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UTILIZATION OF NOVEL MUTANTS FOR
DEVELOPMENT OF
BIOLOGICAL FUNCTIONS IN CROPS

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UTILIZATION OF NOVEL MUTANTS FOR
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(31th GF Symposium)

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FOREWORD

Mutation breeding has contributed to the enrichment of genetic resources through the creation of novel genotypes with mutated genes in higher plants and new cultivars serving for present-day demands are being released. On the other hand, in the progress of molecular biology in recent years, mutant plants have played important roles in elucidating functions of biochemical processes of plants. Accordingly, the 31th Symposium was held with a tittle 'Utilization of novel mutatants for development of new biological functions in crops', to discuss and develop the future studies through utilization of induced mutants and mutation researches.

In the special lecture of this Symposium, Dr M. Furuya reviewed the study of analysis of phytochrome-mediated mutants in plants and gave useful suggestions for further advancement and creation of new modified genes in mutation studies. After the lecture, biosynthesis of gibberellins, key enzyme associated with starch biosynthesis, leaf senescence using various induced mutants in rice were discussed. In addition, recent achievement in amino acid and amino acid analog resistant mutants in rice, gravitropism in space botany in barley and alaska pea and phytochrome and its function in phytochrome mutants were reviewed.

We wish to express our sincere thanks to Dr. M. Furuya for his impressive lecture, and to other lectures, chairpersons and those who contributed for the Symposium.

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Tetsuo Masuda
Shigeki Nagatomi
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PROGRAM

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Congratulatory address: T. Hino

Special lecture
Chairperson: S. Tano
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Session I
Chairperson: S. Tano
Biosynthesis of gibberellins in rice and its dwarfism .................. N. Murofushi

Session II
Chairperson: N. Katsura
Characteristics and roles of key enzymes associated with starch
biosynthesis in rice endosperm .............................................. Y. Nakamura
Analysis of leaf senescence through mutation and affinity-labelling ..... S. Kawasaki
Amino acid and amino acid analog resistant mutants in higher plants H. Hasegawa

Session III
Chairperson: H. Kikuchi
Use of gravitropic mutants in barley and pea for the study
of space botany ................................................................. H. Suge
Phytochrome and its functions in phytochrome mutants .................. A. Nagatani
The characteristics of a supernodulating mutant isolated from soybean
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General discussion

Closing address: Y. Futsuhara
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ANALYSIS OF PHYTOCHROME-RELATED MUTANTS IN PLANTS

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Introduction

Both plants and animals can survive only under very limited environmental condition. In contrast to animal behavior, as plants are unable to move their location for avoiding dangerous or unfavorable conditions, plants must safeguard their existence using light as an enviromental information by responding promptly and efficiently to sudden, diurnal or seasonal changes in their environment. Plants capture light as environmental information by phytochromes and blue-ultra violet light absorbing pigments. Phytochromes are the best characterized phototransducer for the photoregulation in plants (Shropshire & Mohr, 1983; Thomas & Johnson, 1991). Phytochromes change photoreversibly their absorption spectra between red-light absorbing form (Pr) and far-red light absorbing form (Pfr) upon actinic irradiation with either visible or far-red light, and mediate a wide variety of red/far-red reversible reactions at molecular, cellular and organ levels (Furuya, 1968 & 1983). In recent, amino acid analysis (Abe et al, 1989) and gene sequence studies (Sharrock & Quail, 1989) of phytochromes have revealed that multiple molecular species such as phytochrome A (PhyA), phytochrome B (PhyB) and phytochrome C (PhyC) exist always in plant tissues irrespective of environmental light conditions.

Hence, a very important question arises as to which phytochrome species result in what red/far-red reversible effects that were known in literature (Furuya, 1989). To answer this question, the study of phytochrome-related mutants has been particularly useful (Adamse el al 1988; Tomizawa et al 1989; Kendrick & Nagatani, 1991) and most promising in future, because it is very difficult to distinguish physiologically, spectrophotometrically or biochemically the roles of each phytochrome molecular species in intact plants. Assuming that mutation could occur at every elementary processes from light capture to final biological responses, we expect to find three different types of phytochrome-related mutants such as photoreceptor mutants, signal transduction mutants and response mutants. If a null mutant of each Phy is available, the comparison of their phenotype with its isogenic wild type (WT) will probably indicate what the phytochrome species results in. Recent advances in studies on such direction will be
summarized in this article.

**Photoreceptor Mutants**

In the past several decades, photomorphogenetic mutants have been genetically isolated and kept mostly in Europe. In recent, attempts were made to analyze these stocked mutants and newly induced mutants immunochemically and molecular biologically. In this connection, I would like to introduce the latest story of phytochrome-related mutants.

**PhyA-DEFICIENT MUTANTS**

The tomato *aurea* (*au*) mutant is the first and best characterized PhyA-deficient mutant (Fig. 1,A). Koornneef and his colleagues (1985) in Wageningen, the Netherlands, found that *au* plant has no *in vivo* spectrophotometrically detectable phytochrome (Phy), and exhibits little light effect on seed germination, anthocyanin synthesis, hypocotyl elongation and chlorophyll content. Crude extracts prepared from etiolated tissues of *aurea* seedlings are immunochemically deficient in phytochrome apoprotein (PHY) normally present in wild type, WT (Parks et al 1989), while normal levels of translatable mRNA for PHY were detected (Sharrock et al 1988). Recently, Sharma et al (1992) in my laboratory discovered that, using monoclonal anti-PHY antibodies raised specifically against PHYA or PHYB, the content of PHYA in etiolated *au* seedlings was ca. 20% of WT, whereas PhyA in light grown *au* tissues showed no photoreversible absorbance changes and no differential endogenous proteolysis of Pr and Pfr *in vitro* as seen in WT, and that both *au* and WT contain spectrophotometrically active PhyB in equal amounts. Hence, tomato *au* mutant is identified as a photoreceptor mutant, which is PhyA-deficient.

If *au* plant has no or least PhyA function, we will be able to estimate PhyA-dependent effects on the basis of the *au* phenotype. The light grown *au* plants show pale-yellow green leaves (Fig.1, A) and somewhat elongated hypocotyls (Koornneef et al 1985), and anthocyanin formation was not detectable in *au* (Lopez-Juez et al 1990). No significant effects of end-of-day far-red light on leaf elongation, chlorophyll, carotenoid and soluble protein levels were observed in *au* and WT, whereas the photoreversible end-of-day far-red response on stem elongation was clearly shown in light-grown *au* (Lopez-Juez et al 1990).

Several indirect evidence indicates that *au* is a leaky mutant. Therefore, we can not conclusively assign any red/far-red effects as the down-stream from PhyA and will have to wait a Null mutant of PhyA.
PhyB-DEFICIENT MUTANTS

It is an epoch making contribution in this field that the *hy3* mutants of *Arabidopsis* (Fig. 2, Center) are very recently proved to be a Null mutant of PhyB (Reed et al., 1993). Namely, all of *hy3* mutations fall in the *phyB* gene locus on the chromosome #2 of *Arabidopsis*, and three alleles such as *hy3*-Bo-64, *hy3*-8-36 and *hy3*-EMS142 have stop codons in the *phyB* coding sequence. Allele *hy3*-4-117 has a missence mutation that cause a histidine to tyrosine change at amino acid residue 283 of PHYB, and allele 464-19 contains a T-DNA insertion in the *phyB*. The *phyB* mutants express mRNA for PhyB apoprotein (PHYB), but do not make PHYB in most cases except *phyB*-4-117. As the HY3 locus encodes PHYB, we refer the *hy3* mutations as *phyB* (Reed et al., 1993).

In contrast to the case of *au*, we now way assign to which red/far-red effects result from PhyB, because that *hy3* are a Null mutant of PhyB. The *hy3* mutants show abnormal elongation of hypocotyls, stems, petioles and root hairs in the light, a reduced number of rosette leaves and flowering earlier than WT, and accumulate less chlorophyll (Reed et al., 1993). Seed germination in the dark is red/far-red reversibly
regulated in WT but not at all in hy3, indicating that PhyB is involved in the dark germination. The germination in the light during imbibition, however, is PhyA-dependent. Thus, PhyA and PhyB independently control the germination rate, depending upon environmental light condition (Shinomura et al, in press). As the hy3 mutants do not display end-of day far-red responses such as petiole growth (Nagatani et al 1991), we conclude that end-of-day far-red effect is resulted from PhyB.

Another well-characterized PhyB-deficient mutant is the cucumber lh (Fig. 1, B), which was originally isolated on the basis of its elongated hypocotyl in white light (Koornneef & Knaap 1983). In Western blot analysis, the lh mutant lacks a PhyB polypeptide but contains normally PHYA (Lopez-Juez et al 1992), so the optically active Phy is ascribed to PhyA. The phenotype of the etiolated lh plant showed an inhibition of hypocotyl growth, whereas the light-grown lh seedlings failed to respond to red light in terms of hypocotyl growth and greening and lacked the end-of-day far-red light response on stem elongation (Adamse et al 1988).
CHROMOPHORE DEFICIENT MUTANTS

All the above described photoreceptor mutants are resulted from the defect of phytochrome apoproteins. Another type of photoreceptor mutant is available when the biogenesis pathway of the chromophore tetra pyrrole pigment is defected. The contents of spectrophotometrically measurable Phy in the etiolated tissue of the Arabidopsis hy1 (Fig.2, right), hy2 and hy6 mutants were significantly lower than those of WT (Chory et al 1989), but the apoproteins of PhyA and PhyB in lightgrown hy1, hy2 and hy6 were present immunochemically in the same quantities as in WT (Parks et al 1989). Because biliverdin, a precursor of phytochromobilin, applied exogenously from the media to hy1 and hy2 seedlings restores normal photomorphogenesis and Phy synthesis, it is thought that the hy1 and hy2 mutants are inhibited in chromophore biosynthesis at steps prior to the formation of biliverdin IXα (Parks & Quail 1991). The phenotypes of hy1 and hy2, however, are not always consistent with each other; for example, hy1 shows late flowering, whereas hy2 flowers earlier, though the both mutants have a reduced number of rosette leaves and the end-of-day far-red effect on the number of rosette leaves (Goto et al 1991). If the chromophore biogenesis is totally blocked, all Phy molecular species must equally lack their chromophore. However, hy1, hy2 and hy6 showed somewhat different phenotypes, indicating that they appear to be leaky mutants, and that hy1, hy2 and hy6 might produce partly spectrally active Phy species in different contents. Hence, it is difficult to assign the roles of each Phy from the data of these hy mutants.

Signal transduction Mutants

At moment we have no idea on the nature of Phy receptors that would receive a signal from each Phy molecule. It is also an open question as to whether each Phy species has unique own signal transduction pathway (s) or share a common pathway. Studies using mutants of Phy signal transduction pathways will warrant, though the experimental results have not yet accumulated enough to answer this question.

The pea lv mutant showed an increased internode length in the light and the lack of response to end-of-day far-red light on stem elongation and retarded de-etiolation in light grown lv plants. This phenotypes looks like those of PhyB-deficient mutants such as hy3 or lh. Spectrophotometrical and immunochemical analysis, however, suggested that both etiolated and light-grown tissue of the lv contained normal levels of PhyA and PhyB. We thus conclude that lv may be a signal transduction mutant (Nagatani et al 1990).

The hp mutant of tomato is characterized by reduced plant height, darker green leaves and an increased level of anthocyanin in the light (Mochizuki & Kamimura
1985). Based on the physiological analysis of the hp and au/hp double mutants and the observation that the etiolated hp tissue contained normal Phy levels (Peters et al 1989), the action of the hp mutation has been proposed as increasing the sensitivity to labile Pfr.

The Arabidopsis det1 mutants partially develops as a light-grown like plant even in the dark (Chory et al 1989). Dark-grown det1 Plants show more developed plastids, constitutive expression of photosynthetic genes and a high level of anthocyanin. The level of PhyA in etiolated det1 tissue was similar to WT, and recessive mutations at the DET1 locus result in cell-type inappropriate accumulation of RNAs for light-regulated nuclear and chloroplast genes (Chory et al 1990). The Arabidopsis det2 mutant shows very similar phenotypes to det1, except the recessive det2 mutations do not result in the initiation of the chloroplast developmental program (Chory et al 1991). The dark-grown cop1 mutants exhibit light-grown characteristics like det1, except that seed germination and diurnal control of cab gene expression is red/far-red photoreversibly regulated (Deng et al 1991). The det1, det2 and cop1 mutations show a very similar phenotype but are derived from different genetic loci (Deng & Quail 1992), exhibiting some distinct phenotypic differences as described above.

DOUBLE-MUTANT EXPERIMENTS

Chory (1992) has investigated the functional interaction between the det1 and det2 gene products and the photoreceptor phytochromes by producing double mutants with photochrome-defect mutants. The resulting phenotypes of double mutant strains suggest a hierarchical regulatory network among these genes in the control of the switch from etiolated to de-etiolated growth strategies. She found that the phenotype of det1 and det2 double mutants is additive, and that the hy1, hy2 or hy6 mutants and det1 and det2 double mutants indicate that det1 is epistatic to hy1, hy2 and hy6. Somewhat additive interactions were observed between det1 and hy5, or between det2 and hy5.

The phenotypic and genetic analysis of det and hy single mutant lines suggests that DET1 and DET2 proteins are required to couple the red-light induced signals to the downstream light-regulated developmental and gene expression responses in Arabidopsis (Chory 1992). She proposed that the gene product DET1 is negatively acting regulatory molecule which is a common element in the light stimulus transduction pathway. Analysis of plants carrying both cop1 and hy1 suggests that the gene product COP1 acts downstream of the Phy signal (Deng & Quail 1992).

It is interesting to note that det1, det2 and cop1 gene products show a similar function but not identical; namely, 1) all three control anthocyanin synthesis, hypocotyl elongation and phytochrome A dependent gene expression; 2) det1 and cop1 regulate
chloroplast development and leaf shape, while det2 has no effect on this phenotype; 3) cop1 seeds normally show red/far-red photoreversible effect on germination, although det2 seeds has no phytochrome response in germination; and 4) only det2 seedlings block photoperiodic flowering responses.

Future Aspect

First, I would like to know as to whether each phytochrome species has a different, independent signal transduction pathway or share a mutual pathway with other species. If we eventually could provide null mutants of each phytochrome species and their double and triple mutants, we will find the answere to this question using such mutants.

Unfortunately, we have no phytochrome-related mutants in monocots. Hence, we are unable to make the general conclusion which phytochrome species result in which phenotypes in the seed plants. I thus plan to produce phytochrome defect mutants with rice as a representative of monocots.

Although I did not discuss at all on the blue- and UV-light dependent photomorphogenetic mutants, such mutants will be very important and useful to understand the roles of photoregulation in plants besides phytochromes. In recent, P. Nick and O. Yatoh have isolated a blue-light dependent mutant with rice, and characterized it as a chytoskelton-related mutant. The direction of such works will be promising in future.

Finally, we have so far been frustrated in studies using transgenic plants with phytochrome genes, because we have failed to get a crucial result with the assignment of phytochrome species to each red/far-red effect (Furuya 1993). However, we might find a new approach in transgene work using phytochrome null mutants.

References


BIOSYNTHESIS OF GIBBERELLINS IN RICE 
AND ITS DWARFISM

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The Gramineae include many important agricultural crops, e. g. rice, wheat, barley, sorghum, sugarcane, etc. An important class of hormones in the Gramineae, the gibberellins has been extensively studied, especially in rice and maize, mainly for vegetative tissues. In studying endogenous gibberellins of maize, the use of dwarf mutants proved quite effective, and yielded important information on gibberellin biosynthetic routes. Unfortunately studies of endogenous gibberellins in rice have lagged behind those in maize even though there are many dwarf mutants in rice and short stature of the rice plant is recognized to be of great importance. Recently, though noticeable progress on gibberellin biosynthesis as well as on the basis of dwarfism in rice has been made. Herein, relationships between biosynthesis of gibberellins and dwarfism in rice are described and comparisons made with those in maize.

1. Biosynthesis of gibberellins

The basic pathways of gibberellin biosynthesis was initially established by using a fungus, Gibberella fujikuroi. In higher plants important biosynthetic pathways have been best studied using cell-free extracts from endosperm and seeds of the Cucurbitaceae and Leguminosae families (see reviews and reports, Crozier 1983, Graebe 1986, Lange et al. 1993a and 1993b). Gibberellins share the basic biosynthetic routes of terpene synthesis. As shown in Fig. 1, mevalonic acid is converted into ent-kaurene, a tetracyclic compound, via geranylgeranyl pyrophosphate and the dicyclic compound, copalyl pyrophosphate. Ent-kaurene is then oxidized to ent-kaureno, ent-kaurenal and ent-kaurenoid acid, successively en route to the gibberellins. Ent-kaurenoid acid undergoes hydroxylation in ring B to form ent-7α-kaurenoid acid, which is then converted into gibberellin A_{12} (GA_{12}) -aldehyde by ring contraction. GA_{12}-aldehyde is the first compound in this pathway with the ent-gibberellane skeleton (Fig. 2) and all gibberellins arise from GA_{12}-aldehyde through a variety of biosynthetic routes. For example, GA_{12}-aldehyde can be oxidized to GA_{13}, the C-10 methyl of which is oxidized to give a hydroxymethyl (yielding the δ-lactone), an aldehyde and a carboxyl groups,
successively. Gibberellins carrying those groups consist of twenty carbons and are called C\textsubscript{20} gibberellins. Further down the pathway, the C-10 aldehyde (but not the carboxyl) is removed to give gibberellins with a γ-lactone (C\textsubscript{19} gibberellins). Hydroxylactation can occur on both C\textsubscript{20} and C\textsubscript{19} gibberellins at numerous positions on the ent-gibberellane skeleton. Other functional groups, such as an epoxide ring, one to two double bonds, and a carbonyl group, and additional carboxyls can be introduced. Consequently, there are many gibberellins (e.g. over ninety, to date).

We now know that there are some differences in biosynthesis of gibberellins among species of fungi (at present at least three species of fungi are known to produce gibberellins, but gibberellin biosynthesis has been studied most extensively in *Gibberella fujikuroi*). However, the widest diversity of gibberellin structures occurs in higher plants.
2. Gibberellins in plants of the Gramineae

Recent progress in analytical methods, i.e., GC/MS and immunoassay, has made it possible to determine even minute amounts of gibberellins from higher plants. Through intensive efforts made mainly by British and Japanese chemists working in the field of plant hormone chemistry, the basis of gibberelin analysis has been established. This has allowed the identification of many gibberellins in shoots of maize (Hedden et al. 1982, Fujioka et al. 1988a) and rice (Kobayashi et al. 1988 and 1989). Also, feeding experiments with labelled precursors using maize has clarified many gibberellin conversion in this plant (Heupel et al. 1985) and has helped to establish the major biosynthetic routes for gibberellins in the vegetative shoot of maize. The most important results were obtained with dwarf mutants of maize, and they offer guidance as to routes of gibberelin biosynthesis in rice and other Gramineae plants. It is now clear that the major biosynthetic pathways of gibberellins in maize and rice shoots are the early-13-hydroxylation pathway and the early-non-hydroxylation pathway, and that these lead to GA$_3$ and GA$_4$ (Fig. 3). However, while it is generally accepted that GA$_3$ (and also GA$_4$) are active per se in promoting shoot growth, the evidence of such a role for GA$_4$ in maize, rice, of other Gramineae, is equivocal. Gibberellins in other Gramineae plants, barley, wheat, sugarcane and bamboo, have also been investigated.

Fig. 3. Biosynthetic pathway of gibberelin (2):
Two main pathways in higher plants
by several research groups, and on the basis of gibberellins identified the presence of
the early-13-hydroxylation, early-non-hydroxylation and early-3β-hydroxylation path-
ways was suggested for shoots of certain in those plants.

3. Dwarfism of maize and rice

Dwarfism in higher plants is an interesting phenomenon and has been studied by
many scientists. Among the several factors covering dwarfism in maize are specific
blocks in gibberellin biosynthesis. In this paper dwarfism in maize and rice is
discussed, focusing on gibberellins.

Maize

Dwarfism of maize and its relation with endogenous gibberellins were first inves-
tigated by Phinney and his coworkers. Dwarfism in several dwarf mutants of maize
was found to be caused by defects in the biosynthesis of gibberellins, and these results
suggest that similar mechanisms of dwarfism also occur for other higher plants.

Four mutants, d-1, d-2, d-3 and d-5, are known to respond to exogenously applied
gibberellins, but their responses to various gibberellins are not necessarily the same.
For example, the d-5 mutant responds to GA₁ and GA₃₀ similarly, but the d-1 mutant
does not. Dwarf-1 mutant, however, responds to GA₁ very clearly, but not to GA₃₀.
These results implied that there was likely a defect at the stage between GA₃₀ to GA₁
in the later stages of gibberellin biosynthesis. This conclusion was further confirmed
by the analysis of endogenous gibberellins, in which the occurrence of GA₃₀ in high
level and GA₁ in very low level was observed (Fujioka et al. 1988a). On the other hand,
the defect of gibberellin biosynthesis in the d-5 mutant was deduced to exist at a much
earlier stage, because all gibberellins in the early-13-hydroxylation pathway, except a
few inactivated ones, and even ent-kaurenoi and ent-kaurenoic acid, showed growth-
promoting activity when applied to the d-5 mutant. Thus, based on the extensive
experiments, each laying the groundwork for the next, the controlling points of
gibberellin biosynthesis in the four maize mutants were determined or inferred, as
shown in Fig. 4 (Fujioka et al. 1988a, with a few modifications). Further, Phinney and
colleagues proposed that only GA₁ was the active gibberellin responsible for stem
elongation in maize, rice and pea, at least, and the stem elongation activity of
gibberellin precursors to GA₁ in the early-13-hydroxylation pathway was ascribed to
GA₁, converted from those precursors (Phinney and Spray 1982). Evidences that this
theory was correct has accumulated since, although GA₅ may have to be added, for
maize at least.
Rice

There are many dwarf mutants of rice known, but basis for their dwarfism is not so clear as that described above for maize mentioned. There are also many dwarf mutants of rice obtained by artificial mutation processes but only a limited number of these have been taken utilized for breeding to obtain practically useful cultivars. Also, while there are many dwarf cultivars of rice, plant hormone researches using chemical methodologies have only rarely been carried out.

Most dwarf cultivars of rice do not show a severely dwarfed character, and they are semi-dwarfs. Some of the semi-dwarfs are utilized not only for practical cultivation but also for academic research. Two dwarf rice mutants, Tan-ginbozu and Waito-C, are thus well-known for their use in gibberellin bioassay. Their patterns of responses to gibberellins indicate that the mechanisms of dwarfism in Tan-ginbozu and Waito-C are likely to be similar to those in dwarf maize mutants d-5 and d-1, respectively. Tan-ginbozu responds to many gibberellins, while Waito-C responds only to gibberellins possessing a 3β-hydroxyl group (GA1, GA4, etc.). Fig. 5 shows that Tan-ginbozu and Nipponbare respond to GA15 (a C20-precursor of GA4) but Waito-C does not respond, while both respond to GA1. Our recent research suggests that the controlling point of gibberellin synthesis in Tan-ginbozu is located quite early (before GA12-aldehyde) in the gibberellin biosynthetic pathway, but is probably not at the stage of ent-kaurene synthesis as earlier reported (Moore et al. 1988). Thus, Tan-ginbozu shows a growth response to many gibberellins, and as such has become probably the most useful rice for use in gibberellin bioassays.
Fig. 5 The response of Tan-ginbozu, Waito-C and Nihonbare (Nipponbare) to GA_{19} (A) and to GA_{1} (B)

Each seedling was treated with 10 ng of GA_{19} or GA_{1} by micro-drop method.

The availability of dwarf rice for gibberellin bioassay in research was first demonstrated by Murakami. He established a very convenient and sensitive bioassay system called the "microdrop assay" (Murakami 1969). By his method, very minute amount of gibberellins (10 ng of GA_{1} to Tan-ginbozu) was detected three days after application. More recent research using Tan-ginbozu pre-treated with a growth retardant (uniconazole which inhibits the oxidation of ent-kaurene to ent-kaurenoic acid, a biosynthetic precursor of gibberellins) has brought the sensitivity up to femtomole levels for detection of GA_{1} and other 3β-hydroxylated gibberellins (Nishijima et al., 1989). Also, by comparison of activities between the two dwarf mutants, Tan-ginbozu and Waito-C, Murakami proposed a convenient method to distinguish whether an applied gibberellin has a 3β-hydroxyl group, as GA_{1}, GA_{4}, etc. (which are highly active) or not.

Tan-ginbozu appeared to be derived from Ginbozu by spontaneous mutation, but it is not a severe dwarf, the final height reaching about 70% of the height of normal rice. Generally the levels of endogenous gibberellins, and especially GA_{1}, in dwarf plants are lower than those of tall plants. Some exceptions occur, however, in wheat and other plants also, indicating that the mechanism is not just a single blocking of the gibberellin biosynthetic pathway. However, most dwarf maize and dwarf rice mutants examined thus far show lowered levels of GA_{1} in their shoot, relative to tall cultivars. Very early in our research on gibberellins in rice, we identified the gibberellins and determined their levels for three rice cultivars, Nipponbare (japonica, normal), Tong-il (hybrid of japonica and indica, semi-dwarf) and Tan-ginbozu (japonica, semi-dwarf) (Kurogochi et al. 1979, Suzuki et al. 1981). Although only GA_{19} and GA_{1} were identified at early on and the analytical methods were not very sophisticated, these two gibberel-
lins were analyzed in shoots and ear at several growth stages. Differences in endogenous gibberellin levels were not correlated with differences between Nipponbare and Tong-il, but a noticeable difference was observed in the level of gibberellins between Nipponbare and Tan-ginbozu. Fig. 6 shows the fluctuation of the levels of GA\textsubscript{1} and GA\textsubscript{19} at several growth stages in the two cultivars as well as their growth profiles. GA\textsubscript{19} was identified in Nipponbare as a major gibberellin throughout its life cycle and is presumed to be a 'pool gibberellin' that could regulate the level of GA\textsubscript{1}.

