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Increased Susceptibility of Rice Following Insertion of Amylopullulanase Gene, to Brown Spot Caused by *Bipolaris oryzae*

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Abstract

Transgenic rice expressing an amylopullulanase (APU) from the bacterium *Thermoanaerobacter ethanolicus* 39E produces grains which are less expensive to process for production of sugar syrup and protein-enriched flour. During risk assessment of the transgenic line in a field test, brown spot disease caused by *Bipolaris oryzae* was found more severe on the transgenic line APU than on its parental line TNG67. When lines APU and TNG67 were inoculated at seedling, tillering or heading stage with *B. oryzae* isolated from line TNG67, the disease was more severe on line APU than on line TNG67 at heading stage, but not at the seedling or tillering stage. However, when *B. oryzae* isolated from line APU was used in the inoculation tests, the disease was more severe on line APU than on line TNG67 at seedling stage, but not at the tillering or heading stage. To our knowledge, this is the first report of an unintended change in a transgenic plant to become more susceptible to a disease than the non-transgenic plant.

Introduction

Rice (*Oryza sativa* L.) is cultivated on 10% of all cropland in the world, ranking only second to wheat in hectares harvested (Webster, 1992). Rice is high in starch and protein, and is a staple food for a large part of the world's population, especially in South and Southeast Asia. An enzymatic process has been developed for production of sugar-enriched rice syrup and protein-enriched rice flour (Shaw and Sheu, 1992). The sugar-enriched rice syrup can be used for food processing and alcoholic beverage production, while protein-enriched rice flour is useful for production of baby and health food (Hansen et al., 1981; Morita and Kiriya, 1993). Recently, transgenic rice has been generated to express thermostable starch hydrolase, amylopullulanase (APU) from a

bacterium *Thermoanaerobacter ethanolicus* Wiegel et Ljungdahl (Chiang et al., 2005). It is simple and less expensive to produce sugar syrup and protein-enriched flour from APU rice grains than from normal rice grains.

In 2004, APU rice and other transgenic rice lines were sent to Taiwan Agricultural Research Institute (TARI) for planting in a restricted area to evaluate their agronomic characteristics in the field and to assess their environmental impact if released for commercial production. During that period, brown spot disease caused by *Bipolaris oryzae* (Breda de Haan) Shoemaker (teleomorph *Cochliobolus miyabeanus* Ito et Kuribayashi) was very prevalent, while other foliar diseases were absent in the test field. It was noticed that brown spot disease was more severe on the transgenic line APU than its non-transgenic parental line TNG67. To our knowledge, the phenomenon of the unintended change of a transgenic plant becoming more susceptible to a disease than the parental plant has not been described previously. We, therefore, studied the effect of the APU gene on the susceptibility of transgenic rice to *B. oryzae*.

Materials and Methods

Disease survey in the field

Observations were made in a field experiment with several rice lines planted as a randomized complete block design with three replicates for each treatment. We surveyed only the transgenic rice line APU and its parental line TNG67. Five small areas each consisting of 60 rice bunches in each replicate were evaluated. Disease severity was assessed visually as percentage of leaf area with brown spot symptoms (International Rice Testing Program, 1980). The survey was conducted before harvest once every 15 days commencing on 5 March 2004 for the first crop with harvest date of 15 July 2004, and 15 August 2004 for the second crop with harvest date of 30 December 2004.

Inoculum production

Cultures of APU-B01 and TNG67-B04 of *B. oryzae* were isolated from diseased rice leaves of transgenic line APU and non-transgenic parental line TNG67 respectively. Conidia of *B. oryzae* were produced on potato dextrose agar incubated for 14 days at 24°C under continuous cool-white fluorescent light. The concentration of conidial suspension was adjusted to 10^5 conidia/ml as described elsewhere (Ko et al., 1973).

