Chapter 2

Literature Survey

2.1 Importance of *B. japonicum* to agriculture

B. japonicum has been used as biofertilizers for soybeans in many parts of the world including Australia (http://www.plantmanagementnetwork.org) and India (http://www.keralaindustry.org). *B. japonicum* has an intrinsic ability to fix atmospheric nitrogen according to the following equation :

$$N_2 + 8H^+ + 16MgATP \xrightarrow{\text{Nitrogenase}} 2NH_3 + H_2 + 16(MgADP + Pi)$$

Nitrogenase consists of an Fe protein and a Mo-Fe protein. The Fe protein which is approximately 64 kDa consists of two identical α subunits each of which is encoded by *nifH*. In *Azotobacter vinelandii* the two identical α subunits are joined by one 4Fe : 4S metal cluster. The crystal structure of the *Azotobacter vinelandii* Fe protein resembles a butterfly with the metal cluster as its head as shown in Figure 2.3.

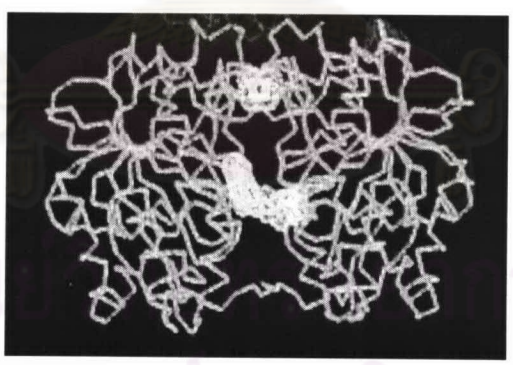
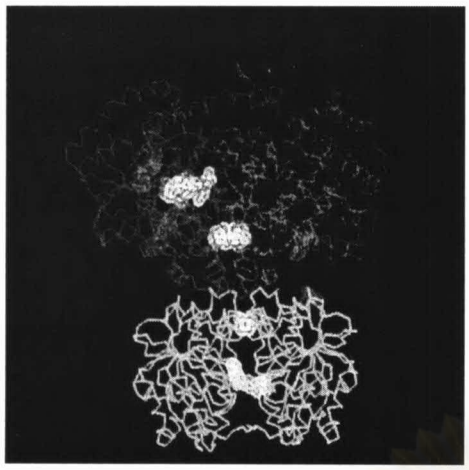


Figure 2.1 Ribbon representation of the Fe protein of nitrogenase of *Azotobacter vinelandii* at 2.9 °A. (Moffat, 1992)

The MoFe protein consists of two αβ subunits each of which is encoded by *nifDK*. The molecular weight of the $\alpha_2\beta_2$ structure is approximately 220 kda. There is one FeMo cofactor embedded in the αβ subunits. The crystallographic structure of the MoFe protein and the FeMo cofactor of *A. vinellandii* is shown in Figure 2.2 and Figure 2.3 respectively.



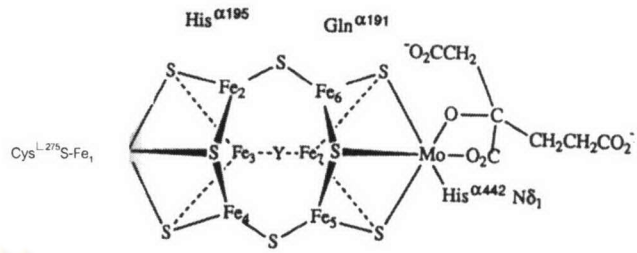


Figure 2.2 Ribbon representation of nitrogenase, made up of an Fe– protein (light blue) and a MoFe-protein. Metal clusters are shown in yellow (Moffat, 1992).

Figure 2.3 Schematic representation of the FeMo cofactor model (Kim & Rees, 1992).

Since the FeMo cofactor is embedded in the αβ subunits, Kim & Rees (1992) suggested that the transfer of two electrons is coupled with the hydrolysis of 2 molecules of MgATP resulting in the change of conformation of the αβ subunits in such a way that the electrons are further transferred to the FeMo cofactor and then to the N_2 substrate which binds to the MoFe protein (Figure 2.4).

