

ECTOPIC EXPRESSION OF A GENE ENCODING *RRM2* INCREASES GRAIN MASS IN TRANSGENIC RICE

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Grain (seed) size and weight are the most important commercial traits in cereal crops, which controlled by multiple loci. In rice, grain size and weight are determined by grain length (GL), grain width (GW) and grain thickness (GT). It has been reported that size and weight of rice grain are complex quantitative traits governed by many genes and alleles at multiple loci (Li *et al.*, 1997 & 2004). While, most of the genes involved in grain weight or fruit size of plants are not yet understood. The *fw2.2*, a quantitative trait locus (QTL) has been found to increase the tomato fruit size (Frary *et al.*, 2000). Mizukami and Fischer had proved that ectopic expression of *Arabidopsis ANT* transgene enlarged embryonic and all shoot organs by increasing cell number in both *Arabidopsis* and tobacco plants (Mizukami and Fischer, 2000). Recently it has been found that the floral homeotic gene *APEALA2* of *Arabidopsis* controlled grain mass and grain yield (Ohto *et al.*, 2005; Jofuku *et al.*, 2005). However, the genes controlling grain size in cereal crops have not been explored. Therefore, we

show here that over expression of *RRM2* can increase dramatically grain size and weight of the transgenic rice lines.

In plants, many genes and cDNA encoding putative RNA-binding proteins have been identified by similarity to vertebrate or yeast proteins (Sylvie *et al.*, 2000). Several of RNA-binding proteins have more than one RRM domain. Several of these proteins containing putative RRM domains have been found and identified in *Arabidopsis* (Macknight *et al.*, 2002). The transition to flowering in *Arabidopsis* is controlling by a posttranscriptional regulator *FCA* gene containing two RRM domains and a WW protein interaction domain (Macknight *et al.*, 1997). Recently, the full-length cDNA of *FCA* (GenBank accession no. AY274928) from rice has cloned and show homologous to *Arabidopsis FCA* (Du *et al.*, 2005). The rice *FCA* (rFCA) encodes the full-length active protein including two RRM domains and a WW-protein interaction domain.

To investigate the functional expression of RRM in cereal crops, we

have constructed a chimeric transgene harbouring a cDNA encoding *RRM2* and transformed into rice embryogenic cells.

MATERIALS AND METHODS

Construction of transgene

For construction of the chimeric transgene, the cDNA of rice FCA (GenBank accession no. AY274928), which encodes *RRM2* was cloned from pGEMT plasmid by PCR amplification primers (5'-ACCAAGCTTATTTAGGTGACACTATAGAA-3') and (5'-TCCTCTAGATAATACCGACTCACTA TAG-3'). The PCR products were purified and digested using *HinIII* and *XbaI*, enzymes, and then inserted into *HinIII* and *XbaI* sites of the plant transgenic vector pBY520 that harbour *bar* gene as a selectable marker (Xu *et al.*, 1996). The *RRM2* cDNA inserted in sense orientation driven by rice *Act1-5* promoter and *Pin2-3* terminator (Fig. 1).

Transformation and transformants selection

The young embryos of japonica rice Zhonghua11 line at the 15 ripening-day old were used to induce calli. Embryogenic calli transformed by micro-projectile bombardment technique, essentially as described by Chen *et al.* (1998). Transformants were selected on a selective MS medium supplemented with 5mgL⁻¹ phosphinothricin (PPT).

Southern and northern analysis

Total genomic DNA was isolated from rice leaves using the CTAB method (Rogers and Bendich, 1985). Approximately 20 µg of genomic DNA from each putative transgenic plant and untransformed (control) plant were digested with *HindIII* enzyme and separated on a 1% agarose gel. Total RNA was isolated from transformed rice leaves using the method of Bugos *et al.* (1995). Total RNA (20 µg) was fractionated on a 1.3% agarose gel. The coding sequences of *RRM2* cDNA was used as labeled probe for blotting hybridizations. All procedures of gel-blot hybridizations were carried out as described previously (Kang *et al.* 1998).

Histological studies

The mature grains of control and transgenic T₀ plants and young seedlings from T₁ seeds were used for histological analysis. The fixation, dehydration, embedding, section of seeds and seedlings were performed with the procedure as described (Tomasi *et al.*, 1997). Mature pollens were collected from T₁ and control plants, respectively. Sections of anthers and pollens were examined under light microscope for the comparison of cell size.