Inoculation

Rice seeds of the transgenic line APU and non-transgenic parental line TNG67 were pregerminated by soaking in water for 3 days followed by exposure to moist conditions for one more day in the laboratory before being planted in soil collected from TARI's experimental farm. For seedling stage inoculation, 10 germinated seeds were planted in soil in a pot (4.5 × 4.5 × 5.0 cm) and grown for 14 days in a greenhouse. For inoculation at tillering and heading stage, germinated seeds were planted in soil in a tray (27 × 22 × 7 cm). After 14 days, three seedlings were transplanted to each pot (16 cm diameter, 20 cm high) with soil filled to 16 cm high. Pots were then flooded with water to 3 cm above the soil surface. Plants were grown for 60 and 90 days in a screenhouse for tillering and heading stage respectively. For inoculation, both isolates APU-B01 and TNG67-B04 of *B. oryzae* were used. Four randomized pots containing rice plants at seedling, tillering or heading stage were sprayed with 40, 60 and 60 ml of conidial suspension respectively. Inoculated plants were sprayed with water before being enclosed in plastic bags in the greenhouse and disease severity was recorded after 10 days. Disease severity was assessed visually as described above. The experiment was carried out twice.

Statistical analysis

Regression analysis was used to determine the relationship between the degree of disease severity and the length of time after transplantation of rice plants. All analyses were conducted using Microsoft Office Excel 2003 Software.

Results

Comparison of disease severity of transgenic and non-transgenic rice in the field

In the first crop season, the disease severity of brown spot was less severe during the first 15 days after transplanting, and there was no difference in disease severity between the transgenic line APU and the non-transgenic parental line TNG67. Disease severity increased as the plants grew through various developmental stages (Fig. 1). The increased rate of disease severity during the growth on line APU was greater than that on line TNG67.

Comparison of disease severity of transgenic and non-transgenic rice in the greenhouse

When transgenic line APU and non-transgenic parental line TNG67 were inoculated at seedling, tillering or

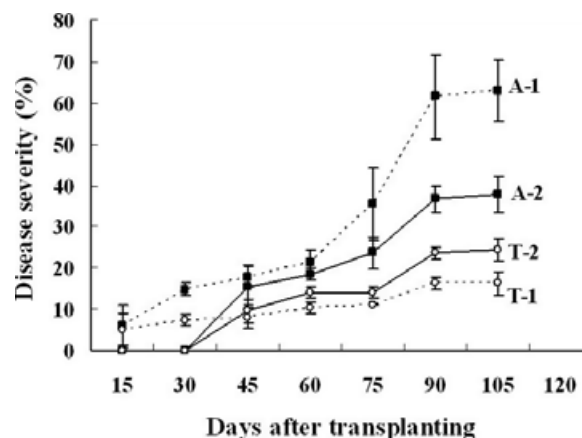


Fig. 1 Severity of brown spot of rice caused by *Bipolaris oryzae* on transgenic line APU (A) and non-transgenic parental line TNG67 (T) during the first (1) and second (2) crop season in the field in 2004

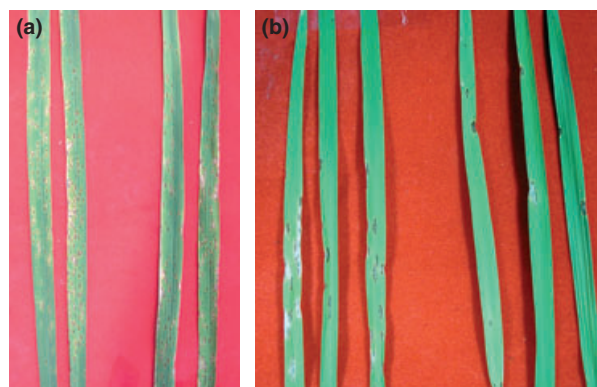


Fig. 2 Disease severity on rice transgenic line amylopullulanase (APU) (left) and non-transgenic parental line TNG67 (right) 10 days after inoculation at the heading stage with APU-B01 isolate obtained from line APU (a) and at the seedling stage with TNG67-B04 isolate obtained from line TNG67 (b)

heading stage with *B. oryzae* isolated from the disease leaf of line TNG67, the disease severity was not significantly different between these two rice lines at either seedling or tillering stage. However, the disease was more severe on line APU than on line TNG67 at heading stage (Fig. 2a) in both experiments I and II (Table 1). When line APU and line TNG67 were similarly inoculated with *B. oryzae* isolated from the diseased leaf of line APU, the disease was significantly more severe on line APU than on line TNG67 at seedling stage (Fig. 2b), but not at either tillering or heading stage (Table 2).