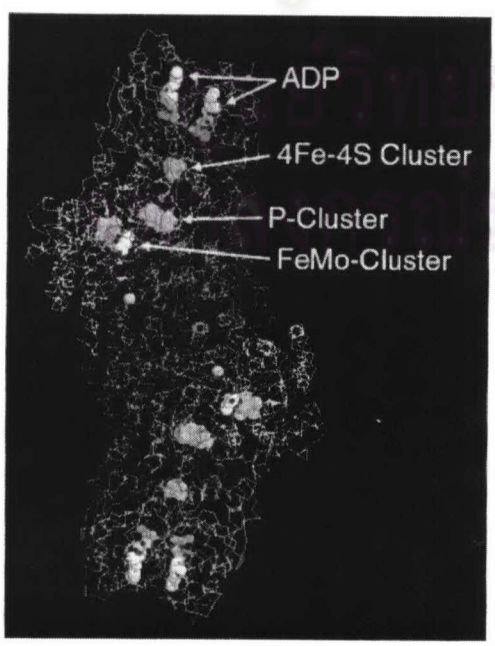
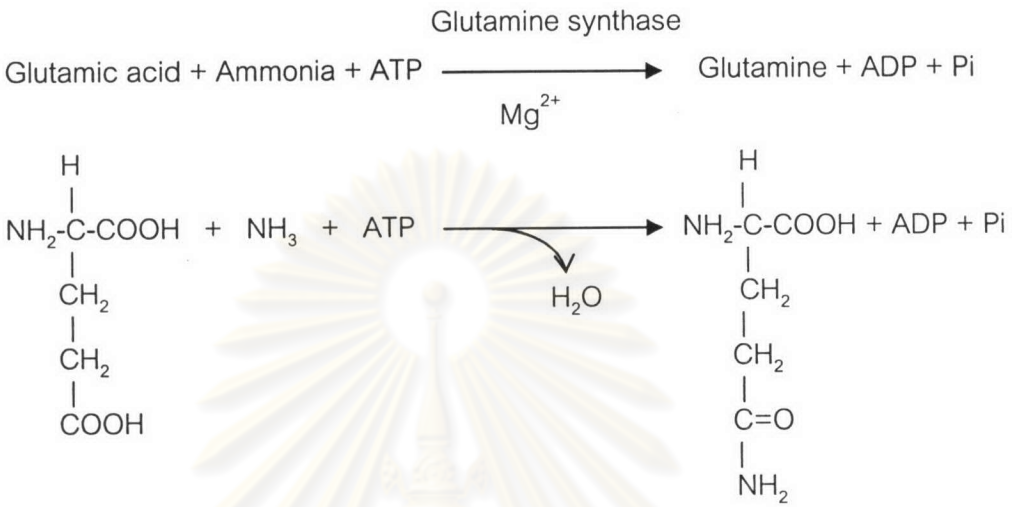


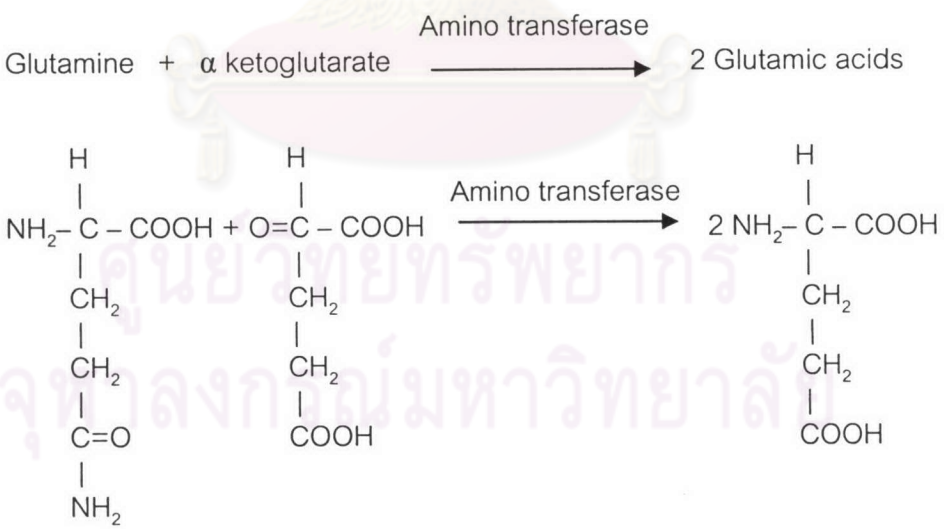
Figure 2.4 Metal clusters involved in the electron transfer in nitrogen fixation. (http://www.rcsb.org/pdb/molecules/pdb26_3.htm.)

Kim & Rees (1992) stated that MgATP and not ATP took part in the reaction possibly to prevent reversible reaction.

