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Physiobiochemical Characteristics of Hybrid Rice

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1代 雜種벼의 生理生化學的 特性

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ABSTRACT : This study was conducted to determine physiobiochemical basis of heterosis using rice hybrids such as Shanyou 63 (Zhenshan 97As × Minhui 63) and Teyou 63 (Longtepu A × Minhui 63) as compared with inbred rice like Milyang 23. Seed protein patterns of rice hybrid showed complementary genetic characteristics inherited from their parents. Hybrid rice had larger embryo and higher α-amylase activity than those of inbred rice. The larger embryo of hybrid was significantly correlated with tillering ability and high number of low node tillers /plant increased by 60~70% in Shanyou 63, leading to higher productive tillers /plant which directly influenced on grain yield of hybrid rice. These characters were further supported by high chlorophyll content in hybrids. Exogenous application of GA_3 (0.02 ppm) on inbred rice like Milyang 23, increased significantly α-amylase activity, but no effect of GA_3 on hybrid rice was observed, indicating that sufficient amount of GA_3 is endogenously present in hybrid rice, showing 1 to 3.5 fold higher activity of α-amylase in hybrid rice, which trigger heterosis from the germinating stage. Further, activity of cytochrome c oxidase was 2.66 to 5.52 fold higher in hybrid rice than that of inbred rice, indicating that rice hybrids have very active metabolism reflecting vigorous growth starting from the germinating stage, in turn leading to higher tillering ability.

Key words : Complementary genetic characteristics, Heterosis, α-Amylase activity, Cytochrome c oxidase

Since Jones [7] first observed heterosis in rice, suggestions for exploiting heterosis commercially by developing F_1 rice seed production were made from time to time. However, difficulties in rice which is strictly self-pollinated crop discouraged most of rice breeders from continuing their efforts. In 1964, spontaneous rice wild abortive cytoplasmic male-sterile plant (indica), known as cms-WA was discovered by Chinese rice breeders, Yuan Longping et al. in Hainam province of China. This in turn resulted in the advent of a new era of heterotic utilization in self-pollinated plants. In addtion, when the three lines, i. e., cms (A line), maintainer (B line) and fertility restorer (R

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line), systems were established in 1973, hybrid rice was succeeded and their growing area was increased from 2 million ha in 1976 to 16 million ha (about 50% of total rice area) in 1993, resulting in an increased production of 24 million tons annually in China [18]. This success facilitated the neighbouring rice growing countries to pay more attention to the development of hybrid rice.

In Korea, with the introduction of wild abortive (WA) type of cms system through IRRI, the breeding program of hybrid rice was initiated in the early 1980s to explore the potential of commercial exploitation of heterosis. Moreover, it has been necessary to study the heterotic effect in rice and the physiobiochemical mechanism of its produce. Some scientists have showed that outyielding of hybrid was primarily due to the high number of productive tillers/plant with the largest direct effect on grain yield. Hybrid rice also exhibited pronounced heterosis for total dry matter and harvest indices, attributed to higher net photosynthetic rate in single leaf, or net assimilation rate (NAR) and faster leaf area development especially at the late vegetative stage and the reproductive stage[1, 11, 18]. However, heterosis is a complicate genetic phenomenon. Simple explanation of heterosis based solely on dry matter and the like, appears untenable. Some scientists suggested the results of complementary biosynthesis on the genome-plasmon of key enzymes of the organelle systems-ribulose-1,5-biphosphate carboxylase/oxygenase in chloroplasts, cytochrome oxidase and ATPase in mitochondria would provide some new insight into the physiobiochemical and genetic mechanisms responsible for heterotic expression particularly in the early stage.

Based on these clues, this study was conducted to elucidate genetic basis, and its physiobiochemical process aimed at further understanding of heterotic effect and its physiobiochemical mechanism especially in early for heterosis of F_1 rice hybrids. Further, mitochondrial efficiency using cytochrome c oxydase at the seedling stage had been considered to be the ideal one to understand the physiology and biochemistry of heterosis. In addition, the possible role of GA in the regulation of amylase de novo synthesis and its relationship with heterosis in rice were evaluated.

