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Fine mapping and *in silico* isolation of the *EUI1* gene controlling upper internode elongation in rice

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Abstract

Upper internode elongation in rice is an important agronomic trait. Well-known mutants with an elongated uppermost internode (*eui*) are important germplasms for developing unsheathed-panicle male-sterile lines in hybrid rice breeding. We finely mapped the *eui1* gene and identified its candidate gene using *in silico* analysis based on previous research work and rice genomic sequence data. The rice *eui1* gene was mapped to two overlapping BAC clones, OSJNBa0095J22 and OSJNBb0099O15, between the markers AC40 and AC46, that were 0.64 cM apart and spanned approximately 152 kb. A simple sequence repeat (SSR) marker AC41 that cosegregated with *eui1* was located in an intron of a putative cytochrome P450-related gene. *In silico* analysis suggested that this encoded the cytochrome CYP714D1. Allelic sequencing confirmed that *EUI1* corresponded to this P450 gene. A gamma ray-induced *eui1* mutant carried a deletion in exon II of the *EUI1* gene, and resulted in a frame-shift deletion that produced a truncated polypeptide. We conclude that the *EUI1* gene controlling the upper internode elongation in rice is 9804 bp long, and comprises two exons and one intron. The length of the cDNA is 1931 bp containing a 1734 bp ORF, a 110 bp 5′-UTR and a 87 bp 3′-UTR. The ORF encodes an unknown 577 amino acid functional protein, that appears to be a member of the cytochrome P450 family.

Abbreviations: BAC, bacteria artificial chromosome; bp, base pair; cDNA, complementary DNA; cM, centi Morgan; dNTPs, deoxyribonucleotide triphosphates; EST, expressed sequence tags; *eui*, elongated uppermost internode; NCBI, national center of biotechnology information; ORF, open reading frame; PA64 eS1, Pei'ai 64 eS1; PCR, polymerase chain reaction; PDB, protein data bank; SSR, simple sequence repeat; STS, sequence tagged site; UTR, untranslated region; XQZ eB1, Xieqingzao eB1; ZH15, Zhonghua 15

Introduction

Rice internode elongation is an important component of plant architecture in rice breeding. Okuno and Kawai (1978) first reported that recessive rice internode elongation mutants were derived from the Japanese rice cultivar Norin 8 by gamma ray treatment (Okuno and Kawai, 1978). American

scientists Rutger and Carnahan (1981) identified a recessive rice mutant with an upper internode elongation trait derived from the progenies of a *japonica* rice hybrid and named it *elongated uppermost internode* (*eui*) mutant. The mutant, line No.76:4512 possesses dramatically elongated upper internodes during the heading stage (Rutger and Carnahan, 1981). Genetic research indicated that *eui* (*elongated uppermost internode*) is a recessive gene (Rutger and Carnahan, 1981) that maps to Chromosome 5 (Maekawa and Kita, 1983; Librojo and Khush, 1986; Wu *et al*., 1998). The phenotype of the *eui* mutant can be used to overcome the sheathed panicle of the rice male sterile line (Shen *et al*., 1987; Yang *et al*., 2002) and to enhance the plant height and panicle neck length of the rice restorer line (Rutger and Carnahan, 1981; Virmina *et al*., 1988). These features increase the plant's pollen dispersal potential to facilitate hybrid rice seed production (Yang *et al*., 2002). Rutger and Carnahan designated the *eui* trait as the Fourth Genetic Element of hybrid rice seed production. Since then, geneticists and plant breeders worldwide have utilized and studied the *eui* mutant.

We obtained two mutants with different upper internode elongation phenotypes derived from M_2 progenies of the *indica* rice line Xieqingzao B, an elite maintainer of three line hybrid rice systems in China. One of them, Xieqingzao eB1 has the recessive gene *eui1* mapped to chromosome 5, and is allelic to IR50*eui* (Ma *et al*., 2004), which derived from mutant 76:4512 (Virmina *et al*., 1988). Another mutant, Xieqingzao eB2, has the recessive gene *eui2* mapped to chromosome 10 and is non-allellic to Xieqingzao eB1 (Yang *et al*., 1999, 2001; Ma *et al*., 2004). Recently, Xu and coworkers (2004) identified a 98 kb DNA fragment containing the *EUI* gene, and constructed a transgene sub-library.

