Research Article

Endogenous Gibberellic Acids (GAs) Biosynthesis Is Tool to Characterization of Elongated Uppermost Internode (*eui*) Gene for Panicle Exsertion in Rice (*Oryza sativa* L.): A HPLC Method

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Abstract

Gibberellic acids (GAs) play vital role in plant growth processes as stem elongation, leaf expansion, fruit elongation, seed germination and flowering etc. In rice, yield improvement depends upon the large scale adoption of key technology like hybrid rice technology. The gibberellic acid (GA_3) spary on male sterile lines in rice to induce panicle exsertion has shown split grains, quick loses of seed viability and increases the production cost. The introduction of elongated uppermost internode (eui) gene for panicle exsertion in wild abortive male sterile (WA-CMS lines) population is definitely revealed higher seed yield without losing seed viability. The elongated uppermost internode (*eui*) gene exhibits gibberellic acid biosynthesis pathway which was confirmed in the *eui* donor parent, Accession 18 by assessing the quantification of gibberellic acids compounds including Abscisic acid. The native *eui* gene donor, Accession 18 has shown higher amount of endogenous gibberellic acids biosynthesis level than IRRI bred *eui* gene donor, IR91-1591-3.

Keywords: Gibberellic acid, Elongated uppermost internode, Panicle exsertion, Hybrid rice

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Introduction

Use of gibberlic acid (GA_3) at proper stage with proper dose enhances the seed set in hybrid rice, but the use of GA_3 tends to be a costly affair. One gram of GA_3 is costing about Rs.35 - 40 in the local markets. One hectare of seed production requires gibberellic acid spray 90 gm/ha with a total expenditure of about Rs.3500 - 4000. This routine expenditure has to be incurred for the hybrid rice seed production. To overcome this expenditure there is a need of CMS line with complete exerted panicles. This can be achieved by incorporating the elongated uppermost internode (*eui*) gene in the CMS line to avoid the use of GA_3. At present, the IRRI bred *eui* donor, IR 91-1591-3 has been utilizing to develop CMS lines with *eui* gene in research and development programme of hybrid rice technology. A native *eui* donor gene, Accession 18 was developed from the germplasm line, Tella hamsa which has shown the elongated uppermost internode (EUI) trait, since it was taken to characterize the endogenous gibberellic acid expression levels compared with international *eui* donor, IR 91-1591-3.

Gibberellins (GAs) are a group of diterpenoid compounds, some of which act as growth-promoting hormones controlling such diverse processes as stem elongation, leaf expansion, seed germination, and flowering. The method for the extraction and purification of endogenous plant hormones IAA, GA_3, and ABA were carried out as described by Radley (1961) [1], Sponel and Hedden (2004) [2] and Kenji Tanabe *et al* (2007) [3].

Materials and Methods

The field experiments were carried out during 2012 at TamilNadu Rice Research Institute, Aduthurai, Agricultural College and Research Institute, Trichy. The seven rice genotypes *viz*., wild type T18, TKM 9, IR 50, IR 79156B, IR73328B along with *eui* donors Accession 18 (mutant) and IR91-1591-3 were represented the panicle exsertion trait. Among the represented rice genotypes, the rice genotypes TKM 9, IR 50, IR 79156 B and IR 733328 B are showing poor panicle exsertion were taken to assay the endogenous gibberellic acids and Abscisic acid expression in the intercalary meristem/internode elongation during anthesis. Measured endogenous Gibberellic acids (GAs) levels by using high-performance liquid chromatography (HPLC) to determine the specific cause of the *eui* phenotype.

Sampling procedure:

The booting and heading dates of individual panicle were labeled and recorded. Twenty labeled intercalary meristem were sampled at 1, 2, 3, 4, 5, 6,7,8,9 and 10 days after heading in each variety. The intercalary meristems were divided into two parts: one was frozen in liquid nitrogen for hormonal assay and the other fresh one is ground to estimate the hormonal concentration.

