IXth INTERNATIONAL COLLOQUIUM FOR THE OPTIMIZATION OF PLANT NUTRITION

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of the Research Institute of Crop Production Prague-Ruzyně (RICP)

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PLANT MUTANTS AND MOLECULAR SIGNALLING IN LEGUME–*RHIZOBIUM* SYMBIOSIS – A REVIEW

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Under a soil NO_3^- stress unique highly organized structures (nodules) in which compatible rhizobia convert atmospheric N_2 into ammonia develop on the roots of legumes. A specific interaction between bacterial Nod factors and hypothetical plant receptors activates a multi-stepped signal transduction pathway that temporally and spatially coordinates successful development of infection and nodule morphogenesis. It seems likely that a step in the nodule developmental program induced by the Nod factors is a local accumulation of flavonoids (auxin transport inhibitors) in roots. They may mediate a second signal for nodule morphogenesis by disrupting the normal endogenous auxin/cytokinin balance, causing cortical cell divisions and the subsequent formation of a nodule primordium. The number of legume nodules and their relative localization on the root system is tightly regulated both by environmental factors and by internal control mechanisms. The inhibitory effect of plant-mediated autoregulation on nodule development in ontogenetically younger root tissues may be enhanced by a direct or ethylene-mediated interaction between shoot-derived inhibitor (SDI), slowing down the rate of further cortical cell divisions, and nitrate. Elucidation of these complex processes was facilitated by the characterization of specific mutants blocked in different steps of legume nodulation.

Rhizobium–legume symbiosis; Nod factors; lectin; nodulation control; plant nodulation mutants

INTRODUCTION

Under conditions of nitrogen limitation, members of the plant family Leguminosae can establish a symbiosis with compatible species of bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*. The rhizobia–legume symbiosis represents a unique system, since highly organized root nodules are formed in which the bacteria convert atmospheric N_2 into ammonia (S m i t et al., 1992).

Over the past two decades, intensive studies of the rhizobia–legume interaction have preferred research of the bacterial contribution to root nodule formation rather than that of the host plant.

The addition of purified rhizobial Nod factors to legume seedlings of temperate legumes results in the deformation of root hairs and a response of cortical cells identical to that seen in rhizobia-infected roots and in the same zone of the root leading to the genuine nodule formation. In addition, the spatial pattern of the expression of early nodulin genes induced by these Nod factors precisely corresponds to the pattern after rhizobial infection (Vijn et al., 1993).

These observations and in particular the finding that the nodules are spontaneously generated by certain lines of alfalfa (Truchet et al., 1989) suggest that rhizobial Nod factors only trigger events that are preprogrammed in the plant to form nodules and to direct the bacterial infection (Verma, 1992).

Because nodule development is largely under the control of organogenesis program (Verma, 1992), it now becomes apparent that the elucidation of the very complex symbiosis will also require the precise information about the contribution of the plant to the molecular signal transduction pathway controlling this process. This review will focus on the genetically and molecularly characterized plant mutations that block specific steps in the nodule-development pathway and are consequently essential for these studies.

THE CHARACTERIZATION OF SINGLE STEPS IN NODULATION

Root hair deformation and infection thread formation

After rhizobial attachment, only recently emerged root hairs (Bhuvaneswari et al., 1980) are deformed into a number of unusual shapes, including branches, twists, corkscrews, and spirals. Only few of them coil 360 ° and form diagnostic curls known as shepherd's crooks which are essential for subsequent successful infection (Hirsch, 1992).

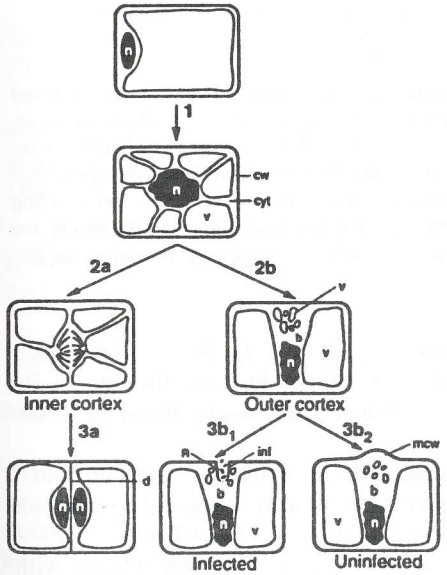
Following by strictly localized dissolution of the plant cell wall, entry of rhizobia occurs by invagination of the plasma membrane of the root hair. A tubular infection thread is formed by depositing new cell wall material around this invaginated membrane with the rhizobia inside it (see references in Kijne, 1992).

In those legumes where root hair curling does not take place such as peanut and *Stylosanthes*, rhizobia enter the roots between epidermal cells where lateral roots emerge (see references in Dart, 1977; Torrey, 1986).

Root nodule initiation and differentiation

Even before the infection thread is initiated, root cortical cells begin to divide, giving rise to a nodule primordium (Caetano-Anollés, Gresshoff, 1991).

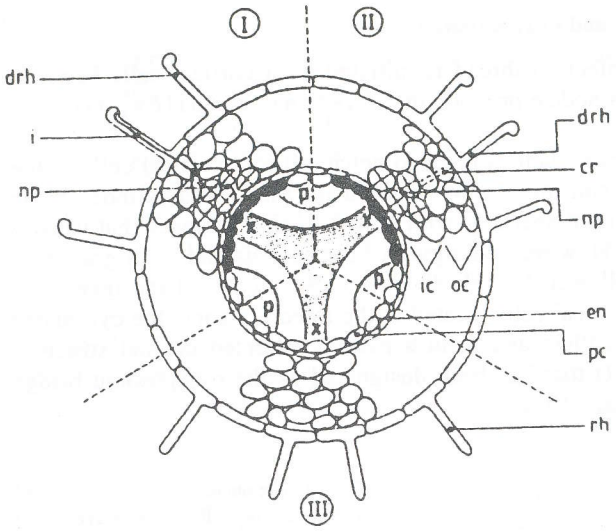
In temperate legumes, such as pea and vetch, all root cortical cells in the sector between a root hair infected by rhizobia and the central cylinder of the root respond almost simultaneously with morphological changes that usually precede cell division. However, enlarging of the nucleus and its migration to the cell center are followed by cell division only in cells of the inner root cortex. In the outer cortical cells located in the infection zone, the cytoplasm and endomembranes collect and form a radially oriented conical structure (Figs. 1 and 2, part II) that has been designated as the cytoplasmic bridge (Van Brussel et al., 1992).



