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Full Length Research Paper

Over expression of *Zmda1-1* gene increases seed mass of corn

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Genetic engineering of seed size and increasing biomass in crop plants has an important significant contribution to the world. Arabidopsis DA1 is one of the key factors that negatively control seed and organ size by restricting the period of cell proliferation, and the mutant of Arabidopsis DA1, da1-1 (DA1R358K) can dramatically increase the size of seed. However, it is not clear whether overexpression of Zmda1-1, the mutant of ZmDA1 which is homology of DA1 in Arabidopsis, has the same biological effect as da1-1 in Arabidopsis. Therefore, in this study, the plant expression vector harboring both *Zmda1-1* driven by the corn ubiquitin promoter and a *PAT* selectable marker gene driven by 35S CAMV promoter was constructed and introduced into maize inbred line 'ji444' using pollen-tube-pathway method. Screened with herbicide phosphinothricin (PPT), 22 seedlings of 2563 transformed samples survived, and 21 independence lines of which were positive in polymerase chain reaction (PCR) analysis, and the transformation rate of T0 generation was about 0.82%. Further PCR-southern blotting results proved that the *Zmda1-1* had integrated into maize genome, and the *Zmda1-1* had expression in low level by reverse transcription-polymerase chain reaction (RT-PCR) analysis. The seed mass of transgenic maize increased at an average of 33.6% of empty vector control lines, and the harvest yield was increased by 23.6 to 114.1% in different lines than empty vector control lines. The result suggests that *Zmda1-1* can be used to engineer higher harvest yield in crops plant, thus providing the first successful example of increasing the harvest yield of maize by transgenic technology.

Key words: Transgenic maize, pollen-tube pathway, *Zmda1-1*, seed mass.

INTRODUCTION

Maize is one of most important crops around the world with a large cultivated area after wheat and rice. Maize is both an important food crop and the main feed crop and as such, how to increase the harvest of maize has been a popular research area. Although the size of the seed and the organ are affected by environmental factors, such as light, temperature and day length, many experiments suggest that the final size of seed and organ are decided by intrinsic information (Conlon and Raff, 1999). The final size of seed and organ is constant within a species, and the size variation usually do not change a lot; however, it was showed that seed and organ develop to the final size dependent on intrinsic information (Tsukaya, 2006). Meanwhile, little is known on how both outside environment factor and intrinsic information affect the final size of seed and organ (Li et al., 2008).

The growth regulators promote organ growth through *Arabidopsis thaliana* auxin-inducible protein (ARGOS) (Hu et al., 2003), which mediates the expression level of

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Abbreviations: PCR, Polymerase chain reaction; **RT-PCR,** reverse transcription-polymerase chain reaction; **ARGOS,** *Arabidopsis thaliana* auxin-inducible protein; **ANT,** AINTEGUMENTA; **BB,** BIG BROTHER; **EOD1,** enhancer of *DA1*; **ABA,** abscisic acid; **ACT,** *actin* gene; **ORF,** open reading frame; **UIM,** ubiquitin interaction conserved motifs; **CK,** control check; **DREB,** dehydration responsive element binding protein; **BT,** *Bacillus thuringiensis*.

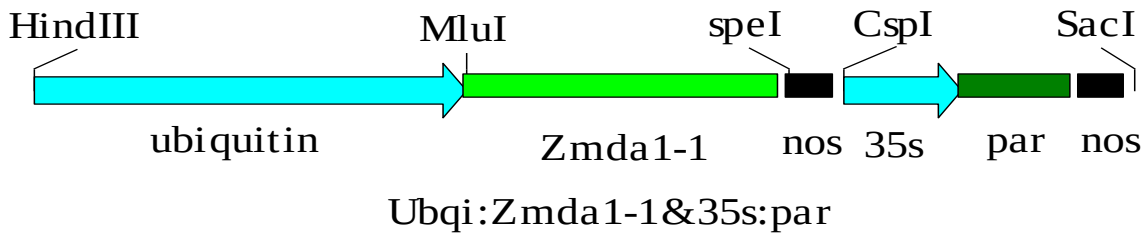


Figure 1. Partial diagram of expression vector PGM-35Sbar-Zmda1-1.

AINTEGUMENTA (ANT), a transcription factor that regulates the expression of cell cycle genes (Mizukami et al., 2000). The high expression levels of ANT increases cell numbers and the organ size, but low ANT levels would restrain the organ size (Mizukami et al., 2000). In leaves and petals, BIG BROTHER (BB) as an organ size regulator, probably depredated the growth stimulators to negatively regulate cell proliferation (Disch et al., 2006). *DA1* gene defined a new growth control pathway that could decide the final size of seed and organs in *Arabidopsis* by dominating the period of cell proliferation (Li et al., 2008). DA1-1, a mutated form of DA1, is allelic to BB, which is identified as an enhancer. When BB /EOD1 (enhancer of *DA1*) has a mutation, the size of seed and organ will be enhanced, showing that DA1 is parallel with BB/EOD1 in deciding the final size of seed and organ. When the growth regulator, abscisic acid (ABA) accumulates, it can induce the *DA1* gene expression, meanwhile organ growth is insensitive to inhibition by ABA in DA1-1 ($DA1^{R358K}$) mutant. The mutant *DA1* gene in *Arabidopsis* plant resulted in the increase of the ovule, seed mass, embryo size and cotyledon areas (Li et al., 2008).

