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OVER-EXPRESSION OF *ZmARG* ENCODING AN ARGINASE IMPROVES GRAIN PRODUCTION IN MAIZE

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Abstract

Arginase, as one of the three key enzymes in nitrogen catabolism, the physiological role of Arg catabolism in cereal crops has not been fully clarified. Studies have shown that arginase-encoding genes play a key role in providing nitrogen to developing seedlings in many plant species.Yield is a primary trait in many crop breeding programs, which can be increased by modification of genes related to photosynthesis, nitrogen assimilation, carbon distribution, plant architecture, and transcriptional networks controlling plant development. In the present study, a maize arginase gene *ZmARG* was cloned and introduced into maize inbred lines by *Agrobacterium tumefaciens*- mediated transformation. Putative transgenic plants were confirmed by PCR, Southern blotting RT-PCR analysis. The expression of the *ZmARG* gene increased arginase activity in several tissues in transgenic lines. Transgenic maize plants had significantly higher ear weight and 100-seed weight as compared with wild-type control. Our results suggested that *ZmARG* was a potential target gene for crop yield improvement.

Key words: *ZmARG* gene; Transgenic maize; Arginase; Grain yield; *Agrobacterium tumefaciens*.

Introduction

Due to the increasing demand for crop production, yield is of the primary trait of interest in breeding programs. Yield is controlled by multiple factors, and involves in various developmental and physiological processes. Despite this complexity, the modification of single genes, including those related to photosynthesis, nitrogen assimilation, distribution of carbon, plant architecture, and transcriptional networks controlling plant development (Ishimaru *et al*., 1998; Ku *et al*., 2001; Kühn 2003; Giroux *et al*., 1996; Ashikari *et al*., 2005; Song *et al*., 2007; Takeda *et al*., 2003), can increase crop yield.

Nitrogen, a crucial nutrient for plant growth and development, plays an important role in yield formation. Large quantities of nitrogen fertilizer are often used to achieve high and stable yield. However, only a third of the nitrogen in soil can be used by plants, and fertilizer runoff pollutes the environment, so this is not a sustainable practice. Promoting absorption of available nitrogen by and improving the nitrogen-use efficiency of crops would be beneficial for improving both grain production and environment. Over-expression or heterologous expression of key enzymes in nitrogen assimilation, combined with physiological and biochemical analysis, is one possible method of achieving both of these goals.

Arginine (Arg) is an amino acid component of proteins that also serves as a medium for the transport and storage of nitrogen (N) (Micallef & Shelp, 1989) and a precursor for the synthesis of nitric oxide (NO), polyamines, and proline (Pro) (Jenkinson *et al*., 1996). Arginase, one of the key enzymes in Arg metabolism, hydrolyzes Arg into urea and ornithine (Orn). Urea is then recycled by urease-catalyzed hydrolysis to ammonia (Jenkinson *et al*., 1996). In soybean, broad bean, pumpkin, and loblolly pine, nitrogen mobilization correlates with large increases in the expression of arginase genes during seedling development (Polacco & Holland, 1993; Todd & Gifford, 2002).

Ma *et al*. (2013) reported that a rice mutant lacking the *OsARG* gene, which encodes an arginine hydrolysis enzyme, had reduced plant height and grain size, and that over-expression of *OsARG* in rice increased grain number per plant under nitrogen-limited conditions. These results showed that the arginase gene is potential a good target for crop improvement. To date, the physiological role of Arg catabolism in cereal crops has not been fully clarified. In the case of rice, arginase activity increases in seedlings, especially at the onset of root growth and at the 2nd day of shoot emergence during germination, corresponding to increased turnover in Arg, although no further evidence of urea accumulation has been reported (Cao *et al*., 2010). Very few reports are regarding the role of arginine produced during the process of remobilization to generative organs until now.

In this study, an arginase gene *ZmARG* was cloned from maize and introduced to an over-expression vector pCAMBIA5300. The objective of this study is to introduce *ZmARG* gene into maize inbred lines by *Agrobacterium tumefaciens*-mediated transformation and to assess gene function for increased yields of the transgenic progenies.

