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Short Communication

Du3, a mRNA cap-binding protein gene, regulates amylose content in Japonica rice seeds

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Abstract Five non-allelic *dull* (*du*) mutants have been isolated and characterized as low amylose mutants in rice (*Oryza sativa*) endosperm. Synthesis of amylose is controlled by *Waxy* the gene that encodes granule-bound starch synthase. We previously showed that the *du1* and *du2* proteins may encode general splicing regulators which affect the expression level and splicing efficiency of $Waxy^b$ (Wx^b), an allele that has a single-base mutation in the 5′ splice site of intron 1. Here we report that the *du*3 mutant has also reduced the splicing efficiency of Wx^b pre-mRNA. Furthermore, map-based cloning revealed that *Du3* encodes the rice homolog of a cap-binding protein 20 kD subunit (CBP20), a component of the heterodimeric nuclear cap-binding complex (CBC) playing a role in pre-mRNA splicing, RNA nuclear export, and nonsense-mediated decay. Transient expression of green fluorescent protein (GFP)-tagged *Du3* proteins in rice protoplasts resulted in localization to the nucleus showing a speckled pattern similar to the pattern displayed by other splicing factors.

Key words: Amylose, Cap-binding protein, *Du3*, pre-mRNA splicing, *Waxy*.

Amylose content in rice endosperm is one of major determinants of its eating qualities. The *Waxy* (*Wx*) locus encodes granule-bound starch synthase, a key enzyme in amylose synthesis in plants (for review see Martin and Smith, 1995; Nelson and Pan, 1995). The *waxy* mutant (called glutinous rice) has a snowy white appearance because its endosperm contains only amylopectin and no amylose. The control of amylose content by regulation of *Wx* expression is an important field of study for rice breeding. Yano et al. (1988) had obtained five non-allelic *dull* (*du*) mutants from N-methyl-N-nitrosourea mutagenized fertilized egg cells of japonica rice (*Oryza sativa* cv Kinmaze). All *du* mutants were recessive and non-allelic to the *waxy* locus on chromosome 6 and exhibited a hazy white endosperm indicative of an intermediate amylose content between *waxy* mutant and wild-type Kinmaze (Yano et al. 1988). Although *Du* genes have been believed to be the regulators necessary for *Wx* expression, it has not been demonstrated at the molecular level.

Rice cultivars have two alleles of the *waxy* locus, Wx^a and Wx^b that differ by their expression levels: the level of the protein encoded by Wx^a is ~10-fold higher than that encoded by Wx^b (Sano 1984). Wx^b has a single-base G-to-T substitution at the conserved 5′ splice site of intron 1 present in the untranslated leader region (Bligh et al. 1998; Cai et al. 1998; Frances et al. 1998; Hirano et al. 1998; Isshiki et al. 1998). This mutation activates two criptic splice sites in exon 1 and leads to reduced splicing efficiency of intron 1 (Isshiki et al. 1998). We previously demonstrated that *du1* and *du2* mutations suppress splicing of Wx^b pre-mRNA (Isshiki et al. 2000). We further found that the effects of *du1* and *du2* are tissue-specific and that they show differential splicing defects in the endosperm and in pollen. These results suggested that *du1* and *du2* may have mutations in pre-mRNA splicing factors for recognition of a weak splice site in Wx^b intron 1 (Isshiki et al. 2000).

One of the two cryptic splice sites (site 2) that is activated in the Wx^b gene is 93 nt upstream of the authentic site and the second is one base upstream of the authentic site (Isshiki et al. 1998). Because these two sites are so close to each other, splicing at the authentic site and at the second cryptic site cannot be distinguished by using RT-PCR; therefore, they are collectively called site 1. RT-PCR analysis showed that expression level of the spliced Wx^b transcripts in the *du3* mutant endosperm was not as obvious as in the *du1* mutant. Amylose content in *du3* mature seeds was 7%, compared with 5% in the *du1* mutant and 16% in the wild-type Kinmaze