Discussion

Results from this study showed that after being transformed with the APU gene from the bacterium *T. ethanolicus*, the TNG67 rice line became more susceptible than the non-transgenic line to brown spot disease caused by *B. oryzae*. To our knowledge, this is the first report of a transgenic plant unintentionally changed to become more susceptible to a disease than

Line	Disease severity (%)					
	Seedling stage		Tillering stage		Heading stage	
	Expt. I	Expt. II	Expt. I	Expt. II	Expt. I	Expt. II
APU	36.8 a	32.3 a	63.9 a	66.7 a	80.6 a	75.0 a
TNG67	24.6 a	25.6 a	69.5 a	63.9 a	61.1 b	61.1 b
LSD	14.8	8.5	11.1	15.0	11.9	11.9

Values followed by the same letter in the same column for each experiment do not differ significantly ($P = 0.05$) according to LSD.

LSD, least significant difference; APU, amylopullulanase.

Line	Disease severity (%)					
	Seedling stage		Tillering stage		Heading stage	
	Expt. I	Expt. II	Expt. I	Expt. II	Expt. I	Expt. II
APU	40.0 a	40.3 a	63.9 a	61.1 a	66.7 a	63.9 a
TNG67	32.8 b	29.4 b	61.1 a	58.3 a	63.9 a	61.1 a
LSD	5.9	9.7	11.9	11.9	15.0	11.9

Values followed by the same letter in the same column for each experiment do not differ significantly ($P = 0.05$) according to LSD.

LSD, least significant difference; APU, amylopullulanase.

the non-transgenic plant. Previously, potato plants has been genetically modified to produce *N*-acylhomoserine lactones to intentionally alter susceptibility to soft rot *Erwinia* for the study of the mechanism of pathogenicity (Toth et al., 2004). *Rhizobium etli* engineered to produce a phenazine antibiotic for biological control of fungal pathogens has also been reported to show an unintended impairment in symbiotic performance (Krishnan et al., 2007). Genetically engineered rice plants have also been shown to induce genetic variation not linked to the transgenic locus (Labra et al., 2001; Shu et al., 2002).

Increase in susceptibility of the transgenic rice to the disease appears to be pathogen specific. Although the transgenic line APU was more susceptible than the parental line to brown spot disease, it was not more susceptible to blast caused by *Magnaporthe grisea* (Hebert) Barr, sheath blight caused by *Thanatephorus cucumeris* (Frank) Donk or bacterial leaf blight caused by *Xanthomonas oryzae* (Uyeda et Ishiyama) Dowson in preliminary tests (Ting, 2005). The reason for the increase in susceptibility to brown spot disease following insertion of APU gene is not known. The effect could be on anatomical features related to resistance or on substances important to defence against invasion by the pathogen (Ou, 1972). Akai et al. (1958) reported the accumulation of starch surrounding the diseased spot caused by *B. oryzae* on rice plants, indicating the possible involvement of starch in defence against disease development. However, whether the increase in susceptibility to brown spot disease on transgenic line APU is due to degradation of starch remains to be investigated.

When inoculated with isolate TNG67-B04, the difference in disease severity between transgenic and

non-transgenic lines was significant only at heading stage (Table 1). However, when inoculated with isolate APU-B01, the difference in disease severity between these two lines was significant only at the seedling stage (Table 2). Misra and Chatterjee (1963) also found great differences in sporulating ability and pathogenicity between two isolates of *B. oryzae*.

Our results suggested that when a new genetically modified crops is subjected to risk assessment, it should also be assessed for possible side effects such as increase in susceptibility to diseases. Recently, increase in disease susceptibility has not been included in the risk assessment (Kjelleeson, 1997).