Gibberellic acid extraction

The frozen intercalary meristems were homogenized and extracted overnight in 80% cold aqueous methanol containing 0.02% butyl hydroxytoluene. At this point, 100 pg deuterated GAs (GA_3 and GA_{4+7}) and 100 pg ^{13}C-ABA were included in the samples as internal standards for recovery estimation after purification. The solution was then filtered and the residue was reextracted twice with 80% cold aqueous methanol. After filtration, the pooled extracts were evaporated in vaccum at 42° C to an aqueous solution. The aqueous residue was then adjusted to a pH of 6 to 7 and was partitioned against hexane three times. The pooled aqueous residue was then adjusted to 2.5 with HCl and partitioned against ethyl acetate. The pooled ethyl acetate phase was partitioned against a potassium phosphate buffer (pH 8.0). Then, insoluable polyvinylpolypyrrolidone was added to the combined aqueous solution and filtered. The filtered aqueous solution was adjusted to pH 2.5 with HCl and partitioned against ethyl acetate again. The pooled ethyl acetate solution was dried over Na_2SO_4 and evaporated in vaccum. The residue was dissolved in a small amount of 80% aqueous methanol, and loaded onto a C18 Presep-Cartridge (Wako Pure Chemical Industries Ltd., Osaka, Japan), which had been prewetted with water, 100% and 80% methanol, followed by drying in vaccum. The residue was dissolved in a small amount of 45% methanol-water containing 0.1% acetic acid and further loaded onto a Bondesil DEA (diethylamino-propyl) column (Varian Associates, Palo Alto, CA). The sample was eluted in succession with distilled water and methanol. The elute was evaporated in vaccum and stored at – 20° C for further purification by high-performance liquid chromatography (HPLC).

High-Performance Liquid Chromatography (HPLC)

The residue was subjected to a reverse-phase Senshu-Pak ODS (OctaDecylSilyl)-4253-D HPLC column (10 mm i.d. x 250 mm; Senshu Scientific, Tokyo, Japan) and eluted with 0.1% acetic acid in 30% aqueous methanol (solvent A) and 100% aqueous methanol (solvent B) at 40° C as follows: 0 to 3 min, elution with solvent A; 3 to 30 min, linear gradient of 0% to 100% solvent B; 30 to 50 min, elution with 100% solvent B. the flow rate of the solvent was 3ml min^{-1}, and the eluate was collected every 1 min as one fraction. The retention times of ABA, GA_3 and GA_{4+7} were identified by running authentic standards under the same conditions. The retention times of ABA, GA_3 and GA_{4+7} were 18 to 19 min, 14 to 15 min and 26 to 28 min respectively.

GCMS – Selected Ion Monitoring

The bioactive GA-like fractions from the intercalary meristem were dissolved in 100 % methanol, transferred to a reaction vial, and dried at room temperature. The samples were then dissolved in 100 % methanol and methylated with ethereal diazomethane followed by trimethylsilylation with N-methyl-N-(trimethysilyl)-trifluoroacetamide (10 µL; Sigma, St.Louis) in glass vial at 80 ° C for GC-MS(6890 N network GC system; Agilent Technologies Santa Clara, CA) analysis. One micro liter of each silylated sample was injected into a DB-1 fused silica chemically bonded capillary column [15 m, 0.25 mm (i.d.), 0.25-µm film thickness; Agilent Technologies]. The Gas Chromatography oven temperature for Gibberellic acids was programmed for 3 min, held at 80° C, then increased at 15°C min^{-1} to 300° C, followed by 5 minutes at 300° C. Helium carrier gas was maintained at a head pressure of 30 kPa. The Gas Chromatography was directly interface and source temperature of 280° C, an ionizing voltage of 70 eV, and a dwell time of 100 ms.

A portion of Abscisic acid (ABA) equivalent fraction from ODS-HPLC was subjected to Gas Chromatography - Mass Spectrometry – selected ion monitoring (SIM) analysis after methylation with ethereal diazomethane. The Gas Chromatography (GC) conditions were as follows: 3 min held at 60° C, then increased at 20° C min^{-1} to 290° C for 5 minutes.