Interestingly, though, the level of GA\textsubscript{19} in Tan-ginbozu was undetectable. It is still unclear why the level of endogenous GA\textsubscript{19} is so low only in Tan-ginbozu except to speculate that GA\textsubscript{19} \rightarrow GA\textsubscript{20} becomes rate-limiting in rice at levels above that occurring in the dwarf, Tan-ginbozu.

It is well known that a shorter plant height is advantageous in cereal crops because it makes the plant more resistant to lodging. In fact, the highest production of rice in Japan, in 1951 was achieved by cultivating Tan-ginbozu (857 kg/10 ares, unpolished grain). One of the reasons for this high yield is probably its dwarf

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**Fig. 6** Growth patterns (a) and seasonal changes of GA\textsubscript{1} and GA\textsubscript{19} contents (b) of Nipponbare and Tan-ginbozu (Suzuki et al. 1981)

(a) I, 1st internode; II, 2nd internode; III, 3rd internode; IV, 4th internode.

(b) PI, panicle initiation stage; H, heading stage.

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- ○--○, GA\textsubscript{19} content in shoots
- ●--●, GA\textsubscript{19} content in ears
- △--△, GA\textsubscript{1} content in shoots
- ▲--▲, GA\textsubscript{1} content in ears
character.

To dwarf crop plants growth retardants are sometimes used. These include chloromequat chloride, ancymidol, paclobutrazol, uniconazole, prohexadione, etc. Most are known to inhibit gibberellin biosynthesis at various steps. While they are effective for retarding the growth of rice, they are not used for rice cultivation on a large scale, both due to prohibitive high cost and environmental reasons.

4. Tissue/organ specificity of gibberellin biosynthesis and dwarfism

Among the various kinds of gibberellin activities, the most clear and dramatic one is shoot growth promotion. In fact there is close relationship between endogenous gibberellin levels and plant height of normal and dwarf plants. Also, the height of dwarf maize seedlings is much shorter than that of normal maize, and dwarf maize seedlings respond proportionately greater (relative to normal maize) to exogenously applied gibberellins. However, there is not big size difference between dwarf and normal maize seeds or embryos, the progenitors of the seedlings. In which stage does their dwarfing start? Are there any changes of gibberellin biosynthesis at different stages of the maize plants' ontogeny? To answer these questions, the levels of endogenous gibberellins (GA$_1$, GA$_3$, GA$_5$, and GA$_{30}$) in the seeds of normal and dwarf maize (d-1) were determined by immunoassay. Although this experiment was a preliminary one, the results showed that there were no significant differences in the levels of the various gibberellins in the immature seeds between tall and dwarf genotypes (Murofushi et al. 1991). This result indicates that the defect causing the dwarfism of the d-1 mutant is not expressed in immature seed.

The tissue/organ specificity of gibberellin biosynthesis can also be observed in rice. Gibberellins contained in the shoots of rice are synthesized by the early-13-hydroxylation pathway. However, analysis of gibberellins in the ears of rice revealed the presence of gibberellins carrying no hydroxyl at C-13, e. g. GA$_3$, GA$_5$ and GA$_{34}$, thereby, indicating the early-non-hydroxylation pathway (see Fig. 1) is operating in ears of rice (Kobayashi et al. 1984). The comparison of endogenous gibberellin levels between shoots of dwarf rice (Tan-ginbozu and Waito-C) and normal rice (Nipponbare) also gave interesting results, ones similar to those obtained from maize (Kobayashi et al. 1989). The level of GA$_1$ in the shoots of Nipponbare at the 10-leaf stage is about twice higher than those of Waito-C and about four times higher than Tan-ginbozu. However, in the ears there was no significant difference in the levels of endogenous GA$_{19}$ and GA$_{28}$, between Nipponbare and Waito-C. In fact, the level of endogenous GA$_5$ in the ear of Waito-C was somewhat higher than that of Nipponbare. Further, although the levels of GA$_{19}$ and GA$_{28}$, in the ear of Tan-ginbozu were very low or
almost undetectable, the level of GA₄ was higher than that of Nipponbare. These results indicate that there is tissue/organ specificity in gibberellin synthesis in rice. The significance of the occurrence of GA₄ in higher levels in dwarf cultivars than in the normal cultivar is obscure, and will probably remain so until the role(s) of GA₄ in rice ears is determined.

There was also no difference in gibberellin-biosynthetic ability in cell-free systems prepared from anthers between a normal rice cultivar and the dwarf rice cultivar (Kobayashi et al. 1990), although this should not be unexpected as the regulation system for biosynthesis is probably lost in cell-free systems.

The results described above suggest that dwarfism in maize and rice is not due to a simple defect (e.g., gene deletion) at some biosynthetic stage, but rather may be due to differences in regulation in gene expression at various growth stages, or in different parts of the plant.

5. Dwarfism by endogenous growth inhibitors

There is also a concept that dwarfism can be caused by endogenous growth inhibitors in some dwarf plants. Although there has been no demonstration that endogenous growth inhibitors are the cause of dwarfism in rice, it seems possible that

![Graph showing seasonal changes of ABA content in three cultivars](image)

**Fig. 7** Seasonal changes of ABA content in three cultivars (Suzuki et al. 1981).

---○---, Nipponbare (N); ---●---, Tong-ill (TI); ---×---, Tan-ginbozu (TG).

Upper parts, in ears; lower parts, in shoots.
there may be dwarf mutants where the short stature is caused by growth inhibitors, e. g. abscisic acid-, jasmonic acid-related compounds or other as yet uncharacterized substances.

Abscisic acid is known to retard the growth of plants and can act as an antagonist to gibberellins. Abscisic acid was analyzed at several stages in the ear of rice and the changes in its levels suggest a possible role in ear growth or development (Suzuki et al. 1981). As shown in Fig. 7, the level of endogenous abscisic acid in the ear increases according to the growth/development of the ear after anthesis/fertilization, and then decreases as the seed matures. This suggests that abscisic acid may play a role(s) in the developing rice grain similar to that postulated for maize and barley, namely as a regulator of premature germination. However, the role for abscisic acid in shoot dwarfism does not, to date, seem likely.

6. Dwarfism unrelated to gibberellins

Some dwarf mutants respond to exogenously applied gibberellins very well, but some do not. One case is a dwarf maize mutant D-8. This mutant not only does not respond to gibberellins but also to any other known plant hormones. Extracts from tall maize which may contain some unknown growth-promoting substances also show no effect to D-8 mutant. Generally, dwarfism in rice, maize and other plants is recessive and, in most cases, controlled by single gene. Surprisingly, the levels of endogenous GA_{20} and GA_{1} in the shoots of D-8 mutant are much higher (over tenfold) than for shoots of normal maize (Fujioaka et al. 1988b). Interestingly, the dwarf character of this mutant is dominant (shown with a capital letter D). This cannot be explained by the deficiency of some promoting factors as plant hormones, but rather suggests the presence of other controlling factors. This mutant has attracted the interests of many researchers but the mechanism of its dwarfism has not been clarified. Dominant or recessive, similar non-gibberellin-responding dwarf rice mutants as D-8 have not been characterized, although a similar mutant is found in wheat.

The non-plant hormone-responding recessive dwarf mutants are possibly important because the dwarfism may be due to a defect in a hormone receptor. They are called 'receptor mutants' in contrast to 'leaky mutants', the name given to hormone-responding dwarf mutants as dwarf maize d-1, d-2, d-3, d-5 and dwarf rice Tan-ginbozu and Waito-C. The receptor mutants may be useful in the investigation of the action mechanism of gibberellins.
7. Search for dwarf rice mutants

As mentioned above, dwarf mutants of rice are important from both research and practical standpoints. It is to be expected that various kinds of dwarf mutants, gibberellin-responding as well as non-gibberellin-responding, will be produced by gamma-ray irradiation. Thus, a survey for dwarf mutants and an examination of their sensitivity to gibberellins have been conducted so as to locate both gibberellin-responding and non-gibberellin-responding dwarfs. If various kinds of dwarf mutants carrying a biosynthetic defect in different biosynthetic stages are obtained, they should be very useful, initially for characterization of unknown gibberellins ("upstream" from the biosynthetic block), secondly for the establishment of exact biosynthetic pathways in rice.

Many lines obtained from Norin No. 8 by gamma-ray irradiation were used in the survey. The seedlings were treated with gibberellin A₃ (as a mimic of GA₁) 10 ng and 100 ng to each seedling) according to the microdrop procedure of Murakami. The response of rice to GA₃ is based on the elongation of the second leaf-sheath. As shown

![Graph showing response of various rice mutants obtained from Norin No. 8 by gamma-ray irradiation to GA₃. Each seedling was treated with 100 ng of GA₃ by micro-drop method. Three days after the treatment the 2 nd leaf sheath length was measured. - rice examined; ○, Nipponbare; □, Tan-ginbozu; △, Waito-C]
in Fig. 8, a large diversity of responses to GA$_3$ was observed. Points near those representing Tan-ginbozu and Waito-C probably represent gibberellin-responding dwarf mutants. To determine the controlling stages in such dwarf mutants, their sensitivity to gibberellins other than GA$_3$ (GA$_4$) was examined. Unexpectedly, all showed insensitivity to GA$_{30}$, the direct precursor of GA$_1$. This suggests that they are dwarf mutants similar to Waito-C (e.g. blocked for 3β-hydroxylation). Hence, gamma-ray irradiation may have a tendency to mutate gene(s) whose products control 3β-hydroxylation. A more intensive examination of non-gibberellin-responding dwarf mutants is now underway.

The dwarfism of higher plants is being studied by several newly developed methodologies, including molecular genetics. Unfortunately Arabidopsis thaliana cannot be investigated very well by analytical chemistry techniques because the very small plants yield only minute amounts of endogenous hormones. For rice and maize, however, breeding research has been extensively carried out. Unfortunately, in the past only a limited number of mutants have been investigated or utilized, with most having ignored or discarded. In conclusion, the dwarfism can be observed easily and the search for dwarf rice mutants should continue, and should include extensive research using chemical methodologies on gibberellins. This future research on dwarf rice mutants will yield both basic scientific advances and practical results which can be utilized in breeding and agriculture.

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References


* The name of Nihonbare was used for Nipponbare in some reports.
イネにおけるジペレリン生合成と矮化

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ジペレリン (GA) 生合成についての研究は、まず微生物を用いて行われ、基本的な経路は解明された。他方、高等植物における GA 生合成は、トウモロコシを対象として行われ、主要な経路として early-13-hydroxylation pathway の存在が確認され、そこにおいて生成される GA₄が茎の伸長を促進することができ明らかにされた。その研究において、GA 欠損による数種の矮性変異種 (d-1, d-5など) が利用され、あわせてそれらにおける GA 生合成欠損段階が決定された。イネにおける GA 生合成についての研究は、主としてトウモロコシにおける研究結果を基に進められたが、全生活環における GA の量的ならびに質的追及を行うことにより、イネの生長生理と GA との関係についての基礎的かつ重要な知見が得られた。とりくにイネにおいては early-13-hydroxylation pathway のほかに early-non-hydroxylation pathway が存在し、そこにおける活性型 GA である GA₄が、栄養生長段階で何らかの役割を演じていることが示唆されたことは注目に値する。イネにおける矮性変異種としては、GA の生物検定用植物として重用されている“短銀坊主”と矮稈 C がよく知られているが、前者は GA 生合成前躯体の段階に、後者は GA₃で GA₄への変換段階に欠損が存在することにより矮性となる。なお両者は、生長の最終段階における茎丈が正常稈のそれの70％程度となることから“半矮性種”ともいわれている。

トウモロコシやイネにおける GA 欠損矮性変異種においては、その GA 生合成欠損は組織/器官特異的であることが示されている。すなわち、トウモロコシの矮性変異種 d 1 と正常株それぞれの未熟種子中の GA レベルを比較すると、有意の差は見られない。また、イネ正常株と矮性変異種のえい花中の GA レベルの分析においても同様の結果が得られている。他方、GA と無関係な矮性変異種がトウモロコシにおいて知られている。この変異種 (D-8) においては、いかなる GA および既知の植物生長調節物質を投与しても茎の伸長は見られず、また GA の内生レベルは正常株のそれに比べて非常に高い。さらにその矮性形質は優性遺伝する。このような矮性変異種は、イネにおいては知られていない。

矮性形質は、イネのみならず多くの農作物の栽培において、倒伏防止などの面から重要である。また、GA 植物検定用植物として、あるいは GA の作用発現機構の追究用の材料としても注目すべきである。放射線処理などによる変異化処理して得られたイネはきわめて多く、その蓄積はきわめて価値あるものである。今後それらを対象として、実用面だけでなく、学術的研究という観点から多面的なスクリーニングを展開することが重要であろう。
CHARACTERISTICS AND ROLES OF KEY ENZYMES ASSOCIATED WITH STARCH BIOSYNTHESIS IN RICE ENDOSPERM

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Introduction

Although biosynthesis of starch in cereals is the most, if not sole, important biochemical events in plant productivity, only a limited information has been available on characteristics, metabolic roles, and both biochemical and genetic regulation of the key enzymes associated with starch metabolism in storage organs.

In rice plants, starch is accumulated in endosperm, where a translocated sucrose is efficiently converted to starch. During development of endosperm amylloplasts increase the number per cell and the volume, and then synthesize and store a great amount of starch. With amylloplasts isolated from developing rice endosperm (Fig. 1), we examined the localization of some enzymes involved in the carbohydrate metabolism in endosperm cells (Nakamura et al. 1989). Table 1 shows that ADPglucose pyrophosphorylase and fructose-1,6-bisphosphatase are located in the amylloplast, while sucrose synthase and UDPglucose pyrophosphorylase are in the cytoplasm. We hypothesize the predominant pathway for starch biosynthesis in rice endosperm, as shown in Fig. 2. In the cytoplasm sucrose is metabolized to form hexose-P by sucrose synthase, UDPglucose pyrophosphorylase and fructokinase reactions. Hexose-P is then converted to dihydroxyacetone-P according to the glycolytic pathway. The triose-P enters into the amylloplast through amylloplast envelopes, possibly via a translocator protein as the phosphate translocator as identified in chloroplast envelopes, and then it is metabolized to glucose-1-P. Now starch is synthesized from glucose-1-P bysequent reactions catalyzed by ADPglucose pyrophosphorylase, starch synthase and
Fig. 1. Micrograph of amyloplasts isolated from developing rice endosperm. The amyloplast were prepared as described by Nakamura et al. (1989).

Fig. 2. Schematic representation of the possible metabolic pathway for carbohydrate metabolism in rice endosperm.

ADPP: ADPglc pyrophosphorylase
STS: Starch synthane
QE: Starch branching enzyme
(Q-enzyme)

\[ \text{Sucrose} \rightarrow \text{Glucose-1-P} \rightarrow \text{ADPglucose} \rightarrow \alpha-1, 4\text{-glucan} \rightarrow \alpha-1, 4/\alpha-1, 6\text{-glucan} \]
Table 1. Distribution of enzymes in amyloplasts isolated from developing rice endosperm

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Crude Suspension&lt;br&gt;(nmol/min per mg of protein)</th>
<th>Amyloplast Fraction&lt;br&gt;(nmol/min per mg of protein)</th>
<th>Yield&lt;br&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADPglucose pyrophosphorylase</td>
<td>957 108</td>
<td>4.43 168</td>
<td>0.46</td>
</tr>
<tr>
<td>FBPase</td>
<td>85.7 9.66</td>
<td>0.18 6.82</td>
<td>0.21</td>
</tr>
<tr>
<td>UGPglucose pyrophosphorylase</td>
<td>16,800 1,894</td>
<td>2.97 112</td>
<td>0.018</td>
</tr>
<tr>
<td>Sucrose synthase</td>
<td>5,500 620</td>
<td>0.42 15.9</td>
<td>0.0076</td>
</tr>
</tbody>
</table>

a The volume and protein content of the crude suspension were 13 ml and 8.87 mg, respectively, and those of the amyloplast fraction were 0.54 ml and 0.0264 mg, respectively.
b The yield was estimated from the total activity in the amyloplast fraction divided by that in the crude suspension.
See Nakamura et al. (1989) for further details.

Fig. 3. Changes in activities of three starch synthesizing enzymes in response with those in basic parameters of rice grain during endosperm development (cv. Fujihikari). ADPP, ADPglucose pyrophosphorylase; STS, soluble and starch granule-bound forms of starch synthase. The data are from Nakamura and Yuki (1992).
starch branching enzyme (Q-enzyme).

As long as the last three enzymes have been examined, they consist of multiple isozymes (Preiss and Levi 1980, Nakamura et al. 1992, Nakamura and Kawaguchi 1992) and are expressed in the tissue-specific manners (Yamanouchi and Nakamura 1992). However, in starch storage organs characteristics and the role of each isozyme mostly remain to be elucidated.

Generally, it is effective for determining the key enzyme to examine in detail the patterns of changes in enzyme activities during development of plant tissues and/or after the alterations in the physiological or environmental conditions, since the key enzyme frequently exhibits the different pattern from the other enzymes. Nakamura and Yuki (1992) demonstrated that the developmental patterns of Q-enzyme and ADPglucose pyrophosphorylase differ from the other 16 enzymes examined, including starch synthase, in that the two enzymes exhibit the greatest enhancement during endosperm development of rice, and that their patterns of increases correlate well with the accumulation patterns of starch in endosperm, as shown in Fig. 3. Thus, they concluded that both Q-enzyme and ADPglucose pyrophosphorylase are key enzymes for starch production in rice endosperm.

In this paper, we summarize the properties of isozymes of Q-enzyme purified from developing endosperm of rice (cv. Fujihikari). We also show the effects of sugary mutation on the levels of major enzymes in rice endosperm (cv. Norin-8) and on the starch structure, and discuss the possible roles of key enzymes which affect the productivity and quality of starch in rice endosperm.

**Characterization of Q-enzyme**

Starch branching enzyme or Q-enzyme is essential for synthesis of amylopectin, a major component of starch, since only this enzyme can introduce α-1,6-glycosidic bonds into starch molecules.

**Table 2. Activities of Q-enzyme in various organs of rice**

<table>
<thead>
<tr>
<th>Organ</th>
<th>Q-enzyme activity AU/g fresh weight</th>
<th>AU/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endosperm</td>
<td>446 ± 31.8</td>
<td>53.3 ± 3.8</td>
</tr>
<tr>
<td>Culm</td>
<td>5.14 ± 1.48</td>
<td>7.97 ± 2.29</td>
</tr>
<tr>
<td>Leaf blade</td>
<td>5.52 ± 1.06</td>
<td>0.965 ± 0.184</td>
</tr>
<tr>
<td>Leaf sheath</td>
<td>1.27 ± 0.37</td>
<td>0.756 ± 0.220</td>
</tr>
<tr>
<td>Root</td>
<td>0.349 ± 0.37</td>
<td>0.196 ± 0.008</td>
</tr>
</tbody>
</table>

Each value is the mean of results from at least three separate experiments ± standard deviation. The data are from Yamanouchi and Nakamura (1992).
Table 2 shows that the activity of Q-enzyme is exceptionally higher in endosperm than any other organs in rice plant. It is also noted that the Q-enzyme activity in the culm, which is known to function as the sink organ immediately before and at the very early stage of the anthesis, is significantly in excess of that in the leaf. Therefore, the level of Q-enzyme activity basically correlates with the capacity for production of starch in rice organs. This suggests that Q-enzyme also plays a key role in accelerating the rate of starch production by increasing nonreducing α-glucan terminals which function as α-glucan acceptors in the starch biosynthesis reaction catalyzed by starch synthase. It has been proposed that ADPglucose pyrophosphorylase regulates the rate of starch biosynthesis in plants. However, it should be stressed that ADPglucose pyrophosphorylase plays the role by producing ADPglucose, an α-glucan donor.

As is the case in a number of plant species including maize endosperm (Boyer and Preiss 1978), pea embryo (Smith 1988) and spinach leaf (Hawker et al. 1974), Q-enzyme activities in rice organs are resolved into two fractions, as designated as QE1 and QEII, when the crude enzyme extracts are applied to a DEAE column (Fig. 4). However, the

Fig. 4. Chromatograms of Q-enzymes from various organs of rice (cv. Fujihikari) on DEAE-5PW. A, endosperm; B, culm; C, leaf blade; D, leaf sheath; E, root. The collection of samples were initiated 3 min after the start of a 30-min linear gradient of NaCl (0-0.5 M), and fractions were collected at 1-min intervals. For further conditions see Yamanouchi and Nakamura (1992).
ratio of the QEII activity to the QEII activity differs greatly depending on the rice organ. For example, in the endosperm the QEII activity is about six times higher than the QEII activity, while in the leaf the activity of QEII accounts for approximately only 45% of that of QEII.

Yamanouchi and Nakamura (1992) have developed a simple but reliable method, the native-PAGE/enzyme activity staining method, for analysis of all the isoforms classified as QEII and QEII, respectively, since they are hardly separated by the conventional DEAE column chromatography. As demonstrated in Fig. 5, the rice endosperm possesses one QEII isozyme, detected as one major but broad band, and two QEII isozymes, as designated as QEIIa and QEIIb. The other organs such as culm, leaf blade, leaf sheath and root contain 5 different QEII isoforms that can be distinguished each other by means of native-PAGE, although the distribution of their contents is specific for each organ (Fig. 5). It is concluded that the rice endosperm is distinct from the other organs in that the QEII activity accounts for the bulk of the total Q–enzyme activity of the tissue, and that the QEIIa isozyme is expressed only in the endosperm.

Plants of wheat, barley and maize store starch in the endosperm like rice plants.

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**Fig. 5.** A. Native-PAGE/activity staining of Q-enzymes from various organs of rice. Lane 1, endosperm; lane 2, culm; lane 3, leaf blade; lane 4, leaf sheath; lane 5, root. QEII isoforms are designated QEIIa–f, respectively, as indicated in the figure. The conditions are those described by Yamanouchi and Nakamura (1992). B. Schematic representation of composition of QEII isoforms detected in various organs of rice. Open and closed rectangles indicate the bands of endogenous phosphorylase and QEII isoforms, respectively.
However, it should be noted that the composition and distribution of isozymes of Q enzyme are markedly specific for plant species. Fig. 6 shows that in barley the total activities of the QEII isoforms in endosperm are markedly higher than that of the QEI isoform, while in wheat those are comparable with the QEI isoforms. It is also known that in maize endosperm the total activity of QEII is substantially higher than that of QEI (Boyer and Preiss 1978; Nakamura unpublished). Therefore, it is concluded that isozyme patterns of Q enzyme are distinct in the tissue- and species-specific manners.

Fig. 7 shows the nucleotide sequence of a cDNA encoding the rice endosperm QEII and the amino acid sequence (Nakamura and Yamanouchi 1992). The complete nucleotide sequence of a cDNA encoding the endosperm QEIIa has been clarified (Nakamura unpublished). These amino acid sequences exhibit little similarity in the N-terminal portions including the transit peptides for amyloplast import, while a number of the partial amino acid sequences which are highly homologous to each other, can be found in the inner portions of the sequences including the possible catalytic sites (Fig. 7). Although this is the first literature on the cDNA structure of higher plant Q enzyme, there exists low but some homology between higher plant Q enzyme and bacterial enzymes from Escherichia coli (Romeo et al. 1988) and Synechococcus (Kiel et al. 1990).
Fig. 7. Nucleotide sequence of a cDNA encoding rice endosperm Q-enzyme I (cv. Fujihikari) and its deduced amino acid sequence. The data are from Nakamura and Yamanouchi (1992). The underlines under the amino acids indicate the portions where the partial amino acid sequences of QEI are the same as those of rice endosperm QElA.

The mutant study

The endosperm mutants would be of great use to assess the metabolic roles of isozymes involved in starch biosynthesis since the metabolic pathway of starch metabolism comprises a complex network of reactions catalyzed by a number of
enzymes, e.g. starch debranching enzyme (R-enzyme), α-glucan phosphorylase, α- and β-amylases and α-glucosidase, as well as ADPglucose pyrophosphorylase, starch synthase and Q-enzyme.

Several sugary (su) mutants have been used in our experiments among a variety of mutants which affect the quantity and quality of starch in rice endosperm. The mutants were obtained from the materials planted and irradiated in the gamma field equipped with the radiation source of 2,400 Ci 60Co, of Institute of Radiation Breeding, NIAR, MAFF. Although standard glucans of amyllopectin, amylose, glycogen and pullulan give the distinct colors when stained with iodine, starch solutions of 86GF169 and 82GF3su2 yielded red to orange color, more close to the color by the commercial glycogen rather than that by the starch preparation obtained from parent cultivar, Norin-8 (Fig. 8).

It is evident that the sugary mutation affects the enzyme activities of Q-enzyme and R-enzyme (Table 3), while the activities of the other enzymes associated with carbohydrate metabolism in endosperm of cereals, such as ADPglucose pyrophosphorylase, both soluble and starch granule-bound forms of starch synthase, phosphorylase, UDPglucose pyrophosphorylase and sucrose synthase, are not significantly altered by the mutation (data not shown). Our previous study (Yamanouchi and Nakamura 1992)

Fig. 8. The I2/KI staining of various starch samples. Lanes 1 and 2, starch samples prepared from developing endosperm of rice (cv. Norin-8) and the sugary mutant, 82GF3su2, respectively. Lanes 3-5, standard α-glucans of amyllopectin from potato tuber, amylose from potato tuber, glycogen from mussel, respectively.
Table 3. Activities of Q-enzyme and R-enzyme in developing endosperm of rice genotypes

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Q-enzyme</th>
<th>R-enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>IIa</td>
</tr>
<tr>
<td>Norin-8</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>86GF169</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>83GF3su2</td>
<td>Exp. 1</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Exp. 2</td>
<td>38.4%</td>
</tr>
</tbody>
</table>

Fig. 9. Native-PAGE/activity staining of Q-enzyme from developing endosperm extracts of the sugary mutants of rice. Lane 1, Norin-8; lane 2, 86GF169; lane 3, 82GF3su2.

showed that rice endosperm contains 3 isoforms of Q-enzyme; Q-enzyme I (QEI), Q-enzyme IIa (QEIIa) and Q-enzyme IIb (QEIIb), respectively. In both sugary mutants, 86GF169 and 82GF3su2, the activity of QEI is lowered by the mutation to the greater extent than that of QEIIa and QEIIb (Table 3 and Fig. 9). The most remarkable alteration in the sugary mutation is that the activity of Renzyme is specifically curtailed in endosperm of 86GF169 and 82GF3su2 (Table 3 and Fig. 10).