In recent years, several functional genes that contribute to important agronomic traits in rice have been isolated by map-based cloning strategies (Spiemeyer *et al*., 2002; Sasaki *et al*., 2002; Yamanouchi *et al*., 2002; Li *et al*., 2003a, b). The availability of the *japonica*- and *indica*-rice draft genome sequences (Yu *et al*., 2002; Goff *et al*., 2002), coupled with the ability to explore large new molecular markers (McCouch *et al*., 2002; Shen *et al*., 2004), renders map-based cloning of the rice gene a straightforward process. The *eui1* mutant plays an important role in hybrid rice seed production and its application will bring about large socio-economic benefits (Yang *et al*., 2002). The molecular cloning of the *EUI1* gene will provide valuable information on the function of this protein product, and on the mechanism of stem elongation.

We constructed an F_2 mapping population using the *indica* mutant Xieqingzao eB1 and the *japonica* cultivar Zhonghua 15 as the maternal and paternal parents, respectively. Based on our previous research and the public rice genomic data, we finely mapped the *eui1* gene, analyzed the *EUI1* candidate gene using *in silico* and identified the *EUI1* gene by allelic sequencing.

Materials and methods

Mutants and mapping population

The Institute of Genetics and Crop Breeding in Fujian Agriculture and Forestry University (IGCB, FAFU) kindly provided the *eui1* mutant, Xieqingzao eB1 (XQZ eB1) used in this research. This mutant was derived from an M_2 population of *indica* rice Xieqingzao B induced by gamma ray irradiation. The *japonica* cultivar Zhonghua 15 (ZH15) was released in China and developed by the rice anther culture. The cross between XQZ eB1 and ZH15 was performed and the progeny were grown at the Fujian Agriculture and Forestry University. The F_2 seeds were harvested in the summer of 2000 and sown in the experimental farm in the spring of 2001. At the heading stage, more than 8000 F_2 individuals were phenotyped and 1100 homozygous recessive F_2 individuals with the upper internode elongation phenotypes were identified for mapping analysis.

Pei'ai 64 eS1, allelic to the *eui1* mutant of XQZ eB1, hereafter called PA64 eS1, was used for allelic sequencing in this research. This mutant was derived from an M_2 population of the *indica* rice Pei'ai 64S, an *elite* photo-thermal sensitive male sterile line of two line hybrid rice, induced by gamma ray irradiation.

Markers used in this research

For fine mapping of the *EUI1* gene, we used sequence tagged site (STS) markers and a series of simple sequence repeat (SSR) markers that we

Table 1. The molecular markers showing co-dominant polymorphisms between parents used in this research.