MATERIALS AND METHODS

Two popular indica hybrid rices currently used in China, i.e., Shanyou 63 (Zhenshan 97A × Minhui 63), Teyou 63 (Longtepu A × Minhui 63), their common male parent Minhui 63 as well as Longtepu A, its isogenic Longtepu B (B line) which are composed of three lines including Minhui 63 which is R line also used for Teyou 63 hybrid seed production, were introduced from China, and they were tested with a selected Tongil type, an Milyang 23 which is indica/japonica derivative.

1. Seed protein pattern electrophoresis

This experiment was outlayed to analyse the biochemical genetic basis in heterotic rice hybrid by 2-D gel electrophoresis[19]. For preparation of protein, each 30 seeds were grounded to fine powder in chilling motar and washed with TCA acetone. After 10 min centrifugation at 15,000 rpm, 4℃, the pellet was dried in vacuum and solubilized in lysis buffer (9.5 M Urea). IEF gel electrophoresis was performed according to the modified

method of O'Farrell[6]. In order to get pH gradient, the IEF gels were pre-run and electrophoresed for 14 hours at 400V and for 1 hour at 800V. For 2-D gel electrophoresis, IEF gels were excluded and loaded onto SDS-polyacylamide gel, composed of stacking gel (4% acylamide, 1.0 M Tris-HCl, pH 6.8) and separating gel (12.5% acrylamide, 1.5 M Tris-HCl, pH 8.8). Proteins were visualized by silver staining, following a procedure modified by Morrissey [10].

2. Activity of α-amylase and cytochrome c oxidase

1) α-amylase

All seeds of each entry sterilized in 70% EtOH for 5 min, followed by thorough washing with distilled water, were imbibed in each petridish, with wet paper in the bottom, and then incubated in the growth chamber under the condition of enough light density and room temperature. Imbibed seeds of each entry were analyzed for α-amylase activities at different germinating stages (0, 3, 6, 9, 12, 15 days) by following the procedure described by Shuster and Gifford [12].

The seeds of each entry at different germinating stages were ground with 6 ml of 0.01 M Tris buffer (pH 7.5) in a chilled mortar, and after centrifugation of the homogenate at 15,000 rpm for 10 min at 4℃, an aliquot of supernatant solution was used for the assay of amylase activities. One ml of soluble starch solution (67 mg dissolved in 100 ml 0.06 M KH_2PO_4) diluted with 0.75 ml distilled water in a tube was preincubated at 25℃ for 10 min. After that, it was added with 0.75 ml of the extract enzyme solution to start the reaction. After 5 min, 0.1 ml of KI-I_2 reagent was added and then the mixture was diluted by adding 2.5 ml of H_2O and the decrement of enzyme activity was measured at 620 nm. Boiled enzyme preparation, in order to detect possible presence of β-amylase in the crude extract, served as a control, as a quantitative measure of relative enzyme activity per min unit enzyme activities (Δ OD/grain) were calculated from the analytical data of 5 min incubation at each germination stage. Specific enzyme activity was quantified in terms of change in absorbance of the starch-iodine complex per 1 unit equalling the absorbance change caused by 1 μg α-amylase.

The F_1 hybrid performance was evaluated on the basis of the estimates of heterosis, heterobeltiosis and standard heterosis (comparison of F_1 with the best commercial variety Milyang 23 for the enzyme activities recorded at each different germinating stage [17].

