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Evaluation of Pollen Viability of Nakdongbyeo, Two Transgenic Rice Lines, Its Hybrids with Weedy Rice, and Subsequent Selfed Progenies: F_2 and F_3

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This experiment was conducted to evaluate pollen viability of Nakdongbyeo, transgenic rice lines, an F_1 hybrid from a cross between Milyang weedy rice and ABC-promoter transgenic rice line containing basta-resistant (bar) gene and subsequent selfed progenies, F_2 and F_3. The reaction of pollen with 3-{4,5 dimethylthiazolyl-2}-2,5-diphenyl monotetrazolium bromide (MTT) as a staining chemical immediately after pollen shedding showed maximum pollen viability of 86% in Nakdongbeyo, 75% in ABC-promoter transgenic rice line, 62% in ubiquitin-promoter transgenic line, 68% in F_1, 79% in F_2 and 78% in F_3. Viability gradually declined during subsequent observations at 20-minute intervals. However, there was a drastic decline in pollen viability after 40 minutes of pollen shedding. The mean difference of pollen viability among rice lines and time was highly significant, indicating significantly different pollen viabilities at different time intervals. Maximum viability of 36.2% was observed in F_3 and minimum viability of 3.5% was found in F_2 at 90 min after pollen shedding. Results of this experiment on pollen viability and longevity elucidate potential risks of pollen-mediated flow of herbicide-resistant gene from transgenic rice lines and possible integration of it into the weedy rice population.

Key words : Pollen viability, MTT, ABC-promoter transgenic rice line, transgene, resistant

Introduction

Pollen viability is an ability of pollen to perform its functions of delivering sperm cells for successful fertilization [17]. After compatible interaction between pollen grains and stigma surface, the pollen germinates and forms pollen tube, which grows through stigma and style to deliver sperm cells to the ovule [2]. Evaluation of pollen viability based on observation of those functions is cumbersome and time-consuming [7]. Many short-cut methods that reflect the competence of the pollen to perform pollination and subsequent fertilization have been devised [17]. Pollen viability has been evaluated by various staining techniques such as tetrazolium salts to investigate dehydrogenase activity [12], aniline blue to detect callose in pollen wall and pollen tube [5], observation of pollen germination and pollen tube *in vivo* [16], iodine in potassium iodide (lugol solution) to determine starch content and fluorescein diacetate to determine esterase activity and the intactness of the plasma membrane by *in vitro* and *in vivo* germination tests [6]. In a study conducted in cotton, pollen viability assays showed different estimates of viability and germination of pollen samples taken from the same anther. The authors have asserted that those differences could be due to the specific characteristics of the pollen grains assayed by each method [14]. The choice of method to estimate pollen viability depends on the crop species [1]. In monocot crop species, such as sorghum, maize, and rice, different reliable methods for estimating pollen viability have been reported [11,22]. Khatun and Flowers conducted pollen viability tests in rice and compared a number of staining methods such as aniline blue in lactophenol, tetrazolium salts and fluorescein diacetate to assess pollen viability together with *in vitro* pollen germination and reported that staining with MTT best correlated with pollen germination.

With rapid advancement in transgenic technology, more and more transgenic rice varieties are being released for testing. However, there have been reports that pollen-mediated gene flow occurs from transgenic rice to its cultivated, weedy and wild counterparts [24] causing tremendous bio-safety concerns [18]. A recent study has indicated that pollen management has been regarded as a method to minimize transgene flow in maize [11]. Consequently, knowledge on viability and longevity of pollen in newly generated transgenic lines could be helpful in developing various methods to restrict pollen flow in crop species that have potential of natural out crossing.