Protein homology with DA1 contains the UIM and LIM conserved domains, and the R358K mutation in DA1 is responsible for increased size of seed and organ (Li et al., 2008). ZmDA1 is a DA1 homologue and identified from corn by protein sequence alignment. We synthesized the cDNA of Zmda1-1, which mutated one of the bases making ZmDA1 to Zmda1-1 ($ZmDA1^{R333K}$). In this study, we transformed the *Zmda1-1* gene into maize's genome, and observed that *Zmda1-1* gene can increase the mass of seed in maize. As far as we know, this is the first report that *DA1* gene can affect seed mass in monocotyledonous plant.

MATERIALS AND METHODS

The maize cultivar used in this study was inbred line "ji 444", which is grown as a parent line in northern China.

Transformation element

The empty vector pGreen0229 was used to generate *Zmda1-1* gene expression vector PGM-35Sbar-Zmda1-1 (Figure 1). The 1527 bp of Zmda1-1 cDNA ($ZmDA1^{R333K}$) was synthesized by Sangon Biotech (Shanghai) Co. Ltd. according to the ZmDA1 sequence (Gene Bank Accession no.BT085014.1), and the pGreen0229 with the *PAT* gene conferring resistance to the glufosinate herbicide is from John Innes Center, UK. The full length cDNA of *Zmda1-1* driven by the corn ubiquitin promoter was subcloned into the *Hind*III/ *Spe* I site of the vector pGreen0229. Then another *PAT* driven by the driven by 35S CAMV promoter was subcloned into the *Csp* I and *Sac* I site of the vector as shown in Figure 1. The construction was confirmed by restriction enzyme product analysis and DNA sequencing in Sangon Biotech (Shanghai) Co. Ltd..

Transformation of maize

The transformation method used was according to that of Wu et al. (2008) with minor modification. The transformation experiment was carried out in Yacheng, Hainan Province in January 2011. Maize inbred line "ji 444" which grew well was chosen for artificial pollination and covered by parchment bag. After artificial pollination 22 h, the parchment bag was removed, the top of the spike-stalk was then cut and 200 µL of the vector solution (500 ng/µl purification by Plasmid Maxi prep System from New Industry Company) was then dropped on the wound. Afterward, it was covered with parchment bag again until harvest. At maturity stage, the T_0 seeds that developed from the treated spike were harvested. Seeds from the control plants were harvested as a negative control.

Screening for the transgenic maize

The T_0 seeds grown were screened for transgenic lines in the green house in Beijing from April to September 2011. The four-leaf stage seedlings from T_0 seeds were sprayed with 200 mg/L glufosinate (Sigma 45520) three times every five days and screened for herbicide resistant lines. Two weeks later, the transformed seedlings still survived, but the untransformed seedlings had been dead.

Polymerase chain reaction (PCR) analysis

After selection, total genomic DNA was extracted from the fresh leaves using the Wizard Genomic DNA purification kit (Promega). Two sets of primer pairs (Table 1) were used in different experiments. In 50 µl reactions, 50 ng total genomic DNA was amplified in a reaction mixture containing 1×PCR reaction buffer, 100 µM of each dNTP, 10 pM of each primer (Table 1) and 1.25 U Taq DNA polymerase (TaKaRa). The PCR program of *PAT* gene was 30 cycles of 30 s at 94°C, 30 s at 55°C and 45 s at 72°C with a 6 min extension of the first cycle at 94°C and a 7 min final extension at 72°C. The PCR program of *Zmda1-1* gene was 30 cycles of 30 s at 94°C, 30 s at 53°C and 2 min at 72°C with a 6 min extension of the first cycle at 94°C and a 7 min final extension at 72°C. PCR products

Table 1. Oligonucleotide primers for 35 Sbar, Zmda1-1 and actin gene in Maize.

Primer	Sequence
PGreen0229-35Sbar-F-470	5'- ACTATCCTTCGCAAGACCCTTCCTC -3'
PGreen0229-35Sbar-R-810	5'- TCAACTGTCCAATCGTAAGCGTTCC -3'
Zmda1-1-F-324	5'-TATGTATCATCCGCCTCG-3'
Zmda1-1-R-621	5'- GGTGAAACTGCTCCTTGTA -3'
ACT-F	5'-ACCTCACCGACCACCTAAT-3'
ACT-R	5'-CTGAACCTTTCTGACCCAAT-3'

were analyzed by electrophoresis separation in 1.5% agarose gels.

PCR-southern blotting analyses

The PCR confirmed plants were further subjected to PCR-southern blotting analyses. The PCR program of *Zmda1-1* gene was same as described earlier; the plasmid template was used as the positive contrast and the inbred line "ji444" DNA and ddH_2O was used as the negative contrast. Then, 5 µl of the PCR products separated by gel electrophoresis in 1.5% agarose gels were transferred onto Hybond nylon membranes (Amersham). Hybridizations were performed at 45°C in Easy-Hyb buffer (Roche) following the manufacturer's protocol, and the product of plasmid PCR with DIG-dUTP dNTP mix was used as hybridization probe. The hybridized membrane was washed and detected according to the manufacturer's protocol (Roche DIG DNA Labeling and Detection Kit II). The signals of hybridization were captured using the phosphor image analyzers (FLA 4000, Fuji).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from the fresh leaves using the spin or vacuum (SV) Total RNA Isolation System (Promega), then 200 ng of RNA from different samples was reverse-transcribed into cDNA (10 µl). In 50 reactions, 1 µl of the first-strand cDNA was amplified in a reaction mixture containing 1×PCR reaction buffer 100 µM of each dNTP, 10 pM of each primer and 1.25 U Taq DNA polymerase (TaKaRa). The primers (Table 1) and program followed the PCR analysis of *Zmda1-1*. The PCR products were separated on 1.2% agarose gel and quantified using AlphaView SA software. Maize *actin* gene (*ACT*, GenBank accession number: J01238) amplified with ACT-F and ACT-R (Table 1) were used as the internal control in this experiment, it was amplified with *Zmda1-1* in one PCR amplifier at the same time. The experiments were repeated three times.