The ammonia obtained from the nitrogen fixing reaction is utilized by soybeans in the following reactions :



The Glutamine formed reacts with α ketoglutarate to form two molecules of Glutamic acid which enter the amino acid synthesis pathway catalysed by appropriate amino acid transaminase(s) (Voet & Voet, 1995).



Nitrogen fixation by *B. japonicum* thus provides amino acid building blocks for soybean protein synthesis resulting in reduction in the use of chemical fertilizers. Table 2.1 shows the growing amounts of fertilizers imported into Thailand in the years 1999-2003.

Table 2.1 Quantities and values of fertilizers imported into Thailand during 1999-2003

Year	Quantity (Ton)	Value (Baht)
1999	3,561,593	17,189.93
2000	3,198,290	18,229.97
2001	3,455,702	21,604.95
2002	3,669,353	22,112.20
2003	4,717,586	26,402.94

(Source : Office of Agricultural Economics in co-operation with the Customs Office, 2004)

At present, Thailand relies on increasing amounts of soybean imports as shown in Table 2.2.

Table 2.2 Quantities and values of soybeans imported into Thailand during 1999-2003.

Year	Quantity (Ton)	Value (Baht)
1999	1,007,983	7,954.68
2000	1,320,402	11,473.79
2001	1,363,224	12,381.76
2002	1,528,557	13,927.64
2003	1,689,649	18,317.74

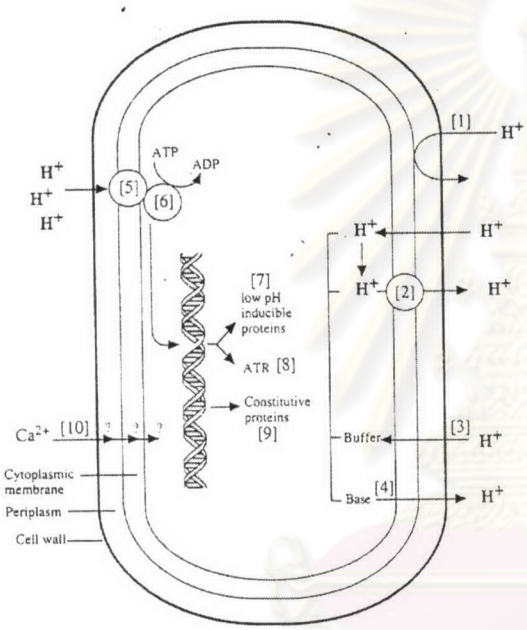
(Source : Office of Agricultural Economics in co-operation with the Customs Office, 2004)

Soybean productivity in Thailand is approximately 248 kg/rai (http://www.oae.go.th) which is far less than those in Brazil, China and the USA which are leading soybean growers and exporters. If Thailand could improve soybean productivity, the country will rely less on the import of fertilizers as well as soybeans resulting in reduction in trade deficit. Moreover, the increase in soybean productivity will result in higher income for soybean growers and other agro-business utilizing soybeans.

2.2 Anticipated types of intracellular proteins that could change upon pH shifts

2.2.1 Proteins involved in the synthesis and secretion of acidic or alkali products

So far the identities of *B. japonicum* acidic or alkali products secreted to the medium are not known. However it is expected that activities and/or quantities of some enzymes in their synthetic pathways should increase. In 1994 Glenn and Dilworth postulated mechanisms for acid tolerance in root nodule bacteria as follows :



(Glenn & Dilworth, 1994)

[1] Decrease in membrane permeability to H^+

[2] H^+ export mechanism (including in pump)

[3] Cytoplasmic buffering

[4] Alkalinization of external medium by base export

[5] Postulated environmental pH sensor(s)

[6] Postulated regulator(s) mediating between sensor(s) and DNA

[7] Transcriptional activation of low pH inducible proteins

[8] Transcriptional activation of ATR genes (Acid Tolerance Responses genes)

[9] Potential transcriptional activation of constitutively expressed *act* genes

[10] Unknown mechanisms involving Ca^{2+}

From the postulated mechanisms listed above, the first anticipated type of *B. japonicum* intracellular proteins that could change upon pH shifts due to liming of soil might consist of an increase in protein(s) that acts as proton pump(s) as well as a decrease in low pH inducible proteins as well as a decrease in acid tolerance responses proteins which are protein products of the as yet to be identified *act* genes. Goodson & Rowbury (1991) reported about *act* genes as follows : the enteric bacteria *Escherichia coli* acclimatized to growth at mild acidic pH responded favorably to other stresses such as UV light when compared to cells previously grown at neutral pH. Such mechanisms

operated when cells were previously grown at mild acidic pH which enabled the bacterial cells to be more tolerant to other stresses had been termed acid tolerance responses (ATR), encoded by *act* genes, which could trigger global response network to environmental stresses. The study of differential gene expression in response to changes of one growth parameter, namely changes in medium pH, might lead to further findings of *B. japonicum* responses to various interactive environmental factors which may have influences on growth, survival, establishment of symbiosis and nitrogen fixing potential.