Fig. 11 shows the distribution of molecular size of the native starch prepared from Norin-8, the parent cultivar, and 82GF3su2. The Norin-8 starch preparation has a large peak (Fractions 9-13) and a small peak (Fractions 18-26), the former and the
Fig. 10. Native-PAGE/activity staining of R–enzyme from developing endosperm extracts of the sugary mutants of rice. Lane 1, Norin-8; lane 2, 86GF169; lane 3, 82GF3su2.

latter corresponding to amylopectin and amylose, respectively. The fractions between the two peaks would contain the amylopectin–like glucan with comparatively small size than the normal amylopectin molecule. The molecular size distribution in the starch preparation from the mutant is quite different from that from Norin-8 in both the molecular size and the shape of the peak in the chromatogram. The molecular size of a large peak (Fractions 10–12) in 82GF3su2 is apparently smaller than that in Norin-8 (Fig. 11b). Following the large peak in 82GF3su2, a shoulder peak is present (Fractions 12–18). It is noted that the sugary glucans in the large and shoulder peaks differ from the Norin-8 amylopectin with respects not only to the molecular size, but also to the chemical structure, since the absorption spectra of the former two peaks are quite different from that of the Norin-8 amylopectin (Fig. 12). It is also stressed that no substantial amount of amylose–like glucans is included in the starch preparation from the sugary mutant (see Figs. 11B and 12). It is interesting that no substantial amount of amylose is detected in the sugary mutant although the activity of a starch granule–bound starch synthase, which is thought to be responsible for amylose biosynthesis, is not affected by the mutation (data not shown).

When the starch of Norin-8 is hydrolyzed with isoamylase and pullu-lanase, amylopectin fractions are decomposed to give the two debranched fractions with
Fig. 11. Gel filtration chromatograms on Sepharose CL-4B of starch samples prepared from developing endosperm of Norin-8 (A) and the sugary mutant, 82GF3su2 (B). The solid and dashed lines indicate the native starch and the treated starch samples with isoamylase plus pullulanase, respectively.
Fig. 12. Absorbance spectra of the iodine-stained starch samples prepared from developing endosperm of Norin-8 (A) and the sugary mutant, 82GF3su2 (B). The samples are the same as in Fig. 11.

smaller sizes, while the amylose peak fractions remain unchanged (Fig. 11A). In contrast to the original Norin-8, the large and the shoulder peaks in 82GF3su2 can be decomposed with the enzyme treatment only to a lesser extent (Fig. 11B). The degree of polymerization (DP) of the soluble oligosaccharides produced after the hydrolysis by isoamylase and pullulanase is examined (Fig. 13). However, it is unlikely that glucans obtained from the sugary mutant contain additional glycosidic linkages besides $\alpha-1,4$ and $\alpha-1,6$ bonds, since most of the sugary glucans can be degraded to glucose by treatment with amylglucosidase (data not shown). The degree of polymerization of the peak glucans is apparently smaller in 82GF3su2 (DP = 9-10) than that in Norin-8 (DP = 12-14).

These results suggest that the sugary mutation causes changes in the ordinary glucans which are found in Norin-8 as amyllopectin and amylose to be more highly branched glucans which are not completely susceptible to attack by isoamylase and/or pullulanase. The changes are probably due to the decrease in the activity of R-enzyme as compared with that of Q-enzyme. As shown in Fig. 14, we postulate that characterization of amyllopectin molecules is not determined by the action of only $Q$
Fig. 13. Distribution of degree of polymerization of α-glucans produced after treatment with isoamylase plus pullulanase of starch samples from developing endosperm of Norin-8 (A) and the sugary mutant, 82GF3su2 (B). Samples were alayzed using a Dionex BioLC model DX-300 HPLC equipped with a pulsed amperometric detector and a CarboPac PA1 column (4 x 250 mm). Linear gradient of sodium acetate (0-0.5 M) in 0.1 N NaOH was used for the elution.
Fig. 14. Schematic representation of the hypothesis concerning the roles of Q-enzyme and R-enzyme in starch biosynthesis. ADPP, ADP-glucose pyrophosphorylase; STS, starch synthase; QE, Q-enzyme (branching enzyme); RE, R-enzyme (debranching enzyme).

-enzyme, but is closely related to a balance of activities of Q-enzyme and R-enzyme.

Acknowledgments

We thank Dr. N. Shibuya, NIAR, for his helpful instructions in analysis of DP of oligosaccharides using a Dionex BioLC. We also thank Drs. K. Suenaga and T. Komatsuda, NIAR, for providing us with wheat and barley plants, respectively.

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that catalytic enzymes are encoded among the biosynthetic genes. *Gene* 70: 363-376.


イネ胚乳におけるデンプン合成キーエンソームの特性と機能

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本研究は、農業生産に関係する最も基本的な生化学反応である植物子実におけるデンプン合成反応を、生化学的かつ分子生物学的に解析することを目的としている。ここでは主として登熟期イネ胚乳におけるデンプン生合成のキー酵素に関するわれわれの知見を要約する。併せてデンプン合成研究におけるミュータントの有用性についても一例をあげて述べる。

デンプン合成代謝は、ADP グルコースヒポフォスフォリラーゼ（以下 ADPP と略す）、スターチシンターゼ（STS）、ブランチングエンザイム（QF）から成ると一般に考えられている（Fig. 2）。登熟中のイネ種子を用いた解析研究の結果、転流物質シュクロースからデンプンにいたる反応を構成すると予想される酵素のうち、特に ADPP と QF の発現が特異的である（Fig. 3）、キ－酵素であると結論された。

以下に QF について明らかになったことを述べる。QF はデンプンの β-1, 6 分岐構造を導入しアミロペクチン合成に不可欠な酵素であるが、デンプンの構造を決定する役割を担うだけでなく、胚乳の高いデンプン合成速度を可能にする役割も有する。

1. イネ胚乳に多量に発現する QF は、タンパク化学的に大きく異なる QF I と QF II から成る。特に胚乳では QF I の存在比が極めて高く（Fig. 5），イネの他の組織（Fig. 5）や他種（ムギ、トウモロコシ、イモ）のデンプン貯蔵器官の結果と比べ（Fig. 6），異なる特徴を示す。このことはイネ胚乳における特異的なデンプン蓄積細胞の存在が示唆され、イネデンプンの構造が決定されることと関係が深いと考えられる。

2. イネ胚乳の QF I 及び QF IIa の cDNA 構造が解明され（Fig. 7），遺伝子発現調節機構を解明する研究での道が開かれた。イネの遺伝子構造は、バクテリア（大腸菌、ランン）の cDNA 構造とは相当に異なっている。また，イネ胚乳 QF I と QF IIa のアミノ酸構造を比較すると，特に N 末端と C 末端領域において顕著な相違があるが，触媒部位を含む内部構造においては両者に共通性の高い領域が認められる。

3. QF の発現はイネ胚乳の発達時期と強く関連し，特に胚乳にデンプンが多量に蓄積される時期に QF I が強く発現される（Figs. 3-5, Table 2）。また ADPP, STS, QF 活性の胚乳発達時期に応じた増加曲線は互いに異なっている（Fig. 3）ことは注目される。
以上述べたように、デンプン合成代謝を構成する酵素は一般に子実特異的であり、また複数のアイソザイムから成る。またアイソザイムパターンは種特異的であり、子実における効率的かつ種特異的なデンプン蓄積過程の存在が推定される。アイソザイムの機能分担の解明は今後に残されている。

デンプン代謝は多数の酵素反応のネットワークから成っているため、デンプンミュータントの持つ有用性は重要である。デンプンミュータントを利用した研究例を述べる。sugary系のイネ胚乳ミュータントを調べた結果、最も特徴的なことはRアイソザイム（枝切り酵素。α-1,6結合を加水分解する）活性が著しく低下し（Fig. 10, Table 3）、アミロペクチンよりも平均鎖長の短い、よりグリコーポンに近いデンプンが合成される（Figs 11-13）原因となっていると推測される。このことは一定構造を有するアミロペクチンが合成・蓄積される際に、Rアイソザイムが（QEとの相対的な活性比率において）重要な役割を演じている可能性を示している（Fig. 14）。従来Rアイソザイムが種子発芽時においてデンプンを分解するという役割は広く認識されているが、本研究によってRアイソザイムがデンプン蓄積時においても、デンプンの構造を決定する上で役割を担う可能性が高いことが示された。
もし事実ならばこの結果は、デンプン代謝過程を解析する研究において、ミュータントが極めて有用であることを示す好例であろう。
ANALYSIS OF LEAF SENESCENCE THROUGH MUTATION AND AFFINITY-LABELING

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Introduction

The process of plant senescence is characterized by a kind of apoptosis for the optimized material-mobilization for the survival of individuals and/or for the growth of reproductive organs. This is especially evident in the case of leaf senescence, which is controlled by environmental factors, primarily light. Leaves put in shade rapidly degrade and mobilize their components to growing parts. These primary environmental signals are translated to secondary signals such as plant hormones. The rejuvenating effect of cytokinins and the opposite effects of ethylene and abscisic acids are well known evidence of the participation of these phytohormones in the regulation of leaf senescence (see review of Thomas and Stoddart 1980). However, the main processes of senescence, the catabolic mobilization of cell components for their transport and reutilization in the growing part and reproducing organs, has not been well characterized, although the participation of some nucleic control was evident from an enucleation experiment (Yoshida 1961).

For the analysis of the elementary steps of this catabolic process, the author used two approaches; analysis of the senescence-mutants and affinity labeling of the catabolic enzymes. Although there have been some reports on the non-yellowing seed-cotyledons of legumes (Woodworth 1921), physiologically analyzed report on non-yellowing (NY) mutant of leaves has been limited to that of a spontaneous mutant of Festuca (see review by Thomas 1987), which is not autogamous and thus cannot be held as a pure line.

Rice has abundant genetic information mainly elaborated in Japan, and amongst crops is smallest in genome size, providing great merit for molecular biological analysis. We have succeeded to obtain senescence mutant of rice. The analysis of the mutant is expected to greatly contribute to the elucidation of the mechanism of plant leaf senescence.

Complementing the genetic analysis, the author tried affinity labeling of the
probable catabolic enzyme responsible for the senescence-accompanied degradation of cell constituents. Although there was no available labeling agent strictly specific for proteases, thio-diisopropylfluorophosphate (DFP) had a promising specificity for mainly catabolic enzymes with serine as the active site. This was proved to be successful for detection of a probable candidate of a senescence-induced chloroplast-protein localized in the thylakoid of spinach (Kawasaki & Takeuchi 1989).

1. Non-yellowing mutants of rice.

This work was done in collaboration with Shuichi Iida; Institute of Radiation Breeding in NIAR.

Material and Methods

Plant and Mutagenesis Japonica rice (*Oryza sativa* ssp. *japonica*) cultiv. Koshihikari was mutagenized by ethylmethanesulfonate (EMS) and γ-ray-irradiation. Dry seeds (190g) were immersed in distilled water for 1.5h, then imbibed in 0.1M EMS for 5h at 25°C, thoroughly rinsed with distilled water, and then continued germination. A non-yellowing mutant was selected at M₂ generation in late autumn at the paddy field, and a homozygote without other apparent traits was selected at M₃ generation and named NY-1.

Another 200g of dry seeds was irradiated by 30kR (7.7×10³ C/kg) of γ-ray, and a non-yellowing mutant was selected at M₂ and M₃ generation as above and named NY-2.

For physiological analysis plants were grown in a paddy field by re-planting in June, and leaves near the top of full grown plants were taken during summer and autumn and used as materials.

Analysis of dark induced yellowing Flag leaves or second leaves from the top were taken and floated on distilled water in sealed styrol boxes and put in the dark at 28°C.

Preparation of leaves for protein and chlorophyll analysis One g of leaves was finely cut, and then ground by mortar and pestle into a fine powder in liqued nitrogen. The powder was dissolved in 1.5ml of homogenizing medium (62.5 mM Tris-HCl pH6.8, 50 µg/ml leupeptin; Peptide Institute, Osaka, 4 mM phenylmethanesulfonyl fluoride (PMSF); Nacalai Tesque, Kyoto, 10 mM MgCl₂), and spun with a horizontal rotor of a microcentrifuge at 3,000rpm at 4°C for 15 second to pellet the powdered tissue, which was then rinsed twice with the homogenizing medium and the same centrifuge to make P₁ fraction. The supernatant was spun at 12,000rpm for 10min to pellet the crude thylakoid membranes (P₂), and the resultant supernatant was named fraction S. The P₁ or P₂ fractions, and S fraction were assumed to represent crude thylakoid and
soluble fractions, respectively, and their protein composition was analyzed with SDS-PAGE (Laemmli 1971). The samples were dissolved in Leammli's sample buffer with 2% SDS at room temperature. Part of P1 was suspended in acetone to make an 80% (v/v) solution, and after brief centrifugation at 12,000rpm the supernatant was assayed for the content of chlorophyll by the method of Arnon (1949).

**Assay of protease activities** To assess protease activity in the leaf sample, 2 μg of Rubisco was added to the 20 μl of supernatant of homogenates prepared in 62.5 mM Tris-HCl pH 6.8, 10 mM MgCl₂ and incubated at 28°C, and the degradation of Rubisco was assessed by SDS-PAGE. For active staining of proteases, SDS-PAGE was performed in a gel with 0.04% of gelatin. The gel was then washed with 250 ml of 25 mM Tris-HCl pH 8.0 and 1% Triton X-100, and then incubated for 16 h at r.t. in the same buffer and the proteolyzed portion was detected as a CBB-non-stained band (Heussen and Dowdle 1980, Ohtsubo et al. 1989).

**Electron microscopy** Leaf tissues were cut into 1 mm squares and fixed with 2% glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.2 at 4°C overnight, washed twice for 1 h in the same buffer, postfixed with 2% OsO₄ in the same buffer overnight at 4°C, and again washed as before. Dehydration was performed with a series of ethanol rinses followed by the rinse of propyleneoxide. Samples were then immersed and embedded with Quetol 635 (Du Pont), which was cross-linked at 70°C for 2 days. Sections were cut out with glass knives and stained with lead citrate.

**Analysis of seed grain properties** Total nitrogen of the milled rice was assayed using the semimicro-Kjeldahl method (Yasui & Tsutsumi, 1982), and the protein content was estimated by multiplying by a factor of 5.95. Properties of seed starch were characterized by the gelatinization temperature, and maximum viscosity by the method of Horiuchi (1967) using a microviscograph, respectively.

**Results**

**Non-yellowing mutants as whole plants** Through mutagenesis with EMS and γ-ray-irradiation, respectively, each one kind of non-yellowing mutant was found in a paddy field in late autumn. The NY-1 was obtained from EMS treatment, and showed no delay in flowering time from the normal control (Table 1), but remained deep green when the normal had turned yellow (Fig. 1). The NY-1 leaves did not turn yellow until the plant died and turned dry with some green pigments still remaining. Its stalks and husks also maintained greenness as long as it retained water, but turned white as in the normal type when it died and desiccated.

NY-2 remained light green at the same date, although the flowering date was slightly delayed (Table 1), and the persistence of green pigment seems to be much
Table 1 Characteristics of mutants as whole plants.

<table>
<thead>
<tr>
<th></th>
<th>normal type</th>
<th>NY-1</th>
<th>NY-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>flowering date*</td>
<td>Aug. 12</td>
<td>Aug. 12</td>
<td>Aug. 22/Sep. 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(128/41)</td>
</tr>
<tr>
<td>plant height (cm)</td>
<td>90±4</td>
<td>89±5</td>
<td>85±3/78±2</td>
</tr>
<tr>
<td>number of spikes/plant</td>
<td>18±3</td>
<td>15±3</td>
<td>16±2/14±2</td>
</tr>
<tr>
<td>weight of seed (g)</td>
<td>32±8</td>
<td>27±5</td>
<td>32±4/24±6</td>
</tr>
</tbody>
</table>

*Seedlings were transplanted to a paddy field on June 12.

Fig. 1 Whole plants of normal typ and NY mutants of Koshihikari in late autumn after maturation of seeds.
A. Whole plants transplanted from paddy field.
B. Senescent leaves of the above plants.
prolonged than to be explained by the delay in the flowering date. In M₄ of NY-2 there remained one recessive gene (ny-2l) that delayed the flowering date by about 20 days (Table 1), but the greenness of the leaves did not differ among NY-2 even in October, when normal type leaves turned yellow.

NY-2 was only slightly stunted but NY-2L was stunted by about 13% from the normal type (Table 1). The yield of the seed was the same in NY-2 but seemed to be slightly lower in NY-1, and was reduced to 2/3 in the NY-2L mutant.

Genetic characteristics of the mutants F₁ and F₂ analysis revealed that both NY-1 and NY-2 were recessive against the normal type (Table 2). Segregation of a very late flowering group (NY-2L) in NY-2 at a ratio of 1:3 (41:128) indicates the presence of another recessive gene (ny-2l), delaying the flowering time and reducing the plant height and seed yield, in the M₄ group.

Chlorophyll content of the mutant leaves Senescent leaves were taken from field grown plants in November and the chlorophyll content was compared between mutants and the normal type (Table 3). They showed from 2 to 5 fold differences in the remaining chlorophyll content, with the chlorophyll a/b ratio being remarkably low in the NY-1 senescent leaves. This was due to the high residual content of chl b which is known to be high in the most intrinsic thylakoid protein LHCPⅡ. There were no significant differences in leaf weight per unit area.

Ultrastructure of senescent chloroplasts Chloroplasts of the above senescent leaves were observed using an electron microscope (Fig. 2). The chloroplasts of the yellowed leaves of the normal type (Fig. 2-a) decreased in volume and were smaller than those

| Table 2  Segregation of F₁ of crossings between NY mutants and normal type Koshihikari. |
|-----------------------------------------------|---------------------|---------------------|
| phenotype | Nomal | Non-yellowing | ratio of normal/NY |
| NY-1×normal | 170 | 55 | 3.09 |
| NY-2×normal | 173 | 50 | 3.46 |

| Table 3 Chlorophyll content of senescent leaves of mutants and normal type |
|-----------------------------------------------|---------------------|---------------------|
| | normal | NY-1 | NY-2 |
| Chl content \( \mu g/cm² \) | 8.35 | 42.9 | 18.1 |
| Chl a/b ratio | 3.06 | 1.48 | 2.97 |
| leaf weight \( mg/cm² \) | 17.5 | 18.4 | 17.0 |

*Leaves were taken on November 8 from a paddy field.
of mutants at the same period, and their thylakoids degraded into a small number of vesicles, and having an accumulation of osmiophilic granules; probably degraded oil droplets.

In the case of NY-1 (Fig. 2-b), the thylakoid membranes did not degrade to vesicles but the grana stacks coagulated to fuse with each other to be finally deposited as a large stack of membranes. Accumulation of osmiophilic granules was also apparent in this case.

This degeneration of the thylakoid seems to occur, first through selective disintegration of intergrana thylakoid (Fig. 3-a), and then proceeds to the coagulation of the remaining thylakoid (Fig. 3-b).
Fig. 3 Chloroplasts of NY-1 on the degradative stage.
A. Chloroplasts of which intergrana-thylakoid is disintegrating.
B. Chloroplasts showing various stages of thylakoid coagulation.

The chloroplasts of NY-2 (Fig. 2-c) were closer to those of normal green leaves, and their size did not diminish as in other cases at this stage. This suggests that NY-2 is delaying its senescence due to the delayed onset of its degradation process.

Dark induced yellowing of detached leaves Dark induced yellowing is often used to study the process of leaf senescence. When the excised leaves were placed in the dark, normal type and NY-2 yellowed at the same pace, while NY-1 did not yellow significantly. This process was followed by measuring their chlorophyll content (Fig. 4), which fell to near 0 in 7 days at the same pace in both normal type and NY-2, but remained at 70% of its initial level in NY-1. The chlorophyll a/b ratios were rather constant in both the normal type and NY-2, but significantly decreased in NY-1, just as in the case of post-flowering senescence (Table 3). This was also due to the selective remaining of chlorophyll b.

Noticeably, this phenotype of non-yellowing is not so apparent in seedlings grown in artificial light or in greenhouses. Thick, fully grown leaves seemed to be necessary for the expression of NY-1 (Data not shown).

Comparison of protein composition of the senescent leaves The composition of proteins of senescent leaves were compared among NY-mutants and the normal type by SDS-PAGE (Fig. 5). In all cases, large (L) and small (S) subunits of Rubisco decreased significantly. NY-2 showed the most persistent existence of Rubisco compared to the
Fig. 4  Comparison of the decrease of chlorophyll in leaves of normal and NY mutants kept in the dark.
A. Change of chlorophyll content in the leaves kept in the dark.
B. Change of chlorophyll a/b ratio.

Fig. 5  Soluble and membrane proteins of senescent leaves on SDS-PAGE.
S : Soluble proteins.  P : Membrane proteins, mainly representing thylakoid proteins.  L, S : large, and small subunits of Rubisco.  LH : LHCP II.  Gel was stained with CBB. Chlorophyll-protein complex (LHCP II) was visible in P fraction of NY-1 mutant. Two samples were taken for each fraction. Outer lanes : Molecular weight markers.
others. The residual content of soluble and membrane proteins was smallest in the senescent leaves of the normal type. NY mutants were especially rich in residual membrane proteins, most prominently LHCP II (light-harvesting chlorophyll protein II) in NY-1. The presence of chlorophyll as a complex with the LHCP protein was apparent in the non-stained gel.

Protease in the senescent leaves In the absence of protease inhibitors, considerable degradation of proteins were observed, especially in the soluble fraction of the senescent leaves. The effect of various protease inhibitors were examined adding Rubisco proteins as a substrate in the homogenate of senescent normal type leaves (Fig. 6). Activation with mercaptoethanol (ME) and SDS, and selective inhibition with leupeptin and, to lesser extent, with \( p \)-chloromercuribenoxy acid (pCMB) indicates that the main protease is the SH-type.

The activity of this strong SH-protease was compared between mutant and normal type of senescent leaves (Fig. 7). Apparently the protease is strongest in the
normal type, and those of NY-2 and NY-1 were much lower and about the same level, corresponding to the remaining proteins in the leaf (Fig. 5). Increase of protease level in the senescent leaves seems to be suppressed in the mutant leaves, in both control and ME- or SDS-added conditions, although it is not yet clear whether this is the cause or result of these NY-mutations. On the other hand, the active staining of protease with gelatin embedded gel revealed a 40 kD band of the same level among mutant and normal types (data not shown). This SDS-resistant protease may be another one from that shown in Fig.7.

Effects of NY mutation on the properties of endosperm As the NY mutants seem to delay the disintegration of leaf proteins, it was expected to affect the mobilization of organic nitrogen from leaves to seeds at the seed maturation stage. The total yield of seeds decreased by about 25% in NY-2L, probably due to the late and nonoptimal maturing period, but also by about 15% in NY-1 (Table 1). The seed protein content was considerably higher than the normal in NY-2, but about the same level as normal in NY-1 (Table 4). The seed protein content was thus not decreased by the delay of leaf protein mobilization. The decreased highest viscosity level and high iodine color indicates an increased ratio of amylose/amylopectin (Table 4), especially in NY-2, corresponding to the delay of leaf protein degradation.
Table 4  Properties of the milled rices of the mutants and normal type.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>NY-1</th>
<th>NY-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kjeldahl protein (%)</td>
<td>5.8</td>
<td>6.0</td>
<td>7.3</td>
</tr>
<tr>
<td>highest viscosity (g · cm)</td>
<td>12.0</td>
<td>9.9</td>
<td>6.4</td>
</tr>
<tr>
<td>Iodine color/</td>
<td>0.35</td>
<td>0.39</td>
<td>0.58</td>
</tr>
<tr>
<td>water soluble material (A_{660}/g)</td>
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<td></td>
</tr>
<tr>
<td>dynamic viscosity (10^3 dyn/cm²)</td>
<td>2.80</td>
<td>2.70</td>
<td>3.41</td>
</tr>
</tbody>
</table>

Discussion

The difficulties in studying the physiology and biochemistry of plant senescence lie in the weakened compartmentation of organelles and the elevated activities of the catabolic enzymes. Mutant analysis can help to circumvent these difficulties, and is indispensable to elucidate the elementary steps of the senescence, as in the case of development.

Two kinds of non-yellowing mutants of rice were obtained by mutagenesis with EMS (NY-1) and γ-ray irradiation (NY-2). NY-1 leaves stayed deep green after seed maturation (Fig. 1), and EM observation indicated that it was unable to degrade thylakoid membranes, especially those of grana thylakoid (Fig. 2,3). Consequently, the most intrinsic chlorophyll protein in the thylakoid membranes (LHCP II) with chlorophyll-b, was the major persistent component in the thylakoid of senescent leaves (Fig. 4, 5, Table 3). The non-yellowing phenotype was also observed in the dark-induced senescence of detached leaves (Fig. 4), as in the case of natural senescence in the paddy field. It is notable that NY-1 does not delay the flowering time (Table 1).

There are some reports on non-senescent types of sorghum (Duncan et al. 1981) and soybean (Woodworth 1921), and delayed leaf senescence of soybean (Pierce et al. 1984). However, only one extensively studied non-yellowing mutant is a spontaneous mutant of a Festuca pratensis strain Bf993 (Thomas 1977, 1987). Recently, its lesion step was shown to be in the degradation of chlorophyll pigments after dephytolation (Thomas et al. 1989). However, there are many differences between rice (NY-1) and Festuca (Bf993) non-yellowing mutants. First, in NY-1, thylakoids disintegrate at the inter-grana site, and then become coagulated and condensed, while in Bf993, thylakoids do not disrupt but seem to swell loosely (Thomas 1977, 1990). Second, as the senescence proceeds, the chl a/b ratio drops to less than half in NY-1, while that ratio increases in Bf993 (Thomas 1983).