RESULTS

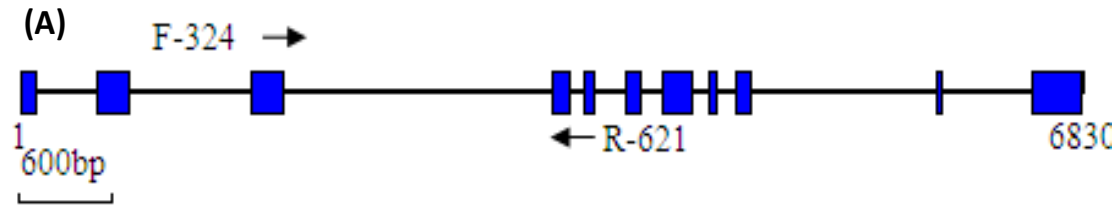
Structure analysis of ZmDA1

The full-length cDNA of ZmDA1 (BT085014.1) was 2268 bp, containing a 1527 bp open reading frame (ORF). By sequence alignment of ZmDA1 cDNA with B73 genome sequence, the full-length *ZmDA1* gene contains 6812 bp with 11 exons and 10 introns as shown in Figure 2A. The *ZmDA1* encodes a protein of 508 amino acids which has 68% identity to Arabidopsis DA1 (At1g19270) in amino acid sequences blast. By multiple sequence alignment of the ZmDA1 (Zmda1-1), DA1, DAR1 and DA1 protein-like (sorghum, *Oryza sativa* and *Hordeum vulgare*), all of the proteins are found to contain two ubiquitin interaction conserved motifs (UIM) typical of ubiquitin receptors (Hicke et al., 2005) and a single zinc-binding LIM domain (Kadrmas et al., 2004). These aforementioned analyses suggest that the ZmDA1 may encode a functional homologue of *Arabidopsis* DA1, encoding a predicted ubiquitin receptor which sets final seed and organ size. Zmda1-1 is a mutated protein of ZmDA1 that changes an arginine to lysine change at position 333, that is the same conserved point of the R358K mutation in DA1 (red ring in Figure 2B). To get Zmda1-1, a single nucleotide G to A transition in the full length cDNA of ZmDA1 at position 998 caused an R333K mutation. The result of RT-PCR analysis (Figure 4) showed that the inbred line (ji 444) can express ZmDA1, which indicates that it is a functional gene.

