Electronic Journal of Plant Breeding, 2(1):1-7 (Mar 2011)
ISSN 0975-928X

Research Article

Molecular and genetic dissection of peduncle elongation in rice

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(Received:30 Sep 2010; Accepted:21 Dec 2010)

Abstract:
Incomplete panicle exsertion in almost all the WA-based CMS lines is one of the major impediments in obtaining higher seed yield in hybrid rice breeding programs. Exploitation of Elongated Uppermost Internode (*eui*) mutants in rice is a viable genetic strategy to solve this problem. Present study was undertaken with an aim of characterizing the elongated uppermost internode mutants in rice germplasm and identifying the molecular markers linked to this trait. Ten IR 64 mutants possessing *eui* morphology were obtained from IRRI, Philippines. Mutants were grown under field conditions and observations on peduncle elongation rate, peduncle length, panicle exsertion and grain number were recorded. All the mutants excepting one mutant (D1027-2-3) possessed elongated peduncle when compared to wild type IR 64. The *eui* mutants exhibited accelerated peduncle elongation rate (8 – 10 cm per day) than the wild type IR 64 (4 – 5 cm per day). At maturity, the *eui* mutants had longer peduncles (44 – 52 cm) than the wild type (27.5 cm) which helped the mutants to bring out their panicles completely outside the boot leaf sheath. Marker genotyping of wild type IR 64 and mutants revealed the genetic association of two *eui1* linked STS markers SAG01 and sMRF19 on chromosome 5 with *eui* trait. Genetic complementation analysis revealed the presence of novel mutation other than *eui1* in the available mutant germplasm.

Key words: Rice, elongated uppermost internode, panicle exsertion, mutant.

Introduction

Rice is a major staple food crop in Asia and Africa. The increased demand for rice is expected to exceed production in many countries in Asia, Africa and Latin America. Increase in rice yield with no additional land available for cultivation depends on the development of high yielding hybrids and varieties. At present, hybrid rice technology for large-scale production has a yield advantage of 15 to 20 per cent or more than 1 tonne of paddy per hectare, over the best bred varieties. Successful commercial hybrid rice production has enabled China to diversify agricultural production on millions of hectares of land.

After the successful demonstration of the use of hybrids developed by making use of the wild abortive (WA) type of cytoplasmic male sterility (CMS) in China, more than 20 countries worldwide have started hybrid rice programmes. As a result of intensive efforts, 56 rice hybrids have been released worldwide. In India, 17 hybrids have been released for commercial production during the last decade (Ahmed, 2003). In rice, the hybrid technology is becoming popular by exploiting various cytoplasmic male sterility systems.

Most of the rice hybrids developed in India is based on utilization of CMS system having the WA cytoplasm. Incomplete panicle exsertion in almost all the WA-based CMS lines is one of the major impediments in obtaining higher seed yield. Almost 30–40% of the panicles are enclosed in the flag leaf and the enclosed spikelets are not available for cross pollination, thus resulting in lower seed yield. Seed set can be increased by applying gibberellic acid (GA3) to promote panicle exsertion and selecting pollinator parents that are 10–20 cm taller than the sterile parent to increase pollen shedding on female panicles (Virmani and Edwards, 1983). Among these practices, GA3 spray is found to be an efficient method. GA3, an efficient and effective plant growth regulator which stimulates cell elongation, has been routinely used to effect panicle exsertion. About 50–75 g/ha of GA3 spraying is recommended in the package developed for hybrid rice production in India. However, Plant breeders have long been looking for a genetic means to solve the panicle exsertion problem.