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Recent investigation has revealed that a recombinant fusion of *Escherichia coli* for trehalose phosphate synthase (TPS) and trehalose phosphate phosphatase (TPP) genes were introduced into Korean rice cultivar Nakdongbyeo; the transgenic lines performed better than the non-transformed cultivar for drought tolerance [8]. The plasmid construct consisted of maize ubiquitin or ABA promoter linked to the TPSP coding region, and the 3′ region of the potato proteinase inhibitor (*pinII*), as well as a gene expression cassette comprised the 35S promoter, the bar-coding region, and 3′-region of the nopaline synthase gene (*nos*).Two similar plasmid constructs, but with different promoters, were then introduced into Nakdongbyeo by agrobacterium-mediated gene transfer. For agrobacterium-mediated transformation, callus induction, co-cultivation with *A. tumefaciens*, and selection of transformed calli were performed [8]. In a similar experiment [3] in transgenic lines of indica rice consisting of two different promoters, it had been reported that transgenic lines were more tolerant to drought and salt stresses than the non-transformed counterpart. Nonetheless, very limited references have been available for pollen viability and longevity of transgenic rice, its hybrid with weedy counterparts and subsequent selfed progenies in order to derive understanding on reproductive fitness of introgressed progenies F_2 and F_3. This study was conducted for evaluating pollen viability and longevity of ABC-promoter and maize ubiqutin promoter transgenic rice lines, Nakdongbyeo, F_1 hybrid, from a cross between Milyang weedy rice as pollen recipient and ABC-promoter transgenic line as pollen donor, and its subsequent selfed generations F_2 and F_3.

Materials and Methods

Plant materials

Transgenic rice lines constructed with drought tolerant TPSP gene and herbicide resistant bar gene along with inserts of either abscisic acid or maize ubiquitin promoters, respectively identified as ABC-promoter transgenic line and Ubi-promoter transgenic lines were acquired from Korea Research Institute for Bioscience and Biotechnology, Daejon, Korea. Milyang weedy rice was obtained from the seed stock of Division of Plant Biosciences, Kyungpook National University because in a previous study performed by Kim et al. 2005, this strain was found susceptible to glufosinate herbicide [10]. Hand pollination was performed between weedy rice as pollen recipient and transgenic lines as pollen donor. F_1 hybrids and subsequent progenies F_2 and F_3 were grown and maintained in the greenhouse [4] for pollen collection and viability evaluation.

Preparation of stain

Sucrose solution of 5% was prepared by dissolving 5 gram of sucrose in water to make final volume of 100 ml. To make 0.9% final concentration of 3-{4,5dimethylthiazolyl-2}-2, 5-diphenyl monotetrazolium bromide (MTT), 0.1125 gram of MTT was dissolved in 5% sucrose solution to make total volume of 12.5 ml of MTT in 5% sucrose solution [9,15].

Collection of pollen and assessment of viability

Panicles, which had more than two-third coming out of flag leaf sheath, were chosen at 9:00-10:00 AM on the third week of August. Selected panicles were detached from the plant and kept with base of panicle immersed in 0.1% ethanol solution for 15-20 minutes. The ethanol solution made simultaneous coming out of anthers from spikelet and shedding of pollen grains from anther sac. Pollen grains were collected in the clean beaker by gently inverting and shaking the spikelet inside the beaker. Collected pollen was mounted on the glass slide at time intervals of 0 min, 20 min, 40 min, 60 min, and 90 min after shedding and a drop of MTT stain was added on the slide. It was then observed under light microscope at 100X magnification and digital images were taken and evaluated for the number of viable and dead on the basis of texture, shape, and stain pattern observed, as described by previous authors [9,23]. Dead pollen was identified and confirmed on the basis of observation on texture, structure and stain patterns of pollen grains after heating it in oven at 80^{o}C for 2 hr and treating with MTT stains, which aided in the reliable confirmation between dead and live pollen (two categories). Photographs of the slides of each time interval and rice lines were taken. Five individual observations with approximately same number of pollen in a microscopic view were randomly selected for image analysis. The statistical analysis was conducted by PROC GLM in Statistical Analysis Systems (SAS). The Error Mean Square was used to test each of the treatment sources of variation based on expected mean squares for fixed treatment effects as described in Steele and Torrie [21].

Results and Discussion

MTT staining immediately after pollen shedding showed

maximum pollen viability of 86% in Nakdongbeyo, 75% in ABC-promoter transgenic rice line, 62% in Ubi-promoter transgenic line, 68% in F_1, 79% in F_2 and 78% in F_3 (Table 1, 3).