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References

- Akai S, Tanaka H, Noguchi K. (1958) On the mechanism of starch accumulation in tissues surrounding spots in leaves of rice plants due to the attack of *Cochliobolus miyabeanus*: I. Observation on starch accumulation in tissues surrounding spots. *Ann Phytopathol Soc Japan* **23**:111–116.
- Chiang CM, Yeh FS, Huang LF, Tseng TH, Chung MC, Wang CS, Lur HS, Shaw JF, Yu SM. (2005) Expression of a bi-functional and thermostable amylopullulanase in transgenic rice seeds leads to autohydrolysis and altered composition of starch. *Mol Breed* **15**:125–143.
- Hansen LP, Hosek R, Callan M, Joens FT. (1981) The development of high-protein rice flour for early childhood feeding. *J Food Technol* **35**:38–43.
- International Rice Testing Program. *Standard Evaluation System for Rice*. Los Banos, Philippines, International Rice Research Institute, 1980.

Table 1

Comparison of disease severity on transgenic rice line APU and non-transgenic line TNG67 inoculated at different developmental stages with isolate TNG67-B04 of *Bipolaris oryzae* isolated from non-transgenic line TNG67

Table 2

Comparison of disease severity of transgenic rice line APU and non-transgenic line TNG67 inoculated at different developmental stages with isolate APU-B01 of *Bipolaris oryzae* isolated from transgenic line APU

- Kjelleeson G. Principles and procedures for ecological risk assessment of transgenic plants. In: Kjelleeson G, Simonsen V, Ammann K (eds), *Methods Risk Assessment of Transgenic Plants: II. Pollination, Gene – Transfer and Population Impacts*. Basel, Switzerland, Birkhauser Verlag, 1997, pp. 221–236.
- Ko WH, Chase LL, Kunimoto RK. (1973) A microsyringe method for determining concentration of fungal propagules. *Phytopathology* **63**:1206–1207.
- Krishnan HB, Kang BR, Krishnan AH, Kim KY, Kim YC. (2007) *Rhizobium etli* USDA 9032 engineered to produce a phenazine antibiotic inhibits the growth of fungal pathogens but is impaired in symbiotic performance. *Appl Environ Microbiol* **73**:327–330.
- Labra M, Savinic C, Bracale M, Pelucchi N, Colombo L, Bardini M, Sala F. (2001) Genomic changes in transgenic rice (*Oryza sativa* L.) plants produced by infecting calli with *Agrobacterium tumefaciens*. *Plant Cell Rep* **20**:325–330.
- Misra AP, Chatterjee AK. (1963) Comparative study of two isolates of *Helminthosporium oryzae* Breda de Haan. *Indian Phytopathol* **16**:275–281.
- Morita T, Kiriya S. (1993) Mass production method for rice protein isolate and nutritional evaluation. *J Food Sci* **58**:1393–1396.
- Ou SH. *Rice Diseases*. Kew, Surrey, UK, Commonwealth Mycological Institute, 1972.
- Shaw JF, Sheu JR. (1992) Production of high-maltose syrup and high-protein flow from rice by an enzymatic method. *Biosci Biotechnol Biochem* **56**:1071–1073.
- Shu QY, Cui HR, Ye CY, Wu DX, Xia YW, Gao MW, Altosaar I. (2002) Agronomic and morphological characterization of *Agrobacterium*-transformed Bt rice plants. *Euphytica* **127**:345–352.
- Ting MY. *Agronomic traits of the amylopullulanase transgenic rice and its effect on microorganisms*. MS Thesis. Wufeng, Taichung, Taiwan, Chaoyang University of Technology, 2005.
- Toth IK, Newton JA, Hyman LJ, Lees AK, Daykin M, Ortori C, Williams P, Fray RG. (2004) Potato plants genetically modified to produce N-acylhomoserine lactones increase susceptibility to soft rot erwiniae. *Mol Plant-Microbe Interact* **17**:880–887.
- Webster RK. Introduction. In: Webster RK, Gunnell PS (eds), *Compendium of Rice Disease*, St Paul, MN, USA, APS Press, 1992, pp. 1–3.