Transcriptional level analysis of MADS-box genes

The rice fifteen putative MADS-box genes (GenBank accession no:

AF091458, AF377947, AF058697, AF345911, OSU78892, AY332477, AF139664, AF141967, AF204063, AY250075, AY551918, OSU78782, AF151693, AF551912, AY553724) were selected to detect their transcriptional level in the *RRM2* lines and control lines. Gene-specific PCR primer pairs for each of the selected MADS-box genes were designed based on the sequences published in GenBank database. The young seedlings from T_1 seeds and the young panicles of these plants were used for detecting transcriptional level of MADS-box genes by quantification PCR amplification. The relative expression level is standardized using *actin* gene as an internal control.

Phenotype analyses of transgenic rice

Primary transformants (T_0) plantlets were grown in a greenhouse at a temperature $30 \pm 2^\circ\text{C}$ during the day (14 h light) and $22 \pm 2^\circ\text{C}$ during the night (10h dark). At five-leaf stage, plantlets were transplanted in the field. Selfed seeds from T_0 lines and the corresponding Control plants were collected and germinated by soaking in water for two days at 37°C . Germinating seeds were sown in pots beetmoth and grown in a greenhouse. At the five-leaf stage, seedlings of each T_1 (first transgenic progeny) lines were transplanted to the field. The phenotypic features were recorded at maturity stage, including heading date (days after germination), the length and width of the flag leaf, plant height; the length and number of panicle, the number of filled seeds per

panicle, the 1000-grain weight and seed mass. Analysis of variance (*t* test; $p < 0.05$) and first degree statistics was carried out using *Statistica* analysis program (Stats, USA).

RESULTS AND DISCUSSION

Molecular and morphological analysis of transgenic T_0 Plants

Screening for transgenic (T_0) plants among plants obtained on resistant calli of five independent T_0 lines was carried out on selection medium supplemented with 5mgL^{-1} PPT. The selected plants were confirmed to have the *RRM2* transgene by PCR, which showed an amplified band ($\sim 450\text{bp}$), which was absent in the Control sample (data not shown). In order to verify stable transformation, the PCR-positive lines were analyzed by Southern hybridization. The result showed that the positive T_0 plants had specific signal bands for transgene *RRM2* (Fig. 2), which confirmed that the gene has stable insertion into the rice genome. Transgenic plants were familiar and successfully transplanted in the field and grew normally. At maturity, transgenic plants flowered one week later than the Control plants (Fig. 3a). But, a striking finding was that the spikelets of transgenic plants showed considerable enlargement in phenotypic characteristics compared to those of the Control plants. The panicles of transgenic plants were shorter in length and contained a fewer seeds than the control (Fig. 3b). Spikelets of transgenic plants had awns (Fig. 3c), large size and heavier seed weight at maturity (Fig. 3d).

Comparing GL, GW and GT of transgenic seeds with that of the control showed that there was an increase in the average of GL (28.6%), GW (17%) and GT (19%) of transgenic seeds compared to the seeds of the control plants (Table 1). In addition, the weight and volume of 100 dry seeds of transgenic plants were greater than the equivalent seeds of Control plants, reaching to 72.7% and 71.3%, respectively (Table 1). These data suggest that the enlargement of seed size of *RRM2* plants was associated with the increase in seed length. Northern blot analysis confirmed the expression level of *RRM2* transgene, which was highly expressed in transgenic plants, while *RRM2* mRNA transcript was not detected in the Control plants (Fig. 4). These results indicated that such significant increase in the phenotypic characteristics of grain size and weight of the transgenic plants was perfectly associated with the presence of the *RRM2* transgene in the plants.

Molecular and morphological analysis of Transgenic T₁ progeny

After selfing, four independent transgenic T₀ lines were characterized in the subsequent T₁ generation using PCR analysis, which detected the *RRM2* transgene in the plants (data not shown). The sequences of the full-length cDNA of the intact *RRM2* transgene from positive T₁ plants were checked against the GenBank database. It was found that the deduced amino-acid sequence of *RRM2* is well conserved in several different plant species, including *Arabidopsis thaliana*

(accession no. NP564336) and *Triticum aestivum* (accession no. AAP36696) (data not shown). Northern hybridization analysis confirmed that the transcriptional level of *RRM2* transgene was highly expressed in T₁ transgenic plants (Fig. 5). This result confirmed that *RRM2* transgene was transmitted genetically to the next generation T₁ plants. At the mature stage, positive T₁ plants also showed the same phenotype system as in T₀ plants. Transgenic T₁ plants showed almost same phenotype of seed size expansion and seed weight as in their parents T₀ plants. As showed in Table (2), there was an increasing of the average of GL (26%), GW (15.7%) and GT (19%) of transgenic seeds as compared to those of control seeds, respectively (Table 2). In addition, the average of 1000-grain weight of dry seeds of transgenic plants was increased to more than 65% comparing with that of control seeds (Table 2). In conclusion, these results proved that the phenotypic characteristics of transgenic T₀ plants was inducing by transgene *RRM2* and transmitted genetically to the next generation of T₁ plants.