The lower estimate of pollen viability in transgenic lines than that of non-transformed cultivar, Nakdongbyeo, was because of greater number of pollen grains with abnormal morphology in transgenic lines. Viability of pollen gradually declined in all rice lines at subsequent observations until 20 min (Table 1, 3). However, there was a drastic reduction in pollen viability after 40 min of pollen shedding except for F_3 (Fig. 1~6). The maximum pollen viability of 36.2% was observed in F_3, 9.4% in Ubi-promoter transgenic line, 8.8% in ABC-promoter transgenic line, 4.4% in Nakdongbyeo and 3.5% in F_2 at 90 min after pollen shedding. The mean difference among rice lines and time interval was found significant suggesting that different lines demonstrated varying pollen viability at different time intervals (Table 2, 4). The interaction of rice line and time interval was also significant suggesting that pollen of different rice line have interaction effects at varying time interval of pollen shedding (Table 2, 4). The pollen viability in Nakdongbyeo was consistently higher until 40 min than both transgenic lines, but the lowest (4.4%) at 90 min among three rice lines (Table 1). This result supports the finding that pollen longevity of cultivar does not depend on initial pollen viability [20]. However, Pollen viability in F_3 was consistently higher than both F_1 and F_2 until 90 min of pollen shedding.

Pacini *et al.* conducted pollen viability study on six angiosperm species including tall fescue. The authors have reported that pollen of this grass species could survive in open air for more than 48 h, but was completely dead at 72 hr of anther opening [13]. Furthermore, the authors found that pollen viability was related to pollination behavior in six angiosperm species and pollen grains withstand changes in volume due to variations in water content, relative humidity and temperature. Similar other studies have reported that the loss of viability in different species has been correlated with water loss and maintenance of the dehydrated state, both in nature and the laboratory [9]. Wang *et al.* in his experiment on studying viability and longevity of transgenic and non-transgenic tall fescue reported that pollen viability of transgenic and non-transgenic pollen reduced to 5% in 30 min, with complete loss of viability in 90 min. However, under cloudy atmospheric conditions, 50% of the pollen remained viable until 60 min and 5% pollen showed viability up to 150 min. The authors further illustrated that relative humidity did not significantly influence pollen viability. The results obtained in our study corresponds with above findings for the patterns of loss in pollen viability in transgenic rice lines and their succeeding out-crossed and selfed progenies with respect to time and environmental conditions such

Table 1. Comparison of pollen viability of transgenic rice lines and their non-transformed check

Time interval	% of viable pollen		
	Nakdongbyo	ABC-transgenic line	Ubi-transgenic line
0 Min	86.0±4.4*	75.0±3.9	62.2±5.9
20 Min	55.6±5.6	41.6±1.2	38.4±3.5
40 Min	43.4±5.5	26.8±1.9	25.2±1.5
60 Min	5.7±1.5	13.4±2.2	11.5±3.9
90 Min	4.4±1.8	8.8±1.4	9.4±4.6

*Mean±S.E. (n=5)

Table 2. ANOVA of pollen viability and longevity of Nakdongbyeo, ABC-promoter and Ubi-promoter transgenic lines

Sources	df	F-value	Pr > F	Remarks
Replication	4	0.40	0.8061	ns[1)]
Rice line	2	24.08	<0.0001	****
Duration	5	183.25	<0.0001	****
Line X Duration	10	5.79	<0.0001	****

[1)]Non-significant; ****Significantly different at 0.01% level.

Table 3. Pollen viability of F_1, F_2 and F_3

Time interval	% of viable pollen		
	F_1	F_2	F3
0 Min	68.2±5.4*	78.8±1.2	78.4±1.9
20 Min	57.2±1.4	63.2±2.8	66.2±1.9
40 Min	39.0±1.6	40.6±2.3	61.4±2.4
60 Min	7.3±0.6	10.0±3.0	45.2±3.7
90 Min	4.7±1.1	3.5±1.2	36.2±2.9

*Mean±S.E. (n=5)

Table 4. ANOVA of pollen viability and longevity of F_1, F_2 & F_3

Sources	df	F-value	Pr > F	Remarks
Replication	4	0.35	0.8403	ns[1)]
Rice line	2	106.25	<0.0001	****
Duration	4	306.75	<0.0001	****
Line X Duration	8	11.76	<0.0001	****

[1)]Non-significant; ****Significantly different at 0.01% level.