Materials and Methods

***ZmARG* gene cloning and binary plant expression vector construction:** In NCBI date base (http://www.ncbi.nlm.nih.gov/), we founded that similarities of nucleotide between three cDNA sequence of maize (BT087608.1, BT040489.1 and AY106166.1) and the mRNA sequence of rice arginase (HM369061.1) was 87%. These three cDNA sequences of maize have the same 1023 bp coding region located in chromosome 10 of maize genome by blasting on MaizeGDB (http://www.maizegdb.org/). Total RNAs were extracted using TRI Reagent® (Tiangen, Beijing, China) from young leaves of maize inbred line Zheng58 seedlings, and DNase-treated RNA were reverse transcribed using the RT Reagent Kit (Transgene, Beijing, China). The cDNA

samples were diluted 5-fold to serve as the templates for the subsequent PCR. Reverse transcription (RT)-PCR was performed using Primers AF1 (R: 5'-ATTCGAGCT CGGTACCCGGGATGGGTGGCGCGGCGGCGGGT-3') and AR1 (5'-GACTCTAGAGGAT CCCCGGGTC ACTTGGAGATCTTAGCAGT-3'). The primers were designed according to the full-length cDNA sequences of the target gene, and the user manual of In-Fusion Cloning Kit [Takara Biomedical Technology (Beijing) Co., Ltd].

Amplification products were resolved on 1% agarose gel, and DNA was purified from the bands. DNA fragments were then inserted into pGEM-T vector plasmid (Promega, Beijing, China), then sequenced by Shanghai Sangon Biotech Company.

The whole length cDNA of *ZmARG* was sub-cloned into the plasmid pCAMBIA5300-ubi-*EPSPS*, which contained a maize constitutive ubiquitin promoter, a nos terminator, and the *EPSPS* gene driven by the *CaMV 35S* promoter according to the standard protocol using the In-Fusion Cloning Kit [Takara Biomedical Technology (Beijing) Co., Ltd]. The recombined plasmid pCAMBIA5300-Ubi-*ZmArg* was introduced into Agrobacterium tumefaciens strain LBA4404 by a freeze-thaw technique (Höfgen & Willmitzer, 1988).

PCR analysis and Southern blotting: Genomic DNA of maize plants was extracted from the upper two leaves using the CTAB procedure (Murray *et al*., 1980). PCR assays were performed with specific primers AF2 (5'-ATTTTGATCTTGATATACTTGGATG -3) and AR2 (5'-TGGCTGTCATCCCATCTACCGTGTC-3'). Due to *ZmARG* gene was an endogenous gene of maize, the primer designed by the sequences of Ubiquitin promoter and *ZmARG* gene.

The PCR cycling parameters were 94ºC for 5 min, followed by 35 cycles of 94ºC for 30 sec, 60ºC for 30 sec and 72ºC for 1 min. The products were separated on 1.0 %-TBE agarose gels.

Southern blotting was performed with the digoxigenin (DIG)-labeled PCR-amplified gene fragment probe from EPSPS gene sequences as described in the DIG System Manual (Roche, Inc., Basel, Switzerland). The DNA of PCR-positive maize plants was digested with *Bam*HIand *Hin*dIII, which has only one cut site in the T-DNA region and no cut site in *EPSPS* region. After *Bam*HI and *Hin*dIII digestion and electrophoresis on 0.8% agarose gel, 20μg of DNA was transferred onto a positively charged nylon membrane (Roche, USA). After 2 h of fixation by drying at 80ºC, the membrane was hybridized with digoxigenin (DIG)-labeled probes as described in the DIG System Manual.

RNA isolation and RT-PCR: Total RNAs were extracted by the Trizol reagent (Tiangen, Beijing, China) from 100 mg of young leaves of maize seedlings, and 500 ng DNase-treated RNA were reverse transcribed using the RT Reagent Kit (Transgene, Beijing, China). The cDNA samples were diluted 5-fold to serve as the templates for the subsequent PCR. The gene-specific primers same as PCR assay from sequences of the conserved region of the *EPSPS* gene were used to amplify a 645bp *EPSPS* transcript. The β-Actin was an internal control for RT-PCR and all experiments were repeated at least three times.

Field experiment to compare phenotypes of WT and transgenic maize plants: Seeds of transgenic lines and their host lines were planted in the field at Harbin (45°45'00"N and 126°37'58"E). Trial plots were arranged in a randomized complete block design with three replications. Forty seeds of each homozygous T3 transgenic and WT line were sown in three-row plots 2.5 m in length, and 0.65 m wide, with an interval of 0.25 m between plants. The plants were thinned at the five-leaf stage, resulting in 10 plants in each plot. Plant and ear heights were measured after flowering. Mature ears were harvested, dried, and threshed to determine hundred-grain weight. Ear weight and the number of grain rows were recorded after harvest from 10 randomly selected ears for each plot. The experimental field was managed according to standard practices for maize cultivation.