Quantification of biologically active endogenous Gibberellic acids and Abscisic acid

The identity of eluted Gibberellic acids, according to retention times was verified by monitoring diagnostic ions of both endogenous Gibberellic acids and deuterated Gibberellic acids. Levels of endogenous Gibberellic acids were

determined by measuring the abundance of the following ion pairs: m/z 504/306 for GA_3, m/z 418/420 for GA_4 and 284/286 for GA_7 and m/z 190/192 for Abscisic acid. In the Gas Chromatography – SIM analysis of Abscisic acid (ABA), characteristic ions m/z 190/1902 were monitored.

Results and Discussion

The higher GA_3, GA_4 and GA_7 levels were measured at seven days after anthesis (DAA) while lower GA_3, GA_4 and GA_7 levels were measured at 10 days after anthesis in all genotypes. The bioactive GA_3, GA_4 and GA_7 levels were proliferated in intercalary meristem region of the culm at 7 days after anthesis thus panicle exsertion is elongated in native *eui* donor Accession 18 (mutant). The bioactive GA_3, GA_4 and GA_7 expression levels was comparatively lesser in IRRI bred *eui* donor, IR91-1591-3 at 7DAA and 10 days after anthesis than native *eui* donor Accession 18 (**Table 1**). The results agree with the findings of Yang *et al* (2000) [4]. They assessed the quantification of gibberellic acids components among the genotypes where expression level had varied based on the allelic variation present in the *eui* gene during different heading stage.

Table 1 Endogenous GAs and ABA expression level in rice genotypes (pg/g^{-1}FW)

S.No	Genotypes	7 DAA			10 DAA		
		GA_3	GA_{4+7}	ABA	GA_3	GA_{4+7}	ABA
1.	Tell hamsa (T18) wild type	934	582	28	86	41	93
2.	Accession 18 EUI donor	3209	2780	17	364	95	58
3.	IR91-1591-3 EUI donor	2983	2384	33	297	43	72
4.	TKM 9	724	184	198	82	46	741
5.	IR 50	718	172	217	67	28	834
6.	IR 79156 B	627	152	213	72	41	697
7.	IR 73328 B	618	139	232	69	36	783

Results shown are the mean of three determinations per genotypes in 7 days after anthesis and 10 days after anthesis (DAA).

The gibberellic acid GA_3 expression level was much better than GA_4 and GA_7 levels in both elongated uppermost internode (*eui*) donors (Figure 2 & 3). Subsequently, a second bioactive gibberellic acids (GAs) peak was measured after first peak, but the GA_3 peak appeared later than that of GA_7 and the second GA_4 peak occurred around the time when cell division ceased in both genotypes. The cell division rate is reduced at 7 days after anthesis where abscisic acid (ABA) expression is proliferated and cell enlargement is happened. The similar plant growth regulation was noticed in rice by Kurata *et al* (2005) [5] and in pear by Kenji Tanabe *et al* (2007) [6].

The occurrence of the first and second peaks of GA_3, GA_4 and GA_7 in native *eui* donor, Accession 18 was higher than wild type, Tella hamsa (T18) (**Figures 1** and **2**). The native *eui* donor, Accession 18 was developed from the wild type Tella Hamsa through mutation breeding approach. The wild type, Tella Hamsa does not reveal the better panicle exsertion than native *eui* donor Accession 18 thus the bioactive gibberellic acids (GAs) expression levels is higher in mutant *eui* donor. The similar findings were already noticed by Yang *et al* (1999) [7] and Kurata *et al* (2005) [8].