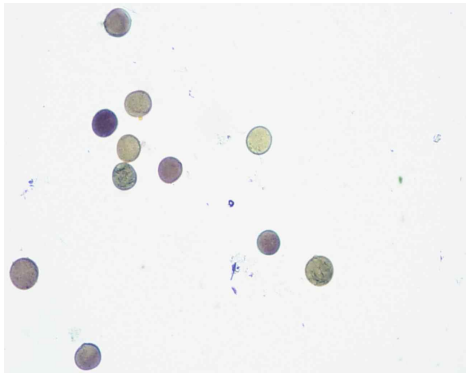

Fig. 1. Viability of ABC-promoter transgenic pollen at 40 minutes after shedding.

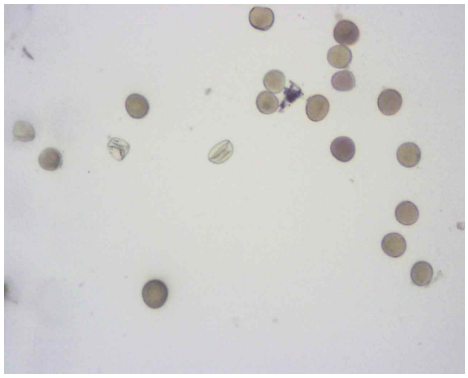

Fig. 2. Viability of Ubi-promoter transgenic pollen at 40 minutes after shedding.

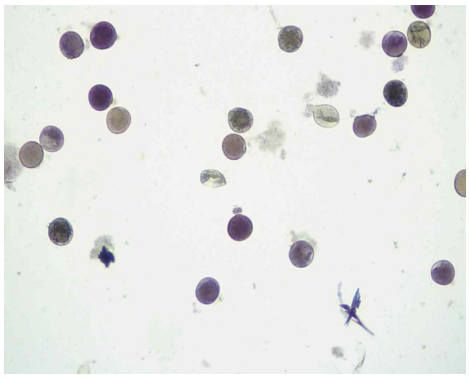

Fig. 3. Viability of Nakdongbyeo pollen at 40 minutes after shedding.

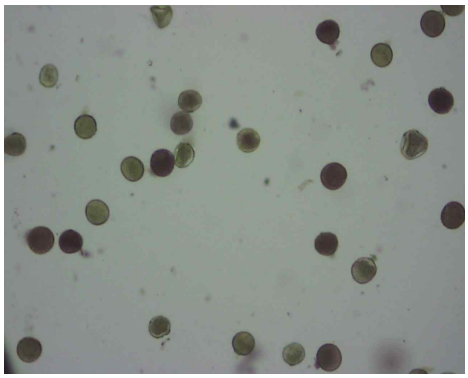

Fig. 4. Viability of F_1 pollen at 40 minutes after shedding.

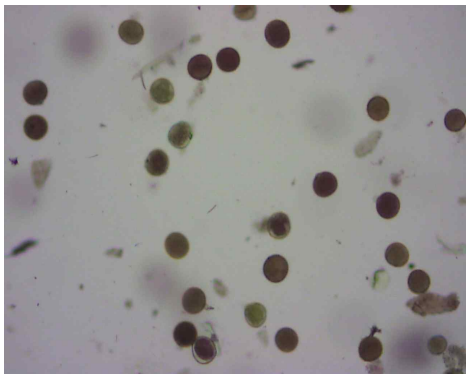

Fig. 5. Viability of F_2 pollen at 40 minutes after shedding.

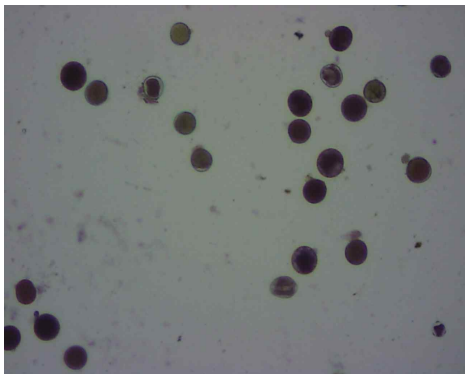

Fig. 6. Viability of F_3 pollen at 40 Minutes after shedding.

as temperature and humidity. During experiment, rice panicles chosen and detached from the main plants were immediately kept in beaker with 0.1% ethanol, brought to the laboratory, which was then maintained at room temperature (20~22°C) and relative humidity of 50~60% until the viability was completed the same day.