Detection of Arginase activity: Tissues were sampled for arginase activity from six developmental stages, including dry seeds, germinating seeds, uppermost leaves at three-leaf and jointing stages, and ears leaves at flowering and seed ripening, from maize plants grown in the field. Tissues from WT and transgenic plants were frozen in liquid nitrogen and ground in a mortar to a fine powder. Each sample was extracted using 1.0 ml ice-cold buffer (pH 9.0) containing 0.1M sodium pyrophosphate and 0.025 mM sodium ascorbate, and the homogenate was centrifuged at approximately 14,000 g for 20 min (4°C) in a 1.5 ml microfuge tube and the supernatant was transferred into a new 1.5 ml microfuge tube. Arginase activity was assayed as described by King & Gifford (1997).

Statistical analysis

All the data are shown as mean ± standard error (SE). Comparisons between transgenic plants and WT plants were performed using Duncan's Multiple Range Test. A value of $p<0.05$ was considered statistically significant. All statistical analysis was done with SAS 9.3.

Results

Plasmid construction and maize transformation: The sequence for the 1023-bp ORF for *ZmArg* gene was obtained from *Zea mays* (Fig. 1) by PCR according to the same ORF in three cDNA sequences of maize (BT087608.1, BT040489.1 and AY106166.1) in NCBI date base(http://www.ncbi.nlm.nih.gov/), and cloned into plasmid p5300-Ubi to create p5530-Ubi-*ZmARG*. The cloned gene was confirmed by sequencing (Sangon Biotech CO., Shanghai, China). The ZmARG protein is comprised of 340 amino acids with a predicted molecular mass of 36.96 kDa and pI of 5.97. CD-Search (add URL) results and sequence alignment indicated that the sequence could encode a conserved arginase domain containing two His and four Asp residues that bind the Mn^{2+} co-factor, as reported by Perozich *et al*. (1998) (Figs. 2 and 3). The ZmARG protein is highly conserved with arginase from other organisms, including those from *Oryza sativa* (HM369061.1), *Arabidopsis thaliana* (NP192629.1), *Solanum lycopersicum* (NP001233851.1), *Glycine max* (AF035671.1), *Pinus taeda* (AF130440.1), and *Triticum aestivum* (AEN92260.1 and JN415197.1) (Fig. 3).

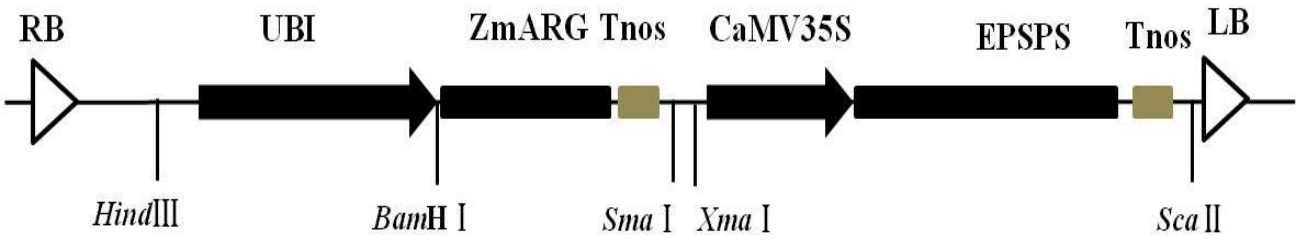

Fig. 1. **T-DNA region of the plasmid p5300-Ubi-*ZmARG*.** The 11023-bp coding sequence of the *ZmArg* (arginase) gene was cloned into vector pCAMBIA3300. *EPSPS*, selectable glyphosate marker gene; Ubi, maize *Ubiquitin* gene promoter; T*nos*, terminator of the *nos* gene from *Agrobacterium*; LB, left T-DNA border; RB, right T-DNA border.

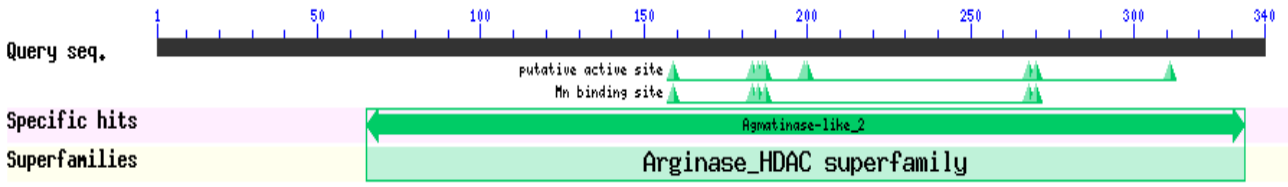

Fig. 2. **Conserved domains of the ZmARG protein predicted by CD-Search.** The ZmARG protein is a member of the arginase gene family and has a conserved arginase domain.