2) Cytochrome c oxidase

Cytochrome c oxidase activity which is related to mitochondrial efficiency was assayed by the following method modified by Senin [11]. Seedling tissues of two hybrids (Teyou 63, Shanyou 63) and Milyang 23 were ground with liquid nitrogen before centrifugation. And they were used to analyse cytochrome c oxidase activity for mitochondrial heterosis. A 25 mg cytochrome c (horse heart cytochrome c, NBC corp.) solution in 75 mM K-phosphate buffer (pH 7.4) was reduced by appropriate amount of 1.2 M aqueous solution of Na-hydrosulfate. Ten or 20 μl of the ferrocytochrome c in a cuvette at 25℃ was added and its oxidation was recorded at 550 nm by means of spectrophotometer with distilled water as reference. All enzyme assays above were repeated at least two times.

The protein contents of each crude ext-

racts for calculating specific enzyme activities were measured at the same time by Bradford method in which Bovin serum albumin (BSA) was used to establish the protein standard curve.

3. Effect of GA on α-amylase activity using embryoless and embryo-attached half seeds

Seeds of each entry were dehulled by hand, and whole seeds were cut into two parts with a razor blade. After sterilized, embryoless and embryo-attached half-seeds of each entry were placed aseptically in 10 ml flasks containing 3 ml of either $2 \times 10^{-2}\mu g$ GA/ml or distilled H_2O as control, previously sterilized by autoclaving at 120℃ for 15 min. Then the contents of the flasks were aseptically incubated at 30℃. No discernible changes occurred in embryoless half-seeds, without the addition of GA. However, the dissolution of starch reserve occurred in the (+GA)system after 5 days of incubation[14]. After 6 days, α-amylase activity was measured by the method as described in the above.

4. Heterosis in embryo weight and seedling growth

Seed embryo as well as endosperm and fresh shoot weights at the early stage (0~15 days) of each entry were measured to know the heterotic effects on them. In addition, plant tillering, net phtosynthetic rates in single leaf and chlorophyll contents were also detected[9] at tillering and booting stages, respectively, by using the half-leaf method (dry matter weight) and spectrophotometer. For this purpose, each entry was transplanted in the pots (3 plants/pot) with at least 5 replications at same time, and after transplanting, observation for rice plant tillers in situ was made in every three days to diagramme the plant tillering curve. Net photosynthetic rates in single leaf attached to main stem of rice plant as well as leaf chlorophyll contents were also determined at tillering and booting stages, respectively, by using the half-leaf method (dry matter weight) and spectrophotometer.

Before harvesting, 5 plants of each entry were randomly sampled from pots to measure yields and its heterotic effects on each traits concerned and their relationships, especially in the earlier rice growth stage[4, 8, 13]

RESULTS

1. Rice seed protein patterns

Seed protein patterns of each entry excluding Milyang 23, separated by 2-D electrophoresis, were shown in Fig. 1. Comparing the protein patterns of A line and B line with that of Minhui 63, the restorer (R) line, it was found that A line showed considerably different from R line (Fig. 1. R), showing the protein spot (a) was only exhibited by A line, and the two protein spots (b, c) were appeared at the location of 20~24 kD and 6~7 pH region and in about 60 kD and pH 6.5 area respectively, which were observed in A and B lines, but these spots not appeared in R line. When A line was crossed with R line, fertile F_1 rice hybrid seeds were made. In this case, the spot (a) in about 15~17 kD and pH 6~7 range was missing in fertile F_1 hybrid seeds.

Accordingly, it is infered that the protein spot (a) present in A line, but absent in all fertile lines including B line, and F_1 hybrid seeds was the product of genome-plasma which may result in sterility.