Histological analysis of transgenic plants

To investigate the reason of the seed enlargement of *RRM2* plants, we did histological analysis of large and small-seeded lines. The transverse sections of mature seeds revealed that the endosperm aleurone cells of transgenic lines seeds have larger volume than that of control line seeds (Fig. 6b). Pollen cells and embryo cells of *RRM2* spikelets have

larger size than that of the control spikelets (Fig. 6a & c). In addition, the young seedling leaves cells of *RRM2* plants have larger size than that of the control plants (Fig. 6d). These results indicated that *RRM2* functions to control the cell size of both somatic and reproductive tissues, including maternal organ, embryo and endosperm in transgenic plants. Therefore, these data suggest that size expansion of transgenic seeds is mainly related to the cell volume increasing, which associated perfectly with the expression of *RRM2* transgene. Similarly, the *PETALA2* transgene genetically acted through the maternal sporophyte and endosperm to control seed weight and yield in *Arabidopsis* (Mizukami and Fischer, 2000; Ohto *et al.*, 2005). It is possible that both *RRM2* and *PETALA2* transgenes may have a common mechanism and have similar regulation fashions for controlling seed mass.

Expression Analysis of MADS-box genes in transgenic plants

In order to study if the over expression of *RRM2* transgene can regulate the expression of some MADS-box genes in transgenic lines, fifteen putative MADS-box genes of rice were selected by analyzing homologous sequences and detected their transcriptional level in transgenic lines. Semi-quantification PCR revealed that six of these fifteen putative MADS-box genes showed differential expression in the shoots and young panicles of transgenic plants (Fig. 7). It is notable that some of MADS-box genes

have alternative expression in transgenic lines, such as up-regulation (Fig. 7b, lane 1 & 2), down-regulation (Fig. 7a, lane 1 & 2), activation (Fig. 7b, lane 3) and silencing (Fig. 7a, lane 3). These indicated that *RRM2* transgene could regulate the expression of some MADS-box genes in transgenic lines. Ohto *et al.* (2005) reported that mutant *ap2* can set large seed in *Arabidopsis* but induces the defective flowers because the main function of *AP2* is to regulate negatively homeotic MADS-box gene *AG* transcription. In contract, we observed that *RRM2* transgene altered expression of some homeotic MADS-box gene without inducing flowers defection or fertility reduction.

We speculate that the polypeptides produced by *RRM2* transgene could independently form the functional expression of *RRM2* domain. Additionally, it could recognize and bind competently to pre-mRNA of target genes. It is possible that the *RRM2* peptides in transgenic cells influence the post-transcriptional procession of some important genes by domain-binding interference and direct or indirect change the pathway setting for cell size, which led to seed enlargement. These results imply the understanding of the molecular mechanisms of controlling seed size and/or fruit size in plant. Based on the high conservation of RRM domains, it is assumed that the *RRM2* transgene has a similar function of controlling fruit and/or seed size in other plant species, including monocot and dicot.

SUMMARY

In this study the first manifestation of ectopic expression of a cDNA encoding the second RNA-recognition motif (RRM2) is shown to play an essential role in determining grain mass in the transgenic rice lines. The *RRM2* gene activity resulted in grain size larger than the control; and grain weight reached to as much as 71% greater than that of the control seeds. The study revealed that the mode of action of the *RRM2* gene in affecting both of somatic and reproductive tissues, including maternal organ, embryo and endosperm. The RNA gel-blotting analyses showed that *RRM2* gene has a high expression in all transgenic lines; proving that size enlargement of transgenic seeds is mainly related to the cell volume increase, which is perfectly associated with the expression of *RRM2* transgene. Additionally, *RRM2* has the function to regulate some homeotic MADS-box genes without any modulation of fertility. The discovery in this study suggest that ectopic-expression of *RRM2* can be as potential tool for searching and isolating the regulated downstream factors that control cell volume and seed size, which will be a useful strategy for identifying new genes for the analysis of yield component of rice and other plant species as well.