Molecular characterization of transgenic *Zmda1-1* over-expressing maize plants

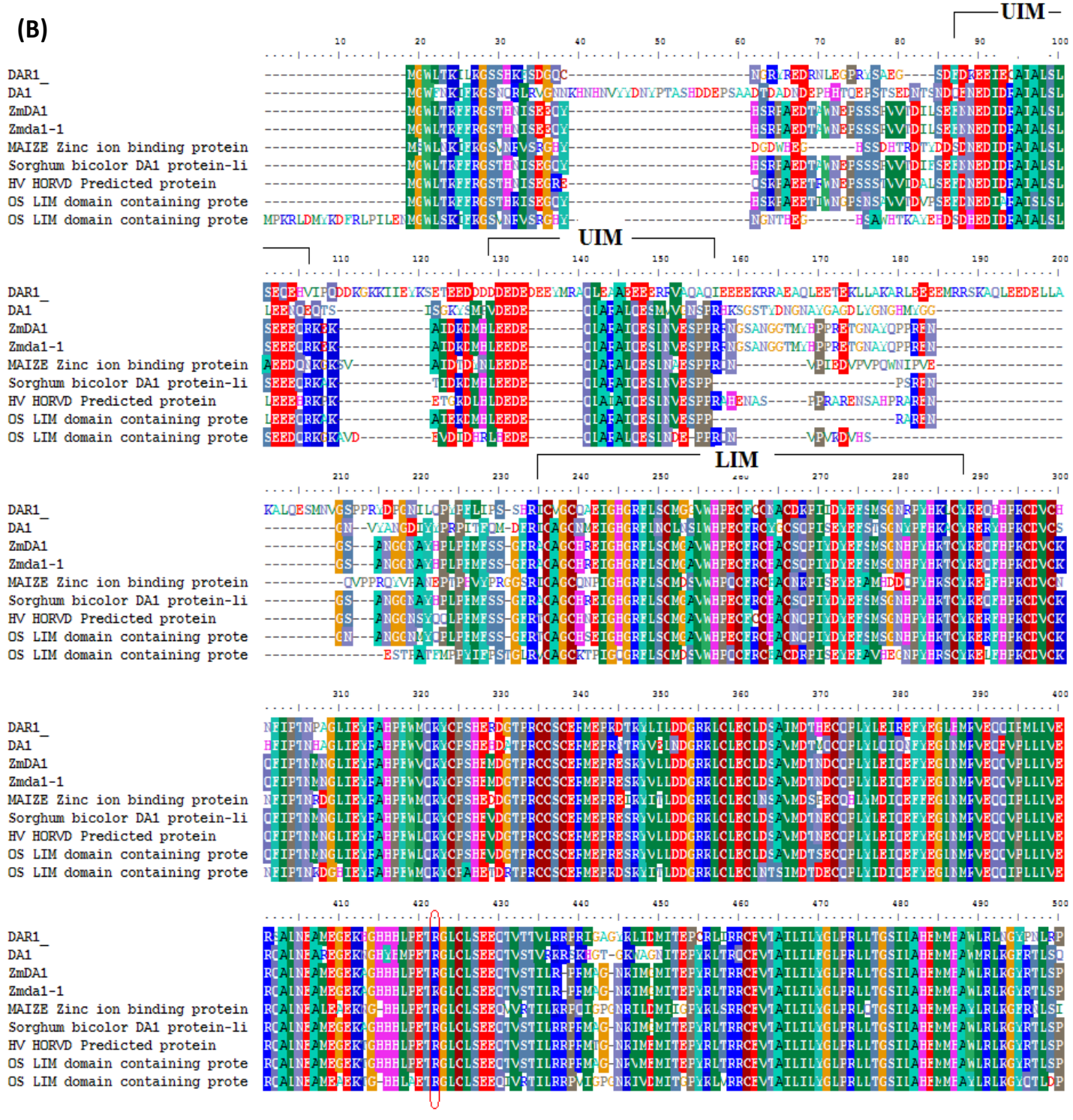
The four leave-stage seedlings from T0 seeds were screened for herbicide resistant plants sprayed with 200 mg/L glufosinate three times, and 22 individual plants survived from the 2564 seedlings. Two weeks later, the herbicide resistant lines still grew normal, but the non-resistant plants withered; their leaves became yellow and died finally (data not shown). Further analysis proved that the *PAT* and *Zmda1-1* genes were integrated into maize inbred line (ji 444)'s genome; the PCR analysis for *PAT* and *Zmda1-1* was carried out. Monitored by PCR of *PAT* (partial results as shown in Figure 3A), 21 transgenic lines were confirmed of the 22 herbicide resistant seedlings. The expected fragment (340 bp) was amplified from total DNA of all the transformed lines except one line, and the transformation rate was about 0.82%. The upper primer of *Zmda1-1* was localized in the third exon of *ZmDA1*, and the lower primer in the fifth exon as shown in Figure 2A, so the 2209 bp PCR product for *ZmDA1* will be expected to show up in the amplification from both maize inbred line and transgenic lines.

The PCR result confirmed our prediction (partial results as shown in Figure 3B); the 2209 bp fragment for *ZmDA1* really existed in the amplification from all the maze samples,



The exon of ZmDA1

(B)



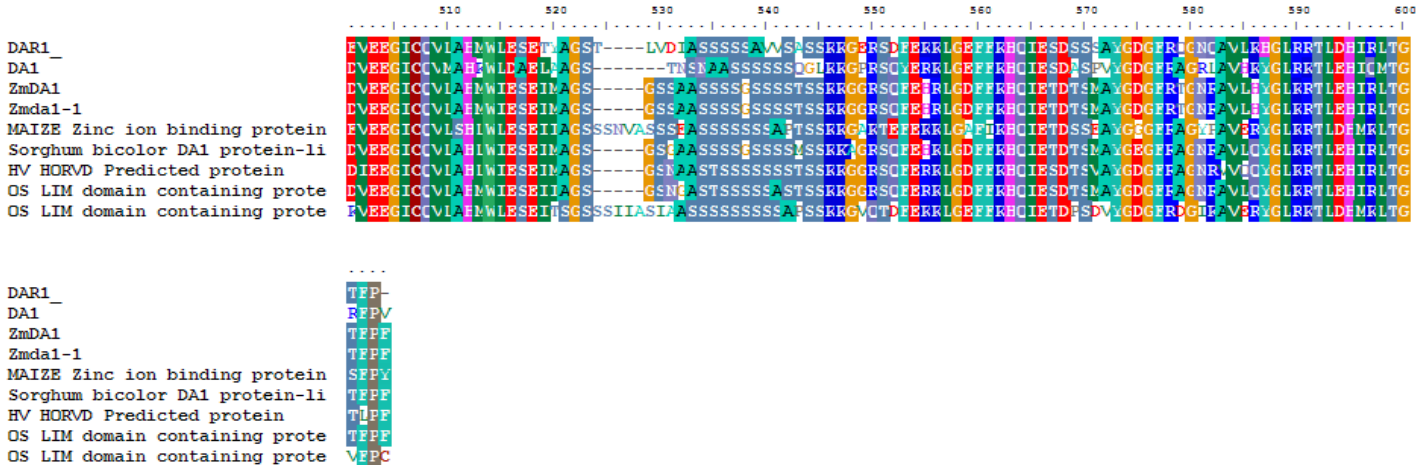


Figure 2. A. Alignment is on plus strand of genomic sequence and on plus strand of ZmDA1's mRNA sequence. The blue rectangles are exons. B. Amino acid sequence alignment of Zmda1-1 with DAR1 (Swiss pro accession no.Q8W4F0) DA1(P0C7Q8), ZmDA1(B4FAN8), MAIZE Zinc ion binding protein (B6SPK3), *Sorghum bicolor* ZmDA1-1 gene protein-like (C5Z5H5), HV HORVD Predicted protein (F2CWS6), OS LIM domain containing protein-like (Q5SMM0), OS LIM domain-containing protein (Q5KQN4).

