Detection of a Protein and Differences in the Populations of Translatable mRNA Specific to Dwarf Rice (*Oryza sativa* L.)

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To isolate genes related to dwarfism in rice, we analysed the differences in the expressions of cellular proteins and translation products of mRNA between normal and dwarf rice of isogenic lines.

By analysis of soluble proteins, one protein of 93 kD was detected exclusively in dwarf rices (Akibare-waisei and Hosetsu-waisei). This protein was detected up 2 weeks after imbibition, however it disappeared after 4 weeks. From analysis of *in vitro* translation products of mRNA by two-dimensional gel electrophoresis, thirty one differences of spots expressed by translatable mRNA were observed among normal rice, Akibare and extreme dwarf rices, Akibare-waisei and Hosetsu-waisei at 2 and 4 weeks after imbibition. In these differences, twenty spots were detected in normal, Akibare and five spots were detected in extreme dwarf rices both Akibare-waisei and Hosetsu-waisei at 2 weeks. Most of the spots specific to normal and dwarf rices almost disappeared at 4 weeks, but two new spots appeared in both normal and dwarf rices.

KEY WORDS: *Oryza sativa*., dwarfism, *in vitro* translation, gene expression, two-dimensional polyacrylamide gel electrophoresis.

Introduction

The breeding of high-yielding cultivars in rice is one of the most important breeding objectives. Dwarfism, especially semidwarf is one of the most important characters in this breeding of high-yielding cultivars because it is accompanied by lodging resistance and thereby have good adaptability to heavy fertilizer application. Although it is known that many dwarfing genes in rice are single recessive genes (FUTSUHARA 1968, KINOSHITA *et al.* 1974, KINOSHITA and SHINBASHI 1982), the expression mechanism of these genes has not been elucidated at all.

Causes of dwarfism have been studied mainly from the point of view of hormonal deficiencies, especially of gibberellin (PHINNEY 1961, SUGE and MURAKAMI 1968, GOTO and ESASHI 1973, REVILLA *et al.* 1988). However, there are some reports that dwarfism is caused not only by a gibberellin deficiency but by auxin shortage (LANTICAN and MUIR 1969, KURAISHI 1974) and increase of peroxidase activity (RIDGE and OSBORNE 1970, 1971). SAKURAI *et al.* (1984a, 1984b) suggested that the change of polysaccharide components of cell wall induced dwarfism in dwarf barley. These reports suggest that the dwarfism has many physiological and biochemical characters and that the gene expression is pleiotropic. On the other hand, in recent years, remarkable progress has been accomplished in the field of plant molecular biology and these advances showed the possibility to isolate and characterize a number of

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novel genes in plants. However, the mechanisms of dwarfism are still unclear. Therefore, as the first step, we speculated that there might be a difference between normal and dwarf rice in the composition of the proteins and in the populations of mRNA. If we identify the proteins and mRNA related to dwarfism and the genes encoding the dwarf phenotype are cloned by using molecular biological techniques, the complex mechanisms of dwarfism can be elucidated.

In the present study, we analysed cellular proteins and translation products of poly(A)⁺ RNA isolated from normal and dwarf rice seedlings, to determine the differences between normal and dwarf rices.

Materials and Methods

Plant materials: Rice (*Oryza sativa* L.) cultivar Akibare, Akibare-waisei: a dwarf mutant from Akibare, and Hosetsu-waisei: a dwarf mutant from rice cultivar Hosetsu were used throughout this experiment. Akibare and Akibare-waisei are isogenic except the dwarf gene (gene symbol: *d-18*). Hosetsu-waisei has the same dwarf gene as Akibare-waisei (FUTSUHARA 1974). Three semidwarf mutants, namely, Reimei (*d-19*), Fukei 71 (*d-50*) and Tanginbozu (*d-35*), and their original cultivar, Fujiminori and Ginbozu were also used.

Plant growth and growth measurements: Seeds were soaked in 0.1% solution of methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate (Benlate, Du Pont Japan Ltd., Tokyo, Japan) for three days. After rinsed in distilled water, seeds were planted in moistened soil (Super soil No.1, Mitsui Toatsu Chemicals Inc., Tokyo, Japan). Plants were grown either in a growth chamber under a 15 h photoperiod, or in a greenhouse under natural photoperiod between June and July. The growth conditions in the growth chamber were at $32 \pm 1^\circ\text{C}$ during the day (15 h) and $26 \pm 1^\circ\text{C}$ during the night (9 h). The light source was white fluorescence lamps (FL 40S W-G, Hitachi Ltd., Tokyo, Japan) and the luminous intensity was 15,000 lux. Height and fresh weight of the seedlings were measured at one week intervals after the start of imbibition.