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Abbreviations: CBP20, cap-binding protein 20kD subunit; CBP80, cap-binding protein 80kD subunit; CBC, cap-binding complex
This article can be found at http://www.jspcmb.jp/

(data not shown). Because reduction of the Wx^b transcripts cannot be clearly established by RT-PCR analysis, we used real-time quantitative PCR to show that in *du3* endosperm (Figure 1A). Wx^b transcripts spliced at site 1 were reduced to 20% of the wild-type's levels (Figure 1B). However, the unspliced Wx^b transcript was also reduced to approximately 70% of the wild-type's levels. These data suggested that the *du3* gene could encode an mRNA processing factor that functions at multiple levels such as pre-mRNA splicing, mRNA stability and transcription.

To investigate how the *du3* mutation affects Wx^b pre-mRNA processing, we isolated the *Du3* gene using a map-based cloning approach. We selected 96 *dull* type F_2 seeds derived from crosses between the *du3* mutant (EM23) and indica cv Kasalath and built a linkage map using simple sequence repeat (SSR) and CAPS markers. The *du3* locus was mapped on the long arm of chromosome 2 on a 185 kb region between SSR marker EM3515 and CAPS marker 5300 that is covered by three BAC clones, OJ1476_F05, OJ1004_A05, and OJ2055_H10 (Figure 2A). Analysis of the sequence of this region revealed the presence of a gene, that we named *OsCBP20*, coding for a homolog of a cap-binding protein 20 kD subunit (CBP20). Amino acid comparison of OsCBP20 with full-length human CBP20 showed in an identity scores of 70%. This protein forms a heterodimeric cap-binding complex (CBC) with an 80 kD subunit (CBP80) in human and yeast. Comparison of the *OsCBP20* gene sequence in EM23 and Kinmaze revealed a G-to-A single-base mutation in EM23 resulting in a 121Arg-to-His change in the OsCBP20 protein (Figure 2B). We also sequenced the genomic DNA coding for OsCBP20 on two other allelic *du3* mutants, EM69 and EM363. Both mutants have a single amino acid substitution, 125Gly-to-Asp and 129Arg-to-Trp respectively, caused by a single base mutation in *OsCBP20*. To confirm that the mutation on *OsCBP20* is responsible of the *du3* mutant phenotype, we transformed *du3* rice with the wild type *OsCBP20* gene (a 3.2 kb genomic DNA fragment) under the control of its own promoter (a 1.2 kb DNA fragment). Three fourths of T_1 Seeds from three independent transformed lines showed normal endosperm (Figure 2C). These results indicate that *du3* alleles are responsible for the low amylose phenotype.