In the case of Bf993, because of the lesion at the degradation of chlorophyll and haem, which may be tugging the senescent chl/protein complex, the degradation of the apoproteins of these complexes was supposed to be inhibited (Thomas 1990). However,
in NY-1, the degradation of thylakoid seems to be more directly inhibited at the degradation enzyme(s), because not only LHCP: the major chlorophyll protein complex, but also other proteins, even the soluble proteins seem to become more persistent than those of normal leaves in NY-1 (Fig. 5). Indeed, the strongest protease in leaf, which was activated by ME and SDS was apparently suppressed in NY-1 (Fig. 7), although it is not yet clear whether this enzyme is responsible for degradation of chloroplast. This strong protease has hindered analysis of the hypothetical senescence-induced intrachloroplast protease which was supposed to control protein degradation in senescent chloroplasts (Kimura 1988, Mae 1989).

The NY-1 mutant phenotypes were most apparent in the field grown plants, and not so conspicuous in the plants grown in growth chambers or greenhouses (data not shown). This may be due to the highly developed thylakoids of the field grown plants, in contrast to the poorly developed thylakoid of the indoor grown ones which may be degraded by the remaining “leaky” catabolic activities of NY-1 mutant. This may support the hypothesis that the most probable lesion site may be the protease, or the membrane lipid degrading enzyme in chloroplasts.

NY-2 was found as a pale green mutant in the late autumn field (Fig. 1). NY-2 was about 10 days late in the flowering, and the present strain contains an additional recessive mutation (NY-2L) delaying flowering for another 20 days (Table 1), but in October there was little difference in leaf greenness between NY-2 and NY-2L, and in the dark their detached leaves yellowed at the same pace as the normal type. Cultivar Nipponbare, grown on the same site, flowered around Sep. 1 and yellowed faster than the NY-2, indicating that the persistent greenness of NY-2 in late autumn is not only due to its late flowering time. In NY-2, a rather intact ultrastructure of chloroplast (Fig. 2), and overall protein constituents remain until later stage (Fig. 5). This contrasts to the rather selective protein persistence in NY-1. Therefore, NY-2 mutation seems to be related to a leaf senescence starting mechanism.

Rice is one of the most genetically extensively analyzed crops. As the NY-1 and NY-2 are shown to be a single recessive mutation (Table 2), their locus may be mapped easily with fine accuracy on the genetic and RFLP map (Saito et al. 1991). A series of F₂ individuals was obtained from the crossing between an indica cultivar Kasalath and NY-1, and their RFLP is now being analyzed.

The non-yellowing of leaves in NY-1 slightly lowered the seed yield (Table 1). This is not surprising because the non-yellowing means only the persistence of a part of the chlorophyll protein, not the persistence of photosynthetic activity. And the delay in nitrogen mobilization in NY-mutants was expected to lower the seed protein content (which is known to affect the taste of rice; Iwama et al. 1974, Yamashita & Fujimoto 1974). However, in milled rice of NY-1 the protein content was about the same or
slightly higher than the normal type (Table 4). The other physical properties of the boiled rice were about the same or slightly unfavorable to NY-1, and considerably inferior in NY-2. This may be due to the low-temperature activation of amylose synthase during maturation. These mutants may be useful for analysis to optimize the maturation process for rice taste.


Materials and Methods

Plant Materials and sample preparation Spinach (Spinacia oleracea) was grown in a greenhouse, and leaves at a series of similar stages of development were taken with a green meter GM1, (Fuji Photo Film Co., Tokyo) as a reference. Typically, leaves 3, 5, 6, 8, 10, 11, and 13 of a 15-leaved plant were taken. Sections of 1 cm² or 0.2 g fresh weight were taken and measured for their chlorophyll content or pulverized in liquid N₂ with a mortar and pestle to analyze its protein and DFP-affinity. The frozen powder was thawed in a homogenizing medium (0.5 M sorbitol, 0.1 M TrisHCl pH 6.8, 5 mM MgCl₂, and 5 mM isoascorbate), and centrifuged at 750 × g for 30 s, then at 12,000 × g for 5 min to separate precipitate (P fraction) and supernatant (S fraction).

Changes in proteins and DFP-binding protein Aliquots of S and P fractions of each stage of leaf were incubated with 37 kBq [1,3⁻³H] DFP (130 GBq/m mol) for 1 h, and electrophoresed in SDS-PAGE, stained with CBB and then fluorographed at –90°C. Changes in protein components and DFP-binding protein were quantitated with a densitometer.

Isolation of chloroplast and fractionation of thylakoid complexes Leaves were homogenized with a Waring blender three times for 5 s in Nakatan and Barber’s (1977) medium and intact chloroplasts were isolated by the method of Morgenthaler et al. (1975) using a Percoll (Pharmacia) gradient. Aliquot of intact chloroplast was ruptured in 50 mM TrisHCl pH 6.8 and 5 mM MgCl₂ and centrifuged at 12,000 × g for 15 min to separate the stroma and thylakoid. Aliquot of thylakoid was incubated with [³H] DFP and solubilized with 4% Triton X-100, and centrifuged at 160,000 × g for 1 h to remove insolubles and the resulting supernatant was applied to a 10~30% sucrose density gradient in 1% Triton X-100, 50 mM TrisHCl pH 6.8, 2 mM EDTA, and centrifuged at 196,000 × g for 20 h. In some cases solubilized thylakoid was applied to a 2-dimensional electrophoresis of O’Farrell (1975), and analyzed for [³H] DFP-binding with fluorography.
Results and Discussion

Changes in leaf proteins and DFP-binding proteins  As seen in chapter 1 in leaf cells, the predominant protease activity is that of vacuolar SH-protease, and other minor cytoplasmic proteases are hidden behind this activity. To search for a cytoplasmic protease induced by senescence, which may be responsible for protein mobilization in leaf senescence, affinity labeling of protease by DFP was tried. DFP is a specific inhibitor of serine enzymes and serine-protease, which is the most probable candidate of the senescence-induced protease, from its abundant distribution. Changes in DFP-binding proteins accompanied leaf development and senescence was followed

Fig. 8  Changes in proteins stained with CBB (A) and protein bound DFP (B) in soluble (S) and membrane (M) fractions associated with leaf aging. Fractions from the same weight of leaves at various ages were applied to SDS-PAGE. Protein-bound [3H] DFP was visualized using fluorography (B). LS of Rubisco, CF1α, and LHCP(II) are indicated by arrows.
with SDS-PAGE, in comparison with the major leaf proteins stained with CBB (Fig. 8). Major leaf proteins showed a rather similar pattern from each other, peaking at the mature phase and falling to nearly zero on leaf senescence (Fig. 8a). The membrane proteins seemed to be more persistent than the soluble proteins in the later stages of senescence. In contrast, DFP-binding proteins showed distinctly different patterns from those of the major proteins (Fig. 8B): A) Richest in the young stage and subsequently decrease (band 4). B) Rather constant in most stages (band 1, 3). C) Increases as leaves get senescent (band 38 kD in P fraction). Patterns A and C suggest their regulative functions during development and senescence. The 38 kD DFP-binding protein is of special interest as a candidate for senescent-induced protease. The quantitated change of this and major leaf proteins indicates that the 38 kD protein increases most when degradation of major leaf proteins is fastest. Also, in the case of dark-induced senescence of detached leaves, a similar induction of the 38 kD DFP-binding protein was apparent (Data not shown). Similar changes in leaf proteins and

![Image](image_url)

Fig. 9 Sucrose density gradient centrifugation of thylakoid component and DFP-binding protein solubilized with Triton X-100. The crude thylakoid fraction (P) was solubilized with 4% Triton X-100 and centrifuged at 160,000×g for 1h to precipitate insolubles (TP). The supernatant was applied to a 10-30% sucrose density gradient and centrifuged at 196,000g for 20h at 4°C. The fraction were applied to SDS-PAGE. A : CBB stain. B : fluorography.
DFP-binding proteins were also observed in soybean leaves.

Localization of the DFP-binding protein The intracellular localization of DFP-binding proteins was analyzed using cell fractionation. As the chloroplast occupies the largest proportion of the green leaves' cytoplasm and shows the most apparent volume decrease in senescence (refer Fig. 1), intact chloroplast was taken from senescing leaves and its DFP-binding proteins were analyzed. Bands 1 and 5 in S fraction was shown to be in the stroma fraction, and the 38 kD protein in the thylakoid fraction. Therefore, it is probable that the 38 kD protein may be functioning as a degrading factor of chloroplast components. As most of the thylakoid component is present as protein complexes, Triton X-100 solubilized thylakoid was fractionated on a sucrose density gradient, and the 38-kD DFP binding protein was fractionated between PSI and PSII complexes. This seems to belong to a rather minor complex or may be dissociating from the faster moving complex. As there was apparently no corresponding CBB band, if a molecular stoichiometry is held, the complex may be a very minor one.

2-dimensional electrophoresis of thylakoid proteins This was also confirmed from a 2-D electrophoresis of thylakoid proteins solubilized with Triton X-100 (Fig. 10A). The 38-kD DFP binding protein was detected around pI 6, but no corresponding CBB stained spot was detected. This was also the case in a 2D-electrophoresis after concentration of the near 38 kD region with a preparative SDS-PAGE (Fig. 10B). The

![Figure 10](image_url) 2D-electrophoresis of Triton X-100 solubilized thylakoid. A : Crude thylakoid fraction (P) was incubated with [3H] DFP and pre-fractionated as Fig. 8 and applied to 2D-electrophoresis with ampholine 5-7 and 6-8 1 : 1. B : 1D-EP and 2D-EP of a near 38-kD fraction of a preparative SDS-PAGE. A and B were CBB stained. The DFP-binding protein spots on fluorography is indicated by contours.
38-kD DFP-binding protein was thus supposed to be a very minor component, as was expected from its probable regulatory function.

Effect of various protease inhibitors  Active staining of the protease in SDS-PAGE detected only a soluble SH-dependent activity of 88 kD in spinach (Kawasaki 1989), in contrast to c.a. 40 kD in rice. Therefore, an indirect method of inferring the character of the 38 kD DFP-binding protein was taken by comparing the effect of various protease inhibitors (Fig. 11). As shown, only PMSF, a rather specific inhibitor of serine-protease, completely inhibited the binding of DFP to the 38 kD protein in old leaf P fraction. In younger leaves or in S fractions, PMSF found to inhibit some other

Fig. 11  Effects of various inhibitors on DFP binding to the protein in membrane (P) and soluble (S) fractions. Young and old leaves were fractionated and incubated with $[^3H]DFP$ in the presence of 4 mM PMSF, TLCK, TPCK, α-phenanthroline, 20mM SHAM, or 1mM pCMB after 1h preincubation with them, respectively. After SDS-PAGE both were applied to fluorography.
DFP-binding proteins' activity, suggesting presence of other proteinase-like components. Other inhibitors for SH-(pCMB), metal-protease (o-phenanthroline), and substrate-mimicked inhibitors (TLCK, TPCK) showed no effect, except for a slight inhibition with pCMB. As PMSF is more specific than DFP in inhibition of serine protease (Fahrney and Gold 1963), this is taken as supporting evidence for considering 38-kD protein as a protease.

A 38-kD DFP-binding protein was identified as a senescence-induced thylakoid protein in spinach chloroplast. This protein was induced in dark-induced senescence of detached leaves, and reached a maximum at the stage when leaf major proteins are degrading most rapidly. Similar protein was also seen in senescent soybean leaves. Its protease-like character was indicated from specific inhibition by PMSF. These characters of the 38-kD DFP-binding protein strongly suggest its function as a protease for mobilization of chloroplast protein components.

Isolation and structure analysis of this DFP-binding protein will finally elucidate its biochemical function. Although the quantity of the protein is small, the result of 2D-fluororography indicate a positive possibility of purification, and all the above results suggest a probable important regulative function for this protein.

References


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葉の老化過程の分析
—非塩化変異体とアファニティラベルを用いて—

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農業生物資源研究所

動くことができない植物において、葉の老化過程は環境に対する適応戦略の重要な一環をなしており、光を受けられなくなった下位葉が生長部分に、あるいは冬や乾季を前に葉が種子や貯蔵器官に構成分を移動し回収することは、生存のための重要な営みである。葉の老化は光を始めとする環境要因の変動を感知して、サイトカイニン、エチレン等、植物ホルモンの変動を2次メッセージとして始まると考えられるが、老化過程の主体となる生化学的な過程について知られるところは、はなはだ少ない。分化と並んで、この様に複雑に制御される過程の集合の分析には変異体による遺伝学的アプローチが最も有力と考えられ、放射線育種場で見出されたイネの2種類の葉の老化の変異体、NY-1とNY-2についてその老化の過程を野生型と比較して解析した。

NY-1は穂が完熟しても葉は黄化せず、深い緑を保ち続ける。また切除した葉を暗所においても黄化が起こらない。電顕観察では葉緑体は野生型に見られるような老化に伴うグラナチャコイド膜の崩壊が起こらず、クロロフィル（Chl）の分解も少ない。特にChl bの分解が遅れるため、Chl a/b比の減少が著しい。タンパク質の電気泳動分析ではグラナチャコイドの内在タンパク質である光化学系IIの集光タンパク質（LHCP）の残存が著しく、Chl bの残存はこのタンパク質の分解の遅れによるものと考えられた。NY-1はその他の可溶性及び膜タンパク質もやや分解が遅れる傾向が見られ、老化時に誘導されるべきプロテアーゼ等の分解酵素が欠損しているものと考えられた。

一方NY-2の葉もある穂の完熟時にNY-1より淡緑色であるが緑を保っている。タンパク質の分解や葉緑体の微細構造の崩壊はNY-1よりも遅れており、老化過程全体の遅延が起こっているものと考えられる。切除葉を暗所に置くと、野生型と同様に黄化が起こることから、光周期性あるいは種子の成熟等のシグナルの伝達や認識に異常があると考えられた。NY-2の出穂は野生型より10日ほど遅れていたが、黄化の進行はそれよりもはあるかに遅れる。

葉の老化に際して、葉緑体はその数は減少しないものの著しく体積を減少させ、チャコイド膜も著しい崩壊を起こす。従って、葉緑体内でのタンパク質の分解は葉の老化過程の中心をなす生化学過程と考えられるが、これまでの多くの試みは液胞に由来する強力なプロテアーゼにより阻害されてきた。これをさけて老分化時に誘導される微量のタンパク質分解酵素を検出す手段として、[3H]diisopropylfluorophosphate（DFP）によるアファニ
ティラベルを試みた。ホウレンソウ葉を材料にして、老化の各段階の葉を用いた実験から38kDのチラコイド結合タンパク質が老化時に特異的に誘導されることが見出され、PMSFで特異的に阻害されることからプロテアーゼ様の活性を持つことが示唆された。今後、これらの手段を統合して老化過程の総合的な解析を目指す。
AMINO ACID AND AMINO ACID ANALOG RESISTANT MUTANTS IN HIGHER PLANTS

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In higher plants, amino acid biosynthesis is regulated by a negative feedback control system which prevents overproduction of any one amino acid; too much amino acid blocks the key enzyme(s) in the biosynthetic pathway (GENGENBACH et al. 1978, MIFLIN et al. 1983, LEA et al. 1992). Some amino acids have been shown to be a potent growth inhibitor at whole plant level (MIFLIN 1969) and at cultured cell level (FURUHASHI and YATAZAWA 1970, BONNER et al. 1992). The growth inhibitory effect of amino acid is strengthened by an analog of the amino acid and the particular combination of amino acids (e.g., lysine and threonine equimolar solution; LT). It has been shown that mutants resistant to amino acid or amino acid analog can be isolated at both whole plant level and cultured cell level. The most important characteristic of the amino acid and amino acid analog resistant mutant is that some of the mutants accumulate the corresponding free amino acid in leaves and seeds. Free amino acid accumulation in the resistant mutant has been shown to be due to the lack of feedback inhibition (MIFLIN et al. 1983).

From the beginning, studies on the amino acid analog resistant mutants in crop plants have been carried out to improve nutritional quality of cereal grains. In cereal crops, much attention has been paid to lysine and/or threonine accumulating mutants, because both these amino acids are nutritionally deficient in grain storage protein. Both lysine and threonine are included in the aspartate derived amino acid group, and the interactions of lysine and threonine on plant growth have been described (FURUHASHI and YATAZAWA 1970, BRIGHT et al. 1978). The feedback regulation mechanism in the aspartate pathway has been clarified using the resistant mutants, LT and 2-aminoethyl-L-cystein (a lysine analog, AEC) (MIFLIN et al. 1983). Proline metabolism plays an important role in plants grown under environmental stresses such as saline, dry or cold conditions. There have been several reports on proline-accumulating mutants in relation to stress resistance (KUEH and BRIGHT 1981, VAN SWAAIJ et al. 1987). However, at present, the biosynthetic pathway of proline in higher plants in not established and the role of proline accumulation under stress conditions is still unclear.
### Table 1. Whole plant mutants resistant to amino acids and amino acid analogs.

<table>
<thead>
<tr>
<th>Species</th>
<th>Selection level</th>
<th>Amino acid accumulation</th>
<th>Gene</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>[AEC resistance]</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barley</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R906</td>
<td>seedling</td>
<td>None</td>
<td>s.r.</td>
<td>Bright <em>et al.</em> 1979</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(aec-1)</td>
<td></td>
</tr>
<tr>
<td>Nicotiana sylvestris</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAEC-1, 2</td>
<td>protoplast</td>
<td>Lys</td>
<td>s.d.</td>
<td>Negrutiu <em>et al.</em> 1984</td>
</tr>
<tr>
<td>Pennisetum americanum</td>
<td>callus</td>
<td>Lys</td>
<td></td>
<td>Boyes &amp; Vasil 1987</td>
</tr>
<tr>
<td>Rice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Two lines</td>
<td>callus</td>
<td>High Lys, Ala, Arg, Asg in seed protein</td>
<td></td>
<td>Schaeffer &amp; Sharpe Jr. 1981</td>
</tr>
<tr>
<td>379–2–4C</td>
<td>callus</td>
<td>High Lys</td>
<td>s.r.</td>
<td>Schaeffer &amp; Sharpe Jr. 1987</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(also LT resistant)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>[LT resistance]</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barley</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R2501</td>
<td>seedling</td>
<td>Thr</td>
<td>s.d.</td>
<td>Bright <em>et al.</em> 1982</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Ltlα)</td>
<td></td>
</tr>
<tr>
<td>R3004</td>
<td>seedling</td>
<td>Thr</td>
<td>s.d.</td>
<td>Bright <em>et al.</em> 1982</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(L2)</td>
<td></td>
</tr>
<tr>
<td>R3202</td>
<td>seedling</td>
<td>None</td>
<td>s.d.</td>
<td>Bright <em>et al.</em> 1982</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Lt1β)</td>
<td></td>
</tr>
<tr>
<td>Carrot</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>...</td>
<td>embryoid</td>
<td>Thr, Ileu</td>
<td></td>
<td>Cattoir–Reynaerts <em>et al.</em> 1983</td>
</tr>
<tr>
<td>Chickpea</td>
<td>seedling</td>
<td></td>
<td></td>
<td>Sains &amp; Rao 1982</td>
</tr>
<tr>
<td>S8, PS16, ML9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maize</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LT19</td>
<td>immature embryo</td>
<td>Thr</td>
<td>s.d.</td>
<td>Hibberd &amp; Green 1982</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Ltr19 (Ask–LT19))</td>
<td></td>
</tr>
<tr>
<td>LT20</td>
<td>immature embryo</td>
<td>Thr</td>
<td>s.d.</td>
<td>Diedrick <em>et al.</em> 1990</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Ask2–LT20)</td>
<td></td>
</tr>
<tr>
<td>...</td>
<td>callus</td>
<td>Thr, Lys, Met</td>
<td>s. d.</td>
<td>Miao <em>et al.</em> 1988</td>
</tr>
<tr>
<td>Nicotiana sylvestris</td>
<td>protoplast</td>
<td>Thr</td>
<td>s.d.</td>
<td>Frankard <em>et al.</em> 1991</td>
</tr>
<tr>
<td>RLT70</td>
<td></td>
<td></td>
<td></td>
<td>(ak–LT1)</td>
</tr>
<tr>
<td>Rice</td>
<td>Callus</td>
<td>High Lys</td>
<td>s.r.</td>
<td>Schaeffer &amp; Sharpe Jr. 1987</td>
</tr>
<tr>
<td>379–2–4C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(also AEC resistant)</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
Table 1. (continued)

[HYP resistance]

<table>
<thead>
<tr>
<th>Plant</th>
<th>Stage</th>
<th>Amino Acid</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley</td>
<td>R5201</td>
<td>seedling</td>
<td>Pro</td>
<td>s.s.d. (Hypla)</td>
</tr>
<tr>
<td></td>
<td>R6102</td>
<td>seedling</td>
<td>Pro</td>
<td>s.s.d. (Hyplib)</td>
</tr>
<tr>
<td></td>
<td>R6901</td>
<td>seedling</td>
<td>Pro</td>
<td>s.s.d.</td>
</tr>
<tr>
<td></td>
<td>R6902</td>
<td>seedling</td>
<td>Pro</td>
<td></td>
</tr>
<tr>
<td>Potato</td>
<td>H2-578</td>
<td>callus</td>
<td>Pro</td>
<td></td>
</tr>
<tr>
<td>Rice</td>
<td>HYP101</td>
<td>seedling</td>
<td>None</td>
<td>s.r. (hpr-1)</td>
</tr>
<tr>
<td></td>
<td>HYP202</td>
<td>seedling</td>
<td>None</td>
<td>s.r. (hpr-2(t))</td>
</tr>
<tr>
<td></td>
<td>HYP203</td>
<td>seedling</td>
<td>None</td>
<td>s.r. (hpr-3(t))</td>
</tr>
<tr>
<td></td>
<td>HYP205</td>
<td>seedling</td>
<td>Pro</td>
<td>d. (?)*</td>
</tr>
<tr>
<td></td>
<td>HYP210</td>
<td>seedling</td>
<td>Pro</td>
<td>d. (?)*</td>
</tr>
</tbody>
</table>

[5-MT (α-MT) resistance]

<table>
<thead>
<tr>
<th>Plant</th>
<th>Stage</th>
<th>Amino Acid</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis thaliana</td>
<td>amt-1 seedling</td>
<td>Try</td>
<td>s.s.d.</td>
<td>Kreps &amp; Town 1992</td>
</tr>
<tr>
<td>Maize</td>
<td>callus</td>
<td>Try</td>
<td></td>
<td>Miao et al. 1988</td>
</tr>
<tr>
<td>Rice</td>
<td>MTR1 callus</td>
<td>Try, Phe</td>
<td>s.d. (?)*</td>
<td>Wakasa &amp; Widholm 1987</td>
</tr>
<tr>
<td></td>
<td>TR1 seedling</td>
<td>Try, His, Phe</td>
<td>s.d.</td>
<td>Lee &amp; Kameya 1991</td>
</tr>
<tr>
<td>Wheat</td>
<td>--- (5 plants) seedling</td>
<td>Try</td>
<td></td>
<td>Singh &amp; Widholm 1975</td>
</tr>
</tbody>
</table>

[Methionine sulfoximine resistance]

<table>
<thead>
<tr>
<th>Plant</th>
<th>Stage</th>
<th>Amino Acid</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco</td>
<td>--- haploid cell</td>
<td>Met</td>
<td></td>
<td>Carlson 1973</td>
</tr>
</tbody>
</table>

[Ethionine resistance]

<table>
<thead>
<tr>
<th>Plant</th>
<th>Stage</th>
<th>Amino Acid</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cowpea</td>
<td>--- seedling</td>
<td></td>
<td></td>
<td>Dessauer &amp; Hannah 1978</td>
</tr>
</tbody>
</table>

[Para-fluorophenylanine resistance]

<table>
<thead>
<tr>
<th>Plant</th>
<th>Stage</th>
<th>Amino Acid</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis thaliana</td>
<td>CR seedling</td>
<td>s.r.</td>
<td></td>
<td>James &amp; Jacobs 1976</td>
</tr>
</tbody>
</table>
Table 1. (continued)

[Valine resistance]

<table>
<thead>
<tr>
<th>Species</th>
<th>Method</th>
<th>s.r.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotiana plumbaginifolia</td>
<td>protoplast (n)</td>
<td></td>
<td>Marion-Poll <em>et al.</em> 1988 (valine uptake deficiency)</td>
</tr>
<tr>
<td>Nicotiana sylvestris</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NsVR Tobacco</td>
<td>protoplast (n/2n)</td>
<td></td>
<td>Vunsh <em>et al.</em> 1982</td>
</tr>
<tr>
<td>Valr-1,6,7 Tobacco</td>
<td>protoplast</td>
<td>s.d.</td>
<td>Bourgin 1978</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Vr1)</td>
<td>Bourgin <em>et al.</em> 1985</td>
</tr>
<tr>
<td>Valr-2,3,4,5 Tobacco</td>
<td>protoplast</td>
<td>d.r.</td>
<td>Bourgin <em>et al.</em> 1985</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(vr2, vr3)</td>
<td></td>
</tr>
<tr>
<td>NtVR Tobacco</td>
<td>protoplast (n/2n)</td>
<td></td>
<td>Vunsh <em>et al.</em> 1982</td>
</tr>
</tbody>
</table>

* Resistance is transmitted heterozygously.  
Abbreviations: s.d., single dominant  
ss.d., single semi-dominant  
s.r., single recessive  
d.r., digenic recessive

Tryptophan is a precursor of indole-3-acetic acid (IAA). It has been suggested that a tryptophan overproducing plant selected as a tryptophan analog resistant mutant can be useful for the research on the hormonal regulation of plant growth in relation to the IAA biosynthesis (Widholm 1977, Sung 1979). Furthermore, there have been several reports on valine resistant mutants (Bourgin 1978, Marion-Poll *et al.* 1988). The characteristics of amino acid analog resistant mutants were expressed in the whole plant system as well as in the cultured cell system. This fact indicates that the amino acid analog resistance can be useful as a genetic marker in the research of plant biotechnology (Lee and Kameya 1989). Table 1 summarizes the amino acid and amino acid analog resistant mutants which are expressed at the whole plant level.