Previous experiments on growth of *B. japonicum* in unbuffered YMB medium with initial pHs ranging from 4.0 to 9.0 indicated that when initial pHs were in the acidic range, final pHs were found to increase (Suwat Saengkerdsub, 2000). The results might indicate that base export took place. On the contrary, when initial pHs were in the basidic range, final pHs were found to decrease to around the neutral pH. The results might indicate secretion of acidic products. The nature of basidic and acidic products in rhizobia are unknown. O'Hara et al (1989) only reported that *Rhizobium meliloti* maintained alkaline intracellular pH when the external pH was acidic. The types of *B. japonicum* intracellular proteins which could change qualitatively or quantitatively could be the proteins in the pathway of synthesis of the basidic or acidic products.

B. japonicum is an interesting bacterium to study not only because of its importance in agriculture but also because of a vast number of unknown metabolic and regulatory pathways in the microbe. For example, at present it is unknown what regulatory pathway(s) is regulated by external pH in relation to liming of soils before planting of soybean seeds. The effects of changes in *B. japonicum* protein profiles are further complicated by the fact that (partial) anaerobic conditions under the soils may have effects on changes of intracellular protein profiles.

Anaerobiosis-inducible genes with unidentified products have been referred to as *ani* (Aliabadi et al, 1988). Identified anaerobiosis-inducible genes in *Salmonella typhimurium* include hydrogenase (*hyd*). The *earA* (external acidification regulator) mutations released *aniG* from pH control. Aliabadi et al (1988) reported that *earA* is the first gene reported to be involved in regulating the transcriptional response of enterobacteria to changes in pH. No such regulatory gene has been reported for *B. japonicum*.

2.2.2 Anticipated changes in nodulation gene expression upon pH shifts

Figure 2.5 shows the physical and genetic maps of nodulation genes in *B. japonicum*.

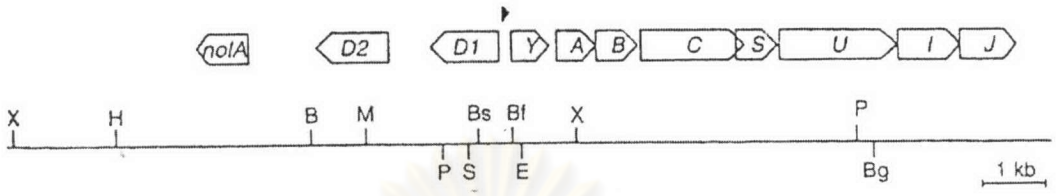


Figure 2.5 Physical and genetic maps of nodulation genes in *B. japonicum*. Arrow heads between $nodD_1$ and *nodY* indicate the direction of *nod* box which is the promoter of *nodYABCSUIJ* operon. B, *Bam*HI ; E, *Eco*RI ; H, *Hin*dIII ; S, *Sal*I ; P, *Pst*I ; X, *Xho*I ; Bs, *Bsp*HI ; Bf, *Bfr*I ; M, *Mlu*I ; Bg, *Bgl*II (Sanjuan et al, 1994)

The common nod genes found in rhizobia are *nodYABCSUIJ* which are arranged in one operon under the control of *nodD1* and *nodVW*. The common nod genes (*nodYABCSUIJ*) encode enzymes in the biosynthetic pathways of lipo-oligosaccharides (Nod factors). Nod factors are essential in the initial root hair curling and plant cortex cell division which lead to the formation of root nodules (Sanjuan et al, 1992 ; Smit et al, 1992 ; Spaink et al, 1991). The functions of NodC, NodB and NodA in the biosynthetic pathway of Nod factors are shown in Figure 2.6 The chemical structure of *B. japonicum* Nod factor is shown in Figure 2.7.