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ZmORG          ..........MGGAAAGTKWIHHIQRLSAA...KVSAEAVERGQSRVIDASLTLIRERAKLKAELLRALGGVKASASLLG   67
Triticum       ..........MGGAAAAAGAARWIQRLSAA...RISTEALERGQSRVIDASLTLIRERAKLKGELLRAMGGVKASATLLG   67
Oryza          ..........MGGVAAGTRWIHHVRRLSAA...KVSADALERGQSRVIDASLTLIRERAKLKAELLRALGGVKASACLLG   67
Arabidopsis    ..........MS.RIIGRKGINYIHRLNSASFTSVSASSIEKGQNRVIDASLTLIRERAKLKGELVRLLGGAKASTSLLG   69
Solanum        ..........MKSAGSMGINYMQKLLTS...NVPKEVVKRGQDRVVEASLTLIRERAKLKGELVRGLGGAVASTSLLG   65
Pinus          ..........MGSMGKMVMRFLQKRSLA...TLPSQMIEKGQNRVVEASLTLIRERAKLKAELVQALGGSIATTCLLG   65
Glycine        MSFLRSFARNKDISKVGRRGIHCMQKLCAE...KISPDSLEKAQNRVIDAALTLVRENTGLRKNLCHSLGGAVATSTLLG   77
Consensus                                                          q rv  a ltl re   l   l    gg  a   llg

ZmORG          VPLGHNSSFLQGPAFAPPRIREAIWCGSTNSSTEEGKELNDPRVLTDVGDVPIHEIRDCGVEDDRLMHVISESVKTVMEE  147
Triticum       VPLGHNSSFLQGPAFAPPRIREAIWCGSTNSSTEEGKELNDPRVLTDVGDVPIQEIRDCGVEDDRLMHVISESVKTVMDE  147
Oryza          VPLGHNSSFLQGPAFAPPRIREAIWCGSTNSSTEEGKELNDPRVLTDVGDVPIQEIRDCGVEDDRLMNVVSESVKTVMEE  147
Arabidopsis    VPLGHNSSFLQGPAFAPPRIREAIWCGSTNSATEEGKELKDPRVLTDVGDVPVQEIRDCGVDDDRLMNVISESVKLVMEE  149
Solanum        IPLGHNSSFLQGPAFAPPLIREAIWCGSTNSTTEEGKILDDQRVLTDVGDLPVQELRDTGIDDDRLMSTVSESVKLVMDE  145
Pinus          VPLGHNSSFLQGPAFAPPRIREAIWCGSTNSATEKGKELKDSRVLSDAGDVPIQEMRDCGIEDERLMKTVSDSVKIVMEE  145
Glycine        VPLGHNSSFLEGPAFAPPFIREGIWCGSANSTTEEGKDLKDLRIMVDVGDIPIQEMRDCGIGDERLMKVVSDSVKLVMEE  157
Consensus       plghnssfl gpafapp ire iwcgs ns te gk l d r   d gd p  e rd g  d rlm   s svk vm e

ZmORG          EPLRPLVLGGDHSISYPVVRAVSEKLGGPVDILHLDAHPDIYDCFEGNTYSHASSFARIMEGGYARRLLQVGLRSITKEG  227
Triticum       DPLRPLVLGGDHSISYPVVRAVSEKLGGPVDILHLDAHPDIYDCFEGNTYSHASSFARIMEGGYARRLLQVGLRSITKEG  227
Oryza          DPLRPLVLGGDHSISYPVVRAVSEKLGGPVDILHLDAHPDIYDAFEGNIYSHASSFARIMEGGYARRLLQVGIRSITKEG  227
Arabidopsis    EPLRPLVLGGDHSISYPVVRAVSEKLGGPVDILHLDAHPDIYDCFEGNKYSHASSFARIMEGGYARRLLQVGIRSINQEG  229
Solanum        NPLRPLVLGGDHSISYPVVRAVSEKLGGPVDILHLDAHPDIYDAFEGNKYSHASSFARIMEGGYARRLLQVGIRSINLEG  225
Pinus          PPLRPLVLGGDHSISYPVVKAVTDHLGGPVDILHLDAHPDIYDAFEGNKYSHASSFARIMEGGHARRLLQVGIRSITKEG  225
Glycine        DPLRPLILGGDPSISYPVVRAISEKLGGPVDVLHFDAHPDLYDEFEGNYYSHASSFARIMEGGYARRLLQVGIRSINKEG  237
Consensus       plrpl lggd sisypvv a    lggpvd lh dahpd yd fegn yshassfarimegg arrllqvg rsi  eg