Extraction of proteins and their analysis: Proteins were extracted from seedlings by the procedure described by MARUYAMA *et al.* (1990). The seedlings were cut into pieces, immediately frozen in liquid nitrogen and ground in a mortar to fine powder. Approximately 1.5g of pulverized seedlings was added to 1.5 ml of an extraction buffer [50 mM Tris-HCl (pH 7.5), 1 mM Na₂-EDTA, 8 mM MgCl₂ and 2% (V/V) 2-mercaptoethanol] and quickly homogenized with a handy micro homogenizer (NS-310E, Nichion-irika, Chiba, Japan). The homogenate was then transferred to an Eppendorf tube and centrifuged at 17,000g for 20 min at 4°C. Soluble proteins in the supernatant were subjected to analysis by SDS-PAGE by the method of LAEMMLI (1970), on 10~20% acrylamide gradient slab gels. Insoluble proteins in the precipitate were washed again with the above described extraction buffer. The washed pellet was suspended in 0.5 ml of a solution containing 50 mM Tris-HCl buffer (pH 6.8), 8 M urea and 3.2% (W/V) SDS, and incubated for 60 min at 40°C to dissolve the insoluble proteins. After incubation, the samples were clarified by centrifugation at 17,000g for 20 min. The protein content of the soluble and insoluble fraction was determined by the Lowry method (LOWRY *et al.* 1951). Eighty µg of soluble and insoluble proteins were sepa-

rated by SDS-PAGE and stained with Coomassie brilliant blue.

Extraction of RNA: Total RNA was extracted from rice seedlings by a modified procedure of the method reported by LOGEMANN *et al.* (1987). Seedlings (10g fresh weight) frozen with liquid nitrogen were pulverized by a mortar and a pestle. The frozen powder was homogenized in 40 ml of 4 M guanidine thiocyanate, 5 mM sodium citrate, 0.07% (V/V) 2-mercaptoethanol and 2% (W/V) sodium lauroylsarcosinate. The homogenate was centrifuged at 20,000g for 10 min at 20°C. The supernatant was layered onto 11 ml pad of 5.7 M CsCl and 0.1 M EDTA (pH 7.5) in polyallomer tubes, and the samples were centrifuged at 113,000g for 16 h at 20°C. The pellet was re-suspended in 3 ml of 50 mM Tris-HCl (pH 9.0) and 1% SDS. Total RNA was extracted from suspension by treatment with phenol/chloroform (1:1, V/V). It was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol at -20°C. After 2 h, RNA was collected by centrifugation and dissolved in distilled water.

High molecular weight RNA was first separated from total RNA by precipitation in 2 M LiCl. Poly(A)⁺RNA was then purified by two rounds of oligo-dT-cellulose (Type VII, Pharmacia, Uppsala, Sweden) column chromatography (AVIV and LEADER 1972).

In vitro translation: Preparation of wheat germ extract and *in vitro* translation were performed according to the protocol of KAWAKAMI and WATANABE (1988). One µg of poly(A)⁺RNA and 0.56 MBq of [³⁵S] methionine (37 TBq/mmol, Amersham Japan, Tokyo, Japan) were added per 10 µl of wheat germ translation mixture (provided by Dr. N. KAWAKAMI of Kihara Institute for Biological Research, Yokohama City University) and incubated for 60 min at 30°C. The translation reaction was stopped by adding 1 µl of 10 µg/ml RNase A (Sigma, St. Louis, U.S.A.) followed by incubation for 15 min at 30°C. The incorporation of [³⁵S] methionine into hot-TCA-insoluble material was measured according to protocol of GRIERSON *et al.* (1985).

One- and two-dimensional gel electrophoresis of translation products and fluorography: Translation products were analysed by one- and two-dimensional gel electrophoresis. One-dimensional gel electrophoresis was carried out by the method of LAEMMLI (1970), on a 12.5% acrylamide slab gel. Two-dimensional gel electrophoresis was carried out by isoelectric focusing (IEF) gel electrophoresis (ampholytes, pH 3.5~10, LKB, Uppsala, Sweden) in the first dimension and a 12.5% (W/V) acrylamide SDS gel in the second dimension as described by O'FARRELL (1975). The samples for the IEF gel electrophoresis were diluted with 8 M urea, 2% (V/V) Nonidet P-40 and 2% (V/V) ampholyte (pH 3.5~10). The anode solution was 0.02 M H₃PO₄ and the cathode solution was 1 M NaOH. IEF was conducted at 200 V for the first 1 h, followed at 300 V for 15 h and at 500 V for the last 1 h. After the second electrophoresis, proteins were detected by fluorography using EN³HANCE^{TM} (Du Pont, Wilmington, U.S.A.) prior to drying. The dried gels were exposed to Kodak X-Omat AR X-ray film for 4 days at -80°C.