In this context, introgression of *eui* (elongated uppermost internode) trait will be of much use in overcoming the problem of poor panicle exsertion in hybrid rice seed production programmes. There are two loci with genes for *EUI*. One locus (*eui1*) was mapped onto chromosome 5 through trisomic analysis by Librojo and Khush (1986) and linked to a RFLP marker RG435 at a genetic distance of 33.6 cM. Later Xhu *et al*. (2004) fine mapped this region on chromosome 5 and found that a gene encoding Cytochrome P450 (involved in GA deactivation) is associated with this trait. Identification of molecular markers closely linked to *eui1 (*SCAR markers namely SAG01 and SMRF19*)* makes it possible to develop hybrid rice parental lines possessing *EUI* leading to higher out-crossing potential, which would reduce the need for application of GA3 (Gangashetti *et al*., 2004). The other locus *eui2* is located in the middle of the long arm of chromosome 10 and it is linked to RM258, RM269, RM271 and RM304 with genetic distances of 12.0, 12.9, 35.1 and 1.4 cM respectively (Yang *et al*., 2002). In this context, identifying suitable elongated uppermost internode mutants in our germplasm and developing linked molecular markers will be useful in deploying these genetic materials and markers in the breeding programs to improve panicle exsertion in male sterile CMS lines.

Against this background, the present study was undertaken with the objectives of collection of elongated uppermost internode mutants from IRRI and Mahyco Seeds Pvt. Ltd., Maharastra and its characterization in identifying molecular markers linked to elongated uppermost internode trait and identifying novel *eui* mutants which are non-allelic to *eui1*.

Material and Methods

Genetic Material used

Collection of *eui* mutants

Ten putative elongated uppermost internode mutants were obtained from International Rice Research Institute, Philippines (Table 1**)**. All the mutants were generated by mutating the popular rice variety IR 64 using different mutagenic agents. BF, an *eui* mutant (allelic to *eui1* located on chromosome 5) was obtained from Mahyco Seeds Pvt. Ltd., Maharastra. Apart from these lines, TNAUCMS2A, TNAUCMS2B, COMS14A, COMS14B and wild type IR64 were also used for genotyping,

Characterization of *eui* mutants

All the mutants were grown under field conditions along with wild type IR 64 during *kharif* season 2007. Seedlings were planted as one per hill by adopting a spacing of 20 x 10 cm^2. Standard agronomic practices were adopted. Observations were recorded on the phenotypic traits such as days to 50% flowering, plant height, peduncle length, panicle length, panicle exsertion and number of grains per panicle. Peduncle elongation rate was recorded by measuring the inter-auricle distance starting from 3DBH until 4-5 days after harvesting. Observations on peduncle length, panicle exsertion, plant height and number of grains per panicle were recorded at the time of maturity.

Identifying novel mutants which are non-allelic to *eui1* through genetic complementation

Hybridization

In order to identify mutants caused due to mutations at other than *eui1* loci, hybridizations were made between different *eui* mutant lines obtained from IRRI which were raised during *kharif* 2007. F_1 generation was raised along with the parents and wild type IR 64. Observations on peduncle length, panicle exertion and plant height were recorded in the parents, wild type and F_1s.

Allelism test for *eui* trait

All the mutants along with the wild type IR64 were screened for molecular polymorphism using two STS markers namely SAG01 and sMRF19 that are reported to be linked to *eui*1 (Gangashetti *et al.,* 2004). Along with these genetic materials, two male sterile A lines (TNAUCMS2A and COMS14A), their respective B lines (TNAUCMS2B and COMS14B) and an *eui* mutant (allelic to *eui1* located on chromosome 5) from Mahyco Seeds Pvt. Ltd., Maharastra were selected and genotyped. Leaf tissues were collected from all the *eui* mutants, IR 64, two A lines, two B lines and Mahyco *eui* line and used for isolating genomic DNA using CTAB method.

DNA quantification

DNA was quantified using a spectrophotometer. 1 µl of crude DNA was diluted to 1 ml deionized water. The absorbance was measured at 260 nm. An optical density (OD) of 1.0 corresponds to 50 µg / ml for double stranded DNA. The final DNA concentration was adjusted to 50ng/µl. Quality of the DNA was assessed by agarose gel electrophoresis.

PCR amplification of STS markers

PCR was carried out in eppendorf master cycler (Eppendorf AG, Germany) machine by adopting a thermal profile as follows: Initial denaturation at 94°C for 5 min, denaturation at 94°C for 45 sec, annealing at 58°C for 45 sec and extension at 72°C for 5 min followed by a final extension at 72°C for

10 min. The profiles 2, 3 and 4 were programmed to run for 40 cycles.