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Table (1): Activity of *RRM2* in transgenic T₀ lines resulting in an increased grain mass.

Measurements	Control Seed	Transgenic Seed	% Increase over Control
Length (mm)	7.34 ± 0.14	9.44 ± 0.23	+ 28.6
Width (mm)	3.23 ± 0.09	3.78 ± 0.13	+ 17.0
Think (mm)	2.27 ± 0.08	2.70 ± 0.10	+ 19.0
Weight (g)	2.20 ± 0.01	3.80 ± 0.19	+ 72.7
Volume (ml)	2.23 ± 0.10	3.82 ± 0.20	+ 71.3

Note: Average of length, width and thickness, weight and volume of transgenic and control grain were given per 100 dry grains. Means ± standard deviation was given.

Table (2): Activity of *RRM2* in transgenic plants resulting in an increased grain mass.

Rice Line	Measurements			
	Length (mm)	Width (mm)	Thickness (mm)	1000-grain weight (g)
Control	7.34 ± 0.14	3.23 ± 0.09	2.27 ± 0.08	22.3 ± 0.10
Act1-5:: <i>RRM2</i> -1	9.44 ± 0.23	3.78 ± 0.13	2.70 ± 0.10	36.1 ± 2.00
<i>Act1-5</i> :: <i>RRM2</i> -2	8.96 ± 0.21	3.78 ± 0.13	2.71 ± 0.11	38.3 ± 2.00
<i>Act1-5</i> :: <i>RRM2</i> -3	9.13 ± 0.22	3.68 ± 0.00	2.66 ± 0.12	35.2 ± 2.21
<i>Act1-5</i> :: <i>RRM2</i> -4	9.47 ± 0.25	3.77 ± 0.11	2.74 ± 0.00	38.1 ± 2.27
% Increase	+ 26.0	+ 15.7	+ 19.0	+ 65.6

Note: the mean ± SD of length, width and thickness is given for 100 dry grains of each T₁ line. Grains weight is given for 1000-grain. Percent increases over Control of grain mass characters were calculated based on the average of all lines compared to that of the Control seeds.

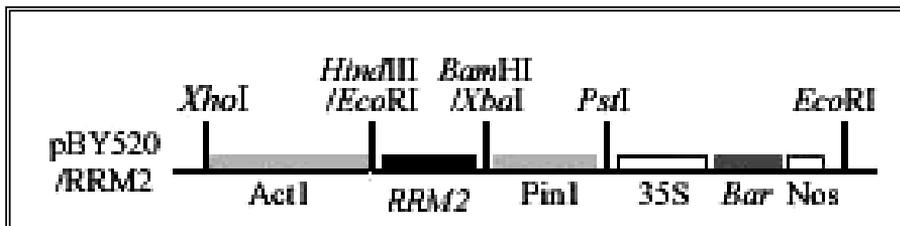


Fig (1): Expression vector pBY520 harboring *RRM2*. *Act1*: rice actin promoter; *Pin1*: pin transcription terminator; *35S*: CaMV 35S promoter; *bar*: coding sequence of the *bar* gene; *Nos*: nos transcription terminator. Restriction sites for insertion are arrowed by vertical lines.

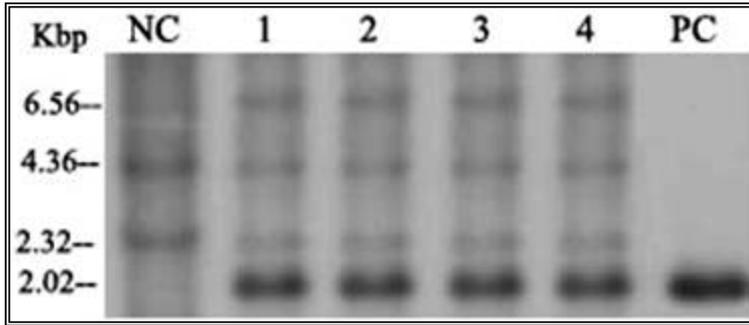


Fig (2): Southern hybridization analyses of transgenic T_0 plants. Lanes from 1-4 are from transgenic lines, lane NC from untransformed plant, lane PC is positive control.

Fig (3): Activity of *RRM2* transgene increases organs size in transgenic plants (on right side) comparing with control plants (on the left side). (a) Whole plants at flowering stage (b) Panicles; (c) Dry mature seeds; (d) Dry grains (e) 5-days old seedlings of T_1 .

