CHAPTER I

INTRODUCTION

Rice, the major food source for approximately one-half of the world's population, is grown for the most part on flooded soil. Annual rice yields seem to be depending on the nitrogen fertilizer supply. Since it is found that nitrogen is usually the limiting nutrient in both flooded soil and wethand. The major role of nitrogen fertilizer in increasing the production of lowland rice is well documented with hundreds of experiments throughout the world showing response to nitrogen. In the United States and other developed countries, rice planted in the fields is always heavily nourished with the nitrogen fertilizer, while rice grown in Asia receives very little fertilizer nitrogen (Buresh et al., 1980). Rice yields are lower in nonfertilized than in fertilized field, but through the years consistent yields of rice in Asia have been obtained in successive rice crops without supplement after of nitrogen fertilizer and with no appearently decrease in the nitrogen content of the soil (Swaminathan, 1984). The maintainance of nitrogen fertility in the rice paddy soils has been attributed to biological nitrogen fixation (App et al., 1978; Koyama and App, 1979).

1.1 Biological nitrogen fixation in paddy rice fields

The principal agents various types of nitrogen fixing microorganisms are reported in paddy rice fields: which are the free-

living blue-green algae (cyanobacteria), the symbiotic water fern; azolla-anabaena complex, the nonsymbiotic free-living soil bacteria and the associative N_2-fixing bacteria in the rhizosphere of rice. Among these four groups, associative nitrogen-fixing bacteria have received much attention during the last few decades since many investigators believed that they may be a major source of nitrogen in those nonfertilized rice fields (Trolldenier, 1977; Barraquio, 1979; Watanabe, 1979). Long term fertility trials with rice in the Temperate and Tropical regions have suggested that, there is a major input of nitrogen (35 to 50 kgN per crop) in flooded rice soils (Koyama and App, 1979). Using acetylene reduction and ^{15}N methods, Yoshida and Ancajas (1973), showed that under field conditions of rice (*Oryza sativa* L.) culture, nitrogen fixation ranging from about 3 to 63 kg/ha occurred in soil, water, and rice plant rhizosphere. The greatest fixation occurred in the rhizosphere and was more pronounced under flooded than under upland conditions. Lee et al., 1977 has found greater nitrogenase activity in the soil of planted areas of a rice field than in the soil of unplanted areas between plant rows. Use of ^{15}N-labeled nitrogen gas in a sealed chamber has confirmed the presence of nitrogen fixation in the root zone and incorporation of the fixed nitrogen into rice plants (Eskew et al., 1979; Ito et al., 1980).

In Thailand, one of the Asian countries where rice has been grown for centuries without applying excessive amount of chemical fertilizers (Swaminathan, 1984). Boonjawat et al.(1982) estimated that the amount of total nitrogen fixed by associative bacteria is in the range of 20-80 kg per hectare per crop.

Associative nitrogen fixation in flooded rice field is carried out by a large number of heterotrophic nitrogen fixing bacteria. The bacteria that have been isolated from the rice root include Azotobacter

(Purushothaman et al., 1976), Beijerinckia (Döbereiner and Rusche., 1961; Diem et al., 1978), Enterobacter (Watanabe et al., 1977), and Azospirillum (Kumari et al., 1976; Nayak and Rajaramamohan Rao 1977; Silva and Döbereiner, 1978). Association between rice and nitrogen-fixing bacteria has been suggested to provide nutrients to the rhizospheric bacteria by means of plant roots' exudate. Simultaneously, rice plants should benefit from associative nitrogen-fixing bacteria by several mechanisms beside fixed-nitrogen such as phytohormone (Okon and Kapulnik, 1986; Patriquin et al., 1983) and in case of protection from other pathogenic microbes.

