Function analysis of a semi-dwarf *sdg* in rice with near isogenic lines*

LI Xiaobo[1,2], LIANG Guohua[3], LI Xiazhen[4], LI Hongchang[1], XU Jichen[1,2**] and ZHU Lihuang[1]

(1. Open Laboratory of Plant Molecular Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China; 2. Xinjiang Technology Institute of Physics and Chemistry, Chinese Academy of Sciences, Urumqi 830011, China; 3. College of Agriculture, Yangzhou University, Yangzhou 225009, China; 4. School of Life Sciences, University of Science and Technology of China, Hefei 230026, China)

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Abstract The physiological phenotype of the rice semi-dwarf mutant, *sdg*, was characterized in details using a pair of near isogenic lines. Neither gibberellin-deficient nor gibberellin-insensitive is characteristic of the sdg phenotype. By analyzing the secretion of α-amylase and the promotion of stem growth caused by exogenous gibberellin (GA), the *sdg* plant was found to have decreased sensitivity to the GA at the seedling stage. The dwarfism stature of the *sdg* mutant can be attributed to the shortened internodes. Increase of the cell number rather than lack of elongation in the *sdg* mutant is responsible for shoot elongation by the treatment of exogenous GA. These results indicate the protein encoded by the wild type gene of *sdg* may be a regulator for cell elongation.

Keywords: semi-dwarf, rice, phenotype, gibberellin.

The semi-dwarf crop cultivars, including those of wheat, maize and rice, have significantly increased grain production that has been known as "green revolution"[1]. The new varieties could raise the harvest index at the expense of straw biomass, and, at the same time, improve lodging resistance and responsiveness to nitrogen fertilizer. Moreover, dwarf traits of plant are crucial for elucidating mechanisms for plant growth and development as well. In many plant species, various dwarf mutants have been isolated and their modes of inheritance and physiology also have been widely investigated. The causes for their dwarf phenotypes were found to be associated with plant hormones, especially, gibberellins (GAs)[2].

GAs are tetracyclic diterperoid plant growth regulators, which control many diverse developmental and growth processes, such as seed germination, stem elongation, flowering and fruit development[3]. The plant stature can be reduced when mutations occur in the genes encoding key enzymes in the biosynthesis of GAs, which are classified as GA-deficient, such as *sd1*[4], and *d18*[5] in rice. There are other dwarf mutants, in which the sensitivity to exogenous GA is modified. They failed to respond to the treatment of higher concentration of exogenous GA, which are classified as GA-insensitive mutants, for instance, *d1*[6] and *gid*[7] in rice. They are involved in GA signal transduction pathway. However, dwarfing mechanisms in some other dwarf and semidwarf mutants are still unknown.

Shuangai[8], an *India* variety, with a stable inherited trait of only 50 cm height, had been screened from the progenies of "Guiyangai" × "NJ11". Analysis of its genetic background had shown that it carried two dwarf genes, *sd1* and *sdg*[9]. Then *sdg* was tagged on chromosome 5 by use of the trisomic lines of IR36[10]. Till recently, by using RFLP markers, *sdg* was found closely linked to a single-copy DNA clone RZ182 on chromosome 5, with a distance of 4.3 cM[11].

Since two dwarf genes coexist in *Shuangai* mutant, they may either function independently resulting in a dwarf phenotype or interact with each other resulting in an extremely dwarf phenotype. In order to study the function of the *sdg* only, *sdg* must be isolated and the function of sd1 should be subtracted from the *Shuangai* background, so a near isogenic line (NIL) was established. Only under the uniform genetic background of the *sdg* NIL, can a dwarfing

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** To whom correspondence should be addressed. E-mail: jcxu@genetics.ac.cn

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phenotype and other physiological characters be attributed to the function of *sdg*.