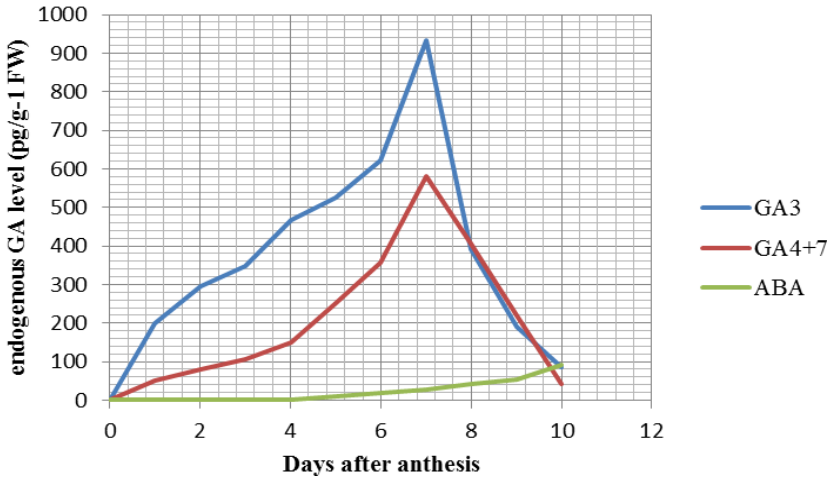

Figure 1 Endogenous GAs and ABA expression level in Wild type Tella hamsa (T 18)

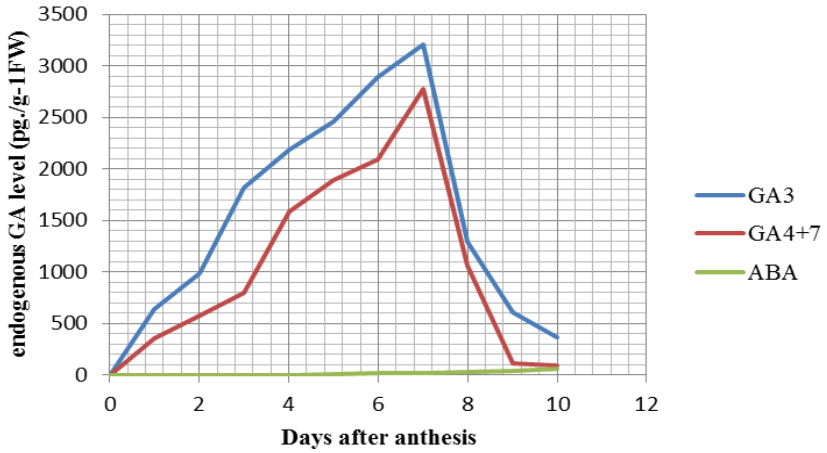

Figure 2 Endogenous GAs and ABA expression level in *eui1*allele mutant, Accession 18

The occurrence of GA_3, GA_4 and GA_7 peaks in the other rice genotypes *viz.*, TKM9, IR50, IR79156B and IR73328B showed poor biosynthesis level of gibberellic acids (GAs) thus poor panicle exsertion is noticed in all rice genotypes due to cell division in the intercalary meristem area have been reduced (**Figures 3-7**).

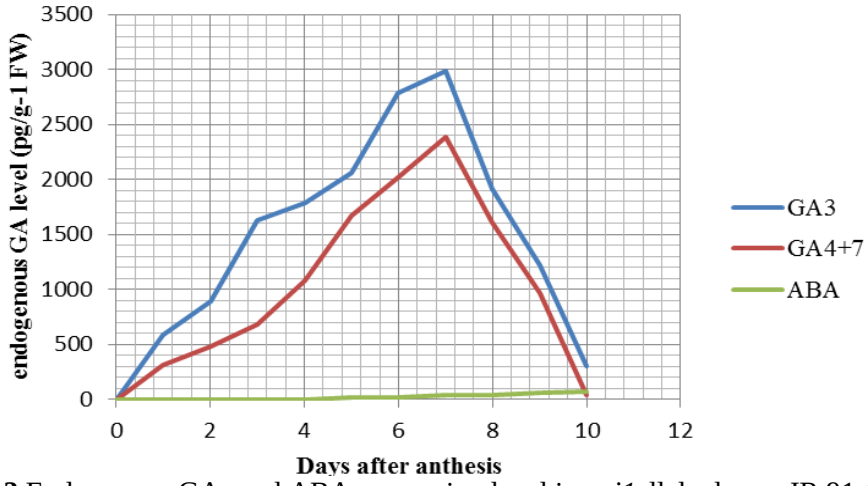

Figure 3 Endogenous GAs and ABA expression level in *eui1*allele donor, IR 91-1591-3