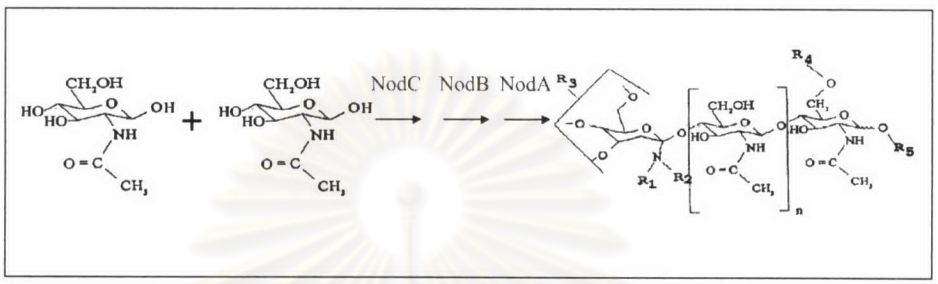
Functions of NodC, NodB, NodA

Nod C = N-acetyglucosaminyl transferase catalyses transfer of N-acetylglucosamine

Nod B = N- deacetylase catalyses removal of acetyl group at non-reducing unit

Nod A = N-acyltransferase catalyses transfer of acyl group

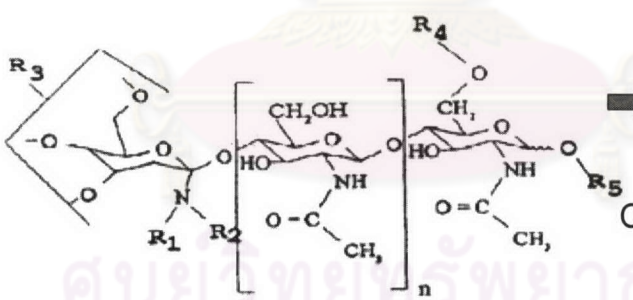
Synthesis of Nod factors



Other Nod proteins catalyse transfer of 2-O Methylfucose etc.

Figure 2.6 Functions of NodC, NodB and NodA in the biosynthesis of Nod factors (Stacey, 1995)

Lipo-chitin nodulation signals (Nod factors)



Root curling

Cortex cell division

Nodules

Strain	R1	R2	R3	R4	R5	n
B.japonicum USDA110	C18 :1	H	H	2-O-MeFuc	H	3

(2-O-MeFuc = 2-O-MethyFucose)

Figure 2.7 Chemical structure of *B. japonicum* Nod factor (Stacey, 1995)

One of the first effects of changes of medium pH on intracellular protein profiles could be the fluctuation in quantity of intracellular proteins involved in the initiation and control of root nodulation process such as NodD1, NodC, NodB and Nod A. Kosslak et al (1987) reported that root hairs of soybeans (*Glycine max*) secreted flavonoids such as Genistein, Daidzein and their derivatives such as 6-O-malonylgenistin, 6-O-malonyldaidzin, ?-O-Acetyldaidzein and Glycitin.

Flavonoids are composed of a C_6-C_3-C_6 core structure as shown in Figure 2.8. The chemical structures of Genistein and Daidzein as well as their derivatives are shown in Figure 2.9.

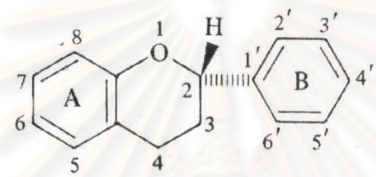


Figure 2.8 The core C_6-C_3-C_6 structure of flavonoids (Goodwin & Mercer, 1983)

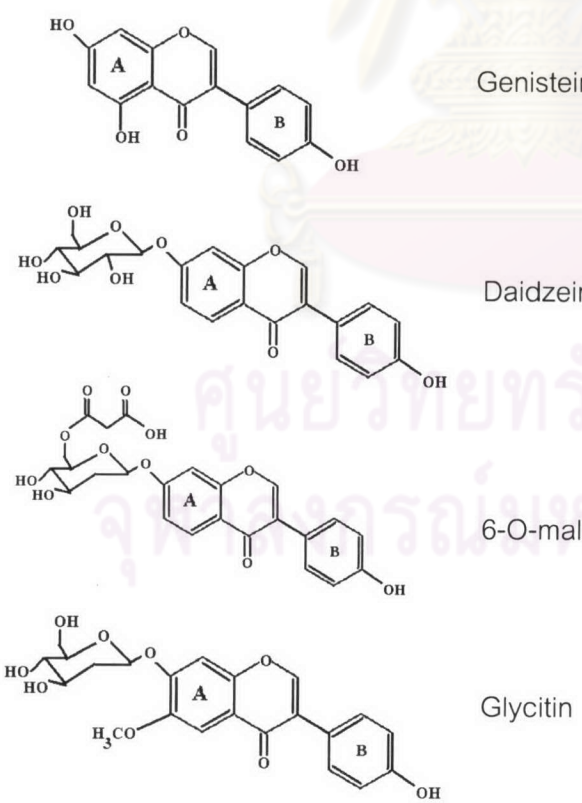


Figure 2.9 Chemical structures of Genistein, Daidzein and their derivatives (Goodwin & Mercer, 1983)