ZmORG          REQGKRFGVEQYEMRTFSKDREKLENLKLGEGVKGVYVSVDVDCLDPAFAPGVSHIEPGGLSFRNVLNILQNLQGDVVAA  307
Triticum       REQGKRFGVEQYEMRTFSRDREKLENLKLGEGVKGVYVSVDVDCLDPAFAPGVSHIEPGGLSFRDVLNILQNLQGDVVAG  307
Oryza          REQGKRFGVEQYEMRTFSKDREKLESLKLGEGVKGVYISVDVDCLDPAFAPGVSHIEPGGLSFRDVLNILHNLQGDVVAG  307
Arabidopsis    REQGKRFGVEQYEMRTFSKDRPMLENLKLGEGVKGVYISIDVDCLDPAFAPGVSHIEPGGLSFRDVLNILHNLQADVVGA  309
Solanum        REQGKRFGVEQYEMRTFSRDRQFLENLKLGEGVKGVYISVDVDCLDPAFAPGVSHFESGGLSFRDVLNILHNLQGDIVGA  305
Pinus          REQGKRFGVEQYEMHSFSKDRDFLENLKLGEGVKGVYISIDVDCLDPAFAPGVSHLEPGGLSFRGVMNLVQNLQGDIVAA  305
Glycine        REQAKKFGVEQFEMRHFSKDRPFLENLNLGEGAKGVYISIDVDCLDPGYAVGVSHYESGGLSFRDVMNMLQNLKGDIVGG  317
Consensus      req k fgveq em  fs dr  le l lgeg kgvy s dvdcldp  a gvsh e gglsfr v n   nl  d v

ZmORG          DVVEFNPQRDTVDGMTAMVAAKLVRELTAKISK..                                               340
Triticum       DVVEFNPQRDTVDGMTAMVAAKLVRELSAKISK..                                               340
Oryza          DVVEFNPQRDTVDGMTAMVAAKLVRELTAKISK..                                               340
Arabidopsis    DVVEFNPQRDTVDGMTAMVAAKLVRELAAKISK..                                               342
Solanum        DVVEYNPQRDTADGMTAMVAAKLVRELAAKMSK..                                               338
Pinus          DVVEFNPQRDTVDGMTAMVAAKLVRELTSKMSKLA                                               340
Glycine        DVVEYNPQREPPDRMTAMVAAKFVRELAAKMSK..                                               350
Consensus      dvve npqr   d mtamvaak vrel  k sk
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Fig. 3. **ClustalX multiple alignment of maize arginase amino acid sequences.** The conserved arginase superfamily domain is indicated by black lines above the sequence. Identical amino acids are enclosed in black boxes, similar amino acids are in gray boxes. Invariant positions within the arginase family (Perozich*et al*., 1998) are denoted with asterisks. ● indicates the amino acid residues involved in binding Mn2+. The other organisms shown as sources of arginase sequence include: Oryza sativa (HM369061.1), Arabidopsis thaliana (NP192629.1), *Solanum lycopersicum* (NP001233851.1), *Glycine max* (AF035671.1), *Pinus taeda* (AF130440.1), *Triticum aestivum* (AEN92260.1 and JN415197.1).

Plasmid p5530-Ubi-*ZmARG* was introduced into *Agrobacterium tumefaciens* strain LBA4404. Transgenic maize plants overexpressing the *ZmARG* gene were successfully generated by *Agrobacterium*-mediated transformation into the maize inbred line KF513. Selection by 0.1% glufosinate and PCR analysis were conducted at each subsequent generation. About 10% of the transformed maize seedlings were herbicide-resistant after spraying with 0.1% glufosinate at the two-leaf stage. More than 20 independent T0 transgenic lines were established and propagated in the greenhouse.

Molecular characterization of transgenic maize plants: The T0 generation transgenic maize lines were screened by PCR using gene-specific primers for the *Ubiquitin* promoter and *ZmArg* gene. PCR product of 1123 bp in size was amplified for *ZmArg* using gene-specific primers (Fig. 4).

The herbicide-resistant and PCR-positive seedlings were transplanted into the field, and self-pollinated for further research. Seeds of the T1 generation were harvested separately from each independent line. T1 lines segregating in the Mendelian ratio of 3:1 for *EPSPS* gene in PCR assays were selected and their progeny were harvested.

Southern blots were performed with DNA isolated from PCR-positive T3 plants by hybridizing *Bam*HI- and *Hin*dIII-digested genomic DNA samples with an *EPSPS*-specific probe. Three transgenic maize T3 lines, AKF58, AKF61, and AKF65 were shown by Southern blot analysis to contain a single integrated copy of *EPSPS*, and no integration of *EPSPS* was detected in the WT plants (Fig. 5).