This paper describes the technique for selecting the whole plant mutants which are resistant to hydroxy-L-proline (a proline analog, Hyp) or LT in rice (cv. Nipponbare) and the nature of the selected mutants. Selection procedures and characterization of Hyp resistant mutants have already been reported elsewhere (Hasegawa and Inoue 1983, Hasegawa and Mori 1986, Mori *et al.* 1989).

**Hydroxy-L-proline resistant mutants**

It is well-known that plants grown under environmental stresses such as saline, dry or cold conditions accumulate free proline in the leaves. However, the role of free proline in relation to the stress resistance is still unclear. Mutants with proline
metabolism can be useful for the understanding of stress resistance in higher plants (MIPLIN et al. 1983).

Mutants resistant to Hyp were obtained in barley and potato. KUEH and BRIGHT (1981, 1982) reported that four Hyp resistant barley mutants accumulated free proline in the leaves and that the resistance was inherited as a semi-dominant trait. VAN SWAAIJ et al. (1986, 1987) isolated Hyp resistant potato clones from the Hyp resistant calli and demonstrated that the mutant clones accumulated free proline in their leaves. It is interesting that the mutant clone also showed increased frost tolerance. In higher plants, Hyp has a vital function as a constituent of glycoprotein in the cell wall (HOOD et al. 1991). Hyp resistant mutants could be useful for understanding biosynthesis and the function of Hyp-rich glycoprotein.

Selection of mutants

In order to determine the selection pressure for isolating Hyp resistant mutants, the seeds, presoaked in distilled water for 4 days, were cultured in the Hyp solution at the concentrations of 0 to $10^{-3}$M in a growth chamber at 25°C. When the plants were grown in the solution of more than $10^{-2}$M Hyp, the seedling growth was severely inhibited, indicating that Hyp resistant mutants could be selected at concentrations of $10^{-4}$M or higher.

The $M_3$ seeds, which had been mutagenized by sodium azide (NaN₃), ethylene imine (EI), ethyl methanesulfonate (EMS) and gamma-ray, were allowed to germinate in distilled water for 4 days at 25°C and then were cultured with $2 \times 10^{-4}$M Hyp in the growth chamber at 25°C. Screening of the mutants was performed using the floating culture system shown in Fig. 1. After culturing for 14 days, seedlings as well-grown as those cultured with distilled water were selected. The selected seedlings were cultured with a nutrient solution containing $2 \times 10^{-4}$M Hyp for 14 days or more. At this stage well-grown seedlings were selected as Hyp resistant variants. The $M_3$ seeds were harvested as an $M_2$ plant progeny.

Frequencies of Hyp resistant variants are shown in Table 2. Twenty-seven resistant variants were obtained from 91,936 $M_2$ seedlings. Of the selected seedlings, 22 were produced by EI treatment. Frequencies for Hyp resistant variants in $M_2$ were $2.5 \times 10^{-4}$ and $1.6 \times 10^{-3}$ per $M_2$ seedling for 0.2 and 0.4% EI treatment for 2h, respectively. These frequencies were about a tenth of those of chlorophyll mutants. The treatment with NaN₃ and EMS each produced one resistant variant. Three variants were selected from the $M_2$ seedlings irradiated with gamma-ray, but two of them were sterile. The $M_3$ seeds were cultured with Hyp in the same way as the $M_2$ seeds for the reexamination of Hyp resistance. Of 25 $M_3$ lines, one line which was a progeny of the gamma-ray induced variant did not recover Hyp resistance. Finally, 24 Hyp-resistant
mutant lines (HYP lines) were established. Genetic and biochemical analysis of the mutants were carried out using the seeds of the $M_s$ and the following generations (Table 2).
Characterization of the mutants

Genetic and biochemical characterization of the Hyp resistant mutants demonstrated that the mutants were divided into two groups, non-proline-accumulating and proline-accumulating mutants. In HYP101, HYP202 and HYP203, Hyp resistance was transmitted as a single recessive trait and free proline accumulation was detected neither in seeds nor in seedlings. These three lines did not show thiopropine (another proline analog) resistance. On the other hand, in the other 21 HYP lines, resistance was transmitted heterozygously. The progenies of HYP205 and HYP210 were characterized further. The most important characteristic of HYP205 and HYP210 was that free proline accumulated in both the seeds and the seedlings. Table 3 shows the difference in free proline content in the leaves and the seeds between the two types of HYP mutants. Free proline contents in the seeds of HYP205 and HYP210 were about 24 and 12 times of that of the wild type, respectively, while 6 and 4 times increase in the leaves of HYP205 and HYP210, respectively. Since amino acid analog resistant mutants which are controlled by a dominant gene overproduce the corresponding free amino acid due to the feedback insensitivity, it is reasonable to conclude that Hyp resistance in HYP205 and HYP210 are controlled by dominant gene(s).

<table>
<thead>
<tr>
<th></th>
<th>Seeds</th>
<th>Seedlings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nipponbare</td>
<td>88± 4*</td>
<td>244± 44</td>
</tr>
<tr>
<td>HYP101</td>
<td>184± 7</td>
<td>206± 4</td>
</tr>
<tr>
<td>HYP203</td>
<td>104± 4</td>
<td>278± 40</td>
</tr>
<tr>
<td>HYP205</td>
<td>2,132±137</td>
<td>1,555± 63</td>
</tr>
<tr>
<td>HYP210</td>
<td>1,085± 62</td>
<td>925±232</td>
</tr>
</tbody>
</table>

*: m ± s.d. (average of three replications)

A clear difference between non-proline-accumulating mutants and proline accumulating mutants was also detected in Hyp contents of the leaf when the seedlings were cultured with 10⁻³M Hyp (Table 4). Hyp contents in non-proline-accumulating mutants were about half of that in the wild type, while the contents in proline-accumulating mutants were about twice of that in the wild type. These results supported the view that recessive gene-controlled amino acid analog resistant mutants have an altered uptake system of the amino acid and/or its analog (BRIGHT et al. 1979a, 1979b). However, there can be other explanations for the decreased level in the leaf Hyp content in non-amino acid-accumulating mutants (e.g., enhanced efflux, detoxification).
Table 4. Free hydroxy-L-proline contents (μmol/g fresh weight) in seeds and 14-day-old seedlings of Hyp-resistant mutant lines and Nipponbare. (Mori et al. 1989)

<table>
<thead>
<tr>
<th>Seeds</th>
<th>Seeded</th>
<th>Hyp O M</th>
<th>Hyp 10⁻⁴M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nipponbare</td>
<td>nd</td>
<td>nd</td>
<td>55.5±26.9*</td>
</tr>
<tr>
<td>HYP101</td>
<td>nd</td>
<td>nd</td>
<td>23.4±2.1</td>
</tr>
<tr>
<td>HYP203</td>
<td>nd</td>
<td>nd</td>
<td>23.9±7.2</td>
</tr>
<tr>
<td>HYP205</td>
<td>nd</td>
<td>nd</td>
<td>62.4±5.8  (S)</td>
</tr>
<tr>
<td>HYP210</td>
<td>nd</td>
<td>nd</td>
<td>122.3±19.0 (R)</td>
</tr>
</tbody>
</table>

*, m ± s.d. (average of three replications)
(S) and (R) ; Hyp-sensitive and -resistant segregants of heterozygous lines
nd; not detected

Inheritance of Hyp resistance in proline accumulating mutants is curious. Homozygous resistant plants have not yet been obtained. In every generation, Hyp-resistant plants were developed from the plants which had shown Hyp resistance at the young seedling stage of the previous generation. No Hyp-resistant plants were developed from the sensitive plants in the previous generation. The segregation ratio of resistant seedlings to sensitive ones in most cases seems to fit 1:1, but in some cases the ratio decreases or homozygous recessive plants develop. At present, there is no explanation for the genetic behavior of the Hyp resistance in these mutants. A similar phenomenon was observed in a tryptophan accumulating rice mutant resistant to 5-methyltryptophan (Wakasa and Widholm 1987).

In both types of Hyp resistant mutants, the mutant characters were expressed in the callus as well as in the whole plant system. Hyp resistance was also recovered in the regenerated plants derived from Hyp resistant callus (Mori et al. 1991). These facts show that Hyp resistant mutants isolated in this study can be useful in plant cell genetics.

**Lysine plus threonine resistant mutants**

Since Green and Phillips (1974) proposed that mutants with increased lysine, threonine and methionine could be selected as LT resistant ones, considerable work has been dedicated to screening for LT resistant mutants at both the cultured cell level and the whole plant level in a number of plant species (reviewed in MiFLIN et al. 1983, Gengenbach 1984). Some practically hopeful mutants which have increased free
threonine in the seeds have been found. In a maize mutant isolated from LT resistant callus, free threonine content (per g dry weight) in kernels was 75-100 times higher than that in the wild type and this content was equivalent to 133-150\% of the total threonine content (HIBBERD and GREEN 1982). BRIGHT et al. (1982) reported that in a barley LT resistant mutant a 12-fold increase in free threonine (per g nitrogen) increased the total threonine content in the seeds by 7\%.

Genetic control of feedback inhibition in the aspartate pathway has been proved using LT resistant mutants. From the mutational analysis, BRIGHT et al. (1982) identified two genes (Lt1 and Lt2) for LT resistance in barley. Two allelic genes were identified at Lt1 locus. Lt1 and Lt2 control the aspartate kinase (AK) isoenzymes, AK II and AK III, respectively (BRIGHT et al. 1982, ARRUDA et al. 1984). In maize, two genes for AK were also identified, but the proposed structure-function relationship of AK was different from that in barley (DOTSON et al. 1990).

On the other hand, there are only a few reports that lysine overproducing mutants are selected as LT resistant mutants (LEA et al. 1992). In the aspartate pathway, lysine inhibits dihydrodipicolinate synthase (DHPS) as well as AK. DHPS has been shown to be another key enzyme of lysine biosynthesis (WALLSGROVE and MAZELIS 1981). In Nicotiana sylvestris mutants which are resistant to AEC, a feedback insensitive form of DHPS was detected and free lysine increased 10 to 20 times of the control (NEGRIUTIU et al. 1984). Recently, FRANKARD et al. (1992) reported that more free lysine was accumulated in F1 plants of DHPS and AK mutants than in lysine accumulating DHPS mutant in Nicotiana sylvestris. This observation supported the hypothesis by HERMANN et al. (1972), who suggested that, in Pseudomonas, double mutants with both AK and DHPS might be required for increasing free lysine contents. For the breeding of increased lysine crops, further screening for lysine overproducing mutants is necessary.

Selection of mutants

Fig. 2 shows the growth inhibiting effect of lysine, threonine and LT in rice at 25°C. The seedling growth was severely inhibited in lysine solution at the concentration of $5 \times 10^{-4}$M or higher, while the growth was not inhibited by threonine. Seedling growth inhibition by LT was severer than that by lysine alone, suggesting the synergistic effect of lysine and threonine. From these results, it is indicated that LT resistant mutants can be selected at the concentration of $5 \times 10^{-4}$M or higher.

The selection of LT resistant mutants was performed by the modified method proposed for the selection of Hyp resistant mutants. The $M_2$ seedlings mutagenized by gamma-ray (200 and 300 Gy) were allowed to germinate and the germinated seeds were cultured with $10^{-4}$M LT in a growth chamber at 25°C for 7 days. At this stage, well-grown seedlings were selected and then were transferred to the culture with
Fig. 2. Growth inhibiting effect of lysine (Lys), threonine (Thr) and lysine plus threonine (LT) in rice. Seedling height was measured on the 7th day after treatment.

nutrient solution containing $10^{-3}$M LT. Fourteen days later, the surviving seedlings were selected as LT resistant variants. Finally, 7 and 13 LT resistant variants were obtained from the $M_2$ population mutagenized by 200 and 300 Gy gamma-rays, respectively (Table 5). The $M_3$ and $M_4$ seeds were harvested as an $M_3$ and $M_4$ plant progeny, respectively.

Table 5. Frequency of lysine plus threonine (LT) resistant seedlings in $M_2$

<table>
<thead>
<tr>
<th>Mutagenic treatment</th>
<th>No. of seeds screened</th>
<th>LT resistant variants</th>
<th>No. of seedlings selected</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamma-rays 200Gy</td>
<td>14,400</td>
<td>7*</td>
<td>4.9 $\times 10^{-4}$</td>
<td></td>
</tr>
<tr>
<td>Gamma-rays 300Gy</td>
<td>14,400</td>
<td>13**</td>
<td>9.0 $\times 10^{-4}$</td>
<td></td>
</tr>
</tbody>
</table>

* LTR 1–7
** LTR 8–20
Free amino acid analysis;

In the M₄ generation, the seeds of the progenies of the LT resistant variants were cultured with 0 and 10⁻³ M LT. Seedling height of the progenies of 14 variants out of 20 were apparently higher than that of the wild type. These 14 progenies were identified as LT resistant lines. Free threonine and lysine contents were determined in the seedlings of LT resistant lines. Table 6 shows the free threonine and lysine contents in leaves of the M₄ plants. The progenies of 4 variants (LTR16, LTR18, LTR19 AND LTR20) showed more than a 2-fold increase in free threonine, indicating that increased threonine mutants can be selected by the method described in this paper. The free threonine contents in leaves of threonine accumulating lines were almost equivalent to that in the LT resistant variety, Shirowase (HASEGAWA 1988). Interestingly, increased lysine lines were segregated among the M₄ lines derived from the same variants isolated in the M₃ generation. In the leaves of the M₄ line, LTR16-7, free lysine was 8 times that in the wild type. From these results, it is indicated that increased lysine plants could be selected as LT resistant mutants in rice. However, further characterization of lysine and/or overproducing mutants has not yet carried out.

Table 6. Free threonine (Thr) and lysine (Lys) contents (nmol/g fresh weight) in 14-day-old seedlings of lysine plus threonine resistant M₄ lines (LTR)

<table>
<thead>
<tr>
<th>LTR</th>
<th>Thr</th>
<th>Lys</th>
<th>LTR</th>
<th>Thr</th>
<th>Lys</th>
<th>LTR</th>
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<tr>
<td>9-2</td>
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<td></td>
</tr>
</tbody>
</table>

Nip ; Nipponbare (wild type)
na ; not analyzed
nd ; not determined
Discussion

In order to obtain amino acid and amino acid analog resistant mutants in higher plants, selection at the cultured cell level has been thought to be efficient, because large cell populations can be screened easily and the cultured cell itself produces genetic variability. On the other hand, these mutants can also be selected from the mutagenized M₂ population, if an appropriate mutagen treatment and screening system are applied. The most important advantage of the whole plant selection system is that the mutants are obtained directly. Furthermore, the whole plant selection system can be applied to plant species for which the cell culture technique has not been developed. In the experiments described in this paper, 24 Hyp-resistant mutants and 20 LT-resistant variants were selected from about 90,000 and about 30,000 M₂ seedlings, respectively. The frequencies described in this paper were almost coincidence with those described in previously reported results (SINGH and WIDHOLM 1975, BRIGHT et al. 1979, KUEH and BRIGHT 1982). These results show that the M₂ seedling selection system is better for isolating the amino acid and amino acid analog resistant mutants. The method described in this paper can be applied to selection for other biochemical mutations.

In the cases of opaque-2 maize (NELSON 1979) and hiproly barley (MUNCK 1992) crops with increased lysine were accompanied by grain quality problems due to the alteration of grain storage protein. In rice, the increased lysine mutants isolated from AEC and LT resistant callus also had problems similar to those demonstrated in high lysine maize and barley (SCHAEFFER and SHARPE 1989). On the other hand, by the use of free amino acid overproducing mutants, the amino acid composition of cereals could be improved without altering the storage proteins. In the experiment described in this paper, it is suggested that high proline mutants and high threonine or lysine mutants are isolated from mutagenized M₂ population as Hyp resistant and LT resistant mutants, respectively. In particular, free proline contents in the seeds of HYP205 was about 24-fold of that of the wild type. In consideration with the fact that, in a maize LT resistant mutant, a 75 to 100-fold increase in free threonine (per g dry weight) in the kernel was equivalent to the total threonine by 130 to 150% (HIBBERD and GREEN 1982), it seems to be necessary to isolate mutants having a 100-fold or more increase in the free amino acid (per g fresh weight) for the improvement of amino acid content in cereals. Althouth, total amino acid contents were not determined in the experiment described in this paper, further screening for amino acid and amino acid analog resistant mutants are necessary for the breeding of increased amino acid rice.

The problem about the selection system proposed in this paper is that whole seeds with endosperm were used for the screening. For this reason, it is inevitable that soluble amino acids transferred from the endosperm interact with exogenously sup-
plied amino acids or amino acid analogs. At present, however, influence of amino acids from the endosperm on the efficiency of mutant selection cannot be evaluated. To avoid this problem, MIFLIN et al. (1983) proposed that mutant screening be performed with the embryos removed from the seeds.

Acknowledgements

The author is greatly indebted to Mr. S. MÖRI, Kyoto Prefectural University, for his help and encouragement throughout the studies on Hyp resistant mutants. The author also wishes his thanks to Dr. A. NISHIMURA, University of Osaka Prefecture, for amino acid analysis. This work was partly supported by a Grant-in-Aid for Scientific Research (No. 60560011) from the Ministry of Education, Science and Culture, Japan.

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高等植物におけるアミノ酸・アミノ酸アナログ抵抗性突然変異

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高等植物におけるアミノ酸生合成には負のフィードバック制御システムが働いており、タンパク質合成に必要な量以上のアミノ酸が生産されないように調節されている。すなわち、最終産物であるアミノ酸はその生合成経路の特定の酵素の働きを阻害する。アミノ酸生合成のフィードバック制御システムに不感性性を示す突然変異体が知られているが、これらの突然変異体では、薬や種子中にアミノ酸を遊離の形で蓄積する。このような突然変異体は高濃度のアミノ酸あるいはアミノ酸アナログに対する抵抗性を指標として、培養細胞レベルでも個体レベルにおいても選抜することが可能である。Table 1 はこれまでにアミノ酸・アミノ酸アナログ抵抗性が個体レベルで確認された例を示したものである。

アミノ酸・アミノ酸アナログ抵抗性の研究は高アミノ酸作物の育種を目的として発展してきている。そのため栽培中のタンパク質に不足しているリジンとスレオニンを対象とした研究報告が多い。また、植物の環境ストレスのかかわり（プロリン）や IAA 生合成のかかわり（トリプトファン）に関してそれぞれのアミノ酸のアナログ抵抗性突然変異の利用が試みられている。

著者らはイネについてアミノ酸・アミノ酸アナログ抵抗性突然変異体の選抜方法を考案し、得られた突然変異体の遺伝・生化学特性を調べており、本稿ではヒドロキシン−L−プロリン（Hyp）抵抗性ならびにリジン+スレオニン（LT）抵抗性突然変異体に関する研究結果について紹介する。

Hyp 抵抗性突然変異

イネ（品種、日本製）における Hyp の生育阻害効果を調べ、4 環間発芽させた種子を 2 × 10^{-4} M の Hyp 溶液で栽培し、生育を続ける個体を選ぶことで Hyp 抵抗性突然変異体が選抜できることを示した。4 種類の突然変異原処理を行った総計約 9 万の M_{2} 個体から 24 個体の突然変異体が得られた。用いた突然変異原ではエチレンイミンが最も効果的であった。

得られた突然変異体の遺伝・生化学特性を調べたところ、劣性突然変異遺伝子による散離プロリンが認められないタイプと抵抗性が高値で維持され変異プロリンの蓄積が認められるタイプが見いだされた。さらに系統 HYPER205 の種子中の変異プロリン含量は原種の 2 倍を示した。ヘテロで維持される突然変異体は優性突然変異遺伝子によるものと考えられるがその遺伝様式は不明である。Hyp 抵抗性突然変異体の特性は両タイプと同一個体。
カルスおよび再生個体レベルで維持されており、Hyp 抗性性は遺伝マーーカーとして有用と思われる。

LT 抗性性突然変異体

Hyp 抗性性突然変異体の選抜方法に従い、LT のイネ（品種、日本晴）における生育阻害効果を調べ、抗性性突然変異体の選抜基準を定めた（10**5 MLT で 7 日間以上栽培）。ガンマ線照射後代の約 3 万の M_2 個体から 20 個体が LT 条件でも生育を続けた。それらのうち 14 個体の後代は M_4 においても LT 抗性性が維持されていた。LT 抗性性を示した M_4 系統の幼植物における遊離アミノ酸を測定したところ遊離スレオニンと遊離リジンがそれぞれ品種の 4, 8 倍に増加した系統が認められた。

以上の結果はイネにおいてもアミノ酸・アミノ酸アナログ抵抗性を指標として遊離アミノ酸含量の高い突然変異体が選抜されることを示したものである。実用的には遊離アミノ酸が 100 倍以上蓄積される必要があり、今後もこれら突然変異体の選抜を継続していく必要がある。リジン、スレオニンが含まれるアスパラギン酸に由来するアミノ酸合成経路の調節機構は LT やリジンアナログの抵抗性突然変異体を用いて解明された。このようにアミノ酸・アミノ酸アナログ抵抗性突然変異体は高アミノ酸含有作物の育種材料のみならず植物生化学の貴重な研究材料としても大きな価値がある。
USE OF GRAVITROPIC MUTANTS IN BARLEY AND PEA FOR THE STUDY OF SPACE BOTANY

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Introduction

Growth orientation of plants on the earth is always under the influence of 1g gravity. Thus, evolution of organisms including plants on the earth took place for adapting this gravity environment. For example, plants developed cell wall for supporting themselves against 1g gravity just as animals developed their skeleton structures.

As a human being started to explore space, it is necessary to study how plants grow under microgravity in space since food production must be needed under such extraterrestrial environments to support activities of human beings there.

Microgravity in space also provide unique experimental condition to study gravitational response of plants. However, opportunity to go such microgravity environment is still very limited now then this is a reason why the use of mutants insensitive to gravity or having modified graviresponsiveness is useful to do experiments on this matter on the earth.

We are using several gravitropic mutants that show agravitropic or abnormal gravitropism. Here, I will give some examples of experiments mainly conducted in our laboratory.

1. Experiments with barley mutant, serpentina

Plant morphology

A barley mutant serpentina was produced by Dr. A. YAMASHITA from a Japanese barley variety, Chikurin Ibaragi No. 1. The mutant was selected from plants grown in the gamma field of the Ministry of Agriculture, Forestry and Fishery, Omiyamachi, Ibaragi Prefecture, irradiated with chronic ray from Co^{60} for 64 days during January to March, at the rate of 8 rentogens per day. One plant which showed diagravitropic nature of growth was selected from about 600,000 M_3-plants, and named serpentina
Fig. 1. Plants of barley mutant showing abnormal response to gravity. A: Normal line B: Mutant line. (after Suge et al., 1989)

Fig. 2. Longitudinal section of leaf sheath pulvinus of mutant line showing differential growth. Cell division and cell elongation were stimulated in the upper part of the pulvinus. (after Suge et al., 1989)
after its growth habit, which resembles a snake crawling on the ground.

However, if potted plants were held on a position higher than the ground level, some stems especially those originating from tillers developed early, grew in the direction of gravity although stems which developed at a much later stage showed the tendency to grow in diagravitropic or plagiogravitropic direction. Thus, cell division and elongation is much higher in upper side of pulvinus. This vening direction is just opposite compared with normal curvature of gravistimulated stems, however, stimulation of cell division and elongation in the opposite side of stem curvature is the same in the case of normal response to gravity. (Suge et al., 1989). (Fig 1 and Fig. 2).

**Seedling growth orientation**

In the mutant line, angle between the first leaf and ground level differed greatly depending on the position of seeds at the time of sowing. On the other hand, in the

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Fig. 3. Direction of shoot growth as affected by seed position. A: Seed was positioned so that embryo was parallel with the earth. B: Seed was positioned so the embryo was parallel with earth but upside down. C: Seed was positioned so the embryo was perpendicular to the earth. From A to C, plants on the left are normal, and those on the right are mutant line. (after Suge and Türkan, 1991)
Table 1. Effect of seed orientation on shoot growth.

<table>
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<tr>
<th>Seed position</th>
<th>Line</th>
<th>Angle between ground level and first leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seed was positioned so the embryo was parallel with the earth</td>
<td>Normal</td>
<td>80.0 ± 7.7*</td>
</tr>
<tr>
<td></td>
<td>Mutant</td>
<td>83.8 ± 4.9</td>
</tr>
<tr>
<td>Seed was positioned so the embryo was parallel with the earth, but the seed was upside down</td>
<td>Normal</td>
<td>81.0 ± 8.1</td>
</tr>
<tr>
<td></td>
<td>Mutant</td>
<td>47.5 ± 9.8</td>
</tr>
<tr>
<td>Seed was positioned so the embryo was perpendicular to the earth</td>
<td>Normal</td>
<td>82.8 ± 4.3</td>
</tr>
<tr>
<td></td>
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<td>48.5 ± 11.4</td>
</tr>
<tr>
<td>Seed was positioned vertically so the embryo was in the upper position</td>
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<td>78.3 ± 9.4</td>
</tr>
<tr>
<td></td>
<td>Mutant</td>
<td>56.3 ± 15.4</td>
</tr>
<tr>
<td>Seed was positioned vertically so the embryo was in the lower position</td>
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<td>73.5 ± 8.4</td>
</tr>
<tr>
<td></td>
<td>Mutant</td>
<td>50.0 ± 18.9</td>
</tr>
</tbody>
</table>

* Mean ± SD

normal line, the angle exceeded 70 degree regardless of seed position because growing direction adjusted in response to gravity. However, plants of the mutant line could not adjust their direction of shoot growth because they did not respond to the gravity. Thus the distribution of first leaf growth depend on the position of the embryo when the seed was sown in the field. (Suge and Türkan, 1991). (Fig. 3, Table 1).

