in *M. truncatula*.

Genome organization and evolutionary dynamics of *NCR* and *GRP* gene families.

Few *NCR* and *GRP* exist as single genes in a locus, but they are mostly clustered with multiple genes per locus. Their genomic organizations and evolutionary scenario with the rapid diversification by local duplications are features that are also common in the innate immunity gene families such as *R*

Fig. 6. Genome scale organization of *GRP* and *NCR* genes in *Medicago truncatula*. *NCR* loci are indicated by squares, whereas *GRP* loci are represented by triangles on the pseudochromosomes according to the chromosome visualization tool from the *Medicago truncatula* website. The number of genes per locus is indicated when more than one gene could be identified in the contig.

genes and defensins (Baumgarten et al. 2003; Meyers et al. 2003; Silverstein et al. 2005). These defensins are also cysteine-rich secreted peptides and members of the CCP family, with their eight cysteines forming four disulfide bridges (Shutte et al. 2002). As observed by dS/dnS amino-acid substitution rates analysis, the signal peptide of defensins displays purifying selection, whereas the mature peptide evolution is driven by diversifying selection (Maxwell et al. 2003; Semple et al. 2003; Silverstein et al. 2005).

Similarly to defensins, the cysteine-rich regions of NCR and the glycine-rich domains of GRP undergo diversifying selection. The evolution of signal peptides is less evident, though there is a tendency for purifying selection in both peptide families. For the recently evolved and highly similar genes, such as those in the *MnodGRP2* or *NCR159/172/200/329* clusters, we cannot draw conclusions with certainty for their future evolution, but we presume that they will follow a similar evolutionary scenario as the other genes of the family.

GRP and NCR families are unique to IRLC legumes.

Besides the nodule specificity of the *GRP* and *NCR* gene families, the expression data suggested that they were restricted to IRLC legumes like *Medicago* spp. (Fedorova et al. 2002; Graham et al. 2004; Györgyey et al. 2000; Mergaert et al. 2003), *Pisum sativum* (Kardailsky et al. 1993; Kato et al. 2002; Scheres et al. 1990;), *Trifolium repens* (Crockard et al. 2002), *Astragalus sinicus* (Chou et al. 2006), *Galega orientalis* (Kajjalainen et al. 2002), and *Vicia faba* (Frühling et al. 2000), in which related sequences have been identified during EST sequencing projects or searches for nodule-enhanced or nodule-specific genes. In spite of the large number of EST available from other distantly related legume species, like *Glycine max*, *L. japonicus*, or *Phaseolus vulgaris*, no homologs of *GRP* or *NCR* were found. In our study, microsyntenic evidences reinforced the hypothesis that *GRP* and *NCR* are unique to IRLC legumes, since they were absent in the syntenic *L. japonicus* regions. Thus, these two families should have appeared between 51 and 25 million years ago,

Fig. 7. Plot of synonymous (dS) versus nonsynonymous (dnS) substitution rates for 23 GRP and 37 NCR in *Medicago truncatula*. The signal peptide (A, GRP; C, NCR) and mature peptide (B, GRP; D, NCR) sequences were analyzed separately. The x axis shows the dnS values, the y axis the dS values. Every point corresponds to the pairwise comparison between two nucleotide sequences. The diagonal line ($dS/dnS = 1$) represents the neutral selection. Points above this line ($dS/dnS > 1$) marked purifying selection, points under the line ($dS/dnS < 1$) displayed diversifying selection.

which corresponds, respectively, to the estimated times for the most recent common ancestor (MRCA) of *Lotus*, *Pisum*, and *Medicago* and the age of separation of the MRCA of *Medicago* and *Pisum*, respectively (Lavin et al. 2005). The availability of nodule EST databases from other IRLC legumes would be beneficial to elucidate the history of these gene families and decipher how rapidly they became diverged among the IRLC plants. One can postulate that these gene families had already started to diversify before the *Medicago*-*Pisum* separation, as most NCR-containing IRLC plants harbor several members of the gene family. Also, phylogenetic analysis of NCR originating from different IRLC species shows higher homology between members of different IRLC species than to other members from the same organism (data not shown). These results indicate that the radiation of this gene family has probably started before the successive speciation events that lead to the actual diversity of the IRLC legumes. In other words, the MRCA of IRLC legumes should have already had NCR in its genome. Despite the limited data set available for the smaller GRP family, the same evolutionary scenario can be predicted, as GRP have, so far, been found exclusively in IRLC legumes. Thus GRP and NCR families should have appeared almost simultaneously during legume diversification.