1.2 Interactions between N_2-fixing bacteria and rice

Interaction between nitrogen fixing bacteria and plant has been extensively studied, especially in the physiological change aspect. Inoculation of 7-day-old rice seedlings cv. RD7 with *Klebsiella* sp. R15 and R17 resulted in the alteration root morphology (Limpananont, 1987) e.g. more branching, denser and longer root hairs comparing to uninoculated roots. Similar findings have also been reported in pearl millet (Tien et al., 1979; Umali-Garcia et al., 1980), Wheat (Patriquin et al., 1983; Jain and Patriquin et al., 1983; Patriquin, 1984) and Sorghum (Okon and Kapulnik, 1986) inoculated with *Azospirillum*. Working with *Klebsiella pneumoniae* as inoculant, Haahtela et al., 1986 inoculation of *P. pratensis* and *T. aestivum* seedlings with these bacteria. They reported that infected roots showed increased number of root hairs and decreased in the length of the zone of elongation.

These physiological changes indicated that plant growth regulators such as auxin, gibberellin- and cytokinin-like substances could be produced by the nitrogen-fixing bacteria (Jain and Patriquin, 1984; Jain and Patriquin et al., 1985; Okon and Kapulnik, 1986 and

Patriquin et al., 1983), and these hormonal effects have been believed to be more important than nitrogen-fixation effect, since it may enhance minerals uptake or improve water status (Vose, 1983; Patriquin, 1983 and Okon and Kapulnik, 1986).

Besides, the deformation of rice root, Limpananont, 1987 and Boonjawat et al., 1991, also reported that after 36 h of R15 and R17 inoculation, formation of numerous spherical structures (~ 10 μm), entrapping lived bacteria inside were formed on the root surface as evident by the Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM). Treatment of micronodulated roots with enzymes which break down glycosidic linkage such as, β-N-acetyl glucosaminidase, neuraminidase, and β-glucosidase and trypsin resulted in the damage of the micronodules structure and liberation of free bacteria. From these findings, some glycoproteins produced by plant had been proposed to play a role in adhesion among bacterial cells and root surface. Moreover, studies on the inoculation effect of various strains of bacteria: *E. coli*, *Klebsiella pneumoniae* M5a1, *Klebsiella oxytoca* NG13, *Klebsiella* strains R15 and R17, *Pseudomonas* H8 and *Azospirillum lipoferum* FS into different varieties of rice, HCCMM, RD5, RD7, IR42 and IR58, on micronodule formation and nitrogen fixing activity (Limpananont, 1987) demonstrated that there were successful associative N_2-fixation and micronodules between bacterial strains R15 and R17 only with the local rice cv. RD5 and RD7.

This complementary pairing between R15 or R17 and rice cv. RD7 and RD5 implies that the host-range specificity in associative relationship may exist in as well as the host specificity of Legume-Rhizobium symbiosis which was proposed that the specificity is mediated by lectin (Bohlool and Schmidt, 1974; Dazzo and Humbbel, 1975). Based on this hypothesis, Limpananont, 1987 has determined the

hemagglutinating activity of lectin (HA) in the root exudate of many rice cultivars and reported the relationship between HA and bacterial agglutination among themselves and rice root.

1.3 Involvement of rice lectin in associative relationship

Lectins are protein (or glycoproteins) of non-immune origin which agglutinate cells or precipitate glycoconjugates (Goldstein and Hayes, 1978). Evidences support for the hypothesis that rice lectin is an associative factor between *Klebsiella* and rice (Limpananont, 1987) are the followings: i) there are high lectin in root exudate of the host plant and high lectin receptors on the epidermal cells of rice root. ii) purified lectins from root, embryo and bran of rice cv. RD7, all exhibit similar sugar-binding specificity with N-acetylglucosamine (GlcNAc), which render the ability to agglutinate *Klebsiella* R15 and R17 via the presence of lectin receptors distributed on the glycocalyx and cell wall of these bacteria, iii) the binding or agglutination mediated by lectin can be inhibited by excess amount of GlcNAc. So these results indicate the functions of rice lectin as an associative factor in the adhesion between *Klebsiella* and rice.