**Endogenous gibberellins**

Contents of endogenous gibberellins were surveyed in a barley mutant showing abnormal response to gravity in comparison with the original normal line. The contents were assayed by using gibberellin-sensitive dwarf rice strains Tan-ginbozu and Waito-C. Gibberellin activities were found to be greatly reduced in the mutat line throughout the growth period, and in different plant parts, i.e., leaves, stems and heads. After heading time, gibberellin activities obtained by the Waito-C assay (presumably GA₃) was especially reduced in the mutat line. These results suggest that biosynthesis, especially the ability in the site of 3β-hydroxylation, is greatly reduced in the mutat line. (Türkan and Suge, 1991). (Fig. 4).

As to the role of gibberellins in the gravitropic response of cereal pulvinus, it is reported that when the pulvinus is gravistimulated, it shows marked changes in the distribution of free, polar GAs and GA conjugates, as compared with those in vertical, control pulvini. Free GAs increase significantly (29-fold) in gravistimulated pulvini with these active, free GAs being two times greater in amounts in the lower halves than in the upper halves, and inactive glucosyl ester conjugated of these GAs being 1.8
times greater in upper halves than in lower halves (Pharis et al. 1981).

We did not examine in the difference between the upper and the lower halves of pulvini, however, activities of free gibberellins were greatly reduced in the mutant line. Then it must be studied whether this difference has some correlation in the difference of gravity response or not.

**Productive structures and seed fertility**

Growth analysis was performed on a mutnat line showing an abnormal gravity response and also the original normal line. The productive structures of mutant plants were quite different from the original normal plants. Analysis by stratified clip method revealed difference in distribution of leaves, stems, and heads, as well as leaf area index (LAI) in the mutant line caused by diagravitropic or plagiogravitropic growth. Seed production of mutant plants was about 60% compared with normal plants, even though net assimilation rate (NAR) and relative growth rate (RGR) were not decreased. Seed fertility of mutnat plants was correlated with the angle between
heads and ground level.

However, when plants were turned upside down, all heads, both mutants and normal, were nearly vertical, and seed fertility recovered to a level that not significantly different from normal plants. From these results, it was predicted that barley plants may not produce seed normally under microgravity. (SUGE and TÜRKAN, 1991). (Fig. 5 and Fig. 6).

In a phytotron experiment, best wheat yields (24 gm⁻² d⁻¹ of edible biomass) are five times good field yields (time basis) and twice the world record. Similar yields have also been obtained with potatoes and lettuce. From these figures it was suggested that each astronaut could be supported (about 3,000 kcal d⁻¹) with a space farm of about 30

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**Fig. 5.** Seed fertility as affected by angle between head and ground level. Vertical column heads presented on the right (normal control, normal upside down and mutant upside down) developed almost vertically by making plants upside down. (after Suge and Türkan, 1991)
to 40 m² under continuous production (Salisbury 1986). However, in those experiments problem of microgravity was completely neglected. Our results suggested the importance of microgravity in extraterrestrial crop production if space base is settled in microgravity environment although lunar base may escape from such problem since there exist about 1/6 gravity of the earth.

II. Experiments with an agravitropic pea mutant, ageotropum

Plant morphology

A pea mutant, ageotropum, was discovered from X ray-irradiated peas, cv. Weibul’s Weitor (Brix et al. 1958). Etiolated shoots of the mutant are agravitropic in the dark but orthogravitropic under light. Roots of ageotropic pea show agravitropic behavior both in the dark and in the light. This agravitropism was thought to be due to a disruption in the root cap (Ekelund and Hemberg, 1966, Olsen and Iversen 1980a, b). As discussed in the paper listed here, the agravitropic growth of the roots may involve a disruption of calcium-root cap interactions. By using the roots of ageotropum pea, we also demonstrated the hydro tropism in roots, tropic response toward the mentioned source, without an interference of gravitropic response (Takahashi and Suge, 1991).

Hydro tropism of roots

Existence of hydro tropism, curvature of roots due to a moisture gradient, have been suggested in many cases; however gravitropic response of plant roots is always hid the root curvature to hydrostimulant since response of plant roots to gravity is
dominant than that to humidity gradient. Use of agravitropic pea mutant provide the opportunity to investigate the root curvature to moisture gradient.

Combination of several experiments provided interesting interaction between hydrotropism and gravitropism in roots of *ageotropum* and normal Alaska peas. (Takahashi and Suge, 1991) (Fig. 7). Roots were placed approximately 5-7 mm away from the hydrostimulant in a chamber. As clearly shown in the diagrammatic figures, when the roots were placed horizontally above the moist cheese cloth in near 100 % relative humidity (RH), ageotropic roots grew straight and Alaska pea roots bent downward (Takahashi and Suge, 1991). When the air RH was lowered to ca 85 %, the roots of both *ageotropum* and Alaska peas placed above the hydrostimulant bent downward, however when they placed below or vertical side of the hydrostimulant, only the roots of ageotropum bent toward the hydrostimulant (Fig. 7).

<table>
<thead>
<tr>
<th>VARIETY</th>
<th>A. 100% RH</th>
<th>B. 85% RH</th>
<th>C. 85% RH</th>
<th>D. 85% RH</th>
</tr>
</thead>
</table>
| Ageotropum| ![Diagram](image)
| Alaska   | ![Diagram](image)

Fig. 7. Diagrammatic interaction between hydrotropism and gravitropism in roots of ageotropum and Alaska peas. Roots were placed approximately 5-7 mm away from the hydrostimulant in chamber system. A : Roots were given only gravistimulation in the humidity-saturated conditions; B and C. roots were stimulated both hydrotropically and gravitropically, but the hydrostimulant was placed below and above the roots placed horizontally, respectively ; D, the hydrostimulant was placed by the side of the root in a vertical position. Percent values indicate RH of air inside the chamber. H. Hydrostimulant (moistened cheese cloth); R, root. The arrows (g) indicate the direction of gravitational force. Curvature was observed 10-14 h after the start of stimulation. (after Takahashi and Suge, 1991)
Acknowledgements

I thank Dr. Atsushi YAMASITA and Hideyuki TAKAHASHI for their kind considerations.

References

宇宙植物学におけるオオムギとエンドウの
重力屈性突然変異体の利用

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微少重力の宇宙環境における植物生育の基礎問題を常に1gの重力が存在する地球上で研究するためには、重力に対して正常な反応を失った突然変異体を、利用することが有用であろう。ここでは、我々の研究の現状を、オオムギとエンドウについて紹介した。

I. オオムギの突然変異 *serpentina* を用いた実験

オオムギの突然変異体は、農水省生物資源研究所のガンマーフィールドにおいて、山下淳博士により茨城竹林1号より選抜されたものである。この突然変異系統は、植物体を地面で強く保持すると、茎は重力方向にむかって伸長する。偏曲部の偏位生長は、方向は全く反対であるが、正常な茎が負の屈地性を示す時と全く同様におこる。

また、この重力反応の異常は、芽生えのステージにおいてもおこり、芽生えの生長方向は種子の中の胚の置かれた位置により決定され、正常な対照系統では、種子がどのような位置におかれても、重力反応により芽生えの生長方向を、垂直に補正するのと異なっていた。

生長のいろいろなステージにおける、内生ジフェリンの含量は、正常系統に比較して突然変異系統では、著しく低下していた。とくに、GA1とと思われる活性型ジフェリンの含量の低下が著しい。これらのジフェリン含量の低下が、重力反応の異常と直接関連があるかどうか興味深い。正常系統と突然変異系統の生産構造を比較すると、穂などの生殖器官が正常系統では、カノピーの上層部に分布するのに対して、突然変異系統では地表面に近い所からカノピー上層まで、全層にわたって分布した。突然変異系統では、純同化率、相対生長率などが、正常系統に比較して低下しないのに、粒重は正常系統の約70％に止まった。その理由として、種子稔性は穂が地表面に接する角度と関係があり、正常系統ではすべて穂が垂直に位置補正されるのに対して、突然変異系統では、穂は水平から垂直まで全層にわたって、分布するためであることが推測された。突然変異系統でも、穂を垂直に位置させると、種子稔性は正常系統と有意差のないところまで回復した。

これらのことから、微小重力の宇宙環境では正常系統でも、重力を利用しての位置補正ができないため、穂の分布は地上における突然変異系統のそれと類似した状態になり、効率の高い種子生産ができない可能性が示唆された。

II. エンドウの突然変異系統 ageotropum を用いた実験

根の重力屈性反応が異常なエンドウの突然変異系統 ageotropum を用いて、特に水分屈性についての実験を行った。この系統のオリジンは、スウェーデンにおいて、Weibul's Weitor から X 線照射により得られた突然変異である。光中では、この系統の地上部は、正常な重力反応を示すが、根の重力反応は光中、暗黒中を問わず異常である。

根が水分の多い方向向かって屈曲する、いわゆる屈水性の存在は、前世紀から議論されていたが、特に重力屈性にマスクされて、この性質の存在を科学的に示すことが困難であったため、最近では屈水性の存在そのものに、疑問を呈する研究者が多いかった。我々は、重力反応を示さない、このエンドウの突然変異系統を使用して、根の屈水性の存在を初めて科学的に証明することに成功した。

この性質は、重力を定位反応（位置の補正反応）に利用できない、微小重力の宇宙環境で、植物を色々な目的のために生育させる時に、有効に利用できる可能性がある。

このように、重力に対する正常な反応能力を、遺伝的に喪失した各種植物の突然変異系統は、常に 1g の重力が存在する地球上で、宇宙環境における植物生育の問題を、基礎的に研究する上で、極めて有用であろう。
PHYTOCHROME AND ITS FUNCTIONS 
IN PHYTOCHROME MUTANTS

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Introduction

Incident light modulates both the development and the physiology of plants (Kendrick and Kronenber 1986). To respond to the light environment, plants have developed different types of photoreceptors. Among these, phytochrome is the best characterized (Furuya 1987). Phytochrome is soluble chromoprotein that contains one molecule of open tetrapyrrole as chromophore. Phytochrome is synthesized as red light absorbing form (Pr, λmax = ca. 660 nm), which is biologically inactive, and is photoconverted to a far-red light absorbing form (Pfr, λmax = ca. 730 nm) upon absorption of red light. The resulted Pfr is biologically active and causes various responses in plants. In addition to phytochrome, at least two other photoreceptors, blue-light photoreceptor and UV-B photoreceptor, are known to be involved in photomorphogenesis in plants. However, molecular identities of the photoreceptors other than phytochrome remain unclear.

Recent biochemical and molecular biological analysis demonstrated that phytochrome is encoded by a small multigene family. Amino-terminal amino acid sequence analysis of phytochrome purified from light grown pea indicated presence of two distinct molecular species (Abe et al 1989). In Arabidopsis, DNA sequences of three distinct phytochrome genes, phyA, phyB, and phyC, were reported (Sharrock and Quail 1989). Amino acid homologies between these molecular species are as low as around 50% (Table 1). Based on the sequence comparison, Dehesh et al proposed phylogeny of known members of the phytochrome family (Dehesh et al 1991). Furthermore, expression patterns of these molecular species are different (Table 1). These observations suggest the possibility that different molecular species of phytochrome has distinct functions (Furuya 1989).

Existence of stable Pfr has long been postulated on the basis of physiological observations (Furuya 1989). However, this seemed to contradict with the biochemical observations that Pfr is rapidly degraded in vivo. Discovery of different molecular species of phytochrome has opened way to understand this discrepancy. Until recently,
biochemical works on phytochrome mostly used dark-grown tissue, in which PhyA (spectrally active phytochrome encoded by phyA gene) is predominant. Immunological analysis suggested that Pfr of PhyA is unstable whereas those of PhyB and PhyC are stable (Table 1). Thus, it is possible that stable Pfr, which was postulated on the basis of physiological experiments, consists of PhyB and / or PhyC. Mutants that lack one or some of the molecular species of phytochrome are the most suitable tools to explore this possibility.

1. Phytochrome in lh mutant of cucumber

Although PhyA appeared to be normal in the lh mutant of cucumber, physiological experiments indicated the possibility that other molecular species are deficient (Adamse et al 1987, Adamse et al 1988c). To characterize phytochrome in this mutant, we used anti-phytochrome monoclonal antibodies. Since the amino acid sequences differs substantially among different molecular species of phytochrome, it is not difficult to prepare antibodies that specifically recognize certain molecular species of phytochrome. However, lack of cross-reactions against phytochrome from other plant species occasionally limits the use of these antibodies (Nagatani et al 1987). For example, our monoclonal antibodies that specifically recognize pea PhyB (Abe et al 1985) did not cross-react with the one from cucumber. Therefore, we raised new monoclonal antibodies against tobacco PhyB polypeptide expressed in E. coli (Lopez-Juez et al 1992). Among several antibodies obtained, monoclonal mAT1 reacted very well with cucumber PhyB. For the detection of PhyA, an anti-pea PhyA monoclonal antibody mAP5 was used.

Western blot analysis of dark-and light-grown tissues with the antibodies clearly indicated lack of PhyB in this mutant, whereas PhyA appeared to be normal (Lopez-
Furthermore, the analysis demonstrated that PhyA detected by the antibody was photo-unstable and PhyB was photo-stable, suggesting that these antibodies recognized proper molecular species of phytochrome. Since little is known about the molecular biological and genetical aspects of the \(lh\) mutation, we can not exclude the possibility that the molecular species other than PhyA and PhyB are affected as well in this mutant. Nonetheless, lack of some of phytochrome responses in this mutant suggests that these responses are not mediated by PhyA.

2. Phytochrome in \(au\) mutant of tomato

In the etiolated tissue of the \(au\) mutant of tomato, no spectral activity of phytochrome was detected, suggesting that this mutant lacks PhyA (Koornneef et al 1985, Parks et al 1987). On the other hand, the spectral activity of phytochrome was detected at about normal level in the light grown tissue of this mutant (López-Juez et al 1990). Thus, it is possible that this mutant lacks PhyA but contains functional PhyB. Physiological analysis supported this view since phenotypes of this mutant were very different from those of the \(lh\) mutant described above.

To examine phytochrome in this mutant, we used molecular species-specific anti-phytochrome monoclonal antibodies. Western blot analysis of the etiolated tissue of the \(au\) mutant with a monoclonal anti-PhyA antibody indicated that the level was reduced to 20\% of the wild type (R. P. Sharma, E. López, A. Nagatani, M. Furuya, pers comm.). Furthermore, the amount of PhyA was not decreased by the irradiation of the seedlings with red light, suggesting that PhyA in this mutant is photochemically inactive. On the contrary, PhyB level in the \(au\) mutant was equal to that of the wild type. Preliminary experiment suggested that PhyB from the light-grown tissue of this mutant was photochemically active. Thus, it seemed that PhyA is missing but PhyB is functional in this mutant. However, genetical and molecular basis of the \(au\) mutation still remains obscure as is the case with the \(lh\) mutant. The mutation is probably not that of a structural \(phyA\) gene (Sharrock and Quail 1989).

3. Phytochrome in \(hy\) mutants of \textit{Arabidopsis}

Long hypocotyl mutants at six different loci (\(hy1\)-\(hy6\)) have been reported in \textit{Arabidopsis} so far (Koornneef et al 1980, Chory et al 1989) and four of them have been demonstrated to be phytochrome-related. Of these, three (\(hy1\), \(hy2\) and \(hy6\)) lack spectrally active phytochrome in the etiolated tissue, suggesting that these mutants lack PhyA. Since the phenotypes of these mutants were restored in the presence of precursors to the phytochrome chromophore, these are most likely chromophore
mutants (Parks and Quail 1991). On the contrary, the hy3 mutant of Arabidopsis contains normal level of spectrally active phytochrome in the dark, suggesting that PhyA is functional in this mutant. Nevertheless, this mutant shows clear morphological phenotype such as elongated petals in the light.

To further characterize the hy3 mutant in terms of molecular species of phytochrome, we used a PhyB specific monoclonal antibody. Western blot analysis of the light grown tissue of the hy3 mutant demonstrated that PhyB is undetectable in this mutant (Nagatani et al 1991). Furthermore, the hy3 mutation and phyB gene were genetically linked (J. Chory, pers. comm.). Thus, it seems likely that the HY3 locus encodes PhyB.

4. Regulation of elongation growth in phytochrome mutants

It is well-known that elongation growth in plants is regulated by phytochrome (Gaba and Black 1983). However, the mode of action is not simple. In dark-grown seedlings, elongation of hypocotyl is inhibited by prolonged irradiation with far red light. This response is often referred to as FR-HIR (far-red high irradiance) response. Based on the kinetic analysis, Hartmann proposed that FR-HIR is mediated by unstable Pfr (Hartmann 1966). In contrast, stem of plants that are grown under light/dark cycles respond to pulses of far-red light given at the end of each light periods. This response is referred to as EOD-FR (end-of-day far red) response. Detailed analysis of this phenomenon indicated that EOD-FR responses are mediated by stable Pfr (Downs et al 1957). Although hypocotyl elongation is inhibited in both cases, the modes of action of phytochrome are clearly different. Thus, it is quite interesting to compare these responses in the mutants described above (Table 2).

Table 2 Characteristics of mutants that lack functional phytochrome

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Plant species</th>
<th>Spectral activity of phytochrome</th>
<th>Inhibition of stem elongation</th>
</tr>
</thead>
<tbody>
<tr>
<td>lh</td>
<td>cucumber</td>
<td>+&lt;sup&gt;a,b&lt;/sup&gt; −&lt;sup&gt;c&lt;/sup&gt; n. d.</td>
<td>+&lt;sup&gt;d&lt;/sup&gt; −&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>hy3</td>
<td>Arabidopsis</td>
<td>+&lt;sup&gt;a&lt;/sup&gt; −&lt;sup&gt;b&lt;/sup&gt; n. d.</td>
<td>+&lt;sup&gt;a&lt;/sup&gt; −&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>hy1, hy2, hy6</td>
<td>Arabidopsis</td>
<td>+&lt;sup&gt;a&lt;/sup&gt; −?&lt;sup&gt;a&lt;/sup&gt; n. d.</td>
<td>−&lt;sup&gt;a&lt;/sup&gt; −&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>au</td>
<td>tomato</td>
<td>+&lt;sup&gt;1&lt;/sup&gt; −?&lt;sup&gt;a,b&lt;/sup&gt; n. d.</td>
<td>n. d.* −&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*not determined yet.

In case of the \textit{lh} mutant of cucumber, no EOD-FR response was observed (Adamse \textit{et al} 1988a) whereas the etiolated seedlings responded quite normally to continuous far red light irradiation (FR-HIR response) (Peters \textit{et al} 1991). Similar observations were made in the \textit{hy3} mutant of \textit{Arabidopsis} (Koornneef \textit{et al} 1980, Nagatani \textit{et al} 1991). As described above, PhyB is missing but PhyA appears to be normal in these mutants. Furthermore, only PhyB is missing in the \textit{hy3} mutant since \textit{HY3} locus most likely encodes PhyB (J. Chory, pers. comm.). Thus, the EOD-FR was suggested to be mediated exclusively by PhyB. On the contrary, FR-HIR does not appear to require PhyB. The FR-HIR responses are presumably mediated by PhyA since only PhyA is known to be light- unstable.

In the cases of the \textit{hy1}, \textit{hy2} and \textit{hy6} mutants, both EOD-FR and FR-HIR responses were defective (Koornneef \textit{et al} 1980, A. Nagatani unpublished). This observation can be explained by the fact that they are presumably chromophore mutants and all the molecular species could be nonfunctional in these mutants (Parks and Quail 1991). In case of the \textit{au} mutant of tomato, presence of EOD-FR responses were reported (Adamse \textit{et al} 1988b), indicating that lack of PhyA does not result in the loss of EOD-FR responses. This observation fits to the conclusions suggested from the analysis of the \textit{lh} and \textit{hy3} mutants.

\section*{Conclusion}

The above findings strongly suggest that different molecular species of phytochrome has distinct functions. Mutants that lack PhyB were quite useful to reveal the specific functions of PhyB. However, the set of mutants available at the moment is clearly insufficient to complete the work. Isolation of mutants deficient only in one of the molecular species other than PhyB is awaited.

\section*{Acknowledgments}

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\section*{References}


THE CHARACTERISTICS OF A SUPERNODULATING MUTANT ISOLATED FROM SOYBEAN CULTIVAR ENREI BY THE ETHYL METHANE SULFONATE (EMS) TREATMENT

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Introduction

Symbiotic nitrogen fixation in nodules of legumes depends on the complex interaction between the legume plant and (Brady) Rhizobium bacteria. Nodule formation and nitrogen fixation are closely regulated by the host plant and the microsymbiont. Plant mutants with altered symbiotic performance are to be useful to gain a better understanding of the plant-microbe interactions in the legume-(Brady) Rhizobium symbiosis (Jacobsen 1984; Carroll et al. 1985a, b; Park and Buttery 1988; Duc and Messager 1989; Gremaud and Harper 1989). Carroll et al. (1985a, b) have isolated the supernodulating mutants of the soybean cv. Bragg which display a very large number of nodules and nitrate-tolerant-synbiotic (nts) characteristics. Recently, Gremaud and Harper (1989) have also isolated similar mutants from the soybean cv. Williams. These mutants not only provide materials that are useful for investigations on the interaction in the nodule formation processes but also for agricultural practice. Therefore, we attempted to isolate the mutants with altered symbiotic phenotypes from the soybean cultivar Enrei one of the most common cultivars in Japan.

We have obtained a supernodulating, some hyernodulating and three non-nodulating mutants from EMS-treated seeds of above-mentioned soybean cv. Enrei. The supernodulating mutant (En6500) possesses properties essentially similar to those of the other supernodulating mutant except for its apparently greater tolerance to nitrate. Then we tried to examine the analysis of mechanism of nodulation and nitrate-tolerance by using the mutants with altered symbiotic performance in soybean.
Materials and Methods

Experiment A: Irradiance and nitrate effects on growth and symbiotic parameters

Plant culture

Seeds of the supernodulating and nts soybean mutant En6500 (Akao and Kouchi 1991) and its wild parent cv. Enrei were surface-sterilized by immersion in 70% ethanol for 20 min, followed by 5 min soaking in 3% hydrogen peroxide and subsequently washed with sterile deionized water (5 times at 1 min interval). Sterilized seeds were germinated in the presence of either 0.5, 5.0, or 15.0 mM KNO$_3$ in vermiculite (pH 5.5) previously inoculated with 10 ml suspension of mixed Bradyrhizobium japonicum strains A1017 and IRj 2101 (Agricultural Research Institute, Tokai Federation of Agricultural Cooperatives, Japan) to reach a bacterial concentration of approximately $10^7$ cells ml$^{-1}$. The seeded vermiculite trays were kept in a growth chamber with 28/25 °C day/night temperature, 80% relative humidity, and a photosynthetic photon flux density (PPFD) of approximately 500 μ mol m$^{-2}$sec$^{-1}$ at the vermiculite surface for 14 h daily. At day 7, seedlings with equal height and vigor were carefully dug out, washed in sterile deionized water, and transferred into fully aerated Wagner pots (2 seedlings per pot) containing 3 L of half-strength N-free nutrient solution (pH 5.5) as described by Akao and Kouchi (1989) supplemented with KNO$_3$ solution to give a final nitrate concentration of either 0.5, 5.0, or 15.0 mM. Solutions were renewed every other day. At day 14, the potted plants in each treatment were divided into 2 groups and transferred into identical growth chambers differing only in light intensities-PPFD of either 150 or 850 μ mol m$^{-2}$sec$^{-1}$ measured on top of the pots. Full-strength N-free nutrient solutions (pH 5.5) amended with KNO$_3$ to achieve the desired nitrate concentrations were used from the day of transfer until harvest.

Measurement of photosynthesis

The apparent photosynthetic rates (AP) of the terminal leaflet of the most recently expanded leaves were measured with SPB-H Portable Photosynthesis and Transpiration Measurement System (Shimadzu Corp., Kyoto, Japan) at 17 and 32 days after the initiation of light treatment (DAL). Air for the leaf chamber maintained at a flow rate of 500 ml min$^{-1}$ was supplied through a mast installed outdoors that drew air from 5 m above the ground.

Acetylene reduction assay

At 17 and 32 DAL, sample plants were measured for main stem length then separated into shoots and roots. The nodulated root systems were enclosed in 200-ml
Erlenmeyer flasks fitted with rubber caps, then subsequently incubated with 10% acetylene (v/v) for 35 min. The acetylene gas used was scrubbed by passing through sulfuric acid traps. Ethylene was assayed from 0.5 ml samples (obtained at 5 and 35 min of incubation) injected into a Shimadzu Gas Chromatograph GC-B4 (Shimadzu Corp., Kyoto, Japan) equipped with hydrogen flame ionization detector and Porapak R column with 99.9995% pure nitrogen gas as the carrier. ARA was calculated from the difference between the amount of ethylene produced at 35 and 5 min incubation. Nodules were subsequently detached from the roots and counted.

Plant analyses

Roots, nodules, and shoots were separately oven-dried for 72 h at 80°C, weighed, and ground in a T1-100 Vibrating Sample Mill (Heiko Corp., Fukushima, Japan). Twenty (20) mg samples were used for tissue N analysis in a Sumigraph NC-80 Oxygen Gas Combustion System (Sumitomo Chemicals Co. Ltd., Osaka, Japan) connected to a Shimadzu Gas Chromatograph GC-4C-IT (Shimadzu Corp., Kyoto, Japan). Acetanilide (Wako Pure Chemicals, Japan) was used as the standard.

Growth and biomass production of uninoculated plants

Seeds of cv. Enrei and En6500 were germinated uninoculated and grown under the conditions described above except that KNO₃ concentration in the solution was either 0.5 or 20 mM and irradiance was 500 μmol m⁻²s⁻¹. At 50 days after sowing, plants were harvested and their nodules counted, separated into shoot, root and nodules, oven dried at 80°C for 72 hrs, and weighed.