1 Materials and methods

1.1 Plant materials

A pair of NILs was developed by Liang Guohua at Yangzhou University, by using NJ6 (Nanjing 6), an indica variety, as a recurrent parent and *Shuangai* as a donor parent. NJ6 backcrossed five times with a *sdg* carring variety, *Xinguiai*, derived from *Shuangai*, resulting in a semidwarf NJ6 line (NJ6-*sdg*), containing *sdg* gene only. Both NJ6 and NJ6-*sdg* formed a pair of NILs for *sdg*.

1.2 Measurement of elongation of second leaf sheath

Elongation of shoots was quantified by a modified version of the microdrop method described by Murakami[12]. Forty rice seeds were surface-sterilized with a 1.5% solution of NaClO that contained 0.1% Tween 20 for 15 minutes, washed with sterile distilled water and soaked in distilled water for 2 days at 30 ℃. Thirty of the germinated seeds were placed on a 1% agar plate (15 for application of GA_3 and 15 as controls) and grown at 30 ℃ under fluorescent light until emergence of the second leaf sheath. After about two days, 1 μL of a solution of GA_3 (3×10^{-2} mol/L) in ethanol was applied to the coleoptiles of rice seedlings at the first-leaf stage. After 3 days, the length of the second-leaf sheaths was measured. The results were shown as averages from 5 tested plants with the standard deviation. The ratio was calculated by the lengths of second-leaf sheath that developed with and without prior application of 1 μL GA_3.

1.3 Measurement of total elongation of shoot

To measure the shoot at seedling stage in response to GA, 50 rice seeds were sterilized, pretreated without or with various concentrations of GA_3 at 37 ℃ for 1 day, washed 3 times with sterilized water, and then imbibed for 1 day. The seeds were germinated in sterilized water and grown in a greenhouse under 12 h light and 12 h dark cycles at 30 ℃. After two weeks, total length of elongated of shoot was measured.

1.4 Assay of α-amylase activity

Preparation of embryoless half seeds and induction of α-amylase were performed. The activity of α-amylase was assayed as described by Yamaguchi et al.[10]. For the agar plate assay of α-amylase induction, 5 embryoless half seeds per plate were sterilized, washed, and positioned perpendicularly on a starch plate (0.2% starch and 2% agar). GA plates were made by adding 10^{-8} mol/L GA_3, and the plates without GA_3 were made as control. Then the plates were incubated in the dark for 4 days at 30 ℃. The plates were developed by incubating the plates in iodine vapor. After a few minutes, the reaction between starch and iodine turned the agar plates a blue-purple color. The agar around half seeds with α-amylase activity remained colorless resulting from the digestion of the starch by amylases.

1.5 Measurement of the length of internodes

Length of upper five internodes and panicles of 10 main culms after heading was measured and averaged in both. The relative contributions of each internode to the total culms length were calculated as percentages.

1.6 Section and cytological observation

The fresh seedling without or with application of 10^{-5} mol/L GA_3 at the three-leaf stage was dissected and observed under an optic microscope. The length of the sample was about 2 cm long, just the part above its root, containing the internode meristem and elongation zone of the shoot. The samples were fixed into 2% glutaraldehyde overnight and then processed into osmium acid. The samples were observed under the Hitachi 175 scanning electronic microscope after they were sprinkled gold.

2 Results

2.1 The phenotype of the sdg

Compared with NJ6, the NJ6-*sdg* plants showed some phenotypes besides dwarfing, namely wilder leaf blades, darker green leaf sheaths and blades, and more tiller number than those of the wild-type plants. But it can set normal seeds as NJ6.

In rice, there are four or five internodes above ground. Each internode is numbered from top to bottom such that the uppermost internode just below its panicle is the first, then the second, third in turn. The stature of rice can be shortened either by the number of internodes or the lengths of internodes.

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Measurement of internode number and length of each internode were performed. The results (Table 1) show that *sdg* does not change internode number, but causes a dramatic reduction in internode length, except the fourth one, resulting in the decrease in plant height in NJ6-*sdg*.

The percentage of each internode to the total culm represents the relative contribution of each internode to the plant height[13], and the numbers are in the parentheses (Table 1). It is obvious that the first (uppermost) internodes in NJ6-*sdg* are comparatively more shortened but the fourth one is relatively longer than that of NJ6-*sdg*. Therefore the whole stature is shortened mainly due to the first internode.