1.4 Characteristics of rice lectin

Rice lectin is similar to other Gramineae lectins in its sugar-binding specificity to N-acetylglucosamine (GlcNAc) and its oligomers (Allen et al., 1973; Rice and Etlzer, 1974; Goldstein and Hayes, 1978). Rice lectin has been purified and characterized from rice bran (Tsuda, 1979, rice embryos (Peumans and Stinissen, 1983 Tabary, 1984; rice germ (Shen et al., 1984 and Poola et al., 1986). Although molecular structure of rice lectin reported by different research groups were different, at present it is generally accepted that the major subunit

of rice lectin is 18-kDa, which are preferentially undergo an additional proteolytic cleavage to yield two subunits of 8-kDa and 10-kDa (Stinissen et al., 1984). Amino acid sequence analysis indicated that rice lectin has high homology to wheat lectin or wheat germ agglutinin, both contain exceptionally high amount of half-cysteine (no free sulfhydryl groups) and glycine (Poola, 1986). Rice lectin does not dissociate of low pH value and it also has a four-fold higher specific agglutination activity than other cereal lectin (Peumans et al., 1983). Antiserum against wheat germ agglutinin cross-reacts with the rice lectin (Peuman et al., 1983). Comparison of amino acids from rice lectin and isolectin A of wheat germ agglutinin (WGA-A) indicated that rice lectin has high homology to wheat lectin since rice lectin exhibits 78% identity with WGA-A. And the amino acid sequence identity between rice lectin and WGA-B and WGA-D is only 44% and 33% respectively (Figure 1.1) (Chrispeels and Raikhel, 1991).

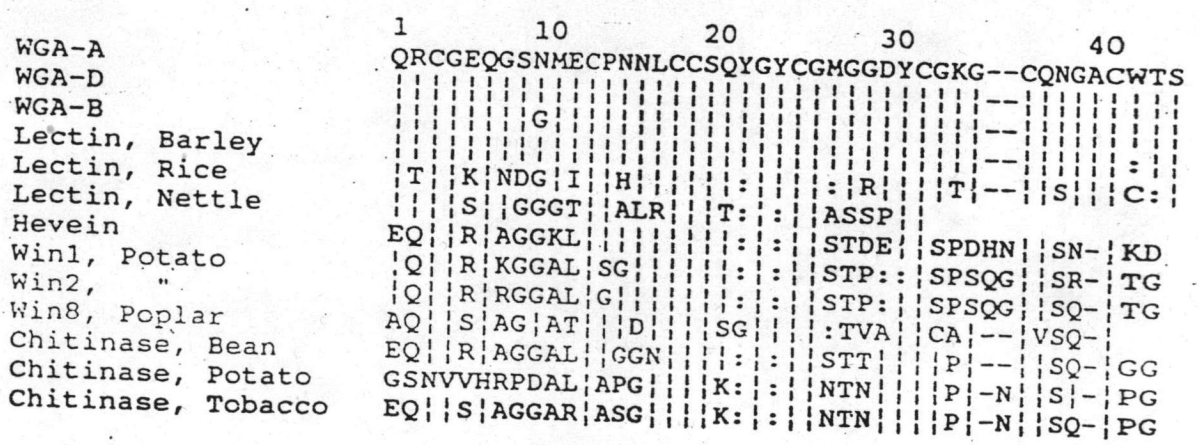

Figure 1.1 Sequences similarity of chitin binding domains in the family of chitin-binding proteins

1.4.1 Rice lectin : cellular, subcellular localization and other physiological role

In rice seed lectin has been localized in embryo, exclusively in the primary axes (Stinissen et al., 1982). In germinating rice seed, rice lectin has been found in peripheral regions, i.e. coleoptile, coleoshiza, root cap and periphery of the radicle which are similar to WGA (Mishkind et al., 1982, Mishkind et al., 1983). By using the fluorescent antibody technique, Chaopongpang, 1989 reported that lectin of 4-d-old rice seedlings distributed in the root cap especially the mucigel and the root hair tip, whereas in flowering stage, lectin was observed only in the root tip. On the leaf surface, lectin has been specifically localized on hydathode and opening stoma of young seedling only.