Stage 1: The nuclei of all cells swell and migrate to the cell center. Stage 2: (a) Cell divisions occur only in cells located in the inner cortex. (b) The outer cortical cells do not continue the cell division cycle but instead form cytoplasmic bridges. Stage 3: (a) In the inner cortex, the daughter cells can enter the cell cycle again. (b) In the outer cortex, the formation of the cytoplasmic bridges is accompanied by local modification of the outer periclinal cell wall, resulting in the formation of an infection thread in the presence of rhizobia ($3b_1$) or in the initiation of root hair growth if only purified Nod factors were applied to the roots ($3b_2$). Symbols: b – cytoplasmic bridge, cw – cell wall, cyt – cytoplasm, d – division wall, inf – infection thread, mcw – modified cell wall, n – nucleus, R – *Rhizobium* bacteria, v – vacuole

1. Diagramatic representation of morphological changes in the vetch root cortex induced by rhizobial mitogenic Nod factors (from Van Brussel et al., 1992).

The aligned cytoplasmic bridges, the so-called pre-infection thread structures, are usually positioned in line with young division walls of the inner cortical cells, so that very typical radial rows of cells with these aligned



Symbols: cr – cytoplasmic rearrangements, drh – deformed root hair, en – endodermis, i – infection thread, ic – inner cortex, np – nodule primordium, oc – outer cortex, p – phloem, pc – pericycle, rh – root hair, x – xylem

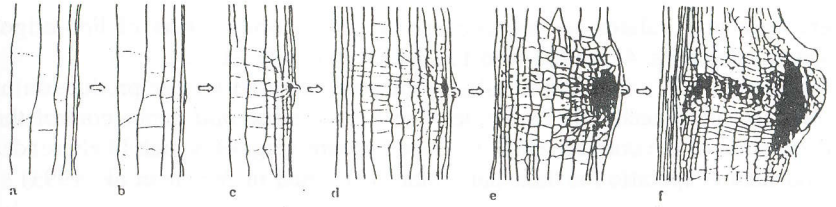
2. The early events of nodule development in a temperate legume root treated by purified Nod factors (part I), inoculated by *Rhizobium* bacteria (part II) or uninoculated (part III) (from Vijn et al., 1993). The Nod factors induce plant responses identical to those found in *Rhizobium*-infected roots, i.e. the differentiation of epidermal cells into root hairs and subsequent hair deformation (drh), cytoplasmic rearrangements (cr) of the cells in the outer root cortex resulting in the formation of aligned cytoplasmic bridges that serve as a track normally followed by the *Rhizobium*-induced infection thread, cell divisions in the inner root cortex leading to the formation of a nodule primordium (np).

radially oriented structures are observed (Fig. 2, part II). Since the infection thread apparently grows through and within these bridges, the site of its formation appears to be determined by the host plant, not by invading rhizobia (Van Brussel et al., 1992).

In contrast, in most tropical legumes, such as soybean, French bean and mungbean, the outer cortical cell regions immediately subjacent to the root epidermis (Fig. 3) are mitotically active (Calvert et al., 1984).

In the development of both types of nodules, the infection threads with proliferating rhizobia inside grow toward these nodule primordia. They ramify, penetrate the cells of the primordium central region and finally release the bacteria within the host cytoplasm enclosed in a peribacteroid host-derived membrane (Newcomb, 1981).

Subsequently, the nodule primordium differentiates into a mature nodule which provides a highly specialized microaerobic environment optimising the process of nitrogen fixation (Sprent, Raven, 1992).



3. Diagram illustrating the root nodule development in soybean (from Mathews et al., 1990). (a) Epidermis (the thin cell layer on the right-hand side of the figure) and the outer cortex of an uninoculated root. (b) Stage I: The outermost cells of the root cortex, subjacent to the epidermis, are mitotically active. (c) Stage II: Divisions of the outer cortical cells progress, root hair deformation and infection thread formation occur. (d) Stage III: The inner cortical cells begin to divide. (e) Stage IV: A nodule meristem becomes visible. (f) Stage VIII: The vascular bundle that connects the nodule vascular system with the root stele starts to differentiate.

Temperate legumes, such as pea, vetch, clover and alfalfa, form cylindrical indeterminate nodules and have a persistent apical meristem (Hirsch, 1992).

The spherical nodules of tropical legumes presented above have a determinate growth pattern. Meristematic activity is limited to an early stage of nodule growth, that is followed by a period of extension growth (Hirsch, 1992).

The rhizobia continue to divide in the infected nodule cells and progressively differentiate into endosymbiotic nitrogen-fixing bacteroids (Newcomb, 1981).

EARLY SIGNAL EXCHANGE(S) BETWEEN PLANT AND BACTERIA

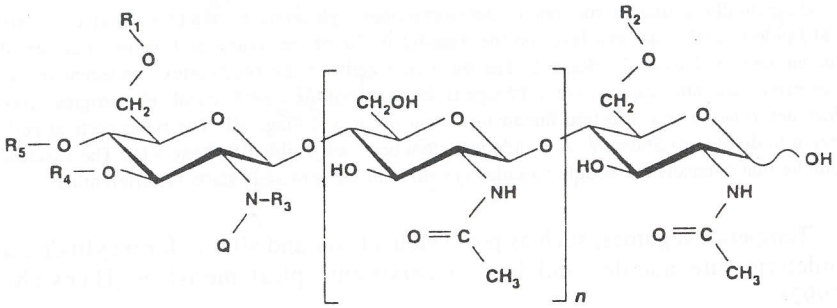
Nod factors as a primary bacterial signal

Nodulation is a host plant-symbiont specific process. Of various species and biovars of (*Brady*) *Rhizobium*, usually only one is able to nodulate a particular set of plants and to fix nitrogen efficiently (Smit et al., 1992). Thus, a precise exchange of molecular signals between these bacteria and the host plant that temporally and spatially coordinate the expression of genes specific for nodulation is essential to the development of effective root nodules (Verma, 1992).