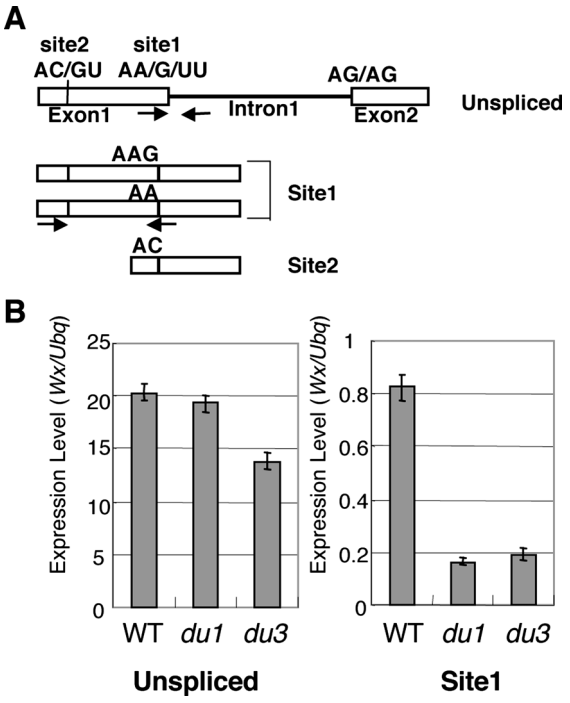

Figure 1. Effect of *du3* mutation Wx^b intron 1 splicing. (A) RT-PCR analysis scheme of Wx^b transcripts in the *du1* and *du3* mutants. Wx^b has a single-base mutation at the 5' splice site of intron 1 and generates two transcripts spliced at two different sites (site1 and site2). Spliced and unspliced Wx^b transcripts were amplified using primers pairs (ACCATTCCTTCAGTTCTTTGTC and GTGGAAGATCTTTGCA-GATG) and (ATCAGGAAGAACATCTGCAAG and AAAGATGC-ATGTGATCGATCTG) respectively. (B) Real-time quantitative PCR analysis of Wx^b transcripts levels in *du1* and *du3* mutants. Reactions were performed using the SYBR Green PCR master mix (Applied Biosystems). Ubiquitin amplified using primer pair (AACCAG-CTGAGGCCCAAGA and ACGATTGATTTAACCAGTCCATGA) was used as a control for normalization. Data were collected using the ABI PRISM 7000 sequence detection system.

Du3 encodes a protein of 243 amino acids similar to a eukaryotic CBP20 as shown in Figure 3A. The human CBP20 has an RNA recognition motif and forms with CBP80 the nuclear CBC which is required for cap-dependent pre-mRNA splicing (Izaurralde et al. 1994), nuclear export of U snRNAs (Izaurralde et al. 1995), 3′-end processing of RNA (Flaherty et al. 1997), and nonsense-mediated decay of RNA (Ishigaki et al. 2001). The rice *Du3* protein is more similar to the CBP20 sequences of human and *Drosophila* than to that of *Saccharomyces cerevisiae* (Figure 3A). The C terminal region of the CBP20 protein is little conserved between rice, Arabidopsis and *S. cerevisiae*. Transcripts of *Du3* were detected in leaf, stem, and immature seed of rice (Figure 3B). Arabidopsis CBP20 and CBP80 are also expressed in all organs (Kmieciak et al. 2002).

To determine whether the amino acid substitution in the *du3* mutant affects intracellular localization of the protein in plant cells, we made *GFP*-tagged *Du3* and *du3* (EM23) constructs, and transformed them into rice protoplasts. These constructs were cotransformed with the control *mOrange* plasmid that harbors a nuclear localizing signal. Our results indicated that localization of *du3* (EM23) was not different than that of *Du3*. *GFP* fluorescence was detected in the nucleoplasm and in nucleoplasmic speckles. In plant, SR splicing factors are localizing in these regions of the nucleus (Tillemans et al. 2005) .

mRNA processing factors were shown to play important roles in plant physiology. Arabidopsis mutants