Name	Repeat	BAC/PAC Clone	Forward Primer Sequence	Reverse Primer Sequence	Tm(°C)	Size(bp)
E30531	STS	OJ1005B11	F 5′-GAATGATAGCCCAGTAACC-3′	R 5′-TGAGATCCCGTACCATTGAC-3′	52	225
C53357	STS	OSJNBa0052K01	F 5′-GGGGATATTTTTTTTCGGGAC-3′	R 5′-CCACAAAAACATTGCCTCTC- 3′	58	188
E10471	STS	OJ1187E11	F 5′-TGAGATGGAAAATCACACCC-3′	R 5′-GCTGAAGTAGCGTTGAGAGG-3′	58	174
AC9	(TTC)20	OJ1525A02	F 5′-CGTGTGGAAAAGGAAGCAT-3′	R 5′-GTCATGTGTATGGCCGGTTA -3′	60	205
AC24	(AACC)9	OJ1057B02	F 5′-ATTCTTCCCGCACTACAC-3′	R 5′-AGTGCGACGGACTCTACT -3′	53	157
AC28	(TATC)6	0J1119H 02	F 5′-AGCTCAGGTGAAACATCCAC-3′	R 5′-ATCCAGAATCCATTGACCCC-3′	58	235
AC29	(CAG)7	OJ1004E02	F 5′-GTGGAGGTGAACGACTACCC-3′	R 5′-CCCAAATCACACATACAGCC -3′	59	216
AC30	(GA)13	OSJNBb0099O15	F 5′-TCACATACGTCCTGCTCGAC-3′	R 5′-TGGTTCAAATTGCTAGAGCC-3′	58	204
AC31	(TC)12	OJ1387F08	F 5′-TAGGCCTCAAACCAAACCAG-3′	R 5′-CAGACGCCCGATTTGTAATC -3′	60	102
AC32	(GA)12	P0486C01	F 5′-GATGCATCCTTCAGGTGATT-3′	R 5′-CGGTATCTTCGGACGAGTTA-3′	57	169
AC34	(CGG)9	OSJNBb0099O15	F 5′-GTCTACTTCCTGGATGACGG- 3′	R 5′-GAACACATCATCGATTGCAC- 3′	57	288
AC40	(GAA)22	OSJNBa0095 J22	F 5′-GCTCTCCTTCTGCCGACCACT-3′	R 5′-CAACCTGCGAGCGAGAATAA-3′	61	214
AC41	(CA)11	OSJNBa0095 J22	F 5′-GGCCATTAACACTAACACGG-3′	R 5′-GGGACCATGTATCATCGAAA-3′	58	260
AC46	(GGATG)5	OSJNBb0099O15	F 5′-AGACCAACCCATTCCACTC -3′	R 5′-GCAGAGTCAGAGAAGGCCAG-3′	57	164

designed according to the rice genome database of Nipponbare. Three out of the five STS markers showed a co-dominant polymorphism between the parents, XQZ eB1 and ZH 15. In 36 SSR primer pairs, two SSR markers showed a dominant polymorphism and 11 SSR markers showed a co-dominant polymorphism between the parents. In this study, only the co-dominant molecular markers were used to analyze the F_2 mutant plants (Table 1).

DNA extraction and PCR amplification for STS and SSR markers

Rice genomic DNA was extracted from the selected 1100 F_2 individuals, mutants and their wild types according to the methods described by McCouch (1998). The PCR amplifications were conducted according to the methods reported by Wu and Tanksley (1993) and Akagi (1996). Briefly, we performed the reactions in a total volume of 20 μl containing 2 μl (15 ng/μl) of DNA template, 2 μl 10× PCR buffer (containing Mg^{2+}), 1.0 μl dNTPs (each 2.5 mmol/l), 0.2 μl Taq DNA polymerase (5 U/μl) (Promega, USA), 1.0 μl each primer (5 μmol/l; Table 1) and by adding 13.8 μl ddH_2O to 20 μl. The mixture was denatured at 94 °C for 5 min, followed by 35 cycles of amplification at 94 °C for 45 s, 50–60 °C for 45 s and 72 °C for 90 s, and a final extension reaction at 72 °C for 10 min. The PCR was performed in a PTC-100 thermal cycler (MJ Research Inc., USA). The amplified products, which were stored at 4 °C until use, were separated on 6% acrylamide gel and stained by the silver dye method.

The PCR product banding patterns in the acrylamide gels were recorded as 'A' or 'B' corresponding to the XQZ eB1 or ZH15 alleles respectively. When the pattern was same as that of the F_1 individual, it was scored as 'H'. The data from SSR and STS analysis were analyzed with the software MAPMAKER/ EXP version 3.0 (Whitehead Institute, USA).