Flavonoids secreted by emerging root hairs represent one class of signals. These phenolic compounds serve as chemoattractants and in concert with the constitutively produced rhizobial NodD protein induce the expression of the other bacterial *nod* genes. Subsequently, the encoded Nod proteins (various

enzymes or regulatory proteins) cause the production of a set of lipo-oligosaccharides (Fig. 4), called Nod factors (Vijn et al., 1993).

The *nod*ABC, common to all rhizobia, are required for the production of the core oligosaccharide molecule. The host-specific *nod* genes control the decoration of the core molecule with side groups (Fig. 4), which likely render Nod factors specific for their particular host (cited in Vijn et al., 1993).



	R. leguminosarum bv. *viciae*	*R. meliloti*	*R.* sp. NGR234
n	2 or 3	1, 2, or 3	3
Q	$C_{18:1}$ or $C_{18:4}$	$C_{16:2}$	$C_{18:1}$ or $C_{16:0}$
R_1	CH_3CO or H	CH_3CO or H	H
R_2	H	SO_3H	2-O-methylfucose or substituted with either 3-O-CH_3CO or 4-O-SO_3H
R_3	H	H	CH_3
R_4	H	H	NH_2CO or H
R_5	H	H	NH_2CO or H

4. Generalized structure of Nod factors, i.e. mono-N-acylated-chitin oligomers (from Vijn et al., 1993). The sugar backbone and the acyl moiety (*Q*) are present in all Nod factors. The number (*n*) of N-acetyl glucosamine residues, the length and the number of unsaturated bonds of *Q* can vary.

Biological activity of Nod factors

These signal molecules induce a complex series of early host plant responses, including root hair deformation (Lerouge et al., 1990; Spaink et al., 1991) and cortical cell divisions that lead to nodule organogenesis (Roche et al., 1991). They are probably also involved in at least some steps of the infection process (Franssen et al., 1992), but by themselves they cannot elicit the infection thread formation (Hirsch, 1992).

Each of these events is marked by the Nod factor-induced expression of a set of nodule-specific plant genes, termed early nodulin genes (Vijn et al., 1993) according to the time of their synthesis.

How do Nod factors induce their effects?

Although it is clear that a few very closely related lipo-oligosaccharides are able to provoke a wide range of morphological responses in legume roots (Franssen et al., 1992), the mechanism by which Nod factors elicit the three processes presented above is unknown.

Nevertheless, recent studies indicate that differences in the amount and molecular structure of purified Nod factors appear to be critical for determining whether only root hair deformation or also cortical cell divisions occur.

For example, the addition of 10^{-11}M of purified *Rhizobium meliloti* Nod factor (NodRm-1) to its host alfalfa roots results in root hair deformation, while a concentration three orders of magnitude greater (10^{-7}M) is required for the stimulation of cortical cell divisions (Truchet et al., 1991).

The structure of the fatty acid moiety and O-acetyl substitution at the nonreducing end appear to be of less importance than the modification at carbon 6 of the reducing terminus (the presence or absence of sulphate group) in determining the root hair deformation of pea, vetch and alfalfa, but they are essential for the host-specific induction of nodule primordia (Franssen et al., 1992).

Since these morphogens have a very specific structure and act at nanomolar to picomolar concentrations, plant receptors or families of receptors are probably required to amplify the signal. At present such receptors have not been directly detected and the nature of possible receptor-Nod factor interaction(s) is quite unclear (Vijn et al., 1993).

Lectins as part of a receptor complex

Lectins as sugar-binding (glyco)proteins appear to be the most likely candidates for these receptor molecules also because some are specifically located at the tips of growing root hairs, the thin unpolymerized cell wall of which is most susceptible to rhizobial infection (Díaz et al., 1986; Hirsch, 1992). Moreover, lectins from different cross-inoculation groups primarily differ in sugar-binding specificity (Díaz et al., 1989).

The first evidence for the involvement of this unique lectin activity in determining host plant specificity has been presented by Díaz et al. (1989). The successful introduction of the pea lectin (*psl*) gene into roots of white clover, which does not belong to the pea cross-inoculation group, resulted in an extension of the clover host specificity range and allowed infection and delayed nodulation by *Rhizobium leguminosarum biovar viciae*.

Recently, Van Eijsden et al. (1992) using site-directed mutagenesis have produced a mutant pea lectin, the sugar binding site of which is modified by the substitution of Asn^{125} for Asp so that it completely loses its binding ability. In view of the highly conserved state of Asn^{125}, the same mutation effects can also be expected in other legume lectins. The repetition of experiments of Díaz et al. (1989) with this mutant *psl* gene (or other mutant lectin genes) could be useful in the identification of the precise role of sugar binding in the recognition of homologous symbionts.

Because legume lectins lack a transmembrane domain, a feature essential for all animal surface receptors, such a multivalent protein itself is unlikely to function as a receptor for Nod factor. However, lectins may be part of a complex that is receptive to Nod factor. Another possibility is that lectins bind Nod factors and interact with transmembrane proteins in the root hair membrane (Hirsch, 1992).

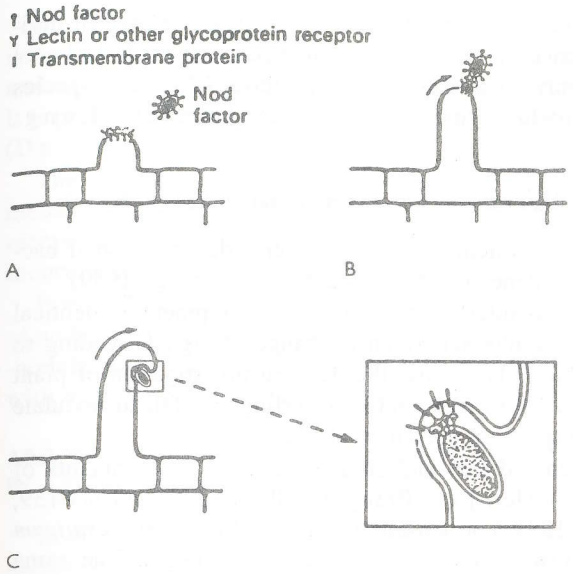
Nod factor-receptor model

How do the amount and molecular structure of purified Nod factors affect differences in plant response by the interaction with presumed plant receptors?

Hirsch (1992) gave an acceptable answer by proposing a very interesting model for rhizobial invasion (Fig. 5).

