NCR PEPTIDES — PLANT EFFECTORS GOVERNING TERMINAL DIFFERENTIATION OF NODULE BACTERIA INTO THE SYMBIOTIC FORM

(review)

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A b s t r a c t

Uptake of mineral nutrients from the soil is the challenge of plant survival. In particular, the availability of such macro-elements as nitrogen and phosphorus is the limiting factor for plant growth and development. Some plant genera overcome this limitation by establishing symbiotic relationships with microorganisms. A remarkable example of such symbiosis is one between legumes and rhizobia — a group of nitrogen fixing soil bacteria. Rhizobial penetration into roots of a specific host plant causes initiation of a specialized organ, symbiotic nodule. Within cells of symbiotic nodule free-living bacteria differentiate into a symbiotic form called «bacteroids». Such organelle-like structures provide plants with fixed nitrogen in exchange for nutrients (B.J. Ferguson et al., 2010). A number of legumes form nodules, in which bacteria terminally (irreversibly) differentiate into bacteroids, thus losing the opportunity to return to the free-living state. Terminal differentiation of bacteroids begins soon after release of the rhizobia into plant cells and leads to morphological, physiological and genetic changes in bacterial cells. It has been shown that a large family of antimicrobial peptides of plants called Nodule-specific Cysteine-Rich peptides (NCR peptides) plays a key regulatory role in this process (P. Mergaert et al., 2003). Its representatives are similar in structure and mode of action to defensins — plant innate immunity factors; however, NCR genes are expressed only in nodules, which fact is reflected in their name. At the moment, about 700 genes encoding NCR peptides that are highly variable in their amino acid sequence but possess a distinct conservative cysteine motif required for the adoption of correct conformation were identified in the genome of the model legume *Medicago truncatula* Gaertn. NCR peptides are delivered to their intracellular target symbiosome (cell compartments containing bacteroides) triggering the process of differentiation by interacting with the components of membranes and various intracellular targets of bacteria (D. Wang et al., 2010). The most studied member of this family in *M. truncatula* is MtNCR247 a cationic peptide with four cysteines forming two disulfide bonds in oxidized form. MtNCR247 affects transcription, translation and cell division processes in *M. truncatula* microsymbiont *Sinorhizobium meliloti* at low concentrations, and also exhibits antimicrobial activity at higher concentrations (A. Farkas et al., 2014). To date, NCR peptides are identified only in plants belonging to IRLC (Inverted Repeat-lacking Clade) legumes which are characterized by terminal differentiation of bacteria into bacteroids. Probably, evolutionary acquisition of the variable gene family encoding NCR peptides has been the selective advantage of this group of plants.

Keywords: rhizobium-legume symbiosis, nitrogen-fixing nodules, differentiation of bacteroides, NCR-peptides, regulation of symbiosis development

The legume family (*Fabaceae*) includes primary food and feed species of cultivated plants such as peas, soybeans, clover, chickpeas and lucerne. It is the third largest group of angiosperms and the second one of food and feed crops grown worldwide [1]. The important environmental advantage of legumes is the

opportunity to grow with virtually no fixed nitrogen in the soil through fixation of atmospheric nitrogen by symbiotic nodule bacteria [2, 3].

When establishing nitrogen-fixing symbiosis, rhizobia selectively penetrate into roots of legumes, which leads to the development of special root structures called nodules [4]. The ontogeny of nodules is a well-organized process based on the coordinated expression of specialized plant and bacterial genes [5[. A large part of a complex system of genes in plants that control the nodule development is needed to control microsymbiont by a host plant.

Inside the nodule, the bacteria differentiate into bacteroids and carry out symbiotic nitrogen fixation, i.e. reduction of molecular nitrogen to ammonium ion using the enzymic complex of nitrogenase [6, 7]. In the legume nodules belonging to the IRLC (Inverted Repeat-lacking Clade) group, the transformation of rhizobia into bacteroids is irreversible (terminal differentiation); in other plants, it is a reversible process [8-10]. If the rhizobia strain is capable of nodulation on the roots of a wide range of plants, the degree of bacteria differentiation into bacteroids and its reversibility strictly corresponds to species of a host plant, from which it follows that the differentiation process is induced through plant signals [11-13].

In *Medicago truncatula* model legume, as such factors there may be at least 800 nodule-specific symbiotic peptides, the majority of which (over 700) belong to the group of nodule-specific cysteine-rich (NCR) peptides [14-17]. NCR peptides have been also described for other legumes belonging to the same IRLC clade, i.e. beans (*Vicia faba*) [18], white clover (*Trifolium repens*) [19], Eastern galega (*Galega orientalis*) [20], and English peas (*Pisum sativum* L.) [21-23]. NCR peptides have been identified in experiments on identification of nodule-specific protein molecules (nodulins) [21], but not all nodulins refer to NCR peptides.