Table 1. Length of each internode and total height (cm)

	NJ6		NJ6-*sdg*	
Panicle	29.89±3.99		22.02±2.37	
1	47.08±5.97	(36.2)	25.90±2.47	(27.8)
2	33.45±1.66	(25.7)	24.07±1.50	(25.8)
3	25.46±1.57	(19.6)	20.10±5.43	(21.5)
4	16.57±5.33	(12.8)	18.35±2.52	(19.7)
5	7.76±1.98	(5.97)	4.80±2.25	(5.14)
Total length (cm)	160.21±5.76		115.29±5.32	

2.2 α-amylase induced by GA_3

For direct characterization of NJ6-*sdg*, starch plate assays for amylases were conducted using the embryoless half seeds from NJ6 and NJ6-*sdg* (Fig. 1). As shown in Fig. 1, neither NJ6 nor NJ6-*sdg* could secrete amylase on starch plate without GA_3. α-amylase activity was induced in all NJ6 embryoless half seeds on the starch plate containing 10^{-8} mol/L GA_3, whereas, at the same concentration, GA_3 induction of α-amylase activity could not be observed in NJ6-*sdg* embryoless half seeds.

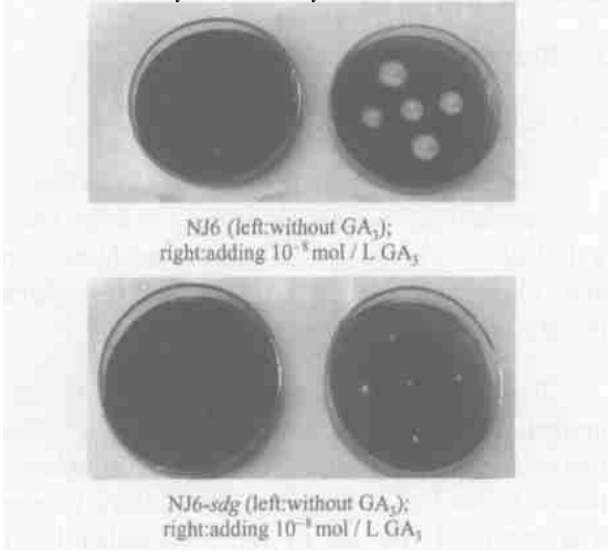

Fig. 1. Agar plate assay of α-amylase induction.

To further investigate the seed response to the exogenous GA_3, α-amylase activities under various concentrations of GA_3 were analyzed and dose-response curves were drawn as in Fig. 2. In the NJ6 seeds, α-amylase activity was induced at a concentration of 10^{-8} mol/L GA_3, and its induction was almost saturated at 10^{-5} mol/L GA_3. In NJ6-*sdg*, however, weak production of α-amylase could be seen at 10^{-7} mol/L GA_3 and the increasing degree was not as sharp as that of the wild type. There was no a saturated point at a higher concentration of GA_3 in the NJ6-*sdg* seeds. The activity of α-amylase in NJ6-*sdg* was still lower than that in the wild type even at 10^{-5} mol/L GA_3, such concentration is higher than the normal physiological concentration *in vivo* by 1000 times. The α-amylase dose-response experiment showed that GA_3 induction of α-amylase activity in the aleurone layer was less sensitive in NJ6-*sdg* than in NJ6.

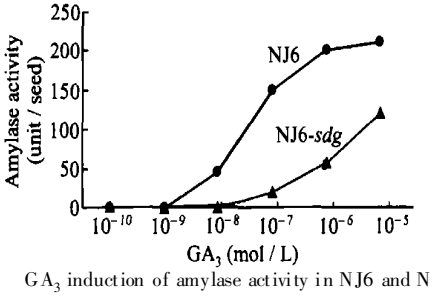

Fig. 2. GA_3 induction of amylase activity in NJ6 and NJ6-*sdg*.