The expression of the *EPSPS* gene in leaves of T3 plants was monitored by reverse transcriptase-polymerase chain reaction (RT-PCR), and the results are shown in Fig. 6. In WT plants, no expression of the introduced *EPSPS* gene was detected with the gene-specific primers for the introduced bacterial gene.

Arginase activities in transgenic plants: Arginase activities were assayed in six different developmental stages in T3 lines and WT KF513. Arginase activities in these three transgenic lines were higher than those in WT lines in both seeds and leaves from different developmental stages, with the highest level of expression in the jointing stage. The arginase activity of line AFK61 was significantly higher level than that of WT in dry seeds and leaves at the three-leaf stage (Fig. 7). Line AFK65 also exhibited an increase in arginase activity relative to that of the WT plants in dry seeds, seedling leaves, and during the grain-filling period.

Plants expressing *ZmARG* gene had improved yield-related traits: Three T3 lines over-expressing *ZmARG* (AKF58, AKF61, and AKF65) and their host line KF513 were grown in the field to explore the impact of *Zm*ARG expression on yield traits. Plant height, ear height, ear weight, seed rows, number of seeds per row and 100-seed weight was measured and the results were shown in Table 1. The three transgenic lines exhibited normal growth and development, and had improved grain yield-related traits compared with the WT control lines.

Among the three transgenic and WT maize line, there were no remarkable differences in plant height, ear height, or seed number of per ear (Table 1). These results indicated that the transgenic plants had normal growth and development throughout their life cycle. The lines overexpressing *ZmARG* had higher 100-seed weight and increased ear weight compared with the WT control (Table 1). Taken together, the phenotypic data indicated that over-expression of *ZmARG* gene in maize increased 100-seed weight and could thereby increase grain yields.

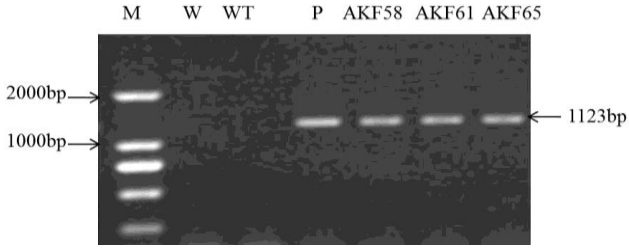

Fig. 4. **PCR analysis of transformed samples using primers specific for the maize *Ubiquitin* promoter and the *ZmArg* gene.** An 1123-bp PCR product was amplified from three transgenic maize lines (AKF58, AKF61, and AKF65) with primers specific for the maize *Ubiquitin* promoter and *ZmARG* gene. M, DNA Marker DL2000 (TaKaRa); W, water (no template DNA) control; WT, wild-type plants of KF513; P, pCAMBIA5300-*Ubi-ZmArg* plasmid; AKF58, AKF61, and AKF65, transgenic maize lines.

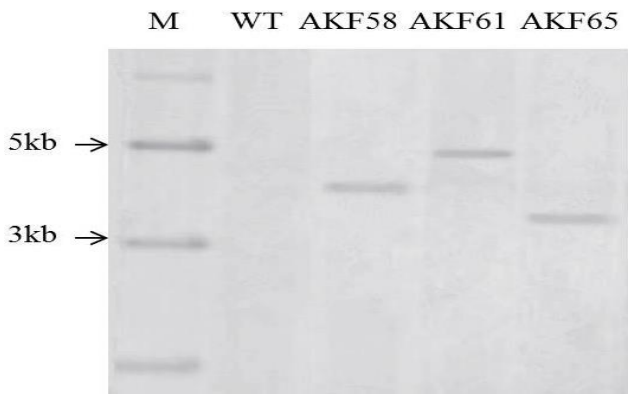

Fig. 5. **Southern blot analysis of transgenic plants and the wild type.** Total genomic DNA of three transgenic maize lines (AKF58, AKF61 and AKF65) and WT was digested with *Bam*HI and *Hin*dIII. A single-copy insertion of the *EPSPS* gene was detected in these three transgenic maize lines, and no integration of *EPSPS* was detected in the corresponding WT plants. M, DNA Marker DL15000 (TaKaRa); WT, wild-type plants of KF513; AKF58, AKF61, and AKF65, transgenic lines.