What are the functions for so many genes?

GRP form a family with at least 23 genes and the NCR family is composed of up to more than 400 genes, while up to 5% of EST databases from *Medicago* nodules are composed of NCR. Yet, no function has been assigned to these two gene families. Mergaert and associates (2006) proposed that NCR, like defensins, could act as antimicrobial peptides and may govern bacteroid differentiation. This hypothesis is in agreement with the differences in morphology and physiology of the bacteroids in IRLC and non-IRLC legumes (Mergaert et al. 2006). In IRLC legumes, bacteroids undergo a multistep irreversible differentia-

tion process manifested by loss of cell division ability and a striking elongation of the cells that is driven by endoreduplication cycles. These modifications are accompanied by membrane permeabilization and the inability of bacteroids to recover growth once they were released from the nodule. In contrast, in non-IRLC legumes, bacteroids are able to divide and resume growth when released from a nodule. They have no cell elongation, genome amplification, or membrane permeabilization. We have shown previously that the observed differences in bacteroid behavior are due to plant factors and not to the bacterial genetic background. This suggests a role for IRLC-specific genes like NCR and GRP that may act as antimicrobial peptides (AMP) especially recruited for bacteroid differentiation. The known AMP are relatively small (<10 kDa), cationic and amphipathic peptides of variable length and structure (Broden 2005). Most AMP are believed to act by disrupting the plasma membrane integrity leading to cell lysis. In plants, the main groups of AMP are cysteine-rich defensins. NCR are also in the CCP group like the defensins but are expressed only in the *Rhizobium*-infected nodule cells in which they might have specific functions in bacteroid differentiation, for example, leading to increase membrane permeability and inhibiting cell division. AMP can be rich in other amino acids as well, such as glycines (Ötvös 2000). Thus, GRP could also be considered as a specific type of AMP acting in symbiosis. AMP act often synergistically. This could explain overlapping expression of several NCR and GRP in the same nodule cell. Moreover, peptides are often involved in cell-to-cell communications. As the different NCR and GRP are expressed in the different stages of symbiotic cell differentiation, they could act, not simply as selective bacteriostatic agents controlling the microsymbionts inside the nodule, but could also monitor differentiation of bacteroids in accordance with the host cells. In addition, the high genetic diversity of isolates from the same rhizobial species in natural ecosystems (combined with the necessity to reinitiate the symbiosis at each generation of the

Fig. 8. Microsyntenic comparison between *Medicago truncatula* and *Lotus japonicus* on *MtnodGRP2* and *NCR062/074/076/128/145/312/313* contigs. The BAC clone numbers corresponding to the *Medicago* and *Lotus* contigs are indicated and followed by the chromosome number. The location and orientation of genes is indicated by arrows. Genes and intergenic regions are not drawn at scale. Black arrows represent the GRP or NCR genes and their underlining indicates the absence of expressed sequence tags. Gray linked arrows show syntenic genes with the same orientation, light gray ones show genes with inversion. Other nonsyntenic genes are shown with white arrows.

plant) might have been an additional driving force in IRLC legumes for diversification of *NCR* and *GRP*. Thereby, the legume host could be armed to confront any kind of microsymbiont isolate, to control their fate and differentiation inside the nodule. This high diversity of *NCR* or *GRP* ensures that the bacterium cannot overcome the effect of single *NCR* or *GRP* and escape the differentiation process imposed by the plant.