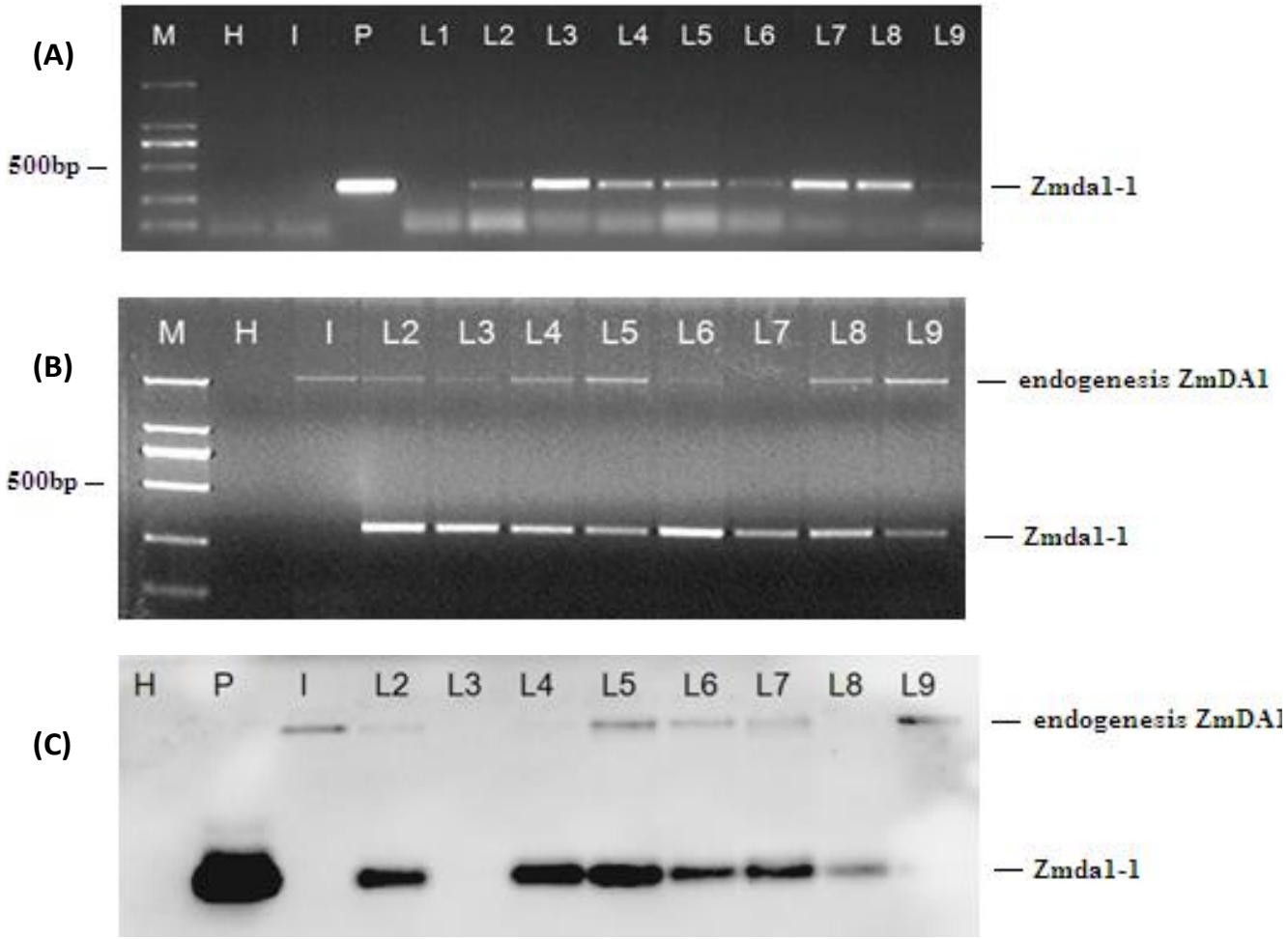


Figure 3. PCR analyses of 35S bar gene (A) and Zmda1-1 gene (2 min at 72°C) (B), Zmda1-1 gene PCR-southern blotting analyses (30 s at 72°C) (C). **A**. M, DL 2000 marker; H, ddH_2O; I, inbred line; P, plasmid; L, T0 herbicide resistant plants (1 to 9). **B**. M, DL 2100 marker; H, ddH_2O; I, inbred line; L2-9: T0 herbicide resistant plants (2 to 9). **C**. H, ddH_2O; P, plasmid; I, inbred line; 2-9, T0 PCR positive plants (2 ti 9).