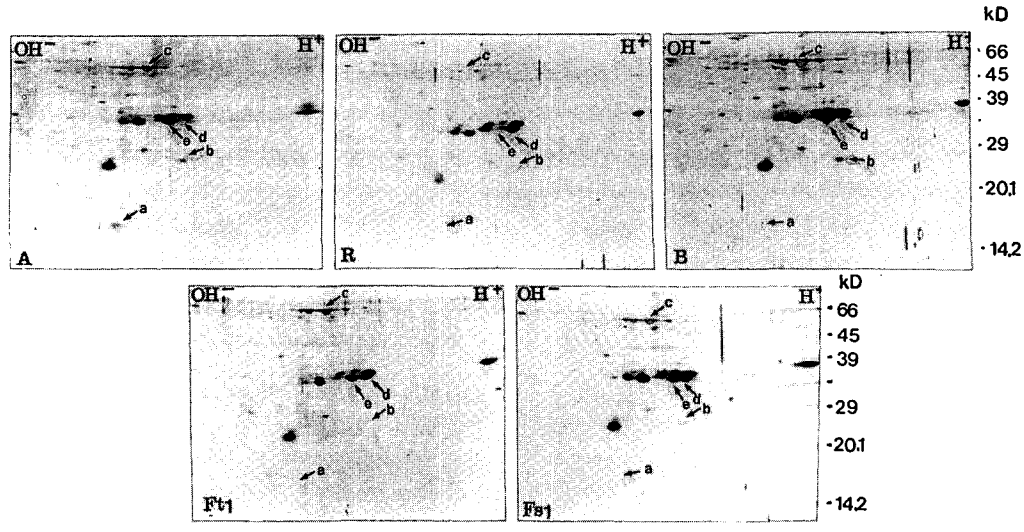

Fig. 1. Seed protein patterns separated by 2-D gel electrophoresis. A; Longtepu A (cms). B; Longtepu B (cms maintainer), R; Minhui 63 (fertility restorer), Ft1; (Longtepu A × Minhui 63), Fs1; Zhenshan 97×Minhui 63.

Additional differences in the protein patterns with unknown functions located in about 30 kD and pH 6.7 region were observed among three lines used (A, B, R lines). No difference was found between A and B lines in this gel area, but the two lines (A and B) different from R line. As shown in Fig. 1. (A, B, R), a protein spot (d) was present only in R lines. One interesting thing to note is that, when A line was crossed with R lines, all protein spots showing different from each other, can be complementally inherited to their progeny F_1 hybrid seeds except for protein spot (a) which was considered a sterile related protein.

2. Heterosis in rice embryo and seedling growth

The results obtained in the study have clearly shown the ocurrence of pronounced heterosis, heterobeltiosis and standard heterosis in rice embryo weight. But slight heterosis can be found in rice endosperms (Table 1). That is all F_1 hybrids used showed higher embryo weight than mid-parent, better parent and standard check cultivar, respectively. Correlation analysis indicates that shoot fresh weight at the initial growth stage of rice (15 days after soaking) was closely related to its embryo weight at same stage was found (Table 1). This phenomenon can be explained as that heterosis at the initial growth of rice is just the maintainance of a growth advantage in the embryo, which was an initial capital to make growth vigor possible at this stage[1]. But why larger embryo promotes seedling vigor needs to be studied in biochemical level.

3. Heterosis in enzyme activity at the early growth stage rice

As analysed in the above, hybrid rice had a higher level initial growth rate than inbred varieties. The growth advantage is expressed as early as in seed germination [3]. The growth advantage is generally expressed as early as

Table 1. Heterotic performance in rice embryos, endosperms and seedlings

	Embryo (mg/5 seeds)	Hmp[1] (%)	Hhb[1] (%)	Hsp[1] (%)	Endosperms (mg/5 seeds)	Hmp[1] (%)	Hhb[1] (%)	Hsp[1] (%)	Shoot fresh weight (mg/5 seelings)	Hmp[1] (%)	Hhb[1] (%)	Hsp[1] (%)
Teyou 63	6.62	30.57 (23.51)[2]	19.92	77.01	114.40	8.59 (9.98)	7.12	38.20	1.34	17.03	15.52	19.64
Longtepu A	4.62				103.92				1.16			
Longtepu B	5.20				104.12				1.17			
Minhui 63	5.52				106.28				1.13			
Milyang 23	3.74				82.78				1.12			
Shanyou 63	6.14				107.80				1.37			
$LSD_{0.05}$	0.89				5.84				0.0890			
$LSD_{0.01}$	1.21				10.81				0.1365			