Experiment B: Autoregulation and nitrate inhibition of nodule formation

Root hair curling and early nodule development

Two separate experiments were conducted, one involving Enrei and its non-nodulating mutants, and the other comparing Enrei with its supernodulating mutant. In both experiments, seeds were inoculated at sowing with a mixed suspension of Bradyrhizobium japonicum strains A1017 and IRj2101 (10⁷ cells mL⁻¹) at a rate of 1 mL inoculum/seed. Seven- and 13-day-old seedlings were harvested and fixed in FAA. Subsequently, 3-cm primary root segments including the root hair zone were stained with 0.5% toluidine blue, embedded in 5% agar medium, transversely sectioned (0.1 mm thick) with a microslicer, washed with 0.1M phosphate buffer (pH 8.0), and re-stained with 0.003% toluidine blue solution overnight at 40°C in a water bath. Randomly sampled sections were mounted in glycerol for light microscopic observation of root hair curling and stages of nodule development of the centers of sub-epidermal cell divisions. Our nodule developmental stages A, B, C, and D roughly correspond to
stages III-V, VI-VII, VIII, and IX, respectively, of Calvert et al. (1984).

In a supplementary study, the presence of infection threads and the early stages of sub-epidermal cell division (stages 1-III of Calvert et al., 1984) in the three non-nodulating mutants were examined. The methods employed were essentially as those of Calvert et al. (1984) where surface-sterilized seeds were grown in pouches containing N-free solution, inoculated at 5 days after sowing, and then 70 mm-primary root segment (30 and 40 mm above and below the root tip mark made at inoculation, respectively) were obtained from 3-5 seedlings 5 days after, divided into 10-mm segments, longitudinally sectioned (10-13 μm) in a rotary microtome, stained with tannic acid-ferric chloride and safranin-fast green according to Jensen (1962), and serially mounted on glass slides for microscopic observation.

Grafting

Five-day-old seedlings grown from seeds inoculated with B. japonicum A1017 (10⁹ cells mL⁻¹) were wedge-grafted (Delves et al. 1987) below the cotyledonary node. In one experiment, Enrei and its non-nodulating mutants were used, while in another, En6500 was included. In a third experiment, seedlings of Enrei and En6500 grown from seeds sown in vermiculite containing N-free-nutrient solution supplemented with KNO₃ to a final concentration of either 0, 7.5, or 15 mol m⁻³ were reciprocally grafted. All grafted plants were harvested for nodule count and nodule dry weight determination at 3 weeks after grafting.

Experiment C: Control of supernodulation in intergeneric grafts of soybean and common bean

Nodule bacteria

B. japonicum strains A1017 and IRj2101 were cultured on YMA medium at 28°C for 1 week in 50ml test tubes. Bacteria of two test tubes of each strain were suspended in 400ml sterile water. Rhizobium leguminosarum bv. phaseoli strain pH6/24 (provided by Tokachi Nokyouren, Hokkaido, Japan) was identically cultured and suspended in the same quantity of sterile water. The roots of soybean and common bean were inoculated with B. japonicum and R. l. bv. phaseoli respectively, by adding 10ml (2×10⁸ cells/ml) of bacterial suspension to each of the beakers in which plants were cultured.

Plant materials

Seeds of soybean cv. Enrei and its supernodulating mutant En6500 and the common bean cv. OAC Rico (hereafter Rico) with its mutant R32BS15 (hereafter RBS15) (Park and Buttery, 1988) were surface sterilized by soaking in 70% ethanol for 2
minutes. Subsequently, they were rinsed with sterile water, immersed in 3% sodium hypochloride for 3 minutes and again rinsed with sterile water. One seed was sown under sterile conditions in an autoclaved 50ml beaker filled with 20g vermiculite and 30ml water. Therefore, beakers were individually covered with dry sterilized parafin paper, which was cut a few days after emergence to allow seedlings to grow through.

**Grafting method**

Soybean seeds were sown 2 days prior to common bean to uniform the developmental stage of cotyledon at the timing of grafting. Seven-day-old seedlings (days after sowing of soybean) were used for grafting and inoculated at 8 days after grafting (Table 1).

Table 1. Experimental conditions.

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Sowing date (DAS)</th>
<th>Grafting method</th>
<th>Inoculation date (DAS)</th>
<th>Culture condition</th>
<th>Harvest date (DAS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>7, I−shaped (Fig. 1a)</td>
<td>15</td>
<td>Water culture in 3−L pot</td>
<td>37</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>5, I−shaped (Fig. 1a)</td>
<td>0</td>
<td>Gravel culture in 50-mL beaker</td>
<td>22</td>
</tr>
</tbody>
</table>

+ DAS means days after sowing of soybean.

**Results**

**Experiment A**

*Growth and dry matter production*

At all light and nitrate treatment combinations, main stem was considerably longer in Enrei, the differences between genotypes being more pronounced at low irradiance (Table 2). Under low light conditions, main stem elongation was promoted in both genotypes by one or both of the higher nitrate levels at 17 DAL, but this effect persisted until 32 DAL only in Enrei. Under high light, the stimulatory effects of higher nitrate levels became more evident in Enrei at 32 DAL while it to tally disappeared in the mutant.

Total dry matter (DM) of the nodules per plant was consistently higher in the mutant in all treatments and increased with nitrate level in the mutant but decreased in Enrei (Table 2). Thus, the difference in nodule DM between genotypes became larger as nitrate concentration in the solution increased. Response to varied irradiance differed between genotypes. In Enrei, high light decreased nodule DM at all nitrate
Table 2. Mean (SE) main stem length and organ dry matter weight (DM) of the supernodulating soybean mutant En6500 and its parent cv. Enrei at various light and nitrate levels. (n=5).

<table>
<thead>
<tr>
<th>Treatment combination [genotype, light, nitrate (KNO₃) conc.]</th>
<th>Sampling date</th>
<th>17 DAL</th>
<th>32 DAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Main stem length (cm)</td>
<td>Nodule DM (mg plant⁻¹)</td>
<td>Root DM (g plant⁻¹)</td>
</tr>
<tr>
<td>Enrei</td>
<td>0.5 mM</td>
<td>49.1(3.1)</td>
<td>59.8(4.7)</td>
</tr>
<tr>
<td></td>
<td>5.0 mM</td>
<td>54.3(2.7)</td>
<td>15.9(0.3)</td>
</tr>
<tr>
<td></td>
<td>15.0 mM</td>
<td>51.7(2.0)</td>
<td>4.3(0.4)</td>
</tr>
<tr>
<td>Enrei</td>
<td>0.5 mM</td>
<td>27.8(0.3)</td>
<td>55.4(13.6)</td>
</tr>
<tr>
<td></td>
<td>5.0 mM</td>
<td>32.0(0.7)</td>
<td>10.4(2.3)</td>
</tr>
<tr>
<td></td>
<td>15.0 mM</td>
<td>27.1(1.3)</td>
<td>0.6(0.3)</td>
</tr>
<tr>
<td>En6500</td>
<td>0.5 mM</td>
<td>25.9(2.4)</td>
<td>126.5(5.7)</td>
</tr>
<tr>
<td></td>
<td>5.0 mM</td>
<td>35.1(3.0)</td>
<td>205.8(23.0)</td>
</tr>
<tr>
<td></td>
<td>15.0 mM</td>
<td>23.1(2.3)</td>
<td>285.2(36.0)</td>
</tr>
<tr>
<td>En6500</td>
<td>0.5 mM</td>
<td>18.1(0.9)</td>
<td>200.1(25.1)</td>
</tr>
<tr>
<td></td>
<td>5.0 mM</td>
<td>17.3(1.8)</td>
<td>250.8(17.8)</td>
</tr>
<tr>
<td></td>
<td>15.0 mM</td>
<td>18.7(0.5)</td>
<td>297.6(42.5)</td>
</tr>
</tbody>
</table>

¹ Days after initiation of light treatment (17 DAL corresponds to 32 days after sowing), ² low light intensity (150 μmol m⁻² sec⁻¹), ³ high light intensity (850 μmol m⁻² sec⁻¹).
levels except at 0.5mM at 32 DAL where the value at low light was doubled at high light. In the mutant, high light consistently increased nodule DM.

Dry matter of the root, shoot, and whole plant in Enrei was between 2 and 7 times the value for the mutant at all treatment combinations (Table 2). However, 15 mM nitrate was supra-optimal for Enrei but was still stimulatory for the mutant in most cases, especially at high light. The differences in the DM of the root, shoot, and whole plant between irradiance levels were greater in Enrei at all nitrate levels, the differences becoming more pronounced as nitrate level increased.

Main stem growth rates between 17 and 32 DAL were higher at low irradiance in both genotypes, but Enrei exhibited faster growth in most cases (Table 2). The retarding effect of 5 mM nitrate on growth was relieved by high irradiance only in Enrei.

Nodule DM accumulation rates between 17 and 32 DAL in the mutant in all cases were at least twice as much as those of Enrei except at 0.5 mM at high light (Table 2). Higher nitrate levels stimulated nodulation at both light levels in the mutant, but depressed it in Enrei. High light promoted nodule DM accumulation rates at all nitrate levels notably in the mutant.

**Dry matter partitioning to the nodules**

Dry matter partitioning (DMP) to the nodules was considerably higher in the mutant, ranging from 5 to 15 times the value for Enrei depending on the treatment (Table 3). Except for the mutant in the low light treatment at 17 DAL, percentage nodule DM decreased with increasing nitrate concentration in both genotypes notably in Enrei where the values at 15 mM nitrate were practically zero. Thus, high nitrate and irradiance levels always decreased percent DMP to the nodules in both genotypes except at 0.5 mM at 32 DAL in Enrei. At high nitrate and irradiance condition, assimilates appeared to be diverted for the development of non-symbiotic plant parts.

**Nodulation and nodule activity**

The number of nodules plant\(^{-1}\) was substantially higher in the mutant than in Enrei at al treatments (Fig. 1). A rise in nitrate concentration resulted in greater nodule numbers in the mutant but caused its reduction in Enrei at both light levels irrespective of sampling dates. High irradiance tended to diminish nodule number in both genotypes in all cases except at 0.5mM for Enrei and at 15 mM for the mutant. Across sampling dates, nodule number plant\(^{-1}\) consistently increased in both genotypes (although insignificant in most cases) except in mutant plants grown at low light, which exhibited significant reduction at all nitrate levels except at 15mM.

Acetylene reduction activity (ARA) plant\(^{-1}\)h\(^{-1}\) was higher in the mutant in all
Table 3. Mean (SE) nodule dry matter (DM) percentages of the supernodulating soybean mutant En6500 and its parent cv. Enrei grown hydroponically at various light and nitrate levels. (n = 5).

<table>
<thead>
<tr>
<th>Treatment combination [genotype, light intensity, nitrate (KNO₃) concentration]</th>
<th>Percentage nodule DM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>17 DAL¹)</td>
</tr>
<tr>
<td>Enrei</td>
<td></td>
</tr>
<tr>
<td>L²) 0.5 mM</td>
<td>1.7 (0.1)</td>
</tr>
<tr>
<td>5.0 mM</td>
<td>0.4 (0.0)</td>
</tr>
<tr>
<td>15.0 mM</td>
<td>0.1 (0.0)</td>
</tr>
<tr>
<td>L³) 0.5 mM</td>
<td>1.3 (0.2)</td>
</tr>
<tr>
<td>5.0 mM</td>
<td>0.1 (0.0)</td>
</tr>
<tr>
<td>15.0 mM</td>
<td>0.1 (⋯)</td>
</tr>
<tr>
<td>En6500</td>
<td></td>
</tr>
<tr>
<td>L</td>
<td></td>
</tr>
<tr>
<td>0.5 mM</td>
<td>16.8 (1.6)</td>
</tr>
<tr>
<td>5.0 mM</td>
<td>12.6 (0.7)</td>
</tr>
<tr>
<td>15.0 mM</td>
<td>14.9 (0.5)</td>
</tr>
<tr>
<td>H</td>
<td></td>
</tr>
<tr>
<td>0.5 mM</td>
<td>14.8 (1.2)</td>
</tr>
<tr>
<td>5.0 mM</td>
<td>12.6 (1.4)</td>
</tr>
<tr>
<td>15.0 mM</td>
<td>8.9 (1.0)</td>
</tr>
</tbody>
</table>

¹) days after commencement of light treatment (17 DAL corresponds to 32 days after sowing),
²) low light intensity (150 μmol m⁻² sec⁻¹),
³) high light intensity (850 μmol m⁻² sec⁻¹)

cases except at 0.5 mM low light at 17 DAL (Fig. 2A). The differences between genotypes, however, were insignificant at the high irradiance and 0.5 mM treatment combination either at 17 or 32 DAL. As the nitrate level increased, ARA plant⁻¹ h⁻¹ declined in Enrei but was enhanced in the mutant in all cases except at 32 DAL when 15 mM nitrate was depressive at both light levels. Although ARA plant⁻¹ h⁻¹ was generally somewhat lowered in Enrei in response to high irradiance, plants grown at 0.5 mM nitrate exhibited a strong increase in symbiotic activity. A similar positive response was consistently recorded for the mutant.

Notwithstanding the 5-to 15-fold higher nodule number plant⁻¹ of the mutant at 0.5 mM (Fig. 1), Enrei had a significantly higher ARA plant⁻¹ h⁻¹ at 17 DAL under low light, while its values in all other cases were more than 50% of the corresponding data for the mutant. At high nitrate levels, the differences in nodule number between genotypes were also not proportional to the differences in ARA plant⁻¹ h⁻¹ (Figs. 1 and 2A9 due to a higher specific nodule activity (ARA g nodule⁻¹ h⁻¹) in Enrei (Fig. 2B). While specific nodule activity was decreased at higher nitrate concentrations in both genotypes, nodule DM was proportionally decreased only in Enrei, indicating that its nodules are more efficient.
Fig. 1. Effects of light intensity and nitrate (KNO₃) concentration on the number of nodules plant⁻¹ of the supernodulating soybean mutant En6500 and its parent cv. Enrei at 17 and 32 days after initiation of light treatment (DAL). Data points are means of 5 replicate plants. Vertical lines indicate standard errors.

Fig. 2. Effects of light intensity and nitrate (KNO₃) concentration on acetylene reduction activity plant⁻¹h⁻¹ (A) and specific nodule activity (acetylene reduction activity g nodule⁻¹ h⁻¹) (B) of the supernodulating soybean mutant En6500 and its parent cv. Enrei at 17 and 32 days after initiation of light treatment (DAL). Data points are means of 5 replicate plants. Vertical lines indicate standard errors.
N concentration, content, and partitioning

Nodule N concentrations were strikingly similar across light and nitrate levels, time, and genotypes. Values for the nodule and combined shoot and root (S+R) ranged from 54.7 to 58.2 and 33.0 to 51.1 mg g\(^{-1}\), respectively at 17 DAL, and from 54.4 to 65.1 and 30.1 to 41.3 mg g\(^{-1}\), respectively at 32 DAL. Nodule N concentration values tended to be higher at 32 DAL and in Enrei and were lower at high light in the mutant at 32 DAL. S+R N concentrations, which were similar to those of whole plant N, were slightly smaller but more variable than nodule N concentration notably at 17 DAL.

Since nodule and S+R N concentrations were not affected by the treatments in either genotype, N contents of the nodules and S+R were dependent on their respective DM (Table 2). Hence, nodule N content was also higher in the mutant while total plant N was higher in Enrei at all treatment combinations. Accordingly, more plant N was partitioned into the nodules in the mutant while Enrei allocated more into its non-symbiotic parts.

Apparent photosynthetic rates

Leaf apparent photosynthetic rates (AP) were comparable for the two genotypes at all nitrate levels under high irradiance at 17 DAL, and under low irradiance at 32 DAL (Table 4). In general, nitrate treatments did not affect APs at low PPF

Table 4. Mean (SE) leaf apparent photosynthetic rates (AP) of the supernodulating soybean mutant En6500 and its parent cv. Enrei grown hydroponically at various light and nitrate levels. (n=5).

| Treatment combination [genotype, light intensity, nitrate (KNO\(_3\)) concentration] | AP (\(\mu\)mol CO\(_2\) m\(^{-2}\)sec\(^{-1}\)) |
|----------------------------------|-----------------------|------------------|------------------|
|                                  | 17 DAL\(^{1}\) | 32 DAL | | |
| Enrei                           |               |       |       |       |
| L\(^{2}\)                       |               |       |       |       |
| 0.5 mM                          | 14.5 (0.2)    | 7.1 (0.5) |   |
| 5.0 mM                          | 14.8 (0.6)    | 8.3 (0.6) |   |
| 15.0 mM                         | 14.4 (0.5)    | 8.7 (0.6) |   |
| H\(^{3}\)                       |               |       |       |       |
| 0.5 mM                          | 18.1 (1.7)    | 14.9 (0.4) |   |
| 5.0 mM                          | 26.2 (1.5)    | 24.2 (1.4) |   |
| 15.0 mM                         | 24.4 (1.5)    | 21.1 (1.2) |   |
| En6500                          |               |       |       |       |
| L                               |               |       |       |       |
| 0.5 mM                          | 7.9 (0.6)     | 7.3 (0.3) |   |
| 5.0 mM                          | 10.5 (0.4)    | 7.2 (0.5) |   |
| 15.0 mM                         | 9.9 (0.5)     | 7.4 (0.4) |   |
| H                               |               |       |       |       |
| 0.5 mM                          | 19.0 (3.5)    | 32.6 (2.8) |   |
| 5.0 mM                          | 25.8 (2.3)    | 32.9 (1.7) |   |
| 15.0 mM                         | 25.6 (1.6)    | 30.7 (1.0) |   |

1) days after initiation of light treatment (17 DAL corresponds to 32 days after sowing),
2) low light intensity (150 \(\mu\)mol m\(^{-2}\)sec\(^{-1}\)),
3) high light intensity (850 \(\mu\)mol m\(^{-2}\)sec\(^{-1}\))
(photosynthetic photon flux) in both genotypes. However, under high PPF, higher nitrate levels initially increased AP in both genotypes to the same extent but this trend was maintained until 32 DAL only in Enrei; values in the mutant for 0.5 and 5 mM nitrate being similar, and those for 15 mM substantially lower. High PPF increased APs in both genotypes notably in the mutant. These observations suggest that the leaves of Enrei were photosynthetically more active than those of the mutant under low light at 17 DAL while the mutant appeared to be more active under high light at 32 DAL. Leaf APs generally decreased from 17 to 32 DAL in all cases except for the mutant at high irradiance.

**Growth of uninoculated plants**

Both Enrei and En6500 did not nodulate in all cases. The mutant exhibited substantially reduced growth and DM production especially the roots. At 0.5 mM nitrate, main stem height and DM of the roots, shoot, and whole plant of the mutant were approximately 92, 84, and 80% of those for Enrei. Except for main stem height, these values became even lower at 20mM, i.e., 60, 49, and 58% for the root, shoot, and the whole plant, respectively. DM partitioning to the root was lower in the mutant at both nitrate levels. These results indicate that growth and DM production are inherently restricted in En6500.

**Experiment B**

**Root hair curling, infection thread formation, and early nodule development**

The parent cv. Enrei exhibited denser primary root hairs, and considerably more markedly-curved root hairs (MCRH) and percentage MCRH than any of the three non-nodulating mutants (Table 5). Among the latter, En115 had the highest values for these parameters while En1314 had the least. The supernodulating mutant En6500 possessed larger number of total hairs/cm primary root than Enrei, and about 30 and

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**Table 5.** Number and percentages of markedly-curved root hairs (MCRH)/cm primary root of the soybean cv. Enrei and its non-nodulating mutants at 7 days after sowing and inoculation with *B. japonicum* (10⁶ cells/mL).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of sections observeda</th>
<th>Total root hair number/cm primary root</th>
<th>MCRH/cm primary root number</th>
<th>percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enrei</td>
<td>27</td>
<td>17785</td>
<td>1830</td>
<td>10.29</td>
</tr>
<tr>
<td>En115</td>
<td>27</td>
<td>16833</td>
<td>181</td>
<td>1.08</td>
</tr>
<tr>
<td>En1282</td>
<td>28</td>
<td>12039</td>
<td>36</td>
<td>0.30</td>
</tr>
<tr>
<td>En1314</td>
<td>35</td>
<td>11268</td>
<td>11</td>
<td>0.10</td>
</tr>
</tbody>
</table>

a Randomly sampled transverse root sections of 5 replicate plants.
50% more total root hairs and markedly curled root hairs, respectively, than Enrei. Percentage MCRH were similar in both genotypes (Table 6).

Although the non-nodulating mutants occasionally produced MCRHs, no sub-epidermal cell division (SCD) was observed even at stage A (Table 7). In contrast, En6500 had about 1.5 times more SCDs than Enrei at 7 and 13 DAS (days after sowing) (Table 8), indicating that the rate of development of the SCDs was faster in the former. The frequency distribution of the development stages of the SCDs also varied between the two genotypes. In En6500, the number and percentage of SCD at more advanced developmental stages progressively increased. Thus, at 7 DAS, only 10% were at stage A while stage D accounted for 48%. This trend became more pronounced at 13 DAS when only 4% were at stage A while 83% were at stage D. In Enrei, this tendency was not as evident at 7 DAS. At 14 DAS, while a little higher than 50% were at stage D, a considerable 36% remained at stage A.

Nodule number per plant in En6500 was 4.5 times that of Enrei at both sampling

Table 6. Number and percentages of markedly-curled root hairs (MCRH)/cm primary root of the soybean cv. Enrei and its supernodulating mutant En6500 at 7 and 13 days after sowing and inoculation with *B. japonicum* (10⁶ cells/mL).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sampling date</th>
<th>Number of sections observed</th>
<th>Total root hairs/cm root</th>
<th>MCRH/cm primary root</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>number</td>
</tr>
<tr>
<td>Enrei</td>
<td>7</td>
<td>47</td>
<td>12695</td>
<td>1278</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>35</td>
<td>9114</td>
<td>929</td>
</tr>
<tr>
<td>En6500</td>
<td>7</td>
<td>13</td>
<td>18154</td>
<td>1546</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>39</td>
<td>13689</td>
<td>1551</td>
</tr>
</tbody>
</table>

*a* days after sowing and inoculation

*b* randomly sampled transverse root sections of 5 replicate plants.

Table 7. Frequency distribution of sub-epidermal cell division stages and nodule number/cm primary root of the soybean cv. Enrei and its non-nodulating mutants at 7 days after sowing and inoculation with *B. japonicum* (10⁶ cells/mL).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sub-epidermal cell division stage</th>
<th>Nodule number/cm root</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Enrei</td>
<td>116</td>
<td>17</td>
</tr>
<tr>
<td>En115</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>En1282</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>En1314</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*a* Stages A, B, C, and D correspond to stages III–IV, V–VII, VIII, and IX of Calvert *et al.* (1984); b >2 mm in diameter. Number of sections observed for each genotype are given in Table 1. Sections were randomly picked from 5 replicate plants.
Table 8. Frequency of sub-epidermal cell division (SCD) and nodule number/cm primary root of the soybean cv. Enrei and its supernodulating mutants En6500 at 7 and 13 days after sowing and inoculation with B. japonicum (10^6 cells/mL).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sampling time</th>
<th>SCD stagea/cm root</th>
<th>Nodulec number/cm root</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Enrei</td>
<td>7</td>
<td>48.4</td>
<td>22.2</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>59.2</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>28.7</td>
<td>44.7</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>10.6</td>
<td>10.6</td>
</tr>
<tr>
<td>En6500</td>
<td>7</td>
<td>9.4%</td>
<td>8.1%</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>35.7%</td>
<td>5.4%</td>
</tr>
</tbody>
</table>

a Stages A, B, C, and D correspond to stages III–IV, V–VII, VIII, and IX of Calvert et al. (1984); b days after sowing and inoculation; c >2 mm in diameter, values are means (S.E.). Number of sections observed for each genotype are given in Table 2. Transverse sections were randomly picked from 5 replicate plants.

The number of nodules produced between 7 and 13 DAS expressed as percentage of the SCDs scored at 7 DAS was very low in both genotypes, suggesting either a cessation or suppression of maturation of many developing nodules from stage D onward.

The supplementary study on root SCDs revealed that the early stages I–II of SCDs, which were abundant in both Enrei and the supernodulating mutant En6500, were not observed in any section of the 70 mm-tap root segment of any non-nodulating mutant (Table 9) indicating mutational blockage of nodule initiation in the current non-nodulating mutants.

Table 9. Frequency of sub-epidermal cell division stages on the primary root of soybean genotypes inoculated with B. japonicum A1017 (10^6 cell/ml). Values represent the means (S.E.) of the data obtained from the middle longitudinal section of 70 mm-primary root segments of 3 randomly sampled plants at 5 days after inoculation.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sub-epidermal cell division stagea/cm root</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I–II</td>
</tr>
<tr>
<td>Enrei</td>
<td>5.8 (0.3)</td>
</tr>
<tr>
<td>En6500</td>
<td>6.7 (0.5)</td>
</tr>
<tr>
<td>En115</td>
<td>0</td>
</tr>
<tr>
<td>En1282</td>
<td>0</td>
</tr>
<tr>
<td>En1314</td>
<td>0</td>
</tr>
</tbody>
</table>

a According to Calvert et al. (1984).
Control of the nodulation phenotypes.

Self-grafts of Enrei exhibited the normal nodule number while those of the non-nodulating mutants failed to nodulate (Table 10). Moreover, only the grafts bearing Enrei as the root stock formed nodules, the reciprocal grafts all failing to nodulate, indicating that the non-nodulating phenotype is controlled by the root, and that the shoot autoregulatory function is not lost in the non-nodulating mutants. Nevertheless, nodule number in the Enrei root was more when self-grafted than when grafted to shoot of any non-nodulating mutants, suggesting that the latter probably produce a greater amount of shoot-derived inhibitors (SDI).