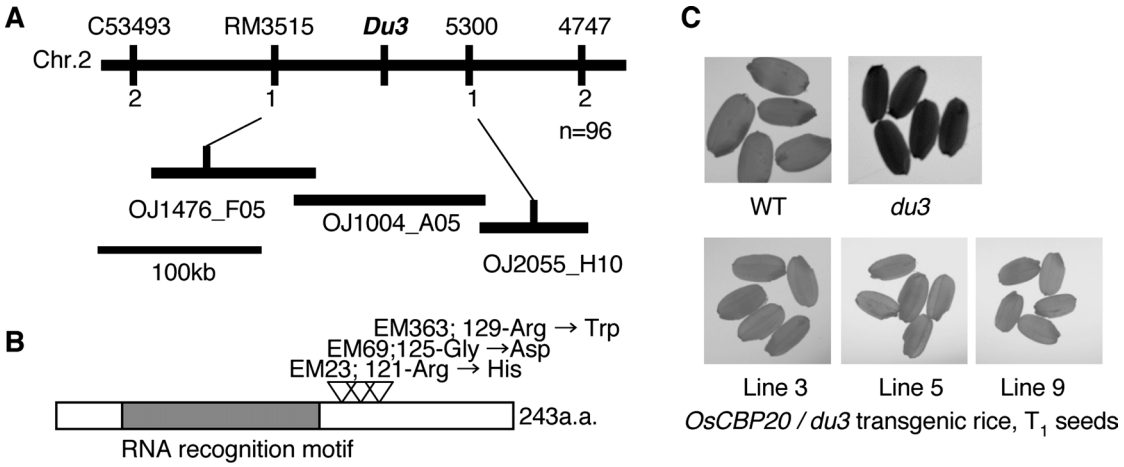

Figure 2. Map-based cloning of *Du3* gene. (A) Linkage map of *Du3* locus. The three BAC clones covering this region are indicated. (B) Positions of mutations in three *du3* alleles. The substituted aminoacids in each mutant line are indicated by arrowheads. Drawings are not to scale. (C) Complementation of the *du3* mutation by the rice *CBP20* genomic DNA. *OsCBP20* was subcloned into the Gateway binary vector pGWB1 (Nakagawa et al. 2007) and introduced into wild type rice (WT) using *Agrobacterium tumefaciens* (EHA101) mediated transformation. Bottom panel shows T_1 seeds, obtained from individual transformant lines, displaying a wild type phenotype.

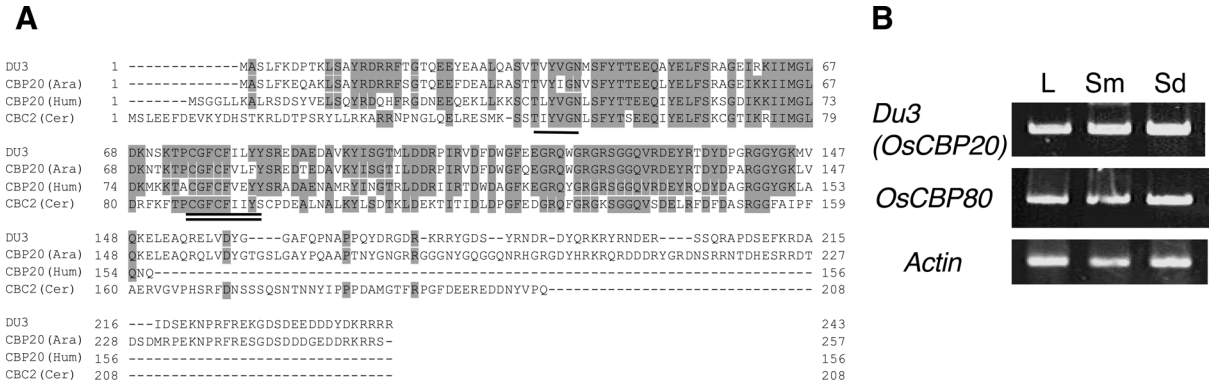

Figure 3. Characteristics of *Du3*. (A) Comparison of the *Du3* amino acid sequence with CBP20s from Arabidopsis (Ara; AK227850), human (Hum; P52298), and *S. cerevisiae* (Cer; Q08920). Identical residues are highlighted. RNP2 and RNP1 sequence are indicated by a single and double line respectively. The amino acid sequences of CBP20s were aligned using the ClustalW program (http://www.ddbj.nig.ac.jp/search/clustalw-j.html). (B) RT-PCR analysis of *Du3* and rice CBP80 homolog (*OsCBP80*) expression in leaves (L), stems (Sm) and immature seeds (Sd). The following specific primers were used: *Du3* (AAACGTTACCGGAACGATGA and CAAGCATGTGGTGATGAACC), *OsCBP80* (CACTTCTGGACATTGGCTCA and CCTTCTTTCGCTTTGTCAGC), and *Actin* (GTATGGTCAAGGCTGGGTTC and CGACCACTGGCATACAGAGA).