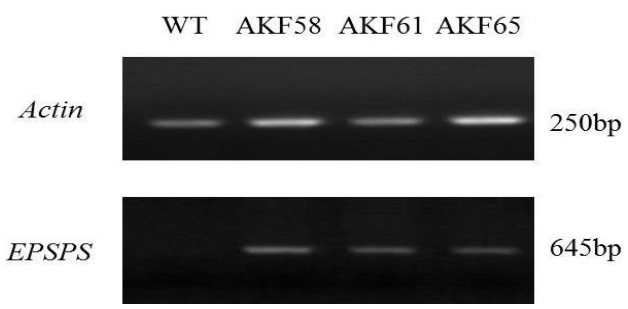

Fig. 6. **RT-PCR analysis of transformed samples using *EPSPS* gene-specific primers.** A 645-bp PCR product was amplified with *EPSPS* gene-specific primers from three transgenic maize lines (AKF58, AKF61, and AKF65) by reverse transcriptase-polymerase chain reaction (RT-PCR). In WT plants, no expression of the *EPSPS* gene was detected. WT, wild-type plants of KF513; AKF58, AKF61, and AKF65, transgenic lines.

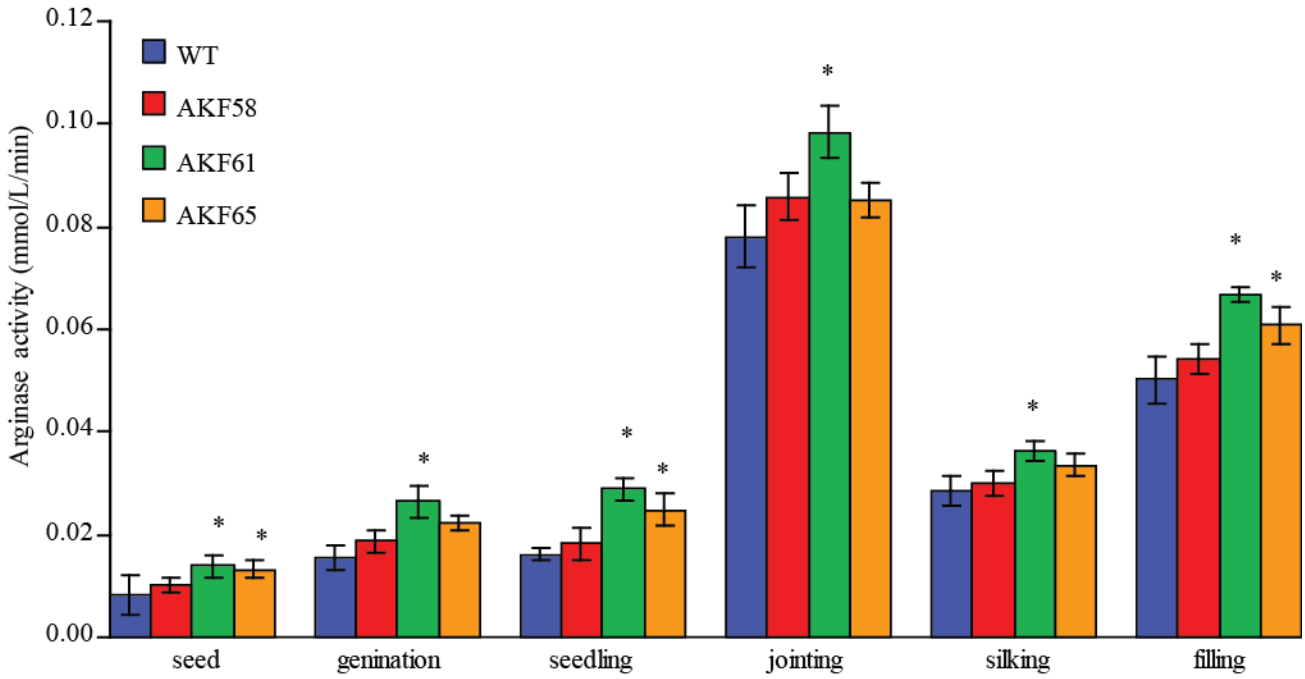

Fig. 7. **Arginase activity in transgenic maize lines at different developmental stages.** Error bars indicate the standard deviation of arginase activity in the WT and three independent transgenic lines (AKF58, AKF61, and AKF65). Asterisks indicate statistically significant differences $p<0.05$ (Duncan's Multiple Range Test). WT, KF513 wild-type seeds and plants; AKF58, AKF61, and AKF65, seeds and plants of respective lines transgenic for *ZmArg*.

Table 1. Agronomic traits in KF513 wild type maize and transgenic lines derived from transformed KF513.