2.3 Effect of GA_3 on shoot elongation

The effect of GA_3 on shoot elongation was analyzed from both second leaf sheaths and total amount of growth in rice seedlings. When the second leaf sheaths of NJ6-*sdg* and NJ6 plants were compared (Table 2), interestingly, under the indicated concentration of GA_3 ($\sim 2\times 10^{-5}$ mol/L), the ratios of elongation in the two types of plants were similar, i.e. 1.41 and 1.32, respectively. It indicates that the sensitivity of second leaf sheath to GA_3 in NJ6-*sdg* is indistinguishable from that in NJ6.

Table 2. Ratio of second sheath length (cm)

Length (cm)	NJ6		NJ6-*sdg*	
	$-GA_3$	$+GA_3$	$-GA_3$	$+GA_3$
	1.60±0.15	2.26±0.44	1.70±0.11	2.24±0.35
$+GA_3/-GA_3$	1.41		1.32	

The total amounts of growth of the rice seedlings were measured by the treatment with the various concentrations of GA_3 (Fig. 3). The NJ6 plants respond-

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ed to GA_3 at its concentration greater than 10^{-8} mol/L to start elongation, and the response was almost saturated at the concentration of 10^{-5} mol/L. NJ6-*sdg* was unable to respond to GA_3 until a higher concentration ($>10^{-7}$ mol/L) reached, and until 10^{-4} mol/L did not reach a saturated concentration yet. This result indicated that a much higher concentration of GA_3 was needed for induction of the action of GA_3 in the NJ6-*sdg* plants than in the NJ6 plants.

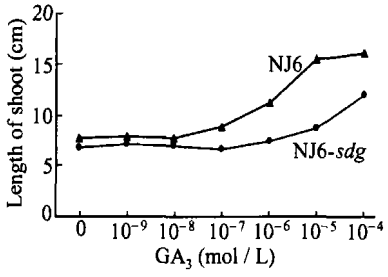

Fig. 3. Shoot elongation response to GA_3 treatment in NJ6 and NJ6-*sdg* plants.

2.4 Cell morphology in NJ6 and NJ6-*sdg*

Usually, internode elongation is caused by cell division in the intercalary meristem (IM), followed by cell elongation in the elongation zone. At the seedling stage, under the natural condition, we examined the architecture of epidermal cells in IM of NJ6-*sdg* and NJ6 by a scanning electronic microscope, respectively. In NJ6 (Fig. 4 (a)), cells were longitudinally elongated, well organized with longitudinal files, and had normal morphology. Similar longitudinal cell files were also seen in NJ6-*sdg* (Fig. 4 (b)), but the cells were shortened, correspondingly, widened, some of them were coved. In addition, the dermal cells on the elongation zone in both NJ6 and NJ6-*sdg* were also observed and compared under an optic microscope. In NJ6 (Fig. 5 (a)), the cells were long and had normal shape, whereas, in NJ6-*sdg* (Fig. 5 (b)), the cells were shorter and wider, some of them had abnormal shape, which might result from asymmetric mitosis. Therefore, the dwarfing of NJ6-*sdg* could be the result of a defect in cell elongation.