The aim of the present article is generalization and structuring of information accumulated over the last decade, in relation to the regulation of symbiotic bacteria differentiation in establishing nitrogen-fixing symbiosis. For the first time, a complete characterization of the numerous protein NCR peptides family has been given. The issues of their structure, functions, targets, modes of action and its intended path of evolution have been also discussed in detail.

The genes of NCR peptides belong to the extensive group of defensin-like genes, various representatives of which are found in the genomes of vertebrates (encode proteins involved in the acquired immunity), invertebrates (e.g., encode a component of the scorpion toxin) and plants [14, 24, 25]. In turn, defensins are a group of antimicrobial peptides (AMP), which are produced by almost all living organisms and play a key role in the innate immunity [26-28]. The general mode of action of antimicrobial peptides is the disruption of microbial membranes and (or) inducing the formation of pores, which leads to lysis of bacterial cells and also makes possible the interaction of peptides with intracellular targets (DNA, RNA and various proteins) [29-31].

NCR peptides, along with plant defensins, by amino acid composition belong to a vast family of cysteine cluster proteins (CCPs) containing a conservative cysteine cluster of 4, 6, 8 or 10 cysteine residues in conservative positions [32-34]. Nodule-specific CCPs, that is, NCR peptides, represent one of 10 CCP subgroups [34, 35]. Like the genes of defensins, the genes of NCR peptides encode short (30-60 amino acid residues) secretable polypeptides with a high variability in amino-acid sequence, which determines their specificity and different mechanisms of action [14, 36, 37]. Unlike defensins, the key function of which is involvement in protective processes and a negative impact on bacteria, NCR peptides play a positive regulatory role in the nodules of legumes controlling the rhizobia differentiation into nitrogen-fixing bacteroids [11, 38, 39]. The antimicrobial activity

of NCR peptides is reflected in the fact that the differentiation into bacteroids under their action is irreversible. Furthermore, the bacteria lose their ability to reproduce.

The expression of the genes encoding NCR peptides is specific to the nodules. However, certain groups of genes are activated together, resulting in several successive "waves" of their expression at different stages of nodule development [40]. Just synthesized NCR peptides contain an N-terminal signal sequence that defines their transport to the endoplasmic reticulum [11, 14]. In the transport of NCR peptides, the key role is played by signal peptidase cutting off the signal sequence from the mature peptide in sorting proteins on the endoplasmic reticulum [41]. In *M. truncatula* mutants of *MtDNF1* gene encoding a nodule-specific signal peptidase subunit, NCR peptides retain the signal sequences and accumulate in the endoplasmic reticulum, without getting into symbiosomes resulting in the absence of bacteroids differentiation [11, 16, 41]. In the case of the normal development of symbiosis, with the gene expression different sets of NCR peptides are delivered to the endosymbiont and mediate the subsequent events of its differentiation [16].
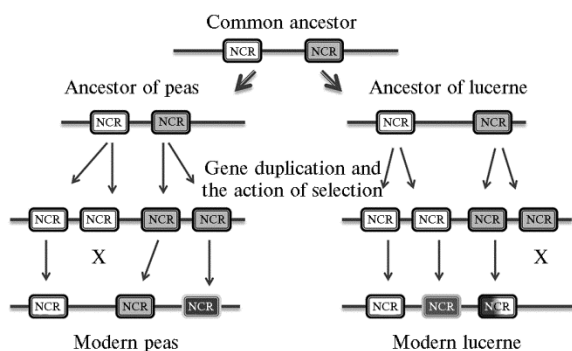
For some NCR peptides (e.g., MtNCR247 and MtNCR335), an ability to interact with the membrane of bacterial cells has been shown, which in vitro leads to the loss of both rhizobia and human pathogens and plants [11, 42]. However, in vivo (in the nodule cells) symbiotic peptides do not violate the permeability of the bacterial membrane so much that it should lead to cell lysis [31]. Probably, the increase in the permeability of the membrane promotes the penetration of NCR peptides into the bacterial cells.

Of bacteroids present in the cells of *M. truncatula* nodules, about 140 different NCR peptides can be identified, which indicates the possibility of their penetration into the bacterial cell, as well as their high stability and potential interaction with intracellular targets [43]. One of MtNCR247 targets is bacterial protein FtsZ, which plays an important role in the formation of the cell wall in the cell division [44-46]. Another partner of MtNCR247 is chaperone GroEL, which is required for full activation of nodulation genes and assembly of nitrogenase complex [47]. Also, MtNCR247 exposure changes the expression of some regulatory genes critical to the cell cycle progression (*ctrA, gcrA, dnaA*), which might be related to stopping the proliferation of bacteria in the plant cells [45].

Rhizobia have protective mechanisms to withstand the effects of vegetable NCR peptides [48]. In particular, some protection against NCR peptides is provided by the *BacA* gene encoding the protein of the family of ABC transporters [49-51]. Thus, *S. meliloti* mutant of the *bacA* gene shows hypersensitivity toward MtNCR247 in vitro and in vivo (bacteria are degraded soon after release of the lucerne nodule into cells, while bacterial strains of the wild type remain viable and are differentiated into bacteroids) [52-54]. In addition, the metallopeptidase gene *HrrP* (host range restriction peptidase), encoding enzyme capable to cut NCR peptides, has been described [55, 56]. Some *Bradyrhizobium* strains for a successful nitrogen-fixing symbiosis require the presence of styrole-like lipids (hopanoids) in the membrane, probably, enhancing its strength and providing protection against NCR peptides [57, 58].