In grafts between Enrei and En6500, the supernodulating phenotype was expressed only when the latter was used as the shoot (Table 11). The degree of supernodulation of the En6500/Enrei graft is comparable to that of En6500/En6500. In Enrei/En6500 grafts, Enrei shoot abolished the supernodulating phenotype of En6500 root, although their nodules were twice as many as that of the Enrei self-graft. These results suggest that the expression of the supernodulation phenotype is controlled largely, but not exclusively, by the shoot.

Grafts of En6500 shoot and non-nodulating mutant root stock (En6500/non-nodulating mutant) all exhibited the non-nodulating phenotype (Table 11). The reciprocal grafts, i.e., non-nodulating mutant/En6500, were not supernodulated but rather had nodule numbers comparable to that of Enrei/Enrei. These results confirm the conclusions from the first set of grafts (Table 10) that the non-nodulating mutants possess autoregulatory function similar to that of the parent cv. Enrei, and that the non-

Table 10. Mean (S.E.) nodule number in grafts between the soybean cv. Enrei and its non-nodulating mutants at 3 weeks after grafting. Grafts were done 5 days after sowing and inoculation with *B. japonicum* A1017 (10<sup>6</sup> cells/mL). Means of 5 replicates with 3-4 plants per replicate.

<table>
<thead>
<tr>
<th>Graft (Shoot/Root)</th>
<th>Nodule number/grafted plant</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primary root</td>
<td>Lateral root</td>
<td>Total</td>
</tr>
<tr>
<td>Enrei/Enrei</td>
<td>0.11 (0.09)</td>
<td>17.28 (0.83)</td>
<td>17.39 (0.83)</td>
</tr>
<tr>
<td>En115/En115</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Enrei/En115</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>En115/Enrei</td>
<td>4.67 (1.44)</td>
<td>5.50 (3.12)</td>
<td>10.17 (3.43)</td>
</tr>
<tr>
<td>En1282/En1282</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Enrei/En1282</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>En1282/Enrei</td>
<td>4.47 (1.05)</td>
<td>11.44 (1.70)</td>
<td>15.92 (0.65)</td>
</tr>
<tr>
<td>En1314/En1314</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Enrei/En1314</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>En1314/Enrei</td>
<td>3.81 (0.70)</td>
<td>10.94 (0.57)</td>
<td>14.75 (0.54)</td>
</tr>
</tbody>
</table>
Table 11. Mean (S.E.) nodule number and dry weight of graft between the soybean cv. Enrei and its nodulelation mutants at 3 weeks after grafting. Grafts were done 5 days after sowing and inoculation with *B. japonicum* A1017 (10^9 cells/ml). Means of 5 replicates at 4-5 plants per replicate.

<table>
<thead>
<tr>
<th>Graft (Shoot/Root)</th>
<th>Nodule no. plant^-1</th>
<th>Nodule dry weight (g plant^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enrei/Enrei</td>
<td>14.60 (1.79)</td>
<td>10.31 (2.03)</td>
</tr>
<tr>
<td>En6500/En6500</td>
<td>686.93 (90.33)</td>
<td>32.70 (2.58)</td>
</tr>
<tr>
<td>Enrei/En6500</td>
<td>22.70 (3.08)</td>
<td>13.26 (3.32)</td>
</tr>
<tr>
<td>En6500/Enrei</td>
<td>609.55 (13.26)</td>
<td>22.01 (2.84)</td>
</tr>
<tr>
<td>En6500/En115</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>En115/En6500</td>
<td>15.75 (1.61)</td>
<td>14.21 (1.96)</td>
</tr>
<tr>
<td>En6500/En1282</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>En1282/En6500</td>
<td>18.97 (3.48)</td>
<td>16.04 (2.20)</td>
</tr>
<tr>
<td>En6500/En1314</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>En1314/En6500</td>
<td>27.33 (4.45)</td>
<td>20.27 (2.28)</td>
</tr>
</tbody>
</table>

Nodulating phenotype is strictly root-controlled. Interestingly, the non-nodulating mutant/En6500 grafts differed in nodule number, which increased in the order En115 < En1282 < En1314, indicating differential expression of autoregulation probably by producing variable amount of SDI.

Nodule dry weight in grafts between Enrei and En6500 were positively correlated with nodule number although the magnitude of the differences between the normally- and super-nodulated grafts in the latter character was greater (Table 11) mainly because of the considerably smaller nodule mass in the supernodulated grafts. The positive correlation between these two nodulation parameters was also evident in all non-nodulating mutant/En6500 graft combinations.

**Shoot control of nitrate tolerance.**

Across increasing nitrate concentration, nodule number per plant decreased in the Enrei self-grafts but increased in the En6500 self-grafts (Table 12). This trend is also evident in nodule dry weight per plant in grafts bearing Enrei shoot. The supernodulating character of En6500 root is abolished by Enrei shoot while the shoot of the former conferred supernodulation in the root of the latter genotype. These data suggest that nitrate inhibition of noduleation, like autoregulation, is controlled by the shoot.

**Supernodulation control in intergeneric grafts.**

Table 13 and 14 summarize the results of experiment-1 and 2. In intraspecific grafts, the grafted plants bearing supernodulating mutants as scions (En6500/Enrei and
Table 12. Mean (S.E.) nodule number and nodule weight in 3-week-old grafts of the soybean cv. Enrei and its supernodulating mutant En6500 grown at various nitrate levels. Grafting was done at 5 days after sowing and inoculation with *B. japonicum* A1017 (10^9 cells/mL). Means of 5 replicates at 3 plants per replicate.

<table>
<thead>
<tr>
<th>Graft (Shoot/Root)</th>
<th>KNO₃ (mol m⁻³)</th>
<th>Nodule number (plant⁻¹)</th>
<th>Nodule dry weight (mg plant⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enrei/Enrei</td>
<td>0</td>
<td>47.2 ( 8.4)</td>
<td>11.58 ( 3.61)</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>35.9 ( 7.4)</td>
<td>7.47 ( 3.26)</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>10.5 ( 3.6)</td>
<td>0.64 ( 0.33)</td>
</tr>
<tr>
<td>Enrei/En6500</td>
<td>0</td>
<td>75.4 (16.8)</td>
<td>9.44 ( 2.25)</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>46.3 ( 7.1)</td>
<td>1.42 ( 0.34)</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>16.4 ( 3.7)</td>
<td>0.16 ( 0.08)</td>
</tr>
<tr>
<td>En6500/En6500</td>
<td>0</td>
<td>850.2 (24.0)</td>
<td>52.98 ( 9.54)</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>915.0 (34.7)</td>
<td>55.34 ( 2.91)</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>1023.8 (31.0)</td>
<td>59.68 ( 6.67)</td>
</tr>
<tr>
<td>En6500/Enrei</td>
<td>0</td>
<td>828.5 (71.5)</td>
<td>75.24 ( 7.18)</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>898.7 (134.9)</td>
<td>70.21 ( 3.87)</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>945.9 (136.4)</td>
<td>68.48 (15.65)</td>
</tr>
</tbody>
</table>

Table 13. Nodulation response of Enrei in 1-shaped (Fig. 1, a) graft experiments.

<table>
<thead>
<tr>
<th>Combination Shoot/root</th>
<th>Nodule number (plant⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. 1</td>
</tr>
<tr>
<td>Enrei/Enrei</td>
<td>17.0 b^(4)*</td>
</tr>
<tr>
<td>En6500/Enrei</td>
<td>230.3 a (3)</td>
</tr>
<tr>
<td>Rico/Enrei</td>
<td>0.0 c (8)</td>
</tr>
<tr>
<td>RBS15/Enrei</td>
<td>0.0 c (8)</td>
</tr>
</tbody>
</table>

* Within columns, means followed by the same letter do not differ significantly at P=0.05 by the t test.
* Figures in parentheses show the number of plants tested.

Table 14. Nodulation response of Rico in 1-shaped (Fig. 1, a) graft experiments.

<table>
<thead>
<tr>
<th>Combination Shoot/root</th>
<th>Nodule number (plant⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. 1</td>
</tr>
<tr>
<td>Rico/Rico</td>
<td>— (0) ^e</td>
</tr>
<tr>
<td>RBS15/Rico</td>
<td>591.3 a ^+ (6)</td>
</tr>
<tr>
<td>Enrei/Rico</td>
<td>0.0 b (7)</td>
</tr>
<tr>
<td>En6500/Rico</td>
<td>0.0 b (7)</td>
</tr>
</tbody>
</table>

* Within columns, means followed by the same letter do not differ significantly at P=0.05 by the t test.
* Figures in parentheses show the number of plants tested.
RBS15/Rico) exhibited the supernodulation phenotype in the inoculation at 8 days after grafting. In case that it was inoculated at time of sowing, the common bean plant bearing supernodulating mutant as a scion did not exhibit the supernodulation phenotype though the nodule number was significantly greater than that of control (Rico/Rico). The intergenerically grafted plants failed to develop nodules, when inoculated after grafting. Although simultaneous inoculation induced plants to develop nodules in grafts having either the wild type (Enrei/Rico) or the supernodulating mutant (En6500/Rico) as scions, supernodulating phenotype was not recognized.

Discussion

Compared to its parent cv. Enrei, the mutant En6500 had remarkably less overall growth as indicated by its much shorter main stem length and less root and shoot DM production and accumulation rates whether it is nodulated or not. When nodulated, it exhibited larger values for nodulation and symbiotic parameters such as nodule number plant\(^{-1}\), nodule DM, percentage nodule DM, and ARA plant\(^{-1}\). Apparently, En6500 has an inherently restricted growth, which is accentuated by supernodulation. Similar properties were observed in nts382 (Carroll et al. 1985a, b; Day et al. 1986), indicating that both mutant are affected in similar genes controlling growth, nodulation, and nitrate tolerance, which is not surprising since both are products of EMS-mutagenesis.

Notwithstanding these similarities, however, there are basic differences between the two supernodulating mutants. One is in their degree of growth restriction relative to their respective parents when grown uninoculated in the presence of nitrate. Growth reduction in nts382 was only slight (Day et al. 1986) while it was considerable for En6500 (Table 4). A more important difference, however, relates to their nodulation in response to elevated nitrate levels. In the present study, En6500 exhibited an unabated increase in nodule number across increasing nitrate concentrations, the highest of which was 15 mM. When grown hydroponically as in this study, nodulation of nts382 was decreased even at a relatively low nitrate level of 5.5 mM (Carroll et al. 1985a). Apparently, En6500 is extremely nitrate tolerant in terms of nodulation and is unique in this character. Whether these dissimilarities reflect mutational differences or are due to inherent genomic differences remains to be studied. One strategy is to compare the loci of the mutated genes, the nts for example, which has been mapped in nts382 using restriction fragment length polymorphism mapping (Landau-Ellis et al. 1991). Alternatively, the mutants can be reciprocally crossed and their progenies examined.

The regulation of nodulation in the current soybean non-nodulating mutants is
essentially similar to that of the non-nodulating mutants of cv. Bragg (Delves et al., 1986, Caetano-Anolles and Gresshoff, 1990) and cv. Williams (Cho and Harper, 1991) in that it is strictly controlled by the root. This is illustrated in the present study by the observation that grafts bearing the non-nodulating mutant as the root stock invariably failed to nodulate. Our results also support the proposal of Mathews et al. (1989) that the non-nodulating phenotype is a consequence of the failure or reduced capacity to initiate sub-epidermal cell divisions. This is shown in our study by the absence of the SCDs from stages A (stages III-IV of Calvert et al., 1984) to D (stage IX).

The highlight of the present investigation is that all the non-nodulating mutants, despite the absence of even the earliest stage of cortical cell divisions in them, were able to elicit a normal autoregulation of nodule formation in both their parent cv. Enrei and in the supernodulating mutant En6500. This would suggest that the mere presence of root hair curling, even if devoid of infection thread, is sufficient to trigger autoregulation. This is contrary to the conclusion of Caetano-Anolles and Gresshoff (1990), who found that the non-nodulating nod49 (Hd−, Hac−, Inf−, Nod−) can induce autoregulation in its parent cv. Bragg, that root hair curling and other events preceding or following initiation of sub-epidermal cell divisions are not essential for the elicitation of feedback control of nodulation.

The supernodulating mutant En6500 appears to have an altered autoregulation of nodulation as in nts382 (Mathews et al., 1989). Presumably, this is a consequence of the reduced production of the SDI (Caetano-Anolles and Gresshoff, 1990; Gresshoff and Delves, 1986). The increased number and density of root hairs in the primary root of En6500 may not be involved in autoregulation. The similarity of the percentage MCRH in En6500 and Enrei indicate that the greater density of markedly curled root hairs in the former is not due to its greater affinity to its bacterial partner. It has been found that seedling extracts or exudates of the cv. Bragg and its supernodulating and non-nodulating mutants have similar competence in inducing the nod genes of Bradyrhizobium japonicum (Sutherland et al. 1990).

En6500 exhibited a clear pattern of increasingly more SCDs at more advanced stages of nodule ontogeny at both 7 and 13 DAS, while a larger proportion in Enrei was at stage A at 13 DAS. Moreover, the nodules formed between 7 and 13 DAS accounted only for 4.2 and 2.3% of the SCDs scored at stage D at 7 DAS in Enrei and En6500, respectively. These data would imply that autoregulation, presumed to target developmental events before nodule emergence (stage IV) also acts at stages beyond. This is in agreement with the findings of Hansen and Akao (1990) on nts1007. Both studies support the proposal of Mathews et al. (1989) that autoregulation acts by slowing the rate of development of the SCDs, and not through a blockage of particular step(s) in nodule ontogeny.
Summary

The supernodulating and nitrate-tolerant soybean (Glycine max [L.] Merr.) mutant ‘En6500’ and its parent cv. ‘Enrei’ were compared in their response to combined irradiance and nitrate treatments. The mutant exhibited substantially less overall dry matter (DM) production but had markedly more nodules plant⁻¹, larger nodule DM, and higher acetylene reduction activity (ARA) plant⁻¹ compared to Enrei at all nitrate (KNO₃) concentrations of 0.5, 5.0, and 15.0 mM and irradiance levels of 150 and 850 µmol m⁻²s⁻¹. In both genotypes, high irradiance increased DM of the shoot and root, and leaf apparent photosynthetic rate, while symbiotic activity and nodule mass were increased in the mutant but essentially unaffected in Enrei. Dry matter production increased with nitrate level in the mutant but tended to be reduced by the highest level in Enrei. Reduction in nodule DM in favor of the roots and shoot at high levels of nitrate and irradiance was more pronounced in Enrei. High nitrate levels stimulated nodule formation only in the mutant regardless of irradiance, but it consistently depressed specific nodule activity, indicating that the tolerance of the mutant to nitrate is essentially limited to nodule formation and does not extend to nitrogenase function.

The regulation and nitrate inhibition of nodule formation in soybean, Glycine max (L.) Merr., was further examined using the nodulation mutants of cv. Enrei. Nodule development occurred at a faster rate in the supernodulating mutant En6500 than in its parent cv. Enrei, indicating that autoregulation affects the maturation of the developing nodules at a lesser degree in En6500. The non-nodulating mutants En115, En1282, and En1314, all produced extremely low number of markedly-curved root hairs devoid of infection threads, but failed to initiate sub-epidermal cell divisions. Since the shoot of any of the non-nodulating mutants grafted onto either Enrei or En6500 root stock induced an apparently normal autoregulation of nodule formation, it is possible that root hair curling is sufficient to trigger the normal autoregulatory process. Reciprocal grafting experiments among the mutants and Enrei showed that the control of the supernodulating phenotype resides in the shoot while the non-nodulating phenotype is strictly controlled by the root. Grafting experiment between Enrei and En6500 grown under various nitrate levels indicate that nitrate inhibition of nodulation, like autoregulation, is also controlled by the shoot. Since autoregulation and nitrate inhibition of nodule formation are expressed together, it appears that they are functionally related, probably acting synergistically to regulate nodule formation in soybean.

The mechanism of nodulation control or substances involved in autoregulation are different between soybean and common bean.
Original Papers for This Report


References

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ダイズの根粒超多量着生ミュータントの特性

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國分牧衛4), 河内宏1), 米山忠克4)
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6) USDA/ARS, University of Illinois

マメ科植物における共生窒素固定は共生窒素固定菌（根粒菌）の窒素固定活性が効率よく発現するように内部構造を特殊化させた組織（根粒）において行われる。ダイズの栽培品種“エンレイ”から、根粒の着生しない（根粒非着生）、極めて多量の根粒が着生する（根粒超多量着生）など、根粒形成に関連した幾つかのミュータントを分離したので、分離したミュータントの特性を詳しく調べるとともに、得られたミュータントを素材として根粒の形成機構ならびに根粒着生数制御機構の解析を試みた。

原品種“エンレイ”の根粒数は培地の硝酸態窒素濃度の増加にともない急激に減少し、15mM 濃度では僅か数粒着生のみとなるが、根粒超多量着生ミュータント（En6500）では窒素濃度を高めても根粒数は減することなく逆に増加した。このようにミュータント En6500 には根粒が極めて多量に着生することの他に培地の硝酸態窒素に強い耐性を示した。

下胚軸で茎葉と根を切り離した後、ミュータントと原品種の茎葉を相互に取り替える接木解析試験から、En6500 の根粒多量着生と硝酸耐性はいずれも根ではなくて茎葉の形質に支配されるが、根粒非着生の表現型は根に支配されることが判明した。また、根粒菌が宿主根に感染して根粒組織を形成するまでの詳細な観察結果から、原品種と En6500 のいずれにもほぼ同程度の根粒分裂組織が形成されるものの、原品種ではこれより後のステップへの進行の抑制されていることが判明した。なお、根粒非着生ミュータントには根粒分裂組織の形成はまったく認められなかった。

これらの結果から、ダイズにおける根粒着生数の制御は根粒分裂組織の形成過程において発現し、なんらかのシグナルが茎葉に送られていることが推定されるとともに、今後の研究のターゲットは根粒分裂組織の形成時になるものと考えている。
総合討論

座長 魚島 宗明（農業生物資源研究所）

座長 魚島（生物研） この総合討論の座長には、5月に岩手大学へご兼ねされた村田孝夫さんが推選されましたが、ご都合で出席できませんでしたので、私が兼ねてヒッターとして座長をとります。それでは発表順に各先生たちからどうぞ。

鵜飼（東大） フィトクロム a と b の染色体上の位置はどうなっているのですか。

古谷：クエールらの報告によるとアラビドプスでは異なる染色体上に存在することです。

鵜飼：日長反応についてはフィトクロム a、b 両方が関与するとの事ですが、大麦の場合日長反応に関する変異体はほとんどが単一子性変性でした。特に日長反応を失う変異体では、すべて劣性でした。このことから日長反応は単一の機構に支配されていると思われます。フィトクロムと日長反応の関係について知見があればお聞かせ下さい。

古谷：わからないというのが答えます。というのは、先のスライドの 1 つに分子種が 3 つ並んでいて、遺伝子から蛋白までの流れがあってその下に安定した形と不安定な形があり、Pfr の下が不安定な形で PhyB の安定な形としていましたが、そのあたりが必要に破綻で矢印がありました。生活学的には日が暮れるとときはみる Pfr で、翌朝までに Pr に戻るのが不安定なフィトクロムですが、ベルツヒルでやられた実験では何月も暗黒中におい たものに近赤外光をあてると反応がいつになっても可逆的におこります。かなり長期にわ たり Pfr のままのフィトクロムが存在することが確かめられています。それと分子種の対 応は量が少ないためにまだわかりません。また分子生物学的な研究と生物学的な研究 の間のギャップが大きく、ちょっと分子種ごとのプローブがそろってきったところです。ただ 顕微顕影による実験によって、フィトクロムは存在する細胞や細胞内の位置でかなり動き が異なっていることがわかりており、植物全体への照射については複雑すぎて正確なこと はいえません。

山口（名大） レセプターのスイッチオンのみでシグナルトランスダクションが動くという仮定で現在までに報告されている生理現象を説明できるのですか。また PhyA, PhyB というレセプターの量的制御のみで光制御現象はどの程度説明できるのですか。またその制御機構としてはどのようなものが考えられるのですか。

長谷：フィトクロムの実体自体がまだ完全にはわかっていないので、シグナルトランスダクションについてはこれからの課題といえます。

古谷：フィトクロムの分光学的な定量は比較的容易ですが、その量と反応量を比較した論文はわずかしかありません。その 1 つはイネの子葉鞘の成長阻害と Pfr の量の関係を明らかにしたものですが、これは子葉鞘という単純で他のフィトクロムによる現象がない場合だからだと思います。他の細胞ではフィトクロムによる様々な現象が同時に起こるため、 1 つ 1 つの現象を個別に明らかにしなければ解明はできません。シグナルトランスダク
ショの際にも様々な制御は働くと思います。
蓬原（名大） わい性遺伝子に関してですが、ジペレリン生成欠損には器官特異性、ステージ特異性があるとのことですが、根についてはどういう情報があるのですか。最近の検査方の進歩により生長中の植物体についてジペレリン合成部位を特定できるようになったのですか。
室伏：根については分析していませんが、かなり以前に行なわれた実験ではほとんど見つかっていません。多くの部位で独立にジペレリンは合成されると考えます。生殖器官である蕷には相当量のジペレリンが存在しますが、それは近傍の栄養性の器官とは異なる経路で合成されています。
古谷：ジペレリンの他のホルモンもわい性化に関与するという説がありますが、どうのですか。
蓬原：ジペレリン以外の植物ホルモンがわい性に関与するという報告は多数あります。オオムギではIAAが関与する例が多く、イネではほとんどジペレリンです。各作物について特異性があるのかも知れません。
菊池（筑波大） イネがわい性遺伝子sd-1を持つ個体は穂間の細胞数が減少し、各穂間長を短縮することにより短稈になっています。ジペレリンは細胞分裂に影響するのですか。またその作用はジペレリンの種類によって異なるのですか。
室伏：一般的にはジペレリンは細胞伸長のみに関与し細胞分裂にはほとんど影響しないとされています。しかし関与すると主張する人もいます。ジペレリンの種類によって効果が異なるというデータはありません。また、すべてのわい性ジペレリンが関与しているわけではありません。
古谷：シダの胞子はジペレリンで造精器へと誘導されます。
蓬原：プロリン抵抗性、感受性はそれぞれ優性と劣性どちらが多いのですか。プロリン抵抗性がM₉まで固定せず分岐した機構は何ですか。プロリン抵抗性カルスはプロリン含量が多いということですが、その場合個体選択を比較して細胞選択の可能性はどうですか。
長谷川：M₉で得た27個の24個体がM₉でも抵抗性を示しました。このうち3個が劣性で残りの21個が優性でした。固定しなかった理由はわかりません。3番目の質問についてですが、選抜の基準をどうとるかにより評価が異なるので、同一の研究者が細胞選抜、個体選抜ともにやった例はなく正確なことは言えません。私の場合スクリーニングの方法が確立しており、多数の試料を扱えることからM₉個体選抜をおこないました。
座長：植物側の根粒形成に関与する機能のどこが変化して超根粒変異体となったのですか。
赤尾：菌が感染することにより出されるシグナルを、地上部が受け取り感染を制御する機構が働くと思われます。変異体ではこの感染制御機構が働くないと推測されます。
丹羽（名大） オオムギの変異体の地下部、エンドウの変異体の地上部の重力に対する反応はそれぞれどうなっているのですか。地上部、地下部における重力反応メカニズムについてどのような見解を持っているのですか。
菅：オオムギの変異体の地下部の重力に対する反応は正常です。エンドウの変異体の地上
部も正常です。ただ暗黒条件下では地上部も重力に反応しなくなります。地上部と地下部では重力に対する反応についてそれぞれ独立した機構を持つものと思われます。
菊池（筑波大） NY-1とNY-2について遺伝分析はなされているのですか。
川崎：インディカと交配して今年中にはF₂ができるので、RFLPによる位置の確定を行う予定です。
菊池：老化の生物学上の適応についてどう考えるのですか。
川崎：下位葉を老化させ上位葉に栄養分を転流させたり、冬に備え老化した葉の栄養分を種子に転流させるなど、老化を効率的に行なうことの適応的理路は重要であると思われます。
座長：放射線育種場で得られた常緑性シバの常緑性の機構はどのようなものですか。
永富（放育場） 常緑性の機構について詳しい研究はまだ行なっていません。
座長：この植物変異の育種以外の分野での利用に関するシンポジウムは何度か開かれてきたと思われますが、単発的に話題になるものの永続的に研究が行なわれたことは少ないと思います。生理分野と突然変異育種との接点は、予め存在する突然変異体を解析する方法と、ある形質についての突然変異体を頼って誘発、選抜する方法、ある連続的な反応系の素過程の物質的基礎を明かにするためにかなり細かくわれたら突然変異体選抜する方法があると思います。この3番目あたりが今日的な課題になるのではないかと思います。その際選抜が非常に重要になると考えられ、誘発および選抜を生理を専門にする人と育種家が、共同して作業を進めていくべきだと思います。この点について突然変異を作り得る側から何か一言あれば。
矢頭（鹿児島県農試） 放射線育種場に赴任して600余りの突然変異系統の管理を任されましたが。育種に使えるような系統はほとんどありませんでしたか生物力学的にみて興味をそそられる系統が多数存在しました。その様な系統について様々な機会に話題に上げていたところ、利用したいという申し出がでてきました。そのうち、具体的な形質についての突然変異体を要求されるようになってきました。ある程度はその様な申し出にも対応してきましたが、こちらの手に負えない場合も生じます。手元に材料が多数あるので提供します。選抜の方法についてできる限りの助言はしますが、基本的には利用する側が選抜するのがよいと思われます。これからの生理学的あるいは分子遺伝学的分野での突然変異の利用には、作る側と利用する側がチームを作って実験を遂行していくべきです。特にイネに関してこの様なチームが組まれた例が少なく、今後ぜひチームが組まれ、よい成果ができることを期待します。
座長：最後を締めくくるのにふさわしい発言をいただきどうもありがとうございました。みなさんのおかげで活発な議論ができたことと思います。分野が広いため不完全燃焼の感があるかもしれませんが、これは座長の不適切なお詫び申しあげます。