Agarose Gel Electrophoresis for separation of amplified products

The PCR products were resolved by using agarose gel electrophoresis. Depending on the size of the amplicon, the PCR products were run on 1.2% and 3.5% agarose gel.

Results and Discussion

Most of the rice hybrids developed in India is based on utilization of CMS system having the WA cytoplasm. Incomplete panicle exsertion in almost all the WA-based CMS lines is one of the major impediments in obtaining higher seed yield. Search for suitable genetic stocks to overcome the problem of poor panicle exsertion in male sterile A lines has resulted in identification and exploitation of novel type of mutants in rice called 'Elongated Uppermost Internode" (*eui*) mutants. Two *eui* mutants were previously identified by their notable elongation of the uppermost internode at the heading stage (Rutger and Carnahan, 1981, Yang *et al*., 2002). The mutants are characterized by a near-doubling in the length of the uppermost internode, extreme panicle exsertion, and an increase in panicle length, with little or no effect on other internodes or plant characters. This recessive genotype with the elongated uppermost internode is a very useful trait in hybrid rice seed production, and was referred to as the fourth genetic element of hybrid rice, in addition to the traits male sterility, maintainer and restorer (Rutger and Carnahan, 1981).

Previous genetic and physiological studies have shown that the *eui* phenotype is due to a recessive mutation in a single gene, which functions similarly in both japonica and indica subspecies and confers highest sensitivity to exogenously applied GA3 at the seedling and heading stages (Xu *et al*., 2004). These results strongly suggested that the *eui* gene is likely to be involved in GA metabolism or signaling. Molecular cloning of the *eui* gene should therefore provide us with valuable information on its function in controlling internode elongation, as well as in GA metabolism and signaling.

One locus (*eui1)* is mapped onto chromosome 5 and linked to a RFLP marker RG435 at a genetic distance of 33.6 cM (Librojo and Khush, 1986). Later Xu *et al*. (2004) fine mapped this region on chromosome 5 and found that a Cytochrome P450 (involved in GA deactivation) is associated with this trait. Identification of molecular markers closely linked to *eui1 (*SCAR markers namely SAG01 and SMRF19 by Gangashetti *et al*., 2004*)* makes it possible to develop hybrid rice parental lines possessing *EUI* through MAS leading to higher out-crossing potential, which would reduce the need for application of GA3. The other locus is located in the middle of the long arm of chromosome 10 linked to RM258, RM269, RM271 and RM304 with genetic distances of 12.0, 12.9, 35.1 and 1.4 cM respectively. In this study, we selected nine different *eui* mutants derived by mutating the popular rice variety IR 64 using different mutagenic agents (Table 1). But we were not having any information on the allelic nature of this mutation. Observations on various quantitative traits in *eui* mutants are presented in Table 2.

Previous studies suggested that rice internode elongation is modulated by endogenous GA levels (Ashikari *et al*., 1999). Excess amount of endogenous GA in the *eui* mutants promoted cell division and cell elongation and thereby resulted in the increased peduncle length. In the present study also, all the *eui* mutants had excessive peduncle length (44- 50 cm) excepting one mutant D1027-2-3. This deviation may be because this mutant is still segregating for this trait and we need to purify it before pursuing further studies. All the *eui* plants were morphologically similar to wild-type plants until flowering stage as reported by He and Shen (1994). Rapid internode elongation happened at the reproductive stage i.e after heading stage. In the present study also, similar observations were made on the peduncle elongation behaviour of *eui* mutants (Figure 1). All the mutants were looking similar like the wild type plants until flowering. In the Wild type IR 64 plants, peduncles elongated at the rate of around 4-5 cm per day and reached the final length of around 27 – 30 cm. Whereas the mutants elongated at the rate of around 8-10 cm per day and the peduncles reached the final length of around 45 – 50 cm. This accelerated elongation of the peduncles may be due to increased rate of cell division and cell elongation (Zhu *et al*., 2006).

