Overexpression of an *Incw2* gene in endosperm improved yield-related traits in maize

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Abstract

High yield is an eternal goal for crop breeding. Incw2 protein is the enzyme in the metabolic pathway that mobilizes photoassimilated sucrose into numerous reactions of the developing plant seeds, associated with grain yield. In the research, an *Incw2* gene driven by 27 kD zein promoter was specifically over-expressed in the endosperm cells of maize inbred line 18-599R by Agrobacterium-mediated genetic transformation. PCR assay displayed that ten of the regenerated plants were integrated with the target gene. By semi-quantitative RT-PCR and invertase activity analysis, five of them showed significantly higher expression of Incw2 transcripts and enzyme activity compared to the wild type. Among them, line 1 stood out because it possessed the highest level of Incw2 mRNA and enzyme activity. The effects of *Incw2* over-expression were reflected in the increased chlorophyll content, improved photosynthesis and delay of leaf senility. In addition, yield-related traits such as ear length, ear diameter, ear weight, grain weight per ear, and hundred-kernel weight appeared to be improved in three of the transformants compared with the wild type. The grain weight per plant of line1 was increased by nearly 10%. The results collectively indicate that it is potentially practical to enhance kernel yield of maize by overexpression of Incw2 in endosperm.

Introduction

High yield is an eternal goal for crop breeding. Photosynthesis, nitrogen assimilation, distribution of photosynthate and plant architecture are commonly accepted to be the most important physiological bases of crop yield (van Camp, 2005). Photosynthate and nutrients are unloaded into the pedicel through vascular elements prior to their entry into the basal endosperm cells and subsequent mobilization into sink organs, the upper parts of endosperm and embryo. This is an essential process for grain yield formation (Miller and Chourey, 1992). As C4 plants possess naturally higher photosynthesis, promoting the transport process is considered an efficient approach for increasing grain yield of maize (Brown, 1977; Crafts-Brandner and Salvucci, 2002).

The capacity of a sink to draw in and metabolize photoassimilates, in competition with the other organs on a plant, influnces the final size of the sink organ (Sonnewald et al, 1997). Cell wall invertase (CWI) is considered to be one of the key enzymes involved in establishing sink-strength in various sinks (Weber et al, 1998; Hirose et al, 2002). The role of CWI was also studied in transgenic carrot plants using antisense suppression technique. Transgenic plants with reduced activity of CWI could not develop taproots,

whereas antisense suppression of sucrose synthase in carrot resulted in the formation of smaller roots and leaves with leaf to root dry weight ratio unchanged (Tang et al, 1999; Tang and Sturm, 1999). Expressing a cell wall invertase gene under a meristem-specific promoter increased CWI activity and caused accelerated flowering and an increase in Arabidopsis seed yield by nearly 30% (Heyer et al, 2004). It has been demonstrated that posttranslational elevation of CWI activity by silencing its inhibitor in tomato delays leaf senescence and increases seed weight and fruit hexose level (Jin et al, 2009). These results collectively reveal that CWIs play important roles in differentiation and early development of sink organs.

Relative studies were also conducted in gramineous crops. *OsCIN1*, encoding a rice CWI and expressed preferentially in the vascular parenchyma of the dorsal vein, integument and its surrounding cells, was suggested to be important for supplying a carbon source to developing filial tissues by cleaving unloaded sucrose in the apoplast (Hirose et al, 2004). *GIF1* an *Incw2* gene in the cultivated rice required for carbon partitioning during early grain-filling, was reported to show a restricted expression pattern during grain-filling compared to the wild rice allele, probably a result of accumulated mutations in the gene's regu-

latory sequence through domestication. Fine mapping with introgression lines revealed that the wild rice *GIF1* is responsible for grain weight reduction. Ectopic expression of the cultivated GIF1 with 35S resulted in smaller grains whereas overexpression of the *GIF1* driven by its native promoter increased grain production (Wang et al, 2008).