B. japonicum, with one subpolar flagellum, moves towards the root hair via chemotaxis along the flavonoids concentration gradients. The flavonoids get accumulated in the periplasm of the bacterial cell and bind to $NodD_1$ which is constitutively expressed. The flavonoid+$NodD_1$ complex binds to $nodY_1$ promoter (*$nodY_1$* box) and acts as an activator for the transcription of *nodYABCDSUIJ* operon. Nod C, Nod B, and Nod A are enzymes in the biosynthesis pathway of a Nod factor (Figure 2.6) which in essential for the processes of root hair curling and soybean root cortex cell division which lead to the formation of a root nodule. The Nod D_1+flavonoid complex also binds to the promoter of *$nodD_1$* (*$nodD_1$* box) to activate the transcription of Nod D_1 . The control of the nodulation gene expression is summarized in Figure 2.10.

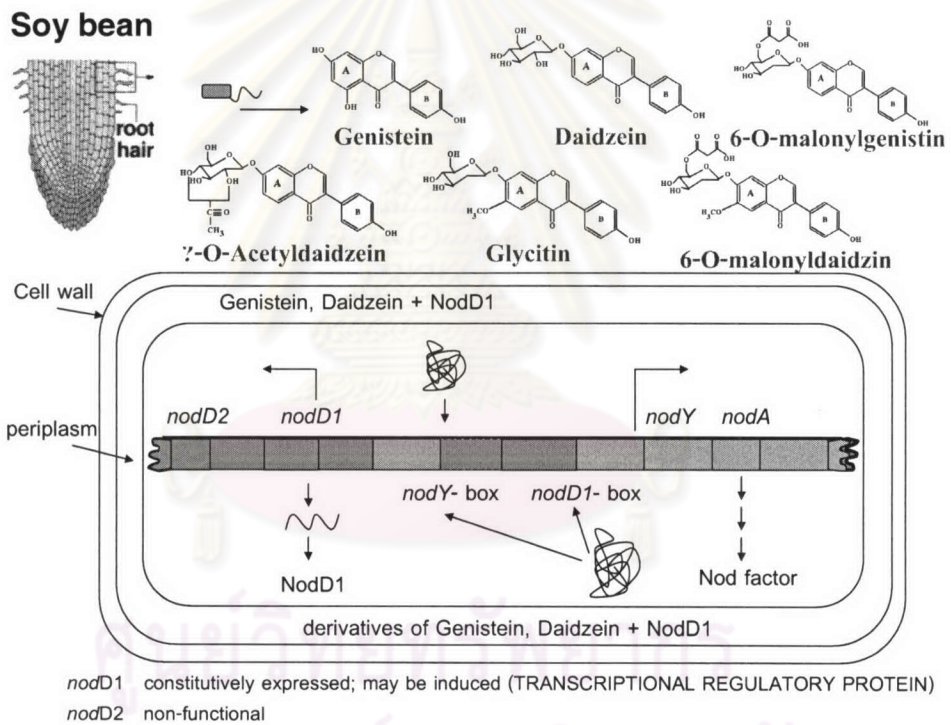


Figure 2.10 The transcriptional regulatory protein $NodD_1$ control of nodulation gene expression in *B. japonicum* (Smit et al, 1992 ; Stacey, 1995)

Richardson et al (1988) reported the use of *lacZ*-nodulation genes (*nod* genes) to follow *nod* gene expression when fast-growing *Rhizobium leguminosarum* biovar *trifolii* was grown in medium pH 4.8-6.5. The results indicated *nodD* was constitutively expressed in the pH range used in the experiments. So far there has been no report on the effects of pH on *nod* gene expression in the slow-growing *B. japonicum*. It is anticipated that abrupt changes in acidic soil pH due to liming could have effects on *B. japonicum nod* gene expression.