Results and Discussion

Phenotype and growth in dwarf rices: Fig. 1 shows the phenotypes of normal rice (Akibare) and dwarf rices (Akibare-waisei and Hosetsu-waisei) grown in a greenhouse under natural pho-

Fig. 1. Thirty-day-old rice plants grown in a greenhouse. Dwarf mutant line Akibare-waisei (center), the original cultivar, Akibare (left), and another dwarf mutant line Hosetsu-waisei (right).

toperiod. In comparison with Akibare, Akibare-waisei and Hosetsu-waisei have only extreme short culms and leaves, but have dark green colored leaves and many tillers. Seedling heights and fresh weights of the dwarf rices at one week after imbibition were approximately one half that of the normal rice. The difference of the growth rates between normal and dwarf rice gradually increased after one week, especially in seedling height (data not shown).

Detection of a protein specific to dwarf rices: The analyses of cellular proteins were performed by using one- to four-week-old seedlings after imbibition because the difference of the growth rates between normal and dwarf rice was distinctly observed at

early stage after germination. Fig. 2 shows the analyses by SDS-PAGE of soluble and insoluble proteins from two-week-old seedlings. Electrophoretic patterns of soluble proteins derived from normal and dwarf rices were basically identical except one faint but distinct band of 93 kD detected in both Akibare-waisei and Hosetsu-waisei but not in normal rice, Akibare. This band of 93 kD was detected in dwarf rices grown in both the growth chamber and the greenhouse. This result was verified over repeat trials. On the other hand, no difference was observed in electrophoretic patterns of insoluble proteins derived from the normal and the dwarf rices.

Fig. 3 shows the time-course of expression of 93 kD protein during the seedling growth up to 4 weeks. This specific protein of dwarf rices was detected in soluble protein up to 2 weeks after imbibition, but it disappeared after 4 weeks. This protein of 93 kD was not detected in semidwarf rices, Reimei, Fukei 71 and Tanginbozu (data not shown). We do not yet know the function of the protein of 93 kD, however, the results presented in Fig. 2 and 3 show that the protein is specific to dwarf rice, especially to extreme dwarf rice and the expression is time-dependent.

In previous studies, NAKAMURA *et al.* (1991) and HIRANO *et al.* (1991) reported that two basic glycoproteins, designated as SRP-1 (32 kD; pI 7.4) and SRP-2 (32 kD; pI 7.7), related to semidwarfism in rice were detected by using a semidwarf near-isogenic line of Taiwanese variety, Dee-geo-Woo-ten (DGWG). Furthermore, it was indicated that the proteins were controlled by codominant alleles, and the locus was closely linked with the semidwarfing gene locus *sd-1*. On the other hand, KIKUCHI *et al.* (1985) suggested that Reimei has the same semidwarfing gene as DGWG. From these reports, we tried whether or not semidwarfism-related proteins like SRP-1 and SRP-2 are detected in the seedlings of semidwarf mutant variety, Reimei and other semidwarf mutants (Fukei 71 and Tanginbozu). However, no differences in the protein patterns between normal and semidwarf rice were observed (data not

Fig. 2. Electrophoretic patterns of soluble and insoluble proteins from seedlings of normal and dwarf rices. Proteins were extracted as described in "Materials and Methods" and stained with Coomassie brilliant blue. Panels a and b correspond to the soluble and insoluble proteins, respectively. Lane 1, Akibare; lane 2, Akibare-waisei; lane 3, Hosetsu-waisei. Long arrowhead indicates positions of the dwarf specific protein, short arrowheads indicate the large subunit (LS) and the small subunit (SS) of ribulose biphosphate carboxylase in the soluble proteins.

Fig. 3. Time-course of the appearance of the dwarf specific protein of 93 kD on the growth of rice seedling. Panels a, b and c correspond to 7 days, 14 days and 28 days after imbibition, respectively. Lane 1, Akibare; lane 2, Akibare-waisei; lane 3, Hosetsu-waisei. Arrowheads indicate position of the dwarf specific protein.