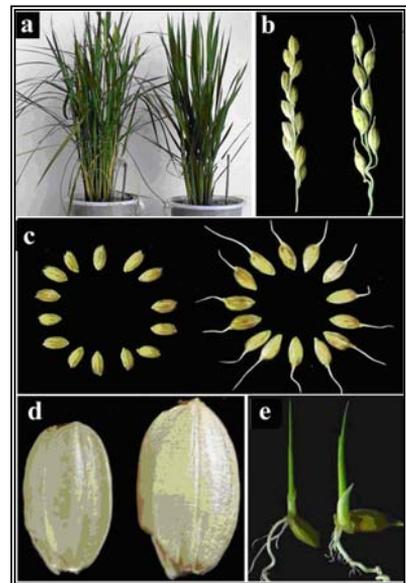
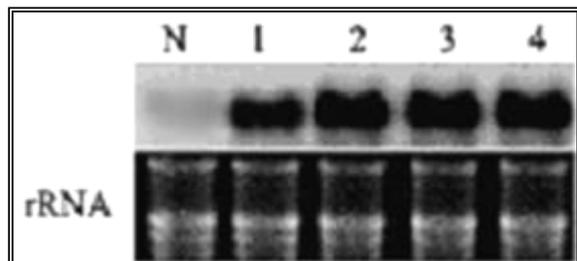


Fig (4): Northern hybridization of T_0 plants (lanes 1-4) as compared to control (lane N).



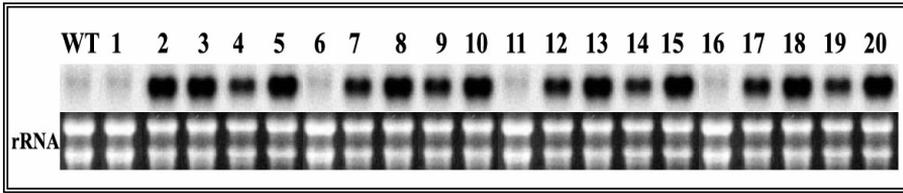


Fig (5): Northern hybridization analyses of T₁ plants, lanes 1, 6, 11 and 16 are negative transformed; lanes WT is negative Control and another lanes are transformed lines.

Fig (6): Activity of *RRM2* in controlling cell size. (a) Mature pollens, (b) Aleurone cells of endosperm, (c) Embryo cells, and (d) Young leaf cells. Notice, in the figure, the control sample is upper, while transgenic sample is lower, respectively. Images for all samples were taken at the same magnification (x100). (Bars: 100 μ m).

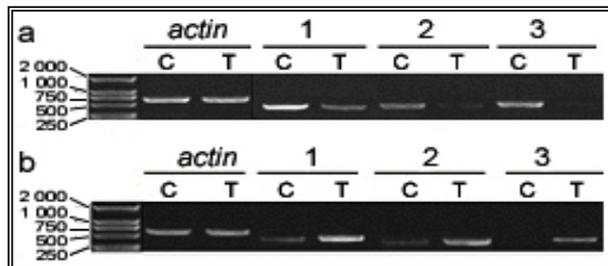
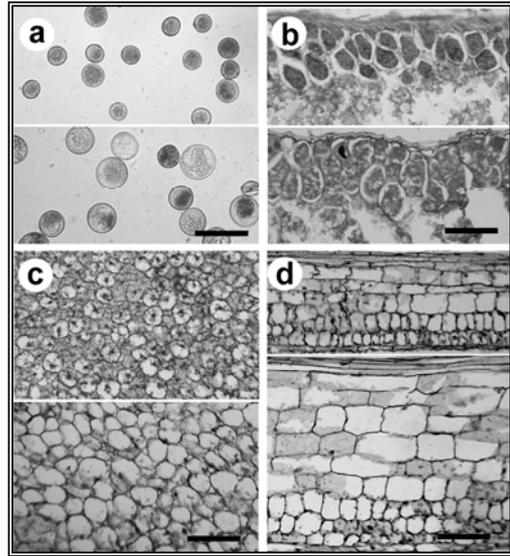


Fig (7): Effect of *RRM2* expression on transcriptional level of homeotic MADS-box genes. Relative transcriptional abundance of homeotic MADS-box genes in both (a): leaves of transgenic plants (lane 1: AF058697; lane 2: AY332476; lane 3: AF345224), and (b): young panicles of transgenic plants (lane 1: AF091458; lane 2: AF058697; lane 3: AF151693) was quantified using *actin* as an internal Control. The molecular marker is in the left lane. C lane: Control line and T lane: transgenic line.