In silico *analysis*

In silico analysis of this research was carried out on-line at the NCBI website (http//www.ncbi.nlm.nih.gov), the TIGR website (http//www.tiger.org), the Japanese website (http//www.rgp.affric.go.jp), and the Cytochrome P450 website (http://drnel-

son.utmem.edu/ CytochromeP450.html). Basic Local Alignment Search Tool (BLAST) approaches and DNAman software (Lynnon corporation, USA) were used to analyze and compare the DNA sequence data.

Exons of EUI1 *candidate gene PCR amplification and sequencing*

The primers used for amplifying exons I and II from the two allelic mutants XQZ eB1 and PA 64 eS1, and their corresponding wild types Xieqingzao B and Pei'ai 64S, were designed based on the putative *EUI1* candidate gene derived from the *japonica* cultivar Nipponbare genomic sequence data available on the Rice Genome Research Project. *Taq* polymerase (LA) and GC buffer (TaKaRA Co., PR. China) were used to amplify the exons because of the high (> 70%) GC content in the exons. The PCR reaction components included: 10 μl 2×GC buffer (containing Mg^{2+}), 3.2 μl dNTPs mixture (each 2.5 mmol/l), 1 μl DNA template (50 ng/μl), 1.6 μl forward primer (5 μmol/l), 1.6 μl reverse primer (5 μmol/l), 0.2 μl LA Taq (5 U/μl) and adding ddH_2O to the total volume 20 μl. The mixtures were denatured at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 45 s, and 72 °C for 2 min. A final extension reaction was performed at 72 °C for 10 min.

The amplified exon products were gel-purified according to the mini-gel reclaim kit protocol (Hua shun company, PR. China) and were ligated into the pMD-18 vector (TaKaRa Co., PR. China). The ligations were transformed into competent *Escherichia. coli* DH5αcells, and positive clones were sequenced using an automated sequencing system (ABI10, Applied Biosystems Inc., USA).

Results

Identification of flanking markers of the eui1 *gene*

The key to mapping a target gene is to identify its linked flanking markers. For this purpose, we used Sequence tagged sites (STS) markers and Simple sequence repeats (SSR) markers. Sequence tagged sites are usually short nucleotide sequences that occur once in the genome and whose location and base sequence are known. These markers are useful for localizing and orienting volumes of mapping and sequence data. Simple sequence repeats are also known as microsatellite DNA and are non-coding nucleotide sequences abundantly distributed throughout eukaryotic genomes. These sequence repeats usually have no biological consequences and are used as markers to identify specific points in the genome.

In the former study, we linked an SSR marker AC9 to the *eui1* gene (Ma *et al*., 2004). The genetic distance between AC9 and *eui1* was 7.9 cM in the F_2 population of the cross between XQZ eB1 and Aijiaonante (Ma *et al*., 2004). AC9 was located on the BAC clone OJ1525A02 (Ma *et al*., 2004). In order to find other flanking markers linked to the *eui1* gene, we downloaded five STS markers from the website (http//www.rgp.affric.go.jp) and synthesized the primer pairs of the STS markers. Three of them, E30531, E10471, C53357, showed DNA polymorphisms between the parents, XQZ eB1 and ZH 15. We selected 180 F_2 individuals with *eui1* morphological phenotypes to identify their marker genotypes and found that 16 and 26 individuals, respectively, showed heterozygous genotypes with markers AC9 and E30531. The genetic distances of AC9 and E30531 to *eui1* were about 4.3 cM and 8.1 cM, respectively (Figure 1A). The STS marker E30531 was mapped to the BAC clone OJ1005B11 by *in silico* analysis. The *eui1* gene was thus defined as being located within a large physical interval between the BAC clones OJ1525A02 and OJ1005B11.