Functional studies of these gene families are of great interest with respect to symbiosis and bacteroid differentiation, but in addition, this high natural diversity of legume peptides might have diverse applications in human, animal, and plant health-care.

MATERIALS AND METHODS

NCR and *GRP* genes identification.

BLASTn (Altschul et al. 1997) analyses were performed against the NCBI nonredundant database, using *GRP* and *NCR* transcripts as query (Kevei et al. 2002; Mergaert et al. 2003). *M. truncatula* BAC clone sequences corresponding to perfect matches with the transcripts were used in further BLASTn analyses to extend the contigs to their maximal size. These contigs were then screened for the presence of other *NCR* or *GRP* using the putative mature or signal peptide sequence in a tBLASTn analysis.

Protein alignments and phylogenetic analyses.

Protein alignments shown in Figure 3 were produced using the ClustalW application of the BioEdit v6.0 pack and were processed by EMBnet's Boxshade 3.21 program. After the alignments, the predicted peptide sequences were processed by the MEGA 3.1 software (Kumar et al. 2004) to produce neighbor-joining trees of *GRP* and *NCR*.

Evolutionary analysis of *NCR* and *GRP* in *M. truncatula*.

In order to estimate the rates of dS and dnS substitutions, the coding sequences of *NCR* and *GRP* were split into the signal peptide and the mature peptide part according to the SignalP prediction server. The coding sequences were aligned by ClustalW. The dS and dnS substitution values were calculated by the Nei-Gojobori method (Jukes-Cantor correction), which, in addition to counting the number of dS and dnS substitutions, takes into account the multiple potential substitutions at the same place. Gaps were removed in each pairwise comparison. All results were computed by MEGA 3.1 software. All pairwise comparisons of dS and dnS values were carried out for the different domains and were used for plot analysis.

Microsynteny study between *M. truncatula* and *L. japonicus*.

Predicted protein sequences of the contigs corresponding to *GRP* or *NCR* clusters and neighboring regions were used in a tBLASTn analysis against a local database composed of *L. japonicus* genomic sequences previously created in BioEdit. *L. japonicus* predicted genes sharing $\geq 80\%$ homology with *M. truncatula* predicted genes were considered as valid anchors.

Gene plotting on *M. truncatula* pseudochromosomes.

Predicted protein sequences from either *GRP* or *NCR* genes were submitted to the chromosome visualization tool on the *Medicago truncatula* Sequencing Resources website and the corresponding BAC hit was localized on the genetic map.

RT-PCR experiments.

Leaf, stem, flower, 21-dpi nodule, and root tissue samples of *M. truncatula* J5 plants were collected, and RNA was extracted, using the RNeasy plant mini kit, and DNase treated (Qiagen S.A., Courtaboeuf, France). First-strand cDNAs were synthe-

sized in the presence of RNasin with SuperScript reverse transcriptase according to the manufacturer's instructions (Promega, Madison, WI, USA). Gene-specific PCR primers were designed and synthesized on the Invitrogen website. RT-PCR were performed for 35 cycles using *Taq* DNA polymerase (Eurogentec, Liège, Belgium) in an iCycler (BIORAD, Marnes la Coquette, France). PCR products were run on 1.5% (wt/vol) agarose gels and were cloned into pGEM-T Easy vectors (Promega) and were sequenced using BigDye Terminator v3.1 in an ABI PRISM 3100 (Applied Biosystems, Courtaboeuf, France). Sequences were then aligned with genomic sequences to verify identity and splicing prediction.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

BioEdit website: www.bioedit.com
 CBS prediction server SignalP program: www.cbs.dtu.dk/services
 EMBnet Boxshade 3.21 program:
www.ch.embnet.org/software/BOX_form.html
 Invitrogen website: www.invitrogen.com
Medicago truncatula website: www.medicago.org
Medicago truncatula Sequencing Resources website:
www.medicago.org/genome/cvut_blast.php
 NCBI nonredundant database: www.ncbi.nlm.nih.gov/BLAST