Fig. 4. *In vitro* translation products of mRNA extracted from seedlings of normal and dwarf rice. Panels a and b correspond to mRNA extracted from 14-day-old and 28-day-old seedlings after imbibition, respectively. Lanes 1, 4 and 7, Akibare; lanes 2, 5 and 8, Akibare-waisei; lanes 3, 6 and 9, Hosetsu-waisei. One μ l (0.06 MBq), 2 μ l (0.11 MBq) and 4 μ l (0.22 MBq) of samples were applied in lanes 1~3, 4~6 and 7~9, respectively. Arrowheads with number indicate positions of normal or dwarf specific proteins.

electrophoreses. Fig. 5 shows fluorograms of translation products of normal and dwarf rice after gel electrophoreses and Table 1 summarizes specific spots detected in Fig. 5. Thirty one spots, indicated by arrows, displayed significant differences among normal and dwarf rice seedlings at 2 and 4 weeks after imbibition. Many specific spots were detected exclusively in normal Akibare. And specific spots in dwarf rice were few in comparison with Akibare. Twenty-two spots No.1~3, 6~8, 10, 12~14, 16~22, 24~25 and 28~30 were detected exclusively in normal Akibare, and five spots No.9, 11, 15, 26 and 31 were observed exclu-

shown).

Differences in the populations of translatable mRNAs in normal and dwarf rice seedlings: To observe the differences of the expression level of dwarf related genes, we analysed translation products of poly(A)⁺RNA isolated from normal and dwarf rice seedlings.

Fig. 4 shows the remarkable differences in the *in vitro* translation products among normal and dwarf rice. Bands No. 1 (72 kD), 2 (55 kD) and 5 (24 kD) were specific in normal rice, Akibare, and No. 3 (40 kD) was specific in dwarf rice both Akibare-waisei and Hosetsu-waisei. Moreover, bands No. 4 (37 kD) and 6 (17 kD) showed remarkable increases of translatable mRNAs in normal rice, and band No. 7 (15 kD) showed notable increase in dwarf rice. Bands No. 6 and 7 were detected up to 4 weeks after imbibition, but the other bands specifically detected in normal or dwarf rice at 2 weeks disappeared after 4 weeks.

Specific bands in Fig. 4 did not correspond to specific band detected in Fig. 2 and 3. This suggests that proteins extracted from plant tissue might be post-translationally modified by polysaccharide *in vivo*. However, nothing is known at present about the detailed relationship between the dwarf specific bands in Fig. 4 and the dwarf specific band in Fig. 2 and 3.

Translation products of mRNA were precisely investigated by two-dimensional gel

Fig. 5. Fluorograms of *in vitro* translation products expressed by mRNA derived from seedlings of normal and dwarf rices and analysed by two-dimensional gel electrophoresis. Panel A and B correspond to mRNA from 14-day-old and 28-day-old seedlings, respectively. Panels a, Akibare; panels b, Akibare-waisei; panels c, Hosetsu-waisei. Arrowheads with number indicate positions of normal or dwarf specific proteins.

sively in dwarf rices both Akibare-waisei and Hosetsu-waisei. Moreover, spots 23 or 27 was detected only in Hosetsu-waisei or Akibare-waisei, respectively. As for time-course of mRNA population, twenty specific spots detected at 2 weeks in normal rice disappeared at 4 weeks. On the contrary, two new spots (No.24 and 25) appeared in normal rice and two other spots (No.4 and 5) appeared in dwarf rices at 4 weeks.

These results correspond to the results from Fig. 4 whereas results from Fig. 2 and 3 did not well correspond to those from Fig. 4. It is suggested that spots No. 16~19 and No. 28~29 in Fig. 5 correspond to bands No.5 and 6 in Fig. 4, respectively, because those spots and bands have equivalent molecular weight and they are all detected in only normal, Akibare. Moreover, spot No.31 in Fig. 5 corresponds to band No.7 in Fig. 4, because the

Table 1. Summary of differences and time-course of translatable mRNA population in normal and dwarf rices in Fig. 5