Fine mapping of the eui1 *gene*

In order to produce a fine map of the *eui1* gene, we downloaded all of the BAC/PAC cloned DNA sequence data between OJ1525A02 and OJ1005B11 in the website, and designed a number of SSR markers according to the SSRs searched by Maskrepeat. Among the SSR markers, five primer pairs, AC24, AC28, AC29, AC31 and AC32, showed co-dominant polymorphisms between the parents, XQZ eB1 and ZH 15. The sixteen and twenty-six recombinant plants identified by AC9 and E30531 were also used for analyzing the above SSR markers. The results were that 12, 4, 0, 12, and 10 individuals showed genotypes heterozygous to AC24, AC28, AC29, AC31 and AC32, respectively. AC24, AC28 and AC29 mapped to the

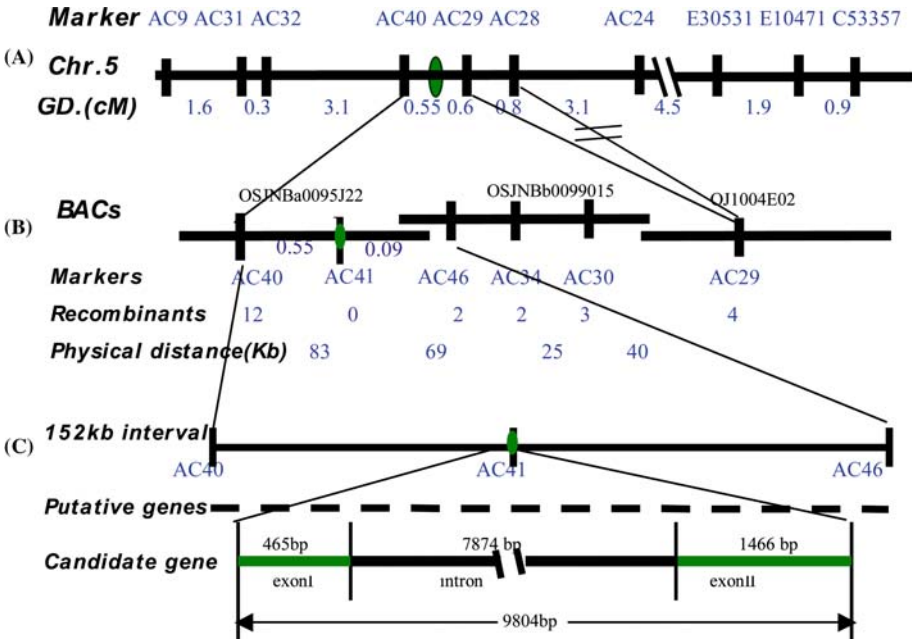

Figure 1. Fine mapping and molecular identification of *EUI1*. (A) The fine mapping of *EUI1* gene, and the closer flanking marker of it was AC40 and AC29; (B) *EUI1* was fine mapped on the three of overlapped BACs, OSJNBa0095J22, OSJNBb0099O15 and OJ1004E02; (C) *EUI1* was further localized within the 152 kb region of the BACs with marker AC40 and AC41 and the candidate gene of *EUI1* was predicted *in silico* analysis.

same side, of the E30531 marker, and their genetic distances to *eui1* gene were 3.8, 0.9, and 0 cM, respectively. AC31 and AC32 mapped to the other side close to AC9, and their genetic distances were 3.3 and 3.1 cM, respectively. It is obvious that the AC29 and AC32 markers are the closer flanking markers of the *eui1* gene (Figure 1A). Further studies showed that 58 and 7 recombinants were identified from 1100 F_2 individuals by using AC32 and AC29 markers, respectively. AC29 and AC32 were located on BAC clone OJ1004E02 and PAC clone P0486C01, respectively. Fourteen SSR markers were further designed according to SSRs identified on the BAC/PAC clone data between OJ1004E02 and P0486C01. Five SSR markers, AC30, AC34, AC40, AC41 and AC46, showed co-dominant polymorphisms between the parents. The recombinants of AC30, AC34, AC40, AC41 and AC46 markers were identified from the 1100 F_2 selected individuals and their relative position to the *eui1* gene was analyzed by Mapmaker (Version3.0; Figure 1B). The closest flanking markers linked to the *eui1* gene were AC40 and AC46, and the genetic distance of them to *eui1* was 0.55 and 0.09 cM, respectively (Figure 1B). AC40 and AC46 were respectively located on the overlapped BAC clones OSJNBa0095J22 and OSJNBb0099O15, which span an approximately 152 kb region between the two markers (Figure 1C). It is noticeable that the SSR marker AC41 was co-segregating with *eui1* gene (Figure 1B).