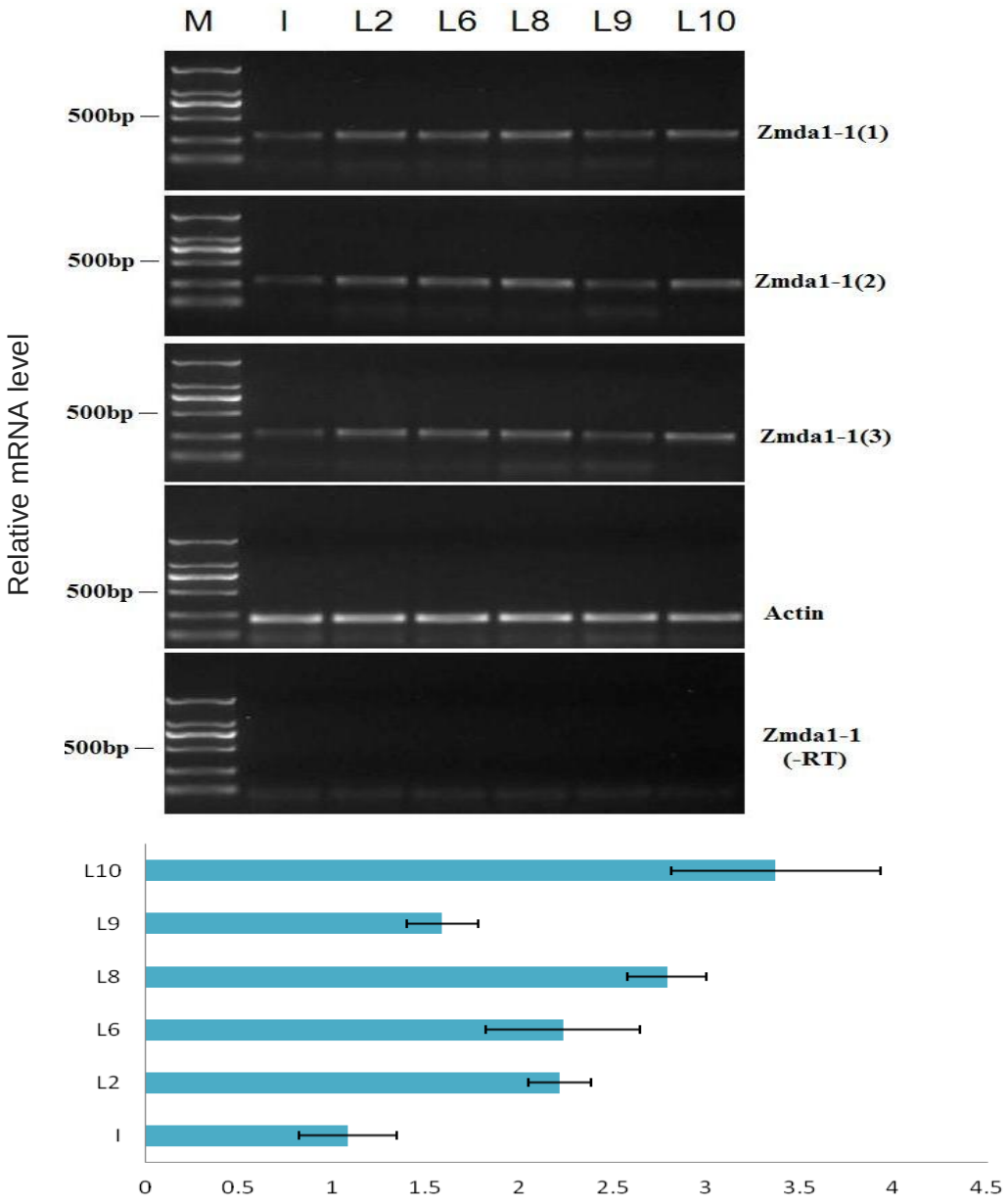


Figure 4. RT-PCR analyses of *Zmda1-1* gene, *ACT* gene and relative mRNA level. I, Inbred line; L2-6, T0 transgenic lines no. 2, 6, 8, 9 and 10.

and the expected fragment (297 bp) for *Zmda1-1* was amplified only in all the 21 transgenic lines but not in ji444 inbred line, which showed that the foreign genes have been integrated into the genome of these lines. For further confirmation of PCR results, PCR-southern blot analysis was performed using genomic DNA isolated from transformed and untransformed plants as templates of PCR; the 1.5-kb fragment of *DA1-1* gene was labeled with DIG as probe. Figure 3C shows that the putative positive lines transformed with vector PGM-35Sbar-Zmda1-1 have both 297 and 2209 bp hybridization signal bands, while the untransformed plant only had 2209 bp hybridization signal band (Figure 3C). The hybridization results (Figure 5) shows that all the 21 positive lines confirmed by PCR except one line, carried predominantly the predicted bands (297 and 2209 bp). All the results together showed that the *Zmda1-1* integrated into the "ji444" maize genome.

RT-PCR was performed to check whether the endogenous *ZmDA1* gene and *Zmda1-1* transgene were transcripted in WT and transgenic plants under normal growth conditions. Both endogenous *ZmDA1* gene and transgenic *Zmda1-1* is expected to be expressed in the transgenic lines, and RT-PCR detection cannot

Table 2. Harvested morphological traits of T0 transgenic Maize.