An important feature of the gene family of NCR peptides is their clustered organization within the genome. The Clusters of genes, which encode NCR peptides, are evenly distributed on eight chromosomes in *M. truncatula* and, apparently, originated from the repeated duplication and subsequent diversification of sequences [9]. Since the promoter regions of genes encoding NCR peptides are also similar, their expression is highly consistent. Sequencing RNA samples from the nodule zones, carved out of the preparation by laser micro-dissection,

showed that the genes of NCR peptides can be divided into several groups based on their spatial-temporal expression profile [16]. Obviously, the expression of certain groups of NCR peptides genes is needed only at the specific stage of nodule development [40].

Despite the previously described interchangeability of antimicrobial molecules [14], there is data on the unique properties and functions of NCR peptides, the absence of which interrupts establishment of nitrogen-fixing symbiosis. Thus, MtNCR211, toxic to *S. meliloti* in vitro, is necessary for the survival of bacteroids in planta, because mutation in its gene leads to the death of bacteroids in the nodule cells [60]. Similarly, to maintain bacteroids, MtNCR169 is necessary, in the absence of which their differentiation practically does not take place and, as a result, nitrogen is not fixed [61]. These two NCR peptides are characterized by similar localization in planta [60, 61]. *M. truncatula* mutants of the *MtDNF4* (=*MtNCR211*) and *MtDNF7* (=*MtNCR169*) genes

Scheme of the proposed evolution of NCR peptides genes of *Medicago truncatula* and *Pisum sativum*. Genes of NCR peptides originated about 25 million years ago in the common ancestor of the IRLC (Inverted Repeat-lacking Clade) group. Subsequently, the duplication, diversification and selection in favor of the highest functionality led to the emergence of a large number of genes encoding antimicrobial peptides with low sequence conservation, which became the basis of the diversity of their activity and physiological functions. Some of NCR peptides gained unique functions distinguishing them from the main group and essential for successful symbiosis; part of the sequences was becoming pseudogenes and was losing a biological function. The color indicates the degree of differences in the variability of the nucleotide sequences of NCR peptides genes and their changes in the evolution (mutation and recombination). X means that the gene, which encoded NCR-peptide, due to mutation evolved in pseudogene and lost its original function.

have similar transcriptomic profiles [62]. Despite this, the role of each of these peptides is unique, because in the mutant by the *MtNCR211* gene, lysis of bacteroids occurs after differentiation, and in the mutant by *MtNCR169* gene, undifferentiated bacteria are exposed to lysis [63]. The sequences of NCR peptides genes are extremely variable even within the same species of legumes. The found homologous sequences in different representatives of the IRLC group have a very low percentage of similarity, so the definition of orthologous pairs becomes impossible [64]. Apparently, these plants diverged from a common ancestor, which already had the genes of NCR peptides, a long time ago (Fig.). Subsequently, in the genome of each of them there was duplication of the genes of NCR peptides, which then evolved independently under the influence of a positive (driving) selection [59]. Some genes of different types due to the accumulation of mutations could turn into pseudogenes and be eliminated, so non-orthologic genes began to perform the same biological function. Finally, the similarity of individual sections of sequences of NCR peptides genes indicates the possibility of their variability increase due to illegitimate recombination. The result of the evolution of this gene family was a wide variety of NCR peptides observed in modern representatives of the IRLC group.

According to research of the efficiency of symbiotic nitrogen fixation, bringing bacteroids differentiation to the terminal phase provides the plant (and the symbiotic system as a whole) a significant benefit [65]. Probably, for this

reason the acquisition in the evolution of the family of NCR peptides genes was a selective advantage of IRLC group plants. In addition, NCR peptides are involved in the background immune response of nodules, the activity of which does not depend on whether there are undesirable (pathogenic) microorganisms in them [63]. The activity in the symbiotic plant organs of the genes of NCR peptides related to the plant immune system and quickly evolving towards diversity, together with the presence of rhizobia systems, which resist the lethal effects of NCR peptides, is a good example of co-evolution of partners in the case of nitrogen-fixing symbiosis.

Thus, to date, in the limited group of legumes, a large gene family has been discovered, which has an organo-specific expression. Its representatives perform a function of a terminal, that is, irreversible differentiation of the symbionts inside the nodule. It is assumed that the genes of NCR peptides arose from related genes of plant defensins in the rapid duplication and diversification of sequences. Many of NCR peptides are interchangeable due to the similarity of the structures, but some acquired new, more specialized functions. The roles, the targets and the modes of action of NCR peptides have been described only for several representatives of this family, and therefore, further research may lead to unexpected and valuable discoveries.

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