EUI1 *candidate gene* in silico *analysis*

According to the genomic data of the BAC clone OSJNBa0095J22 and OSJNBb0099O15 carrying the SSR markers AC40 and AC46, respectively, downloaded from the TIGR website (http://www.tigr.org), there are total 35 putative genes within the 152 kb region bounded by AC40 and AC46. These include 31 open reading frames (ORFs) and four known functional proteins (cdc2 kinase-related gene, F-box domain, putative-Reverse transcriptase gene and putative-cytochrome P450-related gene). Nine of the 35 putative genes have their corresponding full-length cDNA sequences stored in http://cdna01.dna.affrc.go.jp/cDNA.

AC41 co-segregates with *eui1*, and it was located on the intron of the putative cytochrome P450-related gene (Figure 1C). The gene within

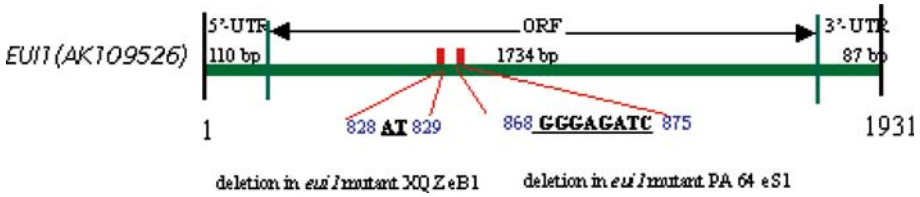

Figure 2. EUI1 cDNA(AK109526) and the mutant site of *eui1*. 828–829, (AT) was the site of the deletion in the *eui1* mutant PA 64 eS1; 868–875, (GGGAGATC) was the site of the deletion in *eui1* mutant XQZ eB1.

which AC41 lies was the obvious first choice for a putative *EUI1* candidate gene.

The genomic DNA sequence analysis showed that the putative cytochrome P450-related gene of Nippobare has roughly 9804 bp long, including two exons and one intron. Exon I has 465 bp in length containing a 110 bp 5′-UTR. Exon II has 1466 bp containing 87 bp 3′-UTR. And the intron of it is up to 7874 bp in length in Nipponbare (Figure 1C). The additional genomic data analysis showed that full-insert cDNA clone AK109526 for Nippobare of the putative cytochrome P450-related gene was already registered in the database (http://cdna01.dna.affrc.go.jp/cDNA), which encodes the unknown functional protein, a member of the cytochrome P450 family, consisting of 577 amino acids.

Allelic sequencing of EUI1 *candidate gene*

Based on the above analysis, we designed two primer pairs to amplify the exonI (F:5′-TCCT AAACATATCCGCTAATCC-3′ and R: 5′-CAT-GAGCGGAGTAATTAACAGG-3′) and exon II (F:5′-CGTCTCTTGAACATGTAAAA—GG-3′ and R: 5′-GTATGCCATGGATGTATG—CT-3′) based on the known exons sequence of Nipponbare candidate gene. The exons of candidate gene were amplified and sequenced using genomic DNA of two allelic mutants (XQZ eB1 and PA 64 eS1) and their wildtpyes (Xieqingzhao B and Pei'ai 64S) as templates. The sequence analysis indicated that the exon I sequence from the mutants XQZ eB1 and PA 64eS1 were identical to that of the wild types Xieqingzao B and Pei'ai 64S, respectively. Furthermore, the exon II sequences from XQZ eB1 and Pei'ai 64eS1, respectively, contained an 8 bp and a 2 bp deletion (aGGGAGATCt → at and gATatc → gatc, respectively) compared to their corresponding wild type alleles (Figure 2).