Transgenic line	100-gain weight (g)	Seed weight of ear	Long of ear (mm)
EK	26.9 ± 1.04	57.3 ± 5.19	1614 ± 26.7
Zmda1-1-4	32.7**	78.1**	2153**
Zmda1-1-6	31.3**	75.5**	1827**
Zmda1-1-8	30.8**	37.6	1763**
Zmda1-1-9	33.5**	70.3**	1819**
Zmda1-1-10	40.3**	76.9**	/
Zmda1-1-11	28.7**	80.4**	2026**
Zmda1-1-12	32.4**	92.1**	1869**
Zmda1-1-14	33.0**	97.8**	/
Zm da1-1-16	27.7**	110.9**	1961**
Zmda1-1-18	35.4**	122.7**	2009**
Zmda1-1-19	31.6**	88.5**	1987**
Zmda1-1-20	47.0**	110.4**	2005**
Zmda1-1-21	32.9**	74.3**	1839**
Zmda1-1-22	34.2**	70.8**	1764*

EK, The transgenic line only transformed *PAT* gene; Zmda1-1-X, the transgenic lines transformed both *Zmda1-1* and *PAT* genes. The empty vector counter lines values are average of 12 well grown plants ± SD. * , **Significant different differences at the 0.05 and 0.01 level using the student's t-test, respectively (Analyzed by SPSS).

separate the expression of these two genes, so amplification of ACT was used as the internal control to analyze the expression level of ZmDA1 between transgenic and control plants. RT-PCR analysis (Figure 4) does detect that the *ZmDA1* gene in "ji444" inbred line has low expression level. That the expression in all the five transgenic lines have 59 - 237% higher than that of "ji444", suggest it could be very possible due to the contribution of the transcription of transgenic *Zmda1-1* driven by a constitutive maize ubiquitin promoter. Based on the fact that T0 generation of transgenic line is heterozygous plant, the higher expression of *Zmda1-1* can be expected when the transgenic lines become homozygous.

Increasing seed mass and harvest in transgenic lines

Transgenic lines sprayed with 200 mg/L PPT three times grew more slowly than the "Ji444" inbred line without herbicide screening. Hence, the 12 well grown T0 transgenic plants transformed only the empty vector without the *Zmda1-1* target gene were set as control check (CK) in this experiment. Different transgenic lines have different expression levels of transgenes due to the position effect in the genome; therefore, they have different tolerance to herbicide. There were 14 *Zmda1-1* transgenic lines of 21 total lines which grew normal and had good seeds setting; the other lines had a few seeds due to the effect of herbicide. As shown in Figure 5, some *Zmda1-1* transgenic lines like L11 and L16 could set two ears of corn. In an average of 12 well grown control plants, all the 14 transgenic lines were heavier than CK in 100-grain weight as shown in Table 2. The 100-grain weight of lowest harvest transgenic line (DA1-1-16) was increased by 9.9% of CK, highest harvest transgenic line (DA1-1-21) was increased by 86.5% of CK, and average of the 14 transgenic lines was increased by 33.6% of CK. The result shows that Zmda1-1 could significantly increase the mass of seeds ($P<0.01$) (Table 2). Also, in the data of seed weight of ear, most of transgenic lines were heavier than CK except one line Zmda1-1-8, only 54.13% of CK. The average weight of ear seeds from all the Zmda1-1 transgenic lines (Table 2) was increased by 25.0% of CK. The result, therefore, suggests that Zmda1-1 can be used to engineer higher harvest yield in crops plant.

DISCUSSION

Most transgenic maize was generated for enhancing drought tolerances or insect resistance, such as dehydration responsive element binding protein (DREB) or *Bacillus thuringiensis* (*Bt*) gene, but there is little report about their direct improvement on the harvest in maize. Many experiments suggested that transforming a gene for enhancing drought tolerances could increase the harvest under drought stress environment, but it would result in the transgenic plant being smaller than wild type (Dubouzet et al., 2003) and decrease the harvest in normal environmental condition. Transgenic crops resistant

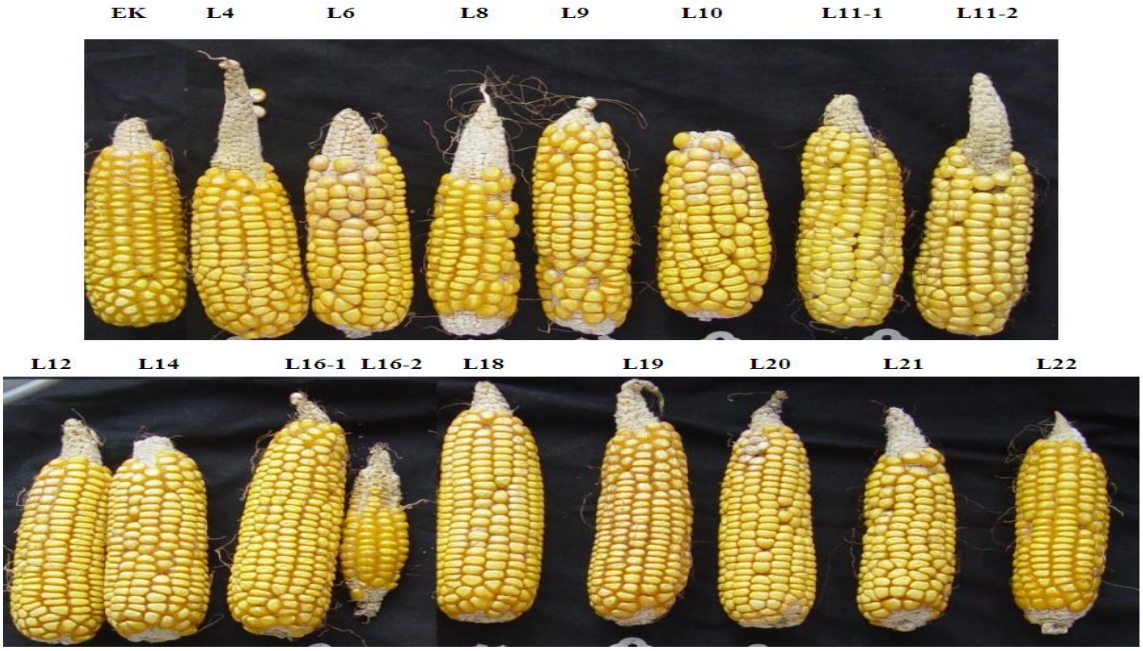


Figure 5. Photographs of maturation ear of T0 transgenic lines.