A random amplified polymorphic DNA marker $\mathrm{OPAG01}_{1000}$ was found to be linked to *eui1* gene which was then converted into a sequence tagged site (STS) marker (Gangashetti *et al.* 2004). Gangashetti *et al.* (2006) developed two STS markers namely SAG01 and sMRF19 which were found to be linked to *eui* trait and this was successfully deployed in the breeding program to select *eui* phenotype at the earlier stage of the segregating population. In the present study, those two STS markers were used to screen the available ten *eui* mutants to identify the mutants that are allelic to *eui1*. PCR analysis revealed that the STS primer pair SAG01 amplified both 1051bp and 1100bp fragments in the *eui* mutants

(GRS7381-1-3, D11324-2, D11031-8 and FNS 4503-3-1) whereas in the wild type IR 64 and two male sterile A lines it produced only the 1051bp fragment. The non-amplification of SAG01 in the five mutants namely D11041-8, FNS7120-5, D2660-1-2, FNS 6171-1 and D1027-2-3 may be due to deletion in the primer region (Plate 1). The STS primer pair sMRF19 amplified a DNA fragment of 300 bp size in the wild type IR 64 and a 300 bp allele in the *eui* mutants (Plate 2). This is similar to the findings made by Gangashetti *et al*., (2004). Preliminary results of genotyping of wild type and mutants using a SSR marker RM304 (linked to *eui* on chromosome 10 as given by Yang *et al.* (2002) revealed that the mutant D11041-8 did not show amplification for both the markers (data not shown). The mutant GRS7381-1-3 was found to be linked to both the markers on chromosome 5 and 10 which indicated the possibility of double mutation.

To identify the novel mutants that are non-allelic to *eui1*, genetic complementation analysis was carried out. The genetic complementation test will allow us to identify the novel genes and to determine its molecular function (Xu *et al*., 2004). Crosses were effected between different *eui* mutants of our collection as described in Table 3. Out of 4 cross combinations evaluated only one combination (GRS7381-1-3 x D11041-8) showed reversal of the phenotype. The F_1s derived between these two mutants possessed the final peduncle length similar to the wild type IR 64. This indicated that the above two mutants are non-allelic and in the F1 the complementation of alleles caused the reversal of the phenotype. Further studies are in progress to identify the *eui2* mutant in our mutant collections to proceed for fine mapping. Efforts are being undertaken to develop independent segregating population using these mutants as male parents and IR64 as female parent.

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Table 1. List of *eui* elements

S.No	Genotypes	Mutagenic agent
1	GRS7381-1-3	Gamma ray
2	D11031-8	Diepoxybutane
3	D11041-8	Diepoxybutane
4	D11324-2	Diepoxybutane
5	D2660-1-2	Diepoxybutane
6	FNS6171-1	Fast neutron
7	FNS7120-5	Fast neutron
8	FNS4503-3-1	Fast neutron
9	D1027-2-3	Diepoxybutane
10	IR64	Wild type

Table 2. Observations on various quantitative characters in IR64 and *eui* mutants

Mutants	Peduncle length (cm)	Panicle exsertion (cm)	Panicle length (cm)	Plant height (cm)	Days to 50% flowering	Number of grains per panicle
IR64	27.54	-1.4	23.3	82	86	83.3
GRS7381-1-3	50.10	15.8	29.2	106.67	121	93.7
D11031-8	44.10	13.3	26.3	104	113	83.3
D11041-8	48.15	16.3	27.5	97.67	119	83.3
D11324-2	44.10	12.1	26.0	100.67	112	73.3
D2660-1-2	47.25	15.6	28.3	106.67	120	85.3
D1027-2-3	28.00	3	20.67	75.33	115	58
FNS6171-1	45.90	14.1	27.9	103.67	115	77
FNS7120-5	52.20	18.8	28.1	97.33	118	85.7
FNS4503-3-1	52.50	21.6	27.8	107	121	85

Table 3. Measurements on peduncle length in the selected mutants and F1s obtained between the *EUI* mutants

F_1 crosses	Peduncle length (cm)
GRS7381-1-3 x D11041-8	25.46
FNS 7120-5 x GRS7381-1-3	36.60
FNS6171-1 x GRS7381-1-3	48.60
D11041-8 x D11324-2	47.46
IR64	27.54
GRS 7381-1-3	50.10
D11041-8	48.15
FNS7120-5	52.20
FNS6171-1	45.90
D11324-2	44.10