The full-length cDNA sequences of *EUI1* were obtained by integrating the sequenced exons I and II of the wild types Xieqingzao B and Pei'ai 64S with the Nipponbare cDNA AK109526 sequence. This cDNA was 1931 bp in length and contained a 110 bp 5′-UTR, a 87 bp 3′-UTR and a 1734 bp ORF (Figure 2). The *EUI1* gene encodes 577 amino acids (Figure 2). The location of the deleted 8 bp in XQZ eB1 is at cDNA position 868–875, and results in a termination codon at position 889–891 (TGA); the deleted 2 bp of PA 64eS1 is located between nucleotides 828–829 and also creates a termination codon at positions 851–853(TGA).

Homology analysis of the EUI1 *gene*

There are 468 putative cytochrome P450 genes in the annotated rice cytochrome P450 database (http://drnelson.utmem.edu/rice.html), according to the rice genomic sequence databases of Nipponbare (*japonica* sequenced cultivar) and 9311 (*indica* sequenced cultivar). Homology analysis indicated that the *EUI1* gene shares 100% similarity and identity over its entire length to the rice cytochrome CYP714D1, which is one of the six members of rice cytochrome CYP714.

When using The NCBI non-redundant protein database, the PDB and SWISS-PROT databases to BLASTP *EUI1*, the homology analysis indicated that all of the known functional proteins homologous to the putative amino acid sequence of the *EUI1* gene belong to the cytochrome P450 family, and the highest homologous protein to *EUI1* is CYP72A1 of *Catharant hus roseus*, of which the identity and similaritywere29%(160/535) and 45% (247/538), respectively. The *Catharanthus roseus* CYP72A1 encodes Secologanin synthase, which is involved in the biosynthesis of indole alkali. We believe that the *EUI1* candidate gene is a new and unknown functional cytochrome

P450 family member. Hence, we propose that *EUI1* gene encodes CYP714D1, an unknown functional member of cytochrome P450 family.

Discussion

The rice genome sequence, as in most eukaryotic genomes, contains many SSRs. The *japonica*- and *indica*-rice genome sequencing project (Yu *et al*., 2002; Goff *et al*., 2002) revealed 2240 new SSR markers that were used to develop other simple and efficient genomic locale markers (McCouch *et al.,* 2002; Shen *et al.,* 2004). Now it is no longer difficult to finely map a target gene. In this study, we explored 36 SSR markers according to the genome sequence of the interval containing the *EUI1* gene. The closest flanking markers of *EUI1* are AC40 and AC46. There were 12 and two recombination events between AC40/*EUI1* and *EUI1*/AC46, while the physical distance of AC40/*EUI1* and *EUI1*/AC46 were respectively 83 and 69 kb (Figure 1B). This region may represent a recombination hot spot. Within the 152 kb region defined by AC40 and AC46, we identified a co-segregating marker AC41. This new marker resides on the intron of a putative P450 gene, as we determined by *in silico* analysis. The sequencing analysis of the putative P450 gene from two *eui1* mutants and their corresponding wide-type genes showed that the putative P450 gene in which AC41 resides may be *EUI1*. The homology analysis indicated that *EUI1* encodes a p450 protein of unknown function, which we term here as CYP714D1.

Plant cytochrome P450s comprise a gene superfamily. Cytochrome P450 is an oxidase that plays an important role in plant biochemistry and is involved in plant hormone synthesis, or in the synthesis and degradation of second metabolites (Gyung and Hirokazu, 2002). The cytochrome P450 family is well-documented in plant gibberellic acid (GA) metabolism, and is closely related to GA synthesis and degradation (Gyung and Hirokazu, 2002). For this study, we cloned the *EUI1* gene, which encodes a new member of the cytochrome CYP714 gene family. The phenotype of the *eui1* mutant that dramatically elongates the upper internodes during the heading stage of rice was considered to be closely to GA biosynthesis (Ji, 2001). Perhaps *EUI1* plays an important role in GA metabolism, and possesses an alternate function, a possibility that requires further investigation.

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