1) Hmp(%)=[(F_1−(A+R)/2)]/[(A+R)/2 or (B+R)/2], Hhb(%)=(F_1-better parent)/better parent, Hsp(%)=(F_1-leading cultivars)/leading cultivar
2) The number in parenthesis was expressed as [((F_1−(B+R)/2)/((B+R)/2)]×100.
3) Correlation coefficient between embryo and shoot fresh weight; r=0.8133*
Correlation coefficient between endosperm and shoot fresh weight; r=0.5704

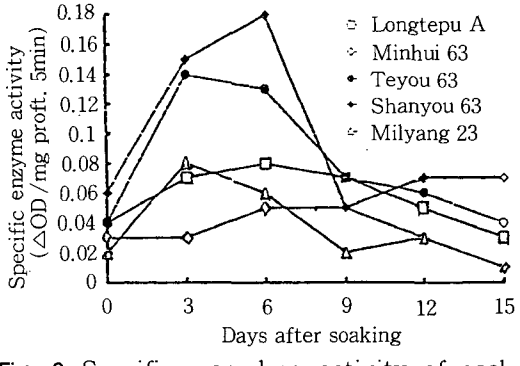

Fig. 2. Specific α-amylase activity of each entry in the initial germinating stages of rice.

in seed germination [3].

The germination speed of hybrids was higher than that of inbred rices used. It was in the order of hybrids used > Milyang 23 > A line > R line in most cases. A similar trend was also observed in specific activities of their α-amylase. During the initial germinating stage (0~15 days), α-amylase activity varied with genotype and time (Fig. 2). At 3 days after soaking, amylase level was increased to 1~3.5 folds and the hybrids tended to have higher amylase levels. At the 6 days after soaking, differences were substantial and statistically significant ($P<0.05$).

In this experiment, heterosis for specific α-amylase activity was statistically significant throughout the time courses (0~15 days) with an exception of Shanyou 63 performed in 9 days after soaking. Namely, rice hybrids, Teyou 63 and Shanyou 63 showed significant heterosis with the mean values of 107.8% and

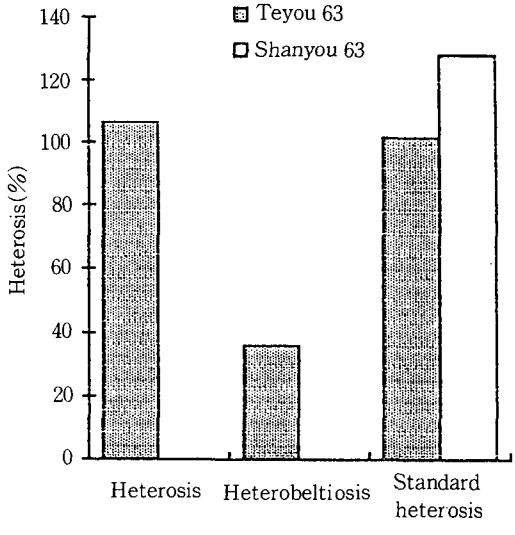

Fig. 3. Heterotic performance in mean specfic α-amylase activities in rice initial germinating stages (0~15 days).

127.9% in specific α-amylase activies respectively (Fig. 3).

The relationship between specific α-amylase activity and fresh shoot weight at initial germinating course was assessed by use of correlation coefficient. The result obtained in this study indicates that fresh shoot weight at this stage had significant correlation with specific α-amylase activity ($r=0.9427^{**}$). It is generally considered that α-amylase is synthesized after germination begins.