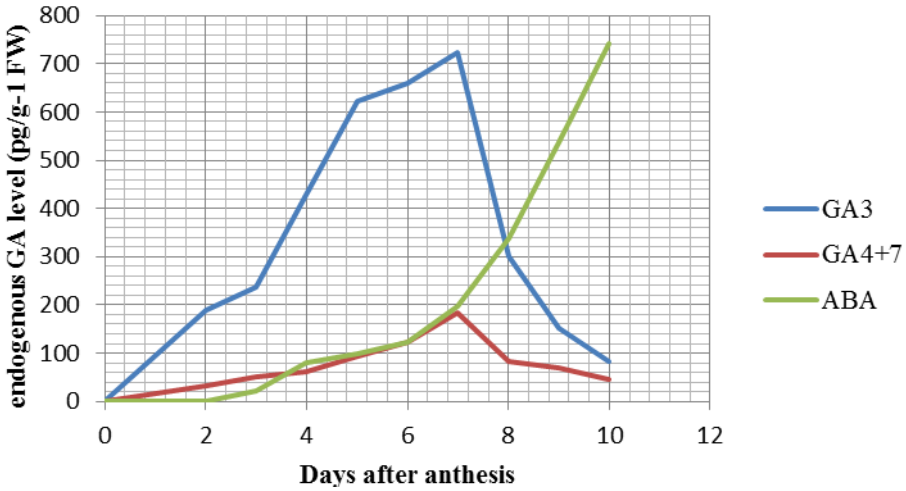

Figure 4 Endogenous GAs and ABA expression level in TKM 9

The Abscisic acid (ABA) levels increased and remained at a high level in rice genotypes *viz.*, TKM 9, IR 50, IR 73328B and IR 79156 B at 3 days after anthesis. However, the Abscisic acid (ABA) concentration remained at a very low level even 7 days after anthesis (DAA) in elongated uppermost internode (*eui*) mutant, Accession 18. During the commencement of anthesis, the ABA concentration in rice genotypes *viz.*, TKM 9, IR 50, IR 73328B and IR 79156B

remained higher than that of *eui* donors *viz*., IR 91-1591-3 and the mutant Accession 18. Although the ABA level decreased when cell enlargement began, it still remained at a relatively high level in rice genotypes *viz*., TKM 9, IR 50, IR 73328B and IR 79156 B at 10 days after anthesis. The results agree with Maekawa and Kita (1983) [9], Luo *et al* (2006) [10].