As mentioned earlier, 10^{-11}M NodRm-1 triggers root hair deformation, while 10^{-7}M stimulates cortical cell divisions in alfalfa roots (Truchet et al., 1991). One possibility is that there are two distinct receptors with a different affinity for NodRm-1, i.e. one receptor only for root hair deformation and another one for cortical cell division. The simplest explanation, however, is that a different plant response occurs depending on the degree of receptor cross-linking. In contrast to root hair deformation, cortical cell divisions (stimulated by the greater concentration of Nod factors) appear to require a larger number of receptors cross-linked and thus a more precise recognition between Nod factors and their receptors.

Nod factors with alterations in the length or the extent of unsaturation of the fatty acid elicit only the initial plant response, root hair deformation, in their own host. It is likely that changes in this lipid moiety which is inserted



5. The Nod factor-receptor model (from Hirsch, 1992). (A) Emerging root hair with equally distributed receptors on the plasma membrane at the tip. (B) Clustering („capping") of receptors and transmembrane proteins attached to them. (C) Growth of root hair continues only on one side, forming a tight curling that sets up a focused site for the initiation of the infection thread and subsequent successful *Rhizobium* invasion.

into the bacterial membrane result in an imprecise orientation of the glucosamine residues reacting with a sugar-binding site of a receptor. Thus, a number of receptors which are able to bind the Nod factors and become cross-linked is too small to generate cortical cell divisions (Hirsch, 1992).

ARE ROOT HAIR DEFORMATION AND CORTICAL CELL DIVISION RESPONSES MEDIATED BY A COMMON RECEPTOR?

The relationship between root hair deformation and cell divisions within the root cortex

The cellular mechanisms that control the earliest observable plant responses, i.e. root hair deformation and cortical cell divisions, at the molecular level are not known.

During nodulation of both soybean and alfalfa, the initial cortical cell divisions occur characteristically as early as the reactions of the epidermal root hair cells (Calvert et al., 1984; Dudley et al., 1987). It is possible to propose common requirements for both processes (for instance, sequential causation of one by other, or common cellular mechanisms or components underlying both phenotypes) (references in Dudley, Long, 1989).

The following observation supports the hypothesis that cortical cell divisions and root hair deformation are tightly coupled processes. Marked root hair curling is observed only on alfalfa infected by those *Rhizobium* species that are able to stimulate nodule organogenesis as well (Dudley, Long, 1989).

Plant mutants lacking root hair curling and failing to nodulate

In many plant–*Rhizobium* systems, both early events do not occur if bacterial strains are mutated in genes *nodABC* (Dudley, Long, 1989).

Plant and bacterial mutants interfere with nodule development at identical stages at which they closely interact by an exchange of signals leading to a cascade of gene expression. Therefore, the detailed investigation of plant non-nodulating mutants that both lack root hair curling and fail to nodulate should help to answer the question presented above.

Such mutants have been induced and studied in detail, e.g. mutants of soybean [*Glycine max* (L.) Merr.] cv. Bragg, *nod*49, *nod*772 and *nod*139, (Mathews et al., 1987, 1989a) or non-allelic mutant of pea [*Pisum sativum* (L.)] cv. Sparkle, R25 (*sym*8) (Markwei, LaRue, 1991). The same characteristics have been found in the spontaneous alfalfa (*Medicago sativa* L.) mutant MnNC-1008 (Dudley, Long, 1989) and soybean mutant rj_1 that is allelic to *nod*49 and *nod*772, and non-allelic to *nod*139 (Mathews et al., 1987, 1989a).

Under controlled experimental conditions, however, these so-called non-nodulating mutants form a few morphologically and anatomically normal nodules in response to certain rhizobial strains, known as overcomers, as shown in mutants rj_1, *nod*49 and *nod*772 (La Favre, Eaglesham, 1984; Mathews et al., 1987, 1989a; Heron, Pueppke, 1987).

It seems likely, therefore, that their genotypes do not influence the normal nodule developmental sequence, the induction of which is only delayed as observed by Pueppke and Payne (1987) in mutant rj_1.

The genetic control of the non-nodulation phenotypes

All of the non-nodulation characters of pea and soybean mutants presented here are associated exclusively with root genotypes and behave as simple monogenic Mendelian recessives (Markwei, LaRue, 1991; Mathews et al., 1989c, 1992).

The fact that the absence of root hair curling is controlled by the same gene as the production of no nodules suggests that these plant responses are cell-autonomous events, but each involves the same host factor such as a receptor.

In a spontaneous mutant of alfalfa, MnNC-1008, the non-nodulation trait is conditioned by two unlinked, tetrasomically inherited, recessive genes (nn_1 and nn_2). The nulliplex condition at both loci is necessary for non-nodulation. It is possible that nn_1 and nn_2 are redundant genes encoding the same function (Dudley, Long, 1989).

The recessive nature of the inheritance of all non-nodulating phenotypes indicates that the mutations might affect Nod factors-recognizing ability of receptors in such a way that the capability of non-nodulating mutants to induce successfully early nodulation events is reduced.

Even inoculation with very high concentrations of rhizobial strains capable of nodulating mutant rj_1 does not completely overcome host resistance. Non--nodulating soybeans had fewer nodules than the wild type plants inoculated with much lower doses (La Favre, Eaglesham, 1984).

ARE THE NON-NODULATING MUTANTS AFFECTED IN A GENE ENCODING A RECEPTOR?

In all non-nodulating mutants of soybean and pea described above it was demonstrated that the absence of root hair curling is not due to the change of bacterial adsorption (Suganuma et al., 1990; Markwei, LaRue, 1992; Mathews et al., 1993).

The exudates of the soybean mutants *nod*49 and *nod*139 (Mathews et al., 1989b; Sutherland et al., 1990), the pea mutant R25 (Markwei, LaRue, 1992) and the alfalfa mutant MnNC-1008 (Peters, Long, 1988) had similar inducing activities of *nod*ABC genes as compared with the wild type plants respectively, so that the nodulation process in these mutants was blocked at a stage subsequent to induction of the rhizobial *nod* genes.

Dudley and Long (1989) proposed the simplest interpretation of the MnNC-1008 non-nodulation phenotype as a block in the perception, or transduction and transmission, of the signals produced by *nod*ABC genes, i.e. the Nod factors.