Lines	Plant height (cm)	Ear height (cm)	Grains per ear	Ear weight (g)	100-grain weight (g)
WT	116.6 ± 2.1	42.9 ± 2.9	312.3 ± 12.3	82.7 ± 3.2a	20.7 ± 1.2a
AKF58	117.2 ± 4.3	43.1 ± 1.7	302.4 ± 21.8	97.9 ± 5.6b	25.2 ± 1.5b
AKF61	118.1 ± 1.2	43.6 ± 2.8	335.8 ± 16.5	124.3 ± 2.5c	31.0 ± 1.8c
AKF65	117.7 ± 2.8	43.2 ± 1.5	326.1 ± 14.6	120.2 ± 4.2c	29.8 ± 1.6c

Values are mean ±SD (n = 10). Values followed by different letters in each column are significantly different as compared with wild type at $p<0.05$

Discussion

Genetic transformation has received considerable attention due to its potential for increasing yield at a time when improving yield using conventional breeding practices is becoming more difficult. Although yield is often considered too complex a trait on which single genes would not have any major effect, in the past few years, significant progress has been made towards improving yield using single genes. Nitrogen recycling and remobilization are also major targets of transgenic strategies for yield enhancement, in addition to increasing source and sink strength, or modifying assimilate partitioning, plant architecture, and development (Van Camp, 2005). The use of nitrogen by plants involves several steps, including uptake, assimilation, and remobilization during plant aging. Although urea is the main source of applied plant nitrogen, it cannot be used directly by plants (Gerendás *et al*., 1998). Arginase, urease, and glutamine synthetase, the three key enzymes in nitrogen catabolism, must act synergistically to make nitrogen available to meet the demands of plant growth (Witte *et al*., 2005).

Martin *et al*. (2006) observed an increase in kernel number in maize over-expressing the *Gln1-3* gene under either high-nitrogen or low-nitrogen growth conditions. Rice lines over-expressing the cytosolic glutamine synthetase (GS1) gene exhibited a 25-35% higher spikelet yield (Brauer *et al*., 2011). These studies have provided important evidence supporting enhancing nitrogen efficiency as a solution to improve crop traits and increase seed yield.

Arginase, as one of the three key enzymes in nitrogen catabolism, the physiological role of Arg catabolism in cereal crops has not been fully clarified. It hydrolyzes Arg to ornithine and urea in plants; and urea is then degraded by urease, while ornithine can be utilized in the urea cycle, or as a precursor for the synthesis of both polyamines and Pro (Zonia *et al*., 1995). Arg constitutes 50% of the nitrogen stored as proteins in seed and 90% of the free nitrogen in vegetative organs of plants. Arginine (Arg) serves as a medium for the transport and storage of nitrogen (N) (Micallef and Shelp 1989) and precursor for the synthesis of nitric oxide (NO), polyamines, and Pro (Jenkinson *et al*., 1996). Studies have shown that arginase-encoding genes play a key role in providing nitrogen to developing seedlings in many plant species, such as loblolly pine (Todd *et al*., 2001), soybean (Goldraij and Polacco, 1999) and tomato (Chen *et al*., 2004). In *Arabidopsis*, such genes also participate in NO accumulation (Flores *et al*., 2008). Ma *et al*. (2013) isolated a rice mutant nglf-1 with reduced plant height, small panicle and grain size, and a low seed-setting rate (10% in nglf-1 compared to 93% in wild-type). The mutant phenotype was caused by loss of function of a gene (*OsARG*) encoding an arginine hydrolysis enzyme. Their

study also indicated that grain yield per plant and filled grain number per plant increased significantly for all transgenic rice plants over-expressing *OsARG* compared to control (Ma *et al*., 2013).

In this study, the *ZmARG* gene was over-expressed in maize inbred lines KF513 under the control of the maize *Ubiquitin* promoter. Southern blotting and RT-PCR examination of transgenic plants indicated that the transgenes were stably inherited and actively expressed. The expression of the *ZmARG* gene increased arginase activity in several tissues in transgenic lines. Transgenic maize plants had higher ear weight and 100-seed weight compared with the WT control. This is the first report to obtain maize with increased 100-grain weight through over-expression of the maize *ZmARG* gene. Yield experiments in the field indicated that the transgenic plants had normal growth and development during their life cycle. Plant height, ear location, seed number, number of seed rows, and the general phenotype of the transgenic plants were not significantly different from those of the untransformed control. Therefore, the transgenic lines should have good potential for use in the breeding of highly productive maize varieties.

In conclusion, our results provide a way to produce new maize inbred lines with improved grain qualities. These results suggest that the *ZmARG* gene can be used to improve grain production in maize through genetic engineering. Further efforts will focus on understanding the coordinated roles of *ZmARG* and other genes in regulating nitrogen metabolism in maize plants.

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