A study conducted to investigate pollen competition between cultivated and wild rice species has revealed that foreign pollen has several disadvantages for pollination and fertilization during pollen germination and pollen tube-style interaction, consequently resulting into significantly low fertilization and seed set [19]. The authors have reported that pollen competition between the compatible species and foreign pollen acts as a substantial barrier for out crossing. However, hybridization could occur if foreign pollen arrives earlier than the compatible species pollen and pollen com-

petition would not prevent out crossing. Given the less chances of outcrossing in self-pollinated weeds like Milyang weedy rice, the amount of seed set in the progeny carrying undesirable herbicide resistant traits could still pose risk [4]. In our experiment, pollen viability of transgenic lines, F_1 and F_2 decreased drastically when time elapsed especially from 40 min to 60 min while that of F_3 decreased gradually (Table 1). At 40, 60 and 90 min after shedding, viability of F_3 pollen was the highest. This result indicates more chances that introgressed F_3 progeny of Milyang weedy rice could pose greater risk to pollen mediated transgene flow than that of F_1 and F_2.

This study was aimed to identify a transgenic line between the two with ABC and Ubiquitin promoters, respectively, that would be relatively safe. Based on the results that F_1 of crossing between weedy rice and ABC-promoter transgenic line showed significantly higher seed set [4] than between weedy rice and Ubi-promoter transgenic line, we chose F_1, F_2 & F_3 of a cross between weedy rice and ABC-promoter transgenic line for further investigation of pollen viability and longevity. Based on those results it is found that out crossing of Milyang weedy rice by ABC-promoter transgenic pollen and subsequent introgression of herbicide resistant gene in the F_2 and F_3 generation with greater pollen viability, poses risk of herbicide-resistant weedy rice in the field condition than that could be possible from pollen of Ubi-promoter transgenic rice line.

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초록 : 낙동벼, 2개의 promoter를 각각 삽입한 유전자변형 계통과 잡초성벼(*Oryza sativa*) 인공수정 한 후 다음세대인 F_1, F_2, F_3의 화분활력 평가

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본 연구는 비유전자변형 계통 (낙동벼)과 2개의 다른 promoter (maize ubiquitin과abscisic acid)를 각각 삽입한 유전자변형 벼와 abscisic acid promoter이용한 유전자변형 계통을 잡초성벼 (*Oryza sativa*)와 인공수정 한 후 다음세대인 F_1, F_2, F_3의 화분활력, 형태형성, 생장차이를 비교하여 평가하였다. 화분이 열개된 후 3-{4,5dimethylthiazolyl-2}-2,5-diphenyl monotetrazolium bromide (MTT)반응을 살펴본 결과, Nakdongbeyo에서 86%, ABC-promoter 이용한 유전자변형 벼에서 75%, maize ubiquitin promoter 이용한 유전자변형 벼에서 62%, F_1에서 68%, F_2에서 79% 및 F_3에서 78%의 각각 최대 화분활력을 보여주었다. 유전자변형 계통과 잡초성벼와의 교잡종의F_1, F_2, F_3 세대간의 화분활력을 비교하였을 때 화분 열개된 후 20분까지는 유의한 차이를 보이지 않았으나, 40분에서 90분 사이에서는 F_3의 화분활력이 다른 두 세대 F_1, F_2보다 높게 나타났다. 화분을 열개된 90분 후 F_3 세대 에서 최대 화분 활력이 36.2% 이었고 F_2 세대 에서는 화분 활력이 최소 3.5% 보였다. 따라서 화분에 의해 벼의 유전자가 다른 계통으로 전이되는가를 조사하기 위하여 연차실험을 수행하였는데, 결론적으로 두 유전자변형 계통으로부터 잡초성벼로 유전자가 전이될 위험성이 나타날 것으로 보였다.