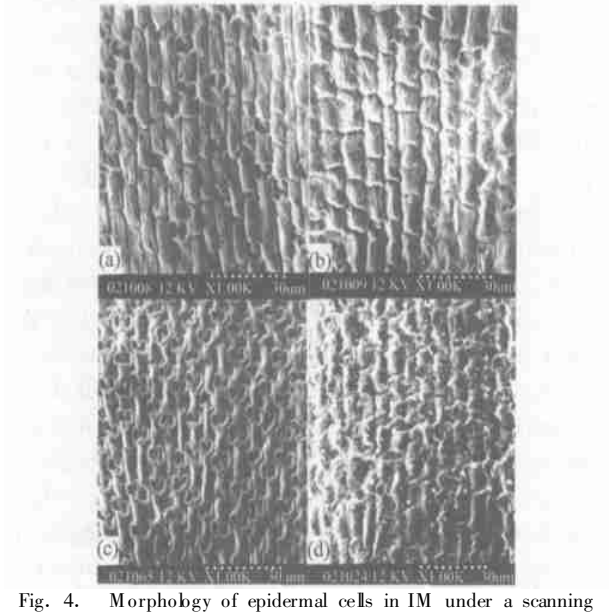

Fig. 4. Morphology of epidermal cells in IM under a scanning electronic microscope in NJ6 and NJ6-*sdg*. (a) NJ6 treated with GA_3(10^{-5} mol/L), (b) NJ6 in normal condition, (c) NJ6-*sdg* treated with GA_3(10^{-5} mol/L), (d) NJ6-*sdg* in normal condition.

The elongation of the shoots in both NJ6 and NJ6-*sdg* occurred at a higher concentration of exogenous GA_3. In order to understand the causes of the stem elongation in NJ6 and NJ6-*sdg*, the epidermal cells in IM from the NJ6 and NJ6-*sdg* treated by exogenous GA_3 were also observed. In the IM zone of NJ6 (Fig. 5 (c)), the cell number dramatically increased, showing that effect of GA_3 on that zone was to accelerate the cell proliferation. Furthermore, in the elongation zone of NJ6 (Fig. 5 (c)), the average longitudinal length of the epidermal cells of the shoot under the GA_3 treatment was about threefold as that without applying GA_3. The results indicate that both the proliferation and elongation are responsible for the elongation of the shoot in NJ6. In NJ6-*sdg* (Fig. 5 (d)), however, when GA_3 was applied, the cell numbers did increase, but the cells were abnormal and still compacted with an irregular shape, in the elongation zone (Fig. 5 (d)), the longitudinal length of the cell almost had no change. Taken together, these results showed that only the proliferation is contributed to the elongation of shoot in NJ6-*sdg*; it is also explained why the phenotype of NJ6-*sdg* could

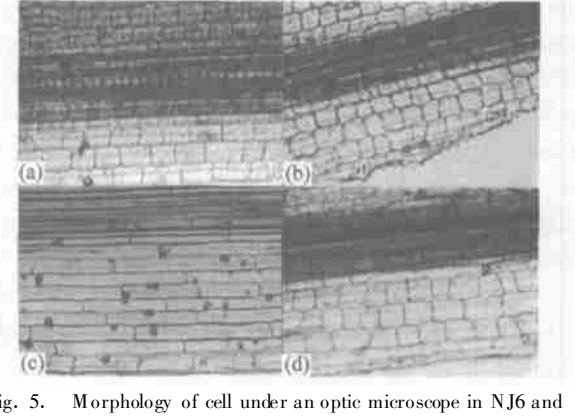

Fig. 5. Morphology of cell under an optic microscope in NJ6 and NJ6-*sdg*. (a) NJ6 in normal condition, (b) NJ6-*sdg* in normal condition, (c) NJ6 treated with GA_3(10^{-5} mol/L), (d) NJ6-*sdg* treated with GA_3(10^{-5} mol/L).

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not be completely recovered by GA_3 treatment.

3 Discussion

The rice dwarf mutants are categorized into six groups based on the elongation pattern of the upper four or five internodes[16]. Each of the six types of mutants is involved in specific reduction of one or more internodes. Table 1 only provides an example of the *sdg* mutant showing schematically the relative length of each internode to the total culm in comparison with the wild type of NJ6. The length of each internode is uniformly reduced in the *dn*-type mutant, so it has an internode elongation pattern very similar to that of the wild type. The *dm*-type mutant reduces the length of its second internode. In contrast, only the first (uppermost) internode is shortened in the *sh*-type mutant. In the *nl*-type mutant, the fourth internode is relatively longer but its first internode is truncated. The genes for *D6* and *dm* type pattern have been cloned and their putative functions have been characterized. As to *sdg*, it is a typical *nl*-type pattern of internode elongation, as demonstrated in the NIL, NJ6-*sdg*.