Further study showed that α-amylase was conserved in the dry seeds in vivo of hybrid rice and inbred cultivars used, especially in the endosperm of them and that the activity of hybrids was much higher than that of inbred rice cultivars used both in embryo and embyoless-attached half seeds (Fig. 4), which in fact has been reported by other scientists recently[1]. This makes it possible

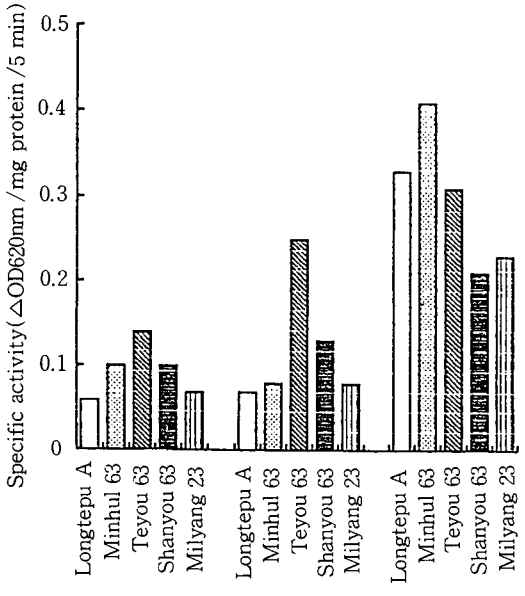

Fig. 4. Specific α-amylase activities in embryo half seed (left 5 bars), embryoless-attached half seed (middle 5 bars) and embryoless-attached half seed treated with 0.02 ppm GA_3 (right 5 bars) in each rice cultivar.

to hydrolyze rapidly the starch in the seeds, providing energy and assimilation for embryo development. The other intriguing thing to note is that when embryoless half-seeds of each entry, in which α-amylase is mostly conserved, were immersed in 0.02 ppm GA_3 for 6 days. The activity of α -amylase was significantly increased, indicating hormone-induced de novo enzyme synthesis, but the increased extent varied with genotypes. Inbred rice cultivars including Minhui 63, Longtepu-A and Milyang 23 increased α-amylase activities by 5.1, 4.7 and 2.9 folds respectively, while hybrids Teyou 63 and Shanyou 63 were only increased by 1.2 and 1.6 folds.

In addition, the results also indicate that the main activity of cytochrome c oxidase which is the product of genome-plasma synthesis[15] in hybrids was much higher than that of parents of the initial growth (Fig. 5). Taking Teyou 63 as an example, the specific activity of α-amylase was 2.66 times as high

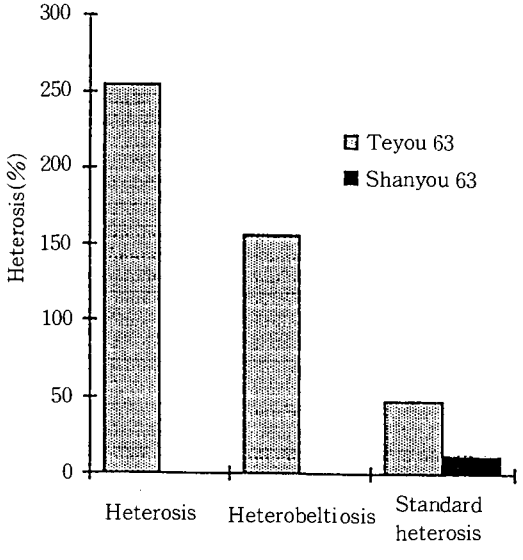

Fig. 5. Heterotic performance for specific cytochome c oxidase activity at the active tillering stage.

as that of A line and 5.5 times as high as that of R line. The heterosis for the enzyme activity was 254.7%.

4. Heterosis performance in the early growth stage of rice

At the initial growth stage, amylase plays an importamt role in hydrolyzing the starch in the seed to provide energy and assimilation for embryo development. After 3 leaf-stage, rice seedling is under autotrophic stage, in which photosynthesis provides energy and assimilation for the young seedling to substantially grow. In this stage, the growth center is, in addition to root development and leaf area expansion which have been intensively studied, rice plant tillering. For practical use, the number of tillers per plant in each genotype was recorded within 1 month after transplanting. The result from this study showed that hybrid rice has stronger tillering ability than their parents or inbred rice. The number of tillers per plant in Shanyou 63 and Teyou 63 was significant different from that of inbred rice including A line. Thus significant heterosis for the number of tillers per plant was exerted by the hybrids (Table 2) responsible for, in addition to growth vigor in the initial growth attributed to higher α-amylase level induced by GA from scutellum of large embryo with less dorminant tiller primordia at heterotrophic stage, high net photosynthetic activity in single leaf resulting from high chlorophyll content (Fig. 6). at the autotrophic stage.