The first physiological role of rice lectin beside that microbe adhesion has been suggested from its distribution in embryonic stage and during development to mature plant to be primary axis specific, maturation-specific (Stinissen and Peumans, 1984) and also appeared to be dormancy-specific (Peumans and Stinissen, 1983). Its synthesis is somehow related to the maintenance of a naturally or ABA-induced resting state, so it is suggested that the role of lectin in primary axes is related to the establishment of the resting state in this organ. Recently Raikhel and Chrispeels, 1991 proposed an alternative role of Gramineae lectins in plant defence mechanism. Since all these Gramineae lectins contain the chitin-binding domain which could effect the growth of infection pathogens such as cowpee weevil. The effect of WGA on the growth of cowpee weevil was recently demonstrated by Murdock et al., 1990. Using an artificial seed system, they showed that 1% of (w/w) WGA increased the time of level development from 32 days to 55 days and that all three isolectins of WGA had similar

effect (Huesing et al., 1991). Similar results have now been obtained with rice lectin and nettle lectin (Huesing et al., 1991)

1.4.2 Biosynthesis, Processing and Intracellular Transport of rice lectin

1.4.2.1 Lectin synthesis in developing embryos and rice seedlings

As rice lectin is abundant in embryos, it was speculated that this protein is most likely synthesized during embryogenesis. Studies by Stinissen and Peumans, 1983 using [^{35}S] cysteine incorporation showed that the relative rate of lectin synthesis is high during the developmental stage between 8 and 16 days post anthesis, coinciding with the rapid lectin accumulation, and biosynthesis decreased dramatically when the accumulation rate of lectin slowed down. Extensive studies by the same research group (Stinissen and Peumans, 1984) found that biosynthesis of rice lectin in developing and germinating rice embryos is under hormonal control. Adding abscisic acid to the nutrient medium of immature embryo culture, could prevent precocious germination of the immature embryos and simultaneously strongly promotes lectin biosynthetic activity. In germinating rice seedlings, rice lectin is also *de novo* synthesized as shown by a combination of high resolution ion-exchange chromatography, and in vivo labelling with [^{35}S]-cysteine and immunocytochemistry, Cammue and Raikhel, 1986 has revealed that developing rice seedlings incorporate reasonable amount of [^{35}S]-cysteine into lectin. The *de novo* lectin synthesis in rice coleoptiles increases as a function of germination whereas it decreases in the other tissues of the seedlings. Moreover, using the [^{35}S]-cysteine incubated with the extract of 3-d-old rice seedlings' root tip, Stinissen and Chrispeels (1985) demonstrated that

root tips of rice seedlings synthesize the respective lectins which proves that root tips are not only a site of lectin location but also of lectin synthesis.

1.4.2.2 Processing and intracellular transport of rice lectin

Rice lectin is firstly synthesized as a preproprotein in high molecular weight precusors (23-kDa), which requires the proteolytic removal of the signal sequence and post-translational processing of COOH-terminal domain to yield the mature 18kDa lectin polypeptides (Stinissen et al., 1983; Raikhel and Wilkins, 1989). Unlike other cereal lectin, rice lectin further undergo the second modification processing the mature polypeptide into smaller polypeptide of 10-kDa and 8-kDa (Stinissen et al., 1983). Which correspond to NH_2-and COOH-terminal portions of the mature 18-kDa protein, respectively (Raikhel and Wilkin, 1989).

Work on the localization of the subcellular site of lectin synthesis in developing rice embryos indicated that newly synthesized rice lectin is indeed transiently sequestered into the endoplasmic reticulum (ER) before transport and processing take place (Stinissen et al., 1984)(a). Still now, it is not clear, whether the lectin precursor is transported from ER to other organelles, and if so, to what kind of organelles, nor can anything be concluded about the site of processing.

1.5 The aim of thesis

As rice lectins are demonstrated as an associative factor in the adhesion of *Klebsiella* R15 to epidermal cells of rice root, and Chaopongpang, 1989 reported that lectin of 4-d-old rice seedlings varied among cultivars. These information leads to the question, whether or

not inoculation of rice seedlings with *Klebsiella* R15 could induce lectin synthesis in rice, and are there different degree of induction among different rice cultivars?

How the lectin content can be related to associative nitrogen-fixing activity?

To answer these questions the following approaches will be performed

1. Comparison of lectin content between inoculated and non-inoculated rice seedlings in three rice cultivars.

2. To localize lectin in root and leaf tissues in order to know the precise localization of lectin at cellular and subcellular levels.

3. To determine nitrogenase activity in the rhizosphere of the rice cultivars, plant dry weight and %N and Total N and study the relationship between nitrogen fixation and lectin.