It has been reported that soybean mutants (*nod*49, *nod*772, *nod*139 and rj_1) are affected in the capacity to initiate very early developmental stages, i.e. root hair curling and sub-epidermal cell divisions (Mathews et al., 1989a). Mathews et al. (1989b) and Sutherland et al. (1990) indicated that the non-nodulation soybean phenotype may be caused by an inability to respond efficiently to rhizobial Nod factors, which normally stimulate cell divisions and concomitant root hair curling.

Suganuma and Satoh (1991), based on their finding that neither the absence nor reduced levels of flavonoids cause the lack of root hair curling in rj_1 soybean mutant, concluded that the mutation occurred in the gene that

regulates root hair curling after the expression of bacterial *nod*ABC genes, and that it may encode a receptor responsive to Nod factors.

HOW DO NOD FACTORS INDUCE DIFFERENTIATED ROOT CORTICAL CELLS TO START DIVIDING AND TO FOLLOW A NEW DEVELOPMENTAL PATHWAY LEADING TO FORMATION OF A NODULE PRIMORDIUM?

Endogenous gradients of both plant growth factors and bacterial stimuli determine further developmental fate of cortical cells that are preparing for cell divisions

In temperate legumes, root nodules are induced predominantly opposite to protoxylem poles of the root vascular system (Newcomb et al., 1979). It is clear that the susceptibility of cortical cells to become mitotically active is determined by their position in the root.

Like nodules, lateral roots usually arise opposite protoxylem poles, but cell divisions occur in the pericycle. An unknown factor that triggers the formation of a lateral root primordium also stimulates the endodermal and cortical cells of the parent root to divide. Moreover, it seems likely that the requirements for the expression of some genes during the initiation of each structure are the same. It is possible that chemically similar signals, i.e. oligosaccharides or glycolipids, may operate through a common signal transduction pathway to generate these different structures (Hirsch, 1992).

Libbenga and Boger (1974) postulated that the information where cell divisions can be stimulated in the root cortex is controlled by a compound of the root stele extract with cytokinin-like activity. The structure of a purified stele factor capable of triggering cell divisions in pea root explants has not yet been elucidated (Vijn et al., 1993). Moreover, Díaz et al. (1986) demonstrated that lectin is specifically located on the surface of root hairs opposite protoxylem points.

It seems likely that the interplay of at least two oppositely oriented morphogen gradients may determine whether cortical cells divide to form the nodule primordium or become arrested in the G2 phase of their cell cycle as preparation for penetration by the growing infection thread. One morphogen is the stele factor released from the protoxylem pole, and the other is the Nod factor itself or a Nod factor-derived, hypothetical secondary signal (Vijn et al., 1993).

Such gradients are different in legumes like soybean and bean, because early root nodule initiation takes place in the outer cortex instead of the inner cortex, another type of infection thread is formed and pre-infection structures may not occur (references in Van Brussel et al., 1992).

ARE NOD FACTORS OR THEIR ACTIVE PARTS TRANSPORTED BY A SPECIFIC MECHANISM TOWARD THE INNER CORTEX OR ARE SECONDARY SIGNAL MOLECULES ELICITED IN ROOT EPIDERMIS?

The signal transduction pathway that starts with the recognition of Nod factors and culminates in nodule formation is unknown. Clues about this process have been obtained from studies in which the activity of the Nod factor is (at least partly) mimicked by other compounds.

Torrey (1986) was first to show that the application of N-(1-naphthyl)phthalamic acid (NPA), known to block auxin transport in stems to clover roots, induces the formation of nodule-like structures. Analogous structures have been observed in response to treatment of alfalfa roots with NPA or 2,3,5-triiodobenzoic acid (TIBA) (Hirsch et al., 1989).

Recently, Van de Wiel et al. (1990) have found that these so-called pseudonodules morphologically resemble bacteria-induced nodules and, moreover, some early nodulin genes are expressed at positions similar to those in regular nodules.

It has been reported that flavonoids might function as endogenous auxin transport inhibitors (ATIs). Since the key enzyme in biosynthesis of flavonoids is chalcone synthase (CHS) and in pea CHS mRNA is present in all cells of the nodule primordium, it is very likely that flavonoids accumulate in these dividing cells (see references in Yang et al., 1992).

Thus, hypothetically, a step in the signal transduction pathway induced by the Nod factors is a local increase in flavonoid concentration which causes a decreased import of auxin into the inner cortical cells. Subsequently the normal endogenous auxin/cytokinin balance would change in such a way that mitotic activity is induced (Yang et al., 1992). Such a model is, however, completely speculative at present.

In general, auxin may initiate DNA replication, while cytokinin may induce mitosis in G2-phase cells. But the mechanism of action of these hormones at the cellular level is unknown. However, it is generally assumed that these phytohormones, like animal hormones, must bind to specific receptor proteins (see references in Taiz, Zeiger, 1991).

A common feature in a great number of metabolic responses to hormonal signals is the involving of membrane G proteins (GTP-binding proteins). Some hormone-receptor complexes in animals act through such G proteins to stimulate adenylate cyclase or phospholipase C, the two different enzymatic systems embedded in the plasma membrane. Activated enzymes, adenylate cyclase and phospholipase C, produce cyclic AMP (cAMP) and inositol triphosphate (IP_3) respectively, functioning as second messengers that relay the signal to the cytoplasm. cAMP stimulates an enzyme called protein kinase

that in turn activates the next enzyme in an enzyme cascade. IP_3 mediates the release of Ca^{2+} from ER and vacuoles into cytosol by opening plasma membrane calcium channels. Ca^{2+}, acting alone or bound to a regulatory protein called calmodulin, regulates activity of a number of enzymes, including protein kinases (Taiz, Zeiger, 1991). A complex of conserved proteins called MPF (maturation-promoting factor) that is required for a cell to progress from late interphase (G2) to mitosis also belongs to a family of protein kinases (Campbell, 1993).

Because G proteins, phospholipase C, protein kinases (see references in Taiz, Zeiger, 1991) and MPF (see references in Verma, 1992) have been recently identified in higher plants, the existence of similar signal transduction pathways in legumes is likely.

With respect to the size and complexity of NodRm-1, the effect on nodulation of which has been in detail studied, Hirsch (1992) proposed that this glycolipid or any similar molecule is unlikely to diffuse intact across plant membranes. Furthermore, the existence of alfalfa Nar^+ (Nodulation in the Absence of *Rhizobium*) phenotype controlled by a single dominant gene suggests that a second signal, the production of which is normally optimized by *R. meliloti*, is permanently generated within plant tissues and propagated to the inner cortical cells (see references in Hirsch, 1992).