Table 2. Performance for the number of tillers/plant in each genotype recorded within 1 month after transplanting

	No. of tiller/plant	Hmp[1] (%)	Hhb[1] (%)	Hsp[1] (%)
Milyang 23	24.5			
Minhui 63	27.4			
Longtepu A	28.2			
Teyou 63	32.0	15.10	13.48	23.44
Shanyou 63	37.0			
LSD 0.05	5.10			
LSD 0.01	7.70			

1) Hmp (%) = [(F_1-(A+R)/2)]/[(A+R)/2 or (B+R)/2],
Hhb (%) = (F_1-better parent)/better parent,
Hsp (%) = (F_1-leading cultivars)/leading culltivar.

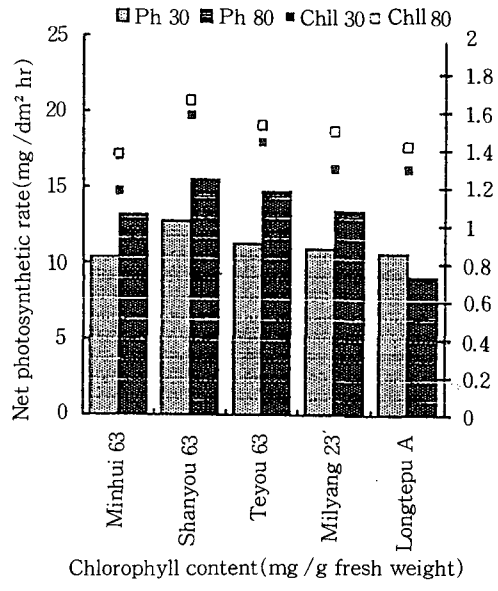

Fig. 6. Net photosynthetic rate (Ph) and chlorophyll content in single leaf (Chll) in each genotype.

Path-coefficient analysis indicates that higher tillering ability and larger number of low-node tillers per plant contribute to increased productive tillers/plant (Table 3). Further study by used of path-codfficient analysis [9] confirmes that higher sink capability (spikelets/plant) in hybrid rice had largest direct effect on grain yield, which was attributed to larger productive til-

Table 3. Number of low-nod tillers /plant at the early stage in each genotype concerned[1)]

Genotypes	Trnsplanting (month /day)	Recording (month /day)	No. of leaf attached in main stem per plant	No. of tillers
Milyang 23	5/26	6/16	3.33	7.02
Minhui 63	5/26	6/16	3.83	6.25
Shanyou 63	5/26	6/16	7.83	9.50
LSD 0.05			2.05	1.60
LSD 0.01			12.78	2.66

1) Each parameter was mean of three replications

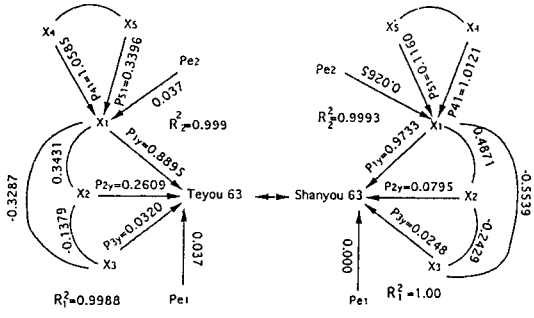

Fig. 7. Diagramatic representation of factors influencing grain yield /plant in hybrid rice used; X_1, total spikelet / plant; X_2, setting percentage; X_3, 1000 grain weight; X_4, productive tillers /plant; X_5, spikelets /panicle; Piy or Pij, direct effect; R_2, determination coefficient; Pe, residual effect.

ler /plant, in turn lead to increased yield (Fig. 7), which was considered an important physiological base [18)].