defective in mRNA processing factors, such as *abh1*, *sad1*, and *ahg2*, show abscisic acid (ABA) hypersensitive inhibition of seed germination. The *ABH1* gene encodes a CBP80 homolog (Hugouvieux et al. 2001). The SAD1 gene encodes a Sm-like snRNP (small nuclear ribonuleoprotein) similar to human LSM5 that assembles the core component of spliceosomal snRNPs (Xiong et al. 2001). AHG2 encodes a poly(A)-specific ribonuclease (PARN) that may function in the control of mRNA degradation (Nishimura et al. 2005). In human, CBP80 inhibits PARN-mediated deadenylation and may regulate mRNA degradation (Balatsos et al. 2006). An Arabidopsis CBP20 knockout mutant displayed the same hypersensitivity to ABA as the *abh1* mutant and had increased tolerance to water deprivation (Papp et al. 2004). The Arabidopsis *sta1* mutant has enhanced stability of unstable luciferase transcripts and is hypersensitive to ABA inhibition during seed germination. *STA1* encodes a nuclear pre-mRNA splicing factor similar to the human U5-102kD protein (Lee et al. 2006). Moreover, the Arabidopsis *SRD2* gene, isolated from a temperature-sensitive mutant, is similar to the human SNAP50, which is required for small nuclear RNA (snRNA) transcription, and may control cell proliferation (Ohtani and Sugiyama 2005). Arabidopsis plants overexpressing an SR-like splicing protein, SRL1, gained salt tolerance (Forment et al. 2002). Identification and analysis of Arabidopsis mutants defective in RNA processing and mRNA 3′ end formation revealed that these processes can influence RNA silencing (Herr et al. 2006). It is well known that RNA processing factors, which are RNA binding proteins such as FCA, FPA, and

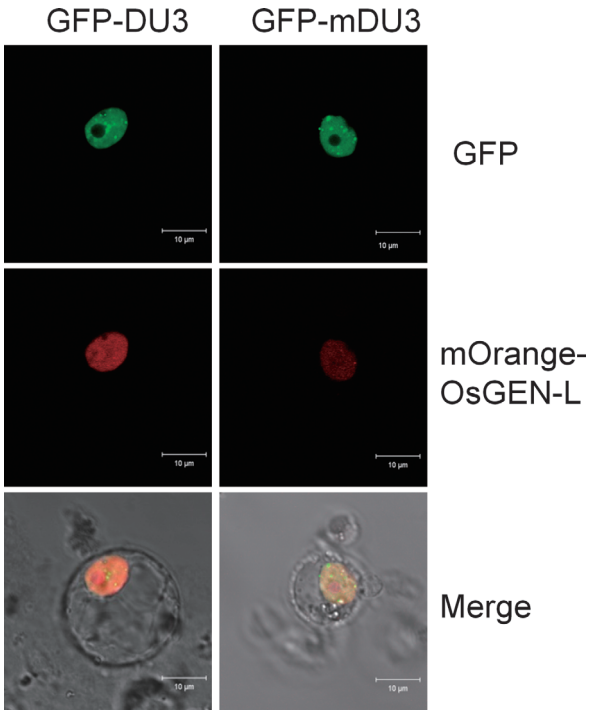

Figure 4. Localization of Wild-type and Mutant *Du3* proteins. *GFP-Du3* or *GFP-mutant Du3* fusions were co-transformed into rice protoplasts with m*Orange-OsGEN-L* a member of the RAD2 nuclease family (Moritoh et al. 2005). Cells transfomed by electropolation were incubated at 30°C for 12 h. Fluorescence signal was observed using a TCS-SP5 microscope (Leica). Bars = 10 μm.

FLK and an mRNA 3′ end processing factor FY, regulate flowering time in Arabidopsis (Macknight et al. 1997; Schomburg et al. 2001; Lim et al. 2004; Mockler et al. 2004; Simpson et al. 2003). The alternative splicing of FCA pre-mRNA is regulated by FCA itself (Quesada et al. 2003). Also, the FCA-FY complex controls the selection of the FCA pre-mRNA poly(A) site (Simpson et al. 2003).

The exact relations between mutant phenotypes and splicing of pre-mRNAs affected by mutation of these mRNA processing factors are not clear. The specific target genes are difficult to identify because of the complex signal transduction pathways of the ABA response and flowering regulation. In this paper, we demonstrated that the mutation of the rice CBP20 homolog gene affected splicing of the Wx^b pre-mRNA. Further studies on these mutants should help clarify how plant splicing factors regulate the expression of endogenous genes.