Spot No.	MW (kD)	pI	Variety (or lines) and detection time (weeks)					
			AB(N)		ABW(D)		HSW(D)	
			2	4	2	4	2	4
1	57	5.4	+	-	-	-	-	-
2	48	5.7	+	-	-	-	-	-
3	48	5.8	+	-	-	-	-	-
4	48	7.4	-	-	-	+	-	+
5	41	11.0	-	-	-	+	-	+
6	38	10.0	+	-	-	-	-	-
7	34	8.8	+	-	-	-	-	-
8	34	6.8	+	-	-	-	-	-
9	33	5.8	-	-	+	-	+	-
10	32	5.8	+	-	-	-	-	-
11	28	11.0	-	-	+	-	+	-
12	27	10.5	+	-	-	-	-	-
13	24	6.4	+	-	-	-	-	-
14	24	10.0	+	-	-	-	-	-
15	23	6.7	-	-	+	-	+	-
16	23	5.0	+	-	-	-	-	-
17	23	10.2	+	-	-	-	-	-
18	23	8.4	+	-	-	-	-	-
19	23	8.7	+	-	-	-	-	-
20	22	6.2	+	-	-	-	-	-
21	22	4.6	+	-	-	-	-	-
22	22	4.8	+	-	-	-	-	-
23	21	4.6	-	-	-	-	+	+
24	20	10.8	-	+	-	-	-	-
25	18	4.4	-	+	-	-	-	-
26	17	8.0	-	-	+	-	+	-
27	17	9.4	-	-	+	-	-	-
28	16	7.3	+	-	-	-	-	-
29	16	7.6	+	-	-	-	-	-
30	16	7.8	+	-	-	-	-	-
31	15	11.0	-	-	+	+	+	+

Spot numbers correspond to those shown in Fig. 6. AB, Akibare; ABW, Akibare-waisei; HSW, Hosetsu-waisei; N, normal type; D, dwarf type. +, detected; -, not detected.

spot and band have similar molecular weight and are intensely detected in dwarf rices.

In the present study, we detected one specific protein of 93 kD in lysate of the seedlings of dwarf rices and the differences of gene expressions between normal and dwarf rice, however, nothing is elucidated at present about the relationship between the specific protein or the differences of translatable mRNA and dwarfism.

FUTSUHARA (1974) reported a lower content of gibberellin-like substances was observed in Akibare-waisei as compared to Akibare and gibberellin (GA₃) applied exogenously to Akibare-waisei restored nearly normal stem and leaf elongation. The gibberellin treatment especially at early stage (a few weeks after germination) was effective to restore normal phenotype from dwarf phenotype (FUTSUHARA 1974). Hosetsu-waisei was also restored nearly normal plant height with gibberellin application, and the gibberellin treatment was effective at early stage after germination similar to Akibare-waisei (data not shown). Majority of the population changes of translatable mRNA in normal Akibare occurred at early stage (2–4 weeks after imbibition). It seems likely, therefore, that a number of changes of translatable mRNA in normal Akibare will be one of the mechanisms involved in gibberellin action during stem and leaf elongation.

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矮性イネに特異的なタンパク質及びmRNAの検出

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矮性関連遺伝子の単離と発現機構の解明を目的として, タンパク質分析及び*in vitro*での翻訳系を用いて矮性関連遺伝子の発現解析を試みた。

供試した材料は, イネ品種秋晴とこれにX線照射して得られた矮性型突然変異体の秋晴矮性と同一矮性遺伝子をもつとされる豊雪矮性の主に3種である(Fig.1)。秋晴と秋晴矮性とは矮性遺伝子(*d18*)以外は同じ遺伝子背景をもった同質遺伝子系統であり, 秋晴矮性と豊雪矮性は, 矮性遺伝子が共通でこれ以外の遺伝子背景は異なっている。各種子を, 0.1% (W/V) ペンレート水溶液中で殺菌発芽させた後, 人工気象器(15時間日照, 15,000 lux, 明期32℃, 暗期26℃)及び温室内で1週間から1ヶ月間生育させた。そしてこの地上部(茎, 葉組織を含む)を分析用試料とした。

タンパク質分析は, MARUYAMA *et al.* (1990)の方法に準じて, 水溶性及び非水溶性画分に分けてSDS-PAGEにより行なった。さらに, *in vitro*での翻訳系を用いた発現解析は, グアニンチオシアネート/C₅Cl法及びアフィニティークロマトにより抽出, 分離したmRNAをコムギ胚芽由来タンパク質合成系を用いてタンパク質に翻訳し, これを一次元及び二次元電気泳動後のフルオログラフィーにより行なった。

その結果, 水溶性タンパク質において, 正常型にはなく矮性型に特異的なタンパク質(分子量約93 kD)が認められた(Fig.2)。さらにこの矮性特異的タンパク質は, 発芽2週間目までは出現し, 4週間目には消失することがわかった(Fig.3)。そこで, この発芽2週間目と4週間目における遺伝子の発現解析を行なった。二次元のフルオログラムから正常型と矮性型間で合計31個の特異的スポットを検出した(Fig.5, Table 1)。この内20スポットが秋晴に特異的で, 5スポットが矮性型(秋晴矮性及び豊雪矮性)に特異的であった。これらのスポットは発芽2週間目に検出されたが, その多くのスポットは4週間目には消失した。そして, この4週間目に新たな特異的スポットが正常型, 矮性型それぞれに2つずつ出現することが認められた。