However, it is also possible that only part of the Nod factor is active in signal transduction, and the remaining part of this molecule allows the Nod factor transport to the receptive cortical cells.

Further investigations to answer to this question will focus in particular on the identification of the putative receptor molecule using the chemically synthesized Nod factors with specific labelling (see references in Vijn et al., 1993).

THE NUMBER OF LEGUME NODULES IS CLOSELY REGULATED BOTH BY PLANTS AND BY ENVIRONMENTAL FACTORS

Inhibition of nodule formation by nitrate and by internal (autoregulatory) mechanism

Development of N_2-fixing nodules on legume roots upon invasion of rhizobia is subject to regulation both by environmental factors and by internal (or autoregulation) control mechanisms.

Several environmental conditions, including light intensity, temperature, soil constituents (e.g. nitrate, trace elements, pH, water), or stress (e.g. browsing, pesticides) influence nodulation (Lee, LaRue, 1992b).

Under optimum conditions for plant growth, soil nitrate represents a major external factor controlling the extent of symbiosis. Small amounts of nitrate stimulate nodulation. However, above these minute concentrations the symbiosis is suppressed (see references in Carroll et al., 1985b). The mechanism of this inhibition by nitrate is not completely known.

In soybean, subterranean clover and alfalfa, it has been demonstrated that the nodule number and their relative localization on the root system is tightly regulated by an autoregulatory mechanism known as feedback. Newly emerging nodules in one part of the root, prior to the onset of nitrogen fixation, systematically suppress subsequent nodule development in ontogenetically younger root tissues (see references in Caetano-Anollés, Gresshoff, 1990).

Plant mutants with diminished autoregulation response

Carroll et al. (1985a) have isolated several nitrate-tolerant symbiotic (*nts*) soybean mutants. Mutant *nts*382 was supernodulating, i.e. it showed significantly enhanced nodulation than the parent cultivar Bragg in both absence and presence of otherwise inhibitory levels of exogenous nitrate.

Supernodulation and tolerance of nodulation to nitrate are controlled by the same gene in nts382 (Carroll et al., 1985b). The *nts* locus was molecularly mapped close to marker pA-132 (less than 1% recombination) on the USDA/ARS soybean RFLP map (see references in Delves et al., 1992).

In addition, this mutant had similar nitrate reductase activity like the wild type and was still sensitive to repression of nodulation at very high NO_3^- levels (Eskew et al., 1989).

It seems probable, therefore, that mutant *nts*382 is affected in an autoregulatory gene controlling the nodule development and not in a gene directly associated with nitrate metabolism, and at least one component is common to the external and internal regulatory systems (Delves et al., 1987).

It has been also postulated that in wild-type plants the inhibitory effect of this autoregulatory signal which originates in the shoot on nodulation may be enhanced at the level of the root by interaction with nitrate. The nitrate tolerance of nodulation in nts382 is thus a consequence of the altered autoregulation signal (see references in Delves et al., 1987).

Plants with the similar supernodulation characters have been also isolated in pea (Jacobsen, Feenstra, 1984; Duc, Messager, 1989) and common bean (Park, Buttery, 1988). The chemical nature of the autoregulatory signal molecule appears to be relatively well conserved and widespread, which indicate a similar autoregulation mechanism in a range of different legumes (Delves et al., 1987).

The general model for regulation of nodule formation in soybean

Recently, on the basis of intensive investigations of the soybean cv. Bragg mutants *nts*382, *nod*49 and *nod*139 that appear to have an altered internal

regulatory mechanism. Caetano-Anollés and Gresshoff (1990, 1991) proposed a general model for the regulation of nodule formation in soybean (Fig. 6).

The central step of this model is the production in response to a translocatable signal *Q* originated in the clusters of dividing hypodermal cells (stages I and II, Fig. 3), of a shoot-derived inhibitor (SDI). This „second messenger" slows down the rate of further cortical cell divisions, and in this way it suppresses the emergence of the developing nodules in ontogenetically younger root tissues. Other systemic signal molecules produced during pre-infection stage, *X*, might act as positive feedback regulators of the formation of first nodules. Their action can be either direct or mediated by the shoot.

The supernodulation phenotype is related to a much reduced ability to convert the root signal *Q* into the SDI, while the non-nodulating mutants result from mutational blocks in early nodule developmental stages (Francisco, Akao, 1993).

It was proposed that, at the root level, nitrate inhibition and autoregulation of nodulation interact, with nitrate probably depending on the SDI, to exert their suppressive effect on nodulation synergistically by slowing down the rate of progress of infection. Nitrate inhibition increases the strength of the shoot signal by either decreasing its metabolism or increasing the roots sensitivity to it (see references in Delves et al., 1992; Francisco, Akao, 1993).

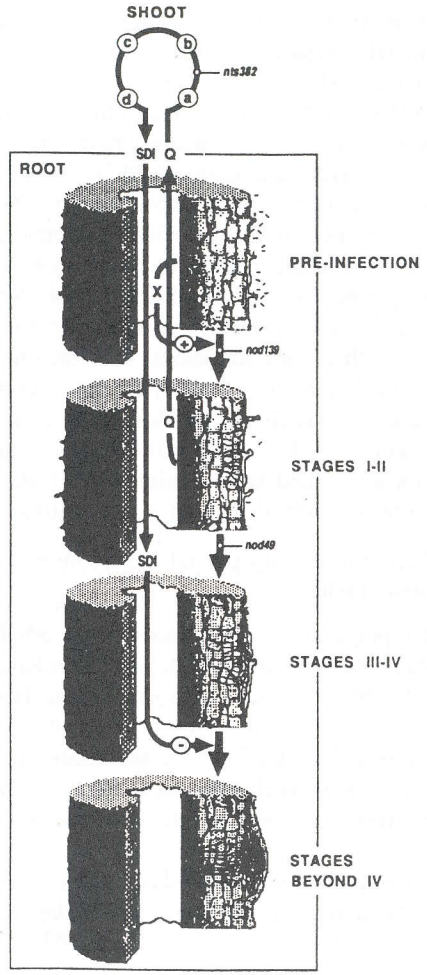
Considering some more recent results it appears to be necessary to reevaluate the nature of supernodulation phenotype of *nts*382, which was interpreted by Olsson et al. (1989) and Caetano-Anollés, Gresshoff (1990) as a substantial decrease in the autoregulation response.