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References

Balatsos NA, Nilsson P, Mazza C, Cusack S, Virtanen A (2006) Inhibition of mRNA deadenylation by the nuclear cap binding complex (CBC). *J Biol Chem* 281: 4517–4522

Bligh HFJ, Larkin PD, Roach PS, Jones CA, Fu H, Park WD (1998) Use of alternate splice sites in granule-bound starch synthase mRNA from low-amylose rice varieties. *Plant Mol Biol* 38: 407–415

Cai XL, Wang ZY, Xing YY, Zhang JL, Hong MM (1998) Aberrant splicing of intron 1 leads to the heterogeneous 5′ UTR and decreased expression of *waxy* gene in rice cultivars of intermediate amylose content. *Plant J* 14: 459–465

Flaherty SM, Fortes P, Izaurralde E, Mattaj IW, Gilmartin GM (1997) Participation of the nuclear cap binding complex in pre-mRNA 3′ processing. *Proc Natl Acad Sci USA* 94: 11893–11898

Forment J, Naranjo MA, Roldan M, Serrano R, Vicente O (2002) Expression of Arabidopsis SR-like splicing proteins confers salt tolerance to yeast and transgenic plants. *Plant J* 30: 511–519.

Frances H, Bligh J, Larkin PD, Roach PS, Jones CA, Fu H, Park WD (1998) Use of alternate splice sites in granule-bound starch synthase mRNA from low-amylose rice varieties. *Plant Mol Biol* 38: 407–415

Herr AJ, Molnar A, Jones A, Baulcombe DC (2006) Defective RNA processing enhances RNA silencing and influences flowering of Arabidopsis. *Proc Natl Acad Sci USA* 103: 14994–15001

Hirano HY, Eiguchi M, Sano YA (1998) Single-base change altered the regulation of the *Waxy* gene at the posttranscriptional level during the domestication of rice. *Mol Biol Evol* 15: 978–987

Hugouvieux V, Kwak JM, Schroeder JI (2001) An mRNA cap binding protein, ABH1, modulates early abscisic acid signal transduction in *Arabidopsis*. *Cell* 106: 477–487

Ishigaki Y, Li X, Serin G, Maquat LE (2001) Evidence for a pioneer round of mRNA translation: mRNAs subject to nonsense-mediated decay in mammalian cells are bound by CBP80 and CBP20. *Cell* 106: 607–617

Isshiki M, Morino K, Nakajima M, Okagaki RJ, Wessler SR, Izawa T, Shimamoto K (1998) A naturally occurring functional allele of the rice *waxy* locus has a GT to TT mutation at the 5′ splice site of the first intron. *Plant J* 15: 133–138

Isshiki M, Nakajima M, Satoh H, Shimamoto K. (2000) *dull*: rice mutants with tissue-specific effects on the splicing of the *waxy* pre-mRNA. *Plant J* 23: 451–460

Izaurralde E, Lewis J, Gamberi C, Jarmolowski A, McGuigan C, Mattaj IW (1995) A cap-binding protein complex mediating U snRNA export. *Nature* 376: 709–712

Izaurralde E, Lewis J, McGuigan C, Jankowska M, Darzynkiewicz E, Mattaj IW (1994) A nuclear cap binding protein complex involved in pre-mRNA splicing. *Cell* 78: 657–668

Kmieciak M, Simpson CG, Lewandowska D, Brown JW, Jarmolowski A (2002) Cloning and characterization of two subunits of *Arabidopsis thaliana* nuclear cap-binding complex. *Gene* 283: 171–183

Lee BH, Kapoor A, Zhu J, Zhu JK (2006) STABILIZED1, a stress-upregulated nuclear protein, is required for pre-mRNA splicing, mRNA turnover, and stress tolerance in *Arabidopsis*. *Plant Cell*