to insects could save 80% of pesticides, decrease of production cost, and improve the harvest 5 to 15%. As one of most important crop plant, whatever transformed a gene is for enhancing drought tolerances or insect resistance in maize, the final result is for increase the harvest in poor environment. In most cases, environment cue was appropriate for maize growth, so over expressing a foreign gene would make transgenic maize expressed useless protein and grew slower than the inbred line. Therefore, it is ideal that engineering maize could increase the harvest grown under both normal and stress conditions.

There were a few reports that intrinsic information could control the size of seed in dicotyledonous plant *A. thaliana* (Mizukami et al., 2000; Hu et al., 2003; Disch et al., 2006; Li et al., 2008), but little report in monocotyledon plant. ANT, BB and DA1 are three independent ways to control the size of organ and seed (Disch et al., 2006; Li et al., 2008). *DA1* gene family as a new family to control both seed and organ over expression of DA1-1 ($DA1^{R358K}$) could increase the size of seed and organ (Li et al., 2008). Dehydration activates a set of genes, and many of them are dependent on the growth regulator ABA signaling (Haake et al., 2002); DA1 expression is induced by the accumulation of ABA. On the other hand, DA1 expression is induced by dehydration. This indicates that over expression of DA1 gene could decrease the seed size. The phylogenetic analysis of the amino acid sequences (Figure 6) has showed that DA1-like protein of monocotyledon plant (maize, sorghum, *O. sativa* and *H. vulgare*) are more likely than dicotyledonous plant (*A. thaliana*); thus suggesting that their function is much more similar. So identifying the function of Zmda1-1 has important sense in crop production.

The Zmda1-1 ($ZmDA1^{R333K}$) mutant as a number of DA1 family in maize, increased both seed and organ size than inbred line as *Da1-1* gene in *A. thaliana*. Over expression of Zmda1-1 could positively regulate cell proliferation and increase seed size harvest. Expression level of Zmda1-1 (Figure 4) was associated with 100-grain weight (Table 2) as the result for100-grain weight and seed weight of ear average of the transgenic lines was increased by 33.6 and 30.3% of CK respectively. This means that Zmda1-1 gene could increase size of seed and did not decrease the seed number of an ear. In this study, our results suggest that *Zmda1-1* was transformed into maize genome, and the Zmda1-1 mutant contributed to an increase in the mass of seed, which has similar function as DA1-1. Further research is necessary to investigate the harvest of the Zmda1-1 transgenic lines grown under stress conditions.

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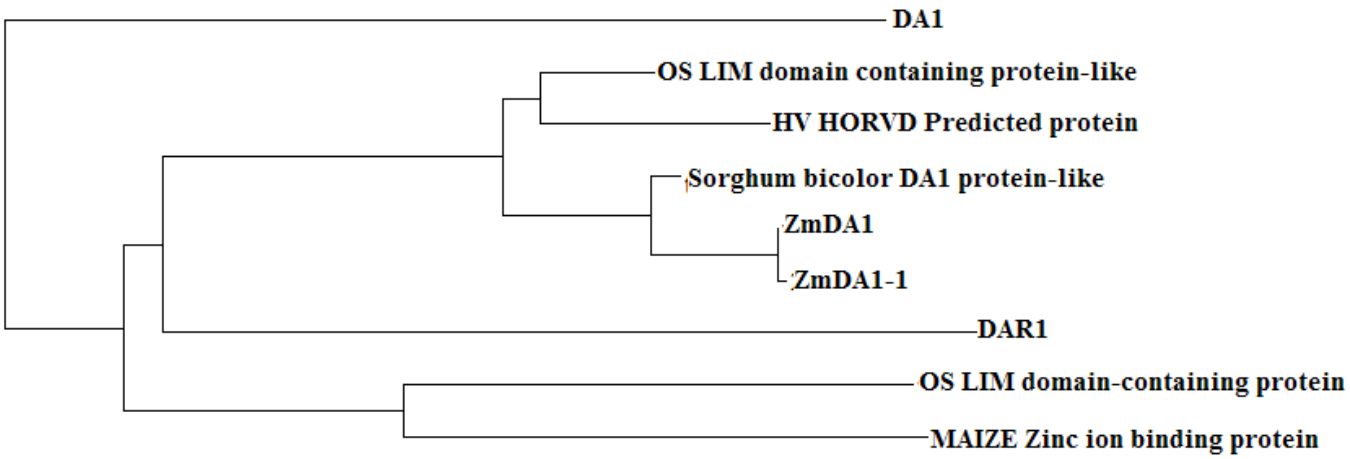

Figure 6. Homolog of DA1.

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