On the other hand, according to the relationship between dwarfism and the gibberellin-mediated control of physiological processes, namely these mutants have been characterized into four different groups[17], N, T, D, and E. Mitsunaga et al.[17] allocated them into a two-dimensional platting of the extent of induction of α-amylase in the endosperm *versus* the extent of enhancement of shoot elongation upon treatment with exogenous GA_3. Group N mutants include normal cultivars and some of semi-dwarf mutants. Group T mutants are highly responsive to exogenous GA_3 with associated lower endogenous GA_3, which was supposed to be GA-deficient. The mutants of group D are only slightly responsive to exogenous GA_3, an indication that they are GA-insensitive. The mutants of group E have responses to GA_3 similar to those of group N, not only in terms of elongation of shoots but also in terms of α-amylase production, an indication that they are dwarf mutants that can be considered as neither GA-deficient nor GA-insensitive mutants. By now the group E has not been well studied and discussed. On the basis of our results of the activity of α-amylase and the elongation of shoot in NJ6-*sdg*, the *sdg* mutant can be put into group E.

Although with the same phenotype as *sd1*, for instance, dark green, wide leaf, *sdg* has different sensitivity to exogenous GA_3. The SD1 gene is located on chromosome 1, encoding an isoenzyme of GA_{20}-1, a key enzyme catalyzing the step of GA_{19} to GA_{20} in the biosynthesis of gibberellin[4], so *sd1* is the GA-deficient. It belongs to the group T, exhibiting sensitivity to the exogenous GA_3 throughout its whole life. Moreover, its phenotype can be completely recovered by the treatment of exogenous GA_3, whereas NJ6-*sdg* is different; it reduced the sensitivity to the exogenous GA_3 in its seedling stage, and during its elongation stage, the shoot of NJ6-*sdg* is unable to respond to the exogenous GA_3 even at the concentration up to 1000-fold the normal level. In addition, the *sdg* phenotype cannot be recovered after applying exogenous GA_3, so NJ6-*sdg* is not GA-deficient.

As to GA-insensitive mutants, most of them are involved in GA signal transduction pathway. Their second sheath cannot elongate even at the concentration of GA_3 up to 1000-fold the normal physiological level *in vivo*, but the *sdg* plants can make the second sheath elongated at such concentration of GA_3, indicating that NJ6-*sdg* is not GA-insensitive. Therefore, the SDG gene might not be involved in GA-signal transduction directly.

In plant, the growth of stem, which can be significantly influenced by signals from external environment or internal growth hormones, is regulated by growth regulators, which accelerate the cell cycle to increase the cell proliferation (pathway A) and, on the other hand, which elongate the cells (pathway B), so the height of plant depends on both pathways[18]. Based on the results of our cytological observations, inhibition of cell elongation accounts for the dwarfing in NJ6-*sdg*, so the pathway B is impaired in someway. At rice seedlings stage, the cell proliferation, pathway A, gives a great contribution to stem growth and development whereas the pathway B affects it weakly, therefore NJ6-*sdg* seedlings are not distinct from their wild counterparts (NJ6) in their statures. As the plant grows, in the elongation phase, the plant growth mainly relies on the cell elongation, so the pathway B plays a key role. That gives the reason why we can see the difference in plant height between NJ6 and NJ6-*sdg*. We deduce that SDG, as a factor, may function in the cell elongation in the period from vegetative to reproductive growth. It should be significant to clone such a gene and to study its function further.

As far as the rice seedling is concerned, the plant

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stature difference can hardly be seen between the wild type and *sdg* mutant, which, as considered by rice breeders, is as good for the *sdg* plants as for the wild type plants to get enough sunlight, air and water. Concomitantly with the change from vegetative to reproductive growth, the NJ6-*sdg* plants become insensitive to GA. Under certain straw biomass, the plants with the shorter and wider stature are more resistant to wind and rain, and can increase the grain yields. Therefore, the *sdg* phenotype may be a valuable trait that can be utilized in rice breeding and production.

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