DISCUSSION

Seed proteins are the physiologically active fractions which constitute the major bulk of enzymes involved in plant metabolism. Seed materials is relatively easy to handle, particulary extraction of protein, and furthermore the seed may be regarded as a fixed physiological state.

Based on this premise, various workers have tried to understand genome and genome-plasma gene relationships by using electrophoretic technique [2, 5, 11, 14, 19)]. These findings reconfirm that the cytoplasmic male sterility in rice currently used in large scale hybrid seed production was attributed to the interaction between genome and cytoplasmic genes. If so, it needs to be intensively studied, how the gene and its product function, this will give a better understanding of sterility mechanism, and in turn would be useful to rogue out off-type seed and to develop near "vigor" R line especially in japonica cultivar by using 2-D gel electrophoretic techniques.

The results from these studies also confirm that heterosis in embryo weight can possibly trigger higher initial growth in F_1 rice[1)] hybrids. Early seedling respiration and hence, growth relies on sugars released from the breakdown of starch from endosperm. This hydrolysis of starch involves α-amylase, which is probably regulated by endogenous GA_3 [14)]. It was determined that hybrid seeds with larger embryo had much higher α -amylase activity than that of inbred rice, which contributed to increased extent of heterosis in shoot growth at the initial stage.

Further study showed that the activity of α-amylase was significantly enhanced by the application of exogenous GA_3. Inbred rices used was more sensitive to exogenous GA_3

than hybrid rice used.

But, few studies reported the relationships among heterosis, GA, and amylase synthesis. So it is necessary to study whether hybrid rice has the higher endogenous GA_3 level or not and whether the suggest of a relationship between GA and heterosis is correct or not continuously.

It is worth mentioning that larger embryo provides initial growth capital to trigger heterosis in heterotic stage, attributed to higher α-amylase level regulated by hormone such as GA moving from scutellum of embryo to aleurone cell in which enzyme de novo synthesis is carried out[16]. Entering autotrophic stage, heterotic individual was supported by higher net photosynthetic rate in single leaf, resulting from higher chlorophyll content and other metabolic level, where the gene products are functioning.

However, heterosis or hybrid vigor is genetic phenomenon resulting from heterozygosity which is a complicate process of physiological system.

Therefore, further study on the metabolic process will shed more light on understanding of heterosis metabolism in rice crop.

摘 要

본 연구는 잡종강세를 나타내는 두 조합의 벼 일대잡종 품종, Shanyou 63, Teyou 63과 inbred 계인 밀양 23호 등을 공시하여 벼의 잡종강세 발현기작을 규명코자 실시되었으며, 수행 후 얻어낸 결과를 요약하면 다음과 같다.

1. 종자 단백질 분석을 통해, 잡종강세 발현의 유전적인 특성들이 부분적으로 모본으로 부터 유전된다는 사실을 규명하였다.
2. Hybrid계 벼는 inbred계보다 배아의 크기가 크고 α-amylase 활성 및 chlorophyll함량이 높게 나타났다. 이러한 특성들은 분얼능력, 특히 수량에 밀접한 영향을 끼치는 하부절간의 분얼수와도 관계가 있었으며, Shanyou 63의 경우 다른 inbred계에 비해 60~70%나 분얼력이 높았다.
3. 인위적으로 GA_3 0.02 ppm을 처리한 후 α-amylase활성 측정시, hybrid계에는 영향을 미치지 못하였으나 밀양 23은 무처리보다 높은 활성을 나타내었다. 이와 같은 결과는, hybrid계 벼의 종자내에는 이미 충분한 GA를 함유하고 있으며 이것이 초기활력과 관계있는 것으로 추정된다.
4. Cytochrome c oxidase의 활성은 inbred계보다 hybrid rice에서 2.7~5.5배나 높게 나타났으며, 따라서 hybrid계 벼의 대사작용이 매우 높았다.

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