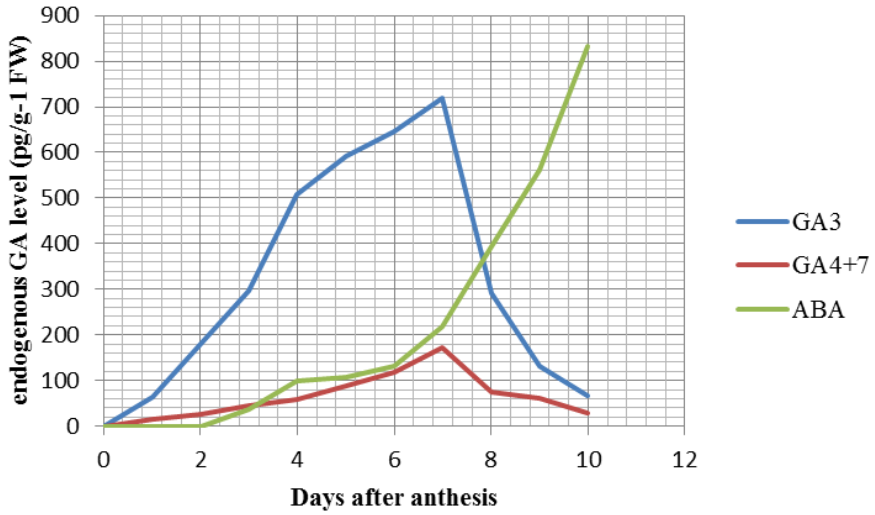

Figure 5 Endogenous GAs and ABA expression level in IR50

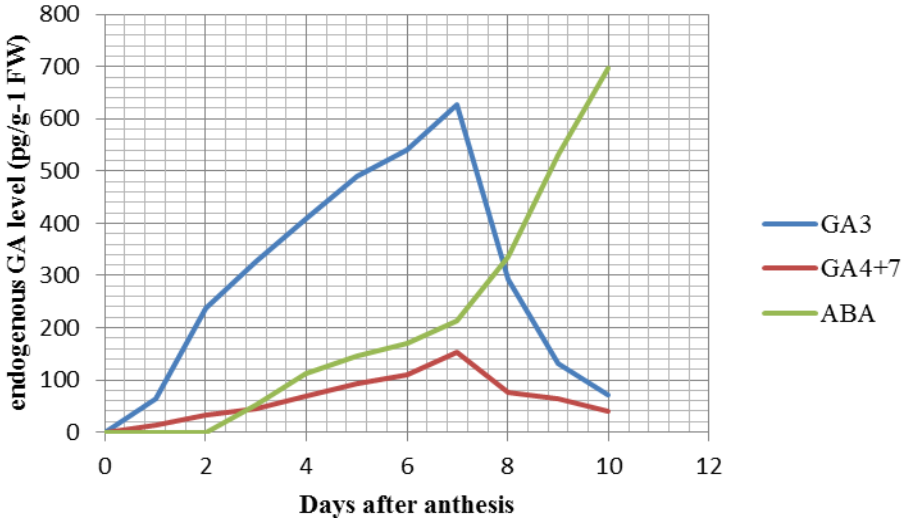

Figure 6 Endogenous GAs and ABA expression level in IR79156 B

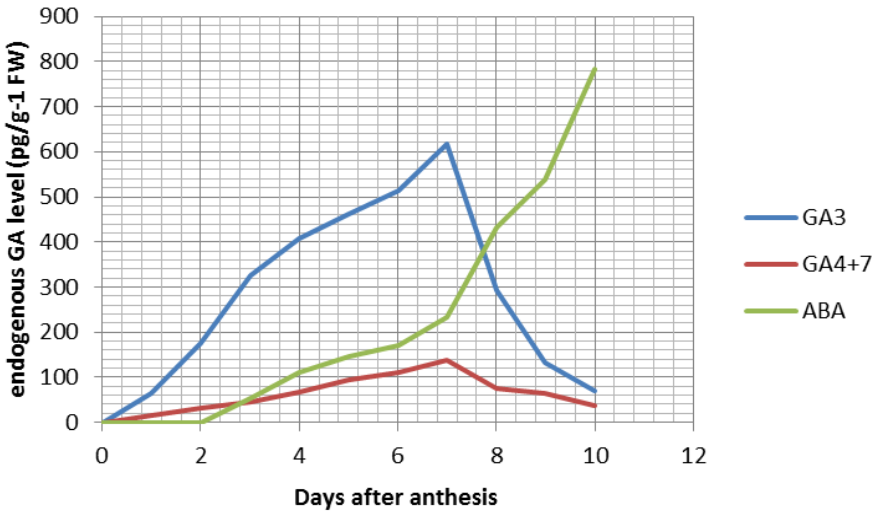

Figure 7 Endogenous GAs and ABA expression level in IR73328 B

Conclusion

During the seedling and tillering stages, *eui* plants were morphologically similar to wild-type plants. However, at the heading stage, the *eui* mutant exhibited an extremely elongated uppermost internode, thus panicle shows lengthy peduncle. Because of the enhanced internode elongation, the stem exposed between the collar of the ear head and the flag leaf sheath (panicle exsertion) is much longer in the *eui* mutant than in wild-type plants. The enhanced internode elongation of the *eui* mutant was due to longitudinally increased cell lengths but not to an increase in the number of cells. These observations suggested that the uppermost internode of the *eui* mutant might accumulate an excessive amount of biologically active Gibberellic acids (GAs) or exhibit enhanced Gibberellic acid sensitivity.

Acknowledgement

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