18: 1736–1749

Lim MH, Kim J, Kim YS, Chung KS, Seo YH, Lee I, Kim J, Hong CB, Kim HJ, Park CM (2004) A new Arabidopsis gene, *FLK*, encodes an RNA binding protein with K homology motifs and regulates flowering time via *FLOWERING LOCUS C*. *Plant Cell* 16: 731–740

Macknight R, Bancroft I, Page T, Lister C, Schmidt R, Love K, Westphal L, Murphy G, Sherson S, Cobbett C, Dean C (1997) *FCA*, a gene controlling flowering time in Arabidopsis, encodes a protein containing RNA-binding domains. *Cell* 89: 737–745

Martin C, Smith AM (1995) Starch biosynthesis. *Plant Cell* 7: 971–985

Mockler TC, Yu X, Shalitin D, Parikh D, Michael TP, Liou J, Huang J, Smith Z, Alonso JM, Ecker JR, Chory J, Lin C (2004) Regulation of flowering time in *Arabidopsis* by K homology domain proteins. *Proc Natl Acad Sci USA* 101: 12759–12764

Moritoh S, Miki D, Akiyama M, Kawahara M, Izawa T, Maki H, Shimamoto K (2005) RNAi-mediated silencing of OsGEN-L (OsGEN-like), a new member of the RAD2/XPG nuclease family, causes male sterility by defect of microspore development in rice. *Plant Cell Physiol* 46: 699–715

Nakagawa T, Kurose T, Hino T, Tanaka K, Kawamukai M, Niwa Y, Toyooka K, Matsuoka K, Jinbo T, Kimura T (2007) Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *J Biosci Bioeng* 104: 34–41

Nelson O, Pan D (1995) Starch synthesis in maize endosperms. *Annu Rev Plant Physiol Plant Mol Biol* 46: 475–496

Nishimura N, Kitahata N, Seki M, Narusaka Y, Narusaka M, Kuromori T, Asami T, Shinozaki K, Hirayama T (2005) Analysis of ABA *hypersensitive germination2* revealed the pivotal functions of PARN in stress response in Arabidopsis. *Plant J* 44: 972–984

Ohtani M, Sugiyama M (2005) Involvement of SRD2-mediated activation of snRNA transcription in the control of cell proliferation competence in Arabidopsis. *Plant J* 43: 479–490

Papp I, Mur LA, Dalmadi A, Dulai S, Koncz C (2004) A mutation in the *Cap Binding Protein 20* gene confers drought tolerance to *Arabidopsis*. *Plant Mol Biol* 55: 679–686

Quesada V, Macknight R, Dean C, Simpson GG (2003) Autoregulation of FCA pre-mRNA processing controls *Arabidopsis* flowering time. *EMBO J* 22: 3142–3152

Sano Y (1984) Differential regulation of *waxy* gene expression in rice endosperm. *Theor Appl Genet* 68: 467–473

Schomburg FM, Patton DA, Meinke DW, Amasino RM (2001) *FPA*, a gene involved in floral induction in Arabidopsis, encodes a protein containing RNA-recognition motifs. *Plant Cell* 13: 1427–1436

Simpson GG, Dijkwel PP, Quesada V, Henderson I, Dean C (2003) FY is an RNA 3′ end-processing factor that interacts with FCA to control the *Arabidopsis* floral transition. *Cell* 113: 777–787

Tillemans V, Dispa L, Remacle C, Collinge M, Motte P (2005) Functional distribution and dynamics of Arabidopsis SR splicing factors in living plant cells. *Plant J* 41: 567–582

Yano M, Okuno K, Satoh H, Omura T (1988) Chromosomal location of gene conditioning low amylose content of endosperm starches in rice, *Oryza sativa* L . *Theor Appl Genet* 76: 183–189

Xiong L, Gong Z, Rock CD, Subramanian S, Guo Y, Xu W, Galbraith D, Zhu JK (2001) Modulation of abscisic acid signal transduction and biosynthesis by an Sm-like protein in *Arabidopsis*. *Dev Cell* 1: 771–781