The fact that autoregulation of nodule number still functions despite shoot apex removal of soybean plants suggests that the shoot tips are not involved in the production of SDI and that the leaf, representating the predominant tissue, is the most likely source (Delves et al., 1992).

The comparison of nodule distribution on the primary and lateral roots of cv. Bragg and mutant *nts*382 in soybean provided evidence that this supernodulating mutant exhibits an active autoregulatory control of nodule formation. This phenotype appears to be related to a defect in the systemic response regulating nodulation and not in autoregulatory or nitrate response mechanisms (Caetano-Anollés, Gresshoff, 1993).

Non-nodulating and temperature-sensitive plant mutants

Temperature also markedly influences the nitrogen-fixing symbiosis. Root hair infection, nodule initiation and development, and nitrogen assimilation



6. A general model of the regulation of nodule formation in soybean (from Caetano-Anollés, Gresshoff, 1990). One or more systemic messengers (a translocatable signal *Q*) originated in the early subepidermal cell division foci (stage I and II, see Fig. 3) trigger in the shoot a cascade of events finally resulting in the production of a shoot-derived inhibitor (SDI). This second messenger arrests the infection development suppressing further nodulation in ontogenetically younger root tissues. During pre-infection, other stimulatory signals are released (*X*) that act systemically as positive feedback regulators of nodulation. These molecules may be of importance during the formation of first nodules. The sites of mutational blockage in the mutants of soybean, *nod*49, *nod*139 and *nts* are indicated. Symbols: a–c = unknown precursors of SDI, + = positive action, – = suppression through blockage

are retarded at lower rhizosphere temperatures, while higher non-permissive temperatures similarly reduce nodulation or accelerate nodule senescence (Fearn, LaRue, 1991a).

Davis et al. (1986) induced two temperature-sensitive, non-allelic recessive mutants of chickpea which do not nodulate under restrictive conditions (29 °C), but produce effective nodules at 24 °C. Cooler temperature permits nodule formation not only in the chickpea mutant (24 °C), but also in pea *sym*5 mutants. This temperature effect did not depend on the strain.

One of these allelic recessive mutants at the *sym*5 locus, designated E2, has an unusual temperature sensitivity. Exposure of its roots to a temperature of 12 °C for 6 h, early in the infection process, significantly increased nodulation. This suggests that once the block is overcome at an early stage of nodule formation, further nodule development can continue at the non-permissive temperature (20 °C) (Fearn, LaRue, 1991a).

Moreover, Fearn and La Rue (1990) have found that four of these *sym*5 mutations are associated with an alteration in the mobility of a constitutively expressed 66-kD peptide which is not cultivar-specific.

The inhibitory effect of different external factors on nodulation may be mediated through ethylene

Several recent reports suggest that endogenous ethylene may be involved in regulating nodule number, but though direct evidence for this is lacking.

The addition of inhibitors of ethylene biosynthesis or action [Co^{2+}, aminoethoxyvinylglycine (AVG) or Ag^{+}] to the substrate increased nodulation of the *sym*5 mutants at 20 °C. Because the roots of these mutants do not overproduce ethylene, it is likely that an early stage in nodule development may be more sensitive to normal levels of ethylene (Fearn, La Rue, 1991b).

AVG and Ag^{+} also partly restored nodulation in another non-allelic mutant in the pea cv. Sparkle, E107 (*brz*) characterized by low nodulation and excessive ion accumulation, most notably Fe^{3+} and Al^{3+}. The plants showed an increase in ethylene production under stress conditions (brought on by metal toxicity in this case) (see references in Guinel, LaRue, 1992).

Recently, Lee and LaRue (1992a) have demonstrated for the first time that low concentrations of exogenous ethylene applied on young intact plants during the time of nodule initiation inhibit nodulation in pea cv. Sparkle. A similar ethylene sensitivity of nodulation has been also observed on sweet clover and on pea mutants that are hypernodulating or form non-fixing ineffective nodules.

In the pea cv. Sparkle, Lee and LaRue (1992a) have determined two stages at which exogenous ethylene blocks nodule development. It aborts

infections at the stage of the passage of the infection thread from epidermis into the inner cortex, and it is likely to inhibit the initiation of the onset of cell division.

The pattern of developmental stages of infections in mutant E107 is similar to that observed on roots of cv. Sparkle treated with exogenous ethylene (Lee, LaRue, 1992a). In the E2 mutant, the infection threads progress to the inner cortex, but the stage of initial cell divisions in the inner cortex is blocked (Guinel, LaRue, 1991).

Similarly, exposure of roots of cv. Sparkle to light induces an increased ethylene production by roots and reduces nodule number, which is restored after treatment with Ag^{+} (an inhibitor of ethylene action). Therefore, endogenous ethylene seems to mediate the inhibitory effect of light on nodulation (Lee, LaRue, 1992b).

Previously, an interaction between NO^{3-} and the soybean autoregulation signal during NO^{3-} inhibition of further nodulation has been suggested. That endogenous ethylene could be also involved in this regulation of nodule number was proposed recently by Ligero et al. (1991), because (a) inoculation with *Rhizobium* and/or the presence of NO^{3-} increased ethylene production in alfalfa roots and (b) the inhibitory effect of NO^{3-} on nodulation was apparently eliminated by AVG. This factor may accelerate the maturation of the root tissue and thus shorten the transient susceptibility of the root cells to infection, resulting in a decreased probability that root cells will be infected or that fewer infections will develop into nodules (Ligero et al., 1991).

The fact that auxin reduces the cell division frequency in root primary meristems by inducing ethylene production (see references in Ligero et al., 1991) supports the idea that ethylene might function as the primary effector also in the process of NO^{3-} inhibition of nodulation.

However, Lee and LaRue (1992b) demonstrated that nitrate affected alfalfa nodulation at an earlier stage than did exogenous ethylene, i.e. before thread formation.

CONCLUSIONS AND PROSPECTS

Root nodule formation is a complex multi-step morphogenic process which is the result of a specific gene to gene interaction between leguminous plants and rhizobia, and their mutual symbiotic nitrogen-fixing relationship is also affected by environmental factors.

At the present it is not still fully clear whether Nod factors control plant morphogenesis either directly or indirectly through more or less complicated transducting mechanisms. The availability of NodRm-1, the total chemical

synthesis of which was recently reported by Wang et al. (1994), will facilitate studies on the precise structure and localization of putative receptor molecules in alfalfa.

Until recently, more than fifty bacterial genes and several plant loci have been characterized (Caetano-Anollés, Gresshoff, 1991), and the frequency of newly induced mutants with a modified symbiosis is high, indicating that a large number of different genes must be involved in the symbiotic process directly or indirectly.

Valuable information about host factors involved in recognition, infection and control of nodule number could be gained by a closer investigation of the host-mediated responses so far proposed both in newly induced legume mutants and in partially characterized legumes. For example, in alfalfa where the formation of nodules is also controlled by an autoregulatory mechanism and a possible involvement of ethylene in the regulation of nodule number was discussed, it would be interesting to see whether some shoot-derived inhibitor slows down further cortical cell divisions as SDI does in soybean.

Although legume nodules are not highly modified lateral roots, the two organs share many similarities. Elucidation of the signal transduction program underlying lateral root organogenesis might be highly beneficial for determining some specific steps in the nodule developmental pathway. Consequently, greater emphasis needs to be placed on defining mutants in lateral root emergence.

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Rostlinní mutanti a přenos molekulárních signálů mezi leguminózami a rhizobii v průběhu symbiotického procesu – přehledná studie.

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Při nedostatku půdního NO_3^- produkují nově vytvořené kořenové vlásky rostlin z čeledi *Leguminosae* flavonoidy, které chemotakticky přitahují kompatibilní bakterie z rodů *Rhizobium, Bradyrhizobium a Azorhizobium* a současně u nich podmiňují syntézu celé řady blízce příbuzných lipooligosacharidů, tzv. Nod faktorů. Hostitelská rostlina koordinuje v čase a prostoru dvě morfogenetické odpovědi indukované Nod faktory v kořenech, tj. deformaci kořenových vlásků nezbytných pro tvorbu infekčních vláken a dělení kortikálních buněk, které vedou k tvorbě nodulových primordií. Bakterie se množí a postupují k centrální oblasti primordia prostřednictvím rostoucího infekčního vlákna. Infikovaná nodulová primordia se diferencují na zralé noduly poskytující velmi specializované mikroaerobní prostředí, ve kterém endosymbiotické bakteroidy přeměňují atmosférický N_2 na NH_4^+.

Mechanismus účinku bakteriálních Nod faktorů na molekulární úrovni je stále ještě diskutovanou otázkou. K objasnění těchto složitých procesů významně přispívá studium rostlinných mutantů, které jsou blokovány v různých stadiích infekce nebo nodulové ontogeneze.

Nod faktory, které mají velmi specifickou chemickou strukturu a působí jako účinné morfogeny již při nízkých koncentracích, jsou zřejmě rozpoznávány molekulami lektinů, které mají afinitu k specifickým cukrům a jsou umístěny na vrcholcích rostoucích kořenových vlásků. Tyto glykoproteiny jsou pravděpodobně součástí komplexu, který představuje vlastní receptor pro Nod faktor, ale po navázání Nod faktorů mohou také interagovat s transmembránovými proteiny v membráně kořenového vlásku. Deformace kořenových vlásků a dělení kortikálních buněk stimulované Nod faktory probíhají téměř synchronně a jsou kontrolovány jediným rostlinným genem. Předpokládá se, že tento gen kóduje receptor pro Nod faktor. Dělení kortikálních buněk, které je stimulováno větší koncentrací Nod faktorů než deformace kořenových vlásků, vyžaduje zřejmě větší počet receptorů s navázanými Nod faktory a tedy přesnější rozlišení mezi těmito bakteriálními signály a jejich receptory.

U leguminóz mírného pásma (hrách, vikev, jetel a vojtěška) může souhra opačně orientovaných gradientů dvou různých morfogenů v sektoru mezi kořenovým vláskem infikovaným bakteriemi a středním válcem kořene ovlivnit další osud kortikálních buněk, které se v této oblasti připravují k dělení. Vnější kortikální buňky se zastavují v G2 fázi buněčného cyklu, reorganizují svou cytoplazmu a vytváří radiálně orientované struktury, nazývané cytoplazmatické mosty, přes které prochází infekční vlákno, zatímco buňky vnitřního kortexu se dělí. Jedním z morfogenů je faktor, který je uvolňován z protoxylémového pólu a má podobnou aktivitu jako cytokinin, druhým je Nod faktor nebo nějaký sekundární signál indukovaný Nod faktorem v kořenové epidermis. Nod faktor nebo hypotetický sekundární signál pravděpodobně způsobují lokální zvýšení v koncentraci flavonoidů, které inhibují transport auxinu do vnitřních kortikálních buněk. Změna v rovnováze auxinu a cytokininu indukuje mitotickou aktivitu těchto buněk. Výsledkem následného složitého morfogenetického procesu jsou válcovité nedeterminované noduly. U tropických leguminóz (sója, bob a fazol) existují odlišné gradienty, protože buněčná dělení probíhají ve vnějším kortexu a kulovité noduly mají determinovaný růst.

Počet nodulů a jejich relativní umístění na kořenovém systému leguminóz je kontrolováno jak faktory prostředí, tak vnitřními (neboli autoregulačními) kontrolními mechanismy. U sóje je inhibiční účinek autoregulace na vývoj nodulů v ontogeneticky mladších tkáních kořene zřejmě na úrovni kořene

zvyšován na základě interakce mezi autoregulačním inhibitorem (SDI), který pochází ze stonku (nebo z listů) a zpomaluje rychlost dělení dalších kortikálních buněk, a nitrátem. Tato interakce může být zprostředkována endogenním etylénem, jehož zvýšená produkce je odpovědí na bakteriální infekci a nebo přítomnost NO_3^-. Protože chemická povaha autoregulační signální molekuly je relativně dosti konzervativní a široce rozšířena, lze očekávat podobné autoregulační mechanismy i u jiných leguminóz.

symbióza mezi leguminózami a rhizobii; Nod faktory; lektin; kontrola nodulů; rostlinní noduloví mutanti

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