A maize (*Zea mays* L) mutant, *miniature-1 (mn1)*, lacking CWI activity in the pedicel and endosperm, showed a marked inhibition of seed growth during early stages of development. Its final grain weight is decreased to only one-fifth of the wild type grain weight. The *mn1* locus encodes a CWI, Incw2, which is expressed exclusively in the basal part of the endosperm at the cell division stage of development. The Incw2 protein, localized in the apoplast between basal endosperm and the pedicel, is necessary for normal development of the endosperm. The authors concluded that the maintenance of a physiological gradient of sucrose between pedicel and endosperm, mediated by the Incw2 protein, is crucial for normal development of maize kernels (Miller and Chourey, 1992). In the present research, an *Incw2* gene was cloned from maize and introduced to maize genome for overexpression in endosperm. As a result, the transgenic plants showed increased grain weight, kernel yield per plant, ear length and ear diameter. This provides us an approach to improve maize yield by specifically overexpressing Incw2 in endosperm.

Materials and Methods

Cloning of Incw2 gene in maize

Genomic DNA was extracted from the leaves of maize inbred line 18-599R (Shen et al, 2012) using Plant DNA Kit (Omega) according to the manufacturer's protocol. Primers were designed according to the Incw2 sequence (GenBank Accession Number: AF050128) using Primer Premier 5 software. The sequences of the primer pairs were respectively: primer F1, 5'-GCGGGATCCAATGAGAGCCCTCG-TAGTCG-3', primer R1, 5'-GCGGAGCTCCAGGC-GCCACTATGTCATG-3'. The nucleotides with underlines donated the introduced endonuclease sites of BamHI and SacI. The PCR amplification programs were performed as follows: 94°C for 4 min; 94°C for 45 s, 56°C for 45 s, 72°C for 2 min, 35 cycles; and followed by a final step of 72°C for 8 min. The resulting products were gel-separated on 1.2% agarose and extracted (Omega), then cloned to the PMD18-T vector (TaKaRa) for sequencing. Three positive clones were subjected to sequencing.

Sequence analysis of Incw2 gene

To identify the nucleic acid sequence homology between the resultant DNA fragment and the reference Incw2 from GenBank, the sequenced fragment was compared with the reference Incw2 using Blastn in NCBI (http://blast.ncbi.nlm.nih.gov/). After removing the introns, the remaining sequence
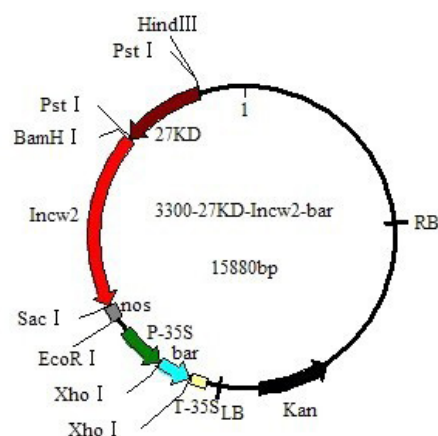

Figure 1 - Profile of endosperm-specific expression vector of maize *Incw2*.

was subjected to deducing the corresponding amino acid sequence by the ExPASy translate tool (http: au.expasy.org/tools/dna.html). The conserved amino acid domain between the two genes was predicted using Pfam program (http://pfam.sanger.ac.uk/). The deduced amino acid sequencing was then submitted to ClustalX software (http://www.ebi.ac.uk/Tools/msa/clustalw2/) for homology analysis and phylogenetic construction with other Incw2 from other plant species.

Vector construction

Plasmid p3300-27kD-bar, kindly provided by Dr JR Zhang (School of Life Science, Shandong University, China), contains the sequence of 27-kD zein promoter which is endosperm-specific promoter, and the resistant marker gene bar. After digested by endonuclease, a 2.9 kb fragment from the above PMD18-T was introduced into the BamHI and SacI sites between the 27 kD promoter and the terminator NOS of p3300-27 kD-bar, thus generating plasmid p3300-27kD-Incw2-bar (Figure 1).

Plant transformation and growth

The immature embryos (1.5 to 1.8 mm) of the maize line 18-599R were aseptically dissected from greenhouse-grown ears harvested 10 to 13 days post pollination. *Agrobacterium tumefaciens* EH105 strains was used to transform maize by the immature embryo transformation procedure (Frame et al, 2002). Embryonic calli of putative transformants were selected in N6 medium (Chu et al, 1975) containing 1.5 mg/L 2, 4-dichlorophenoxyacetic acid and 1.5-3.0 mg/L Bialapos. Then the resistant somatic embryos were transferred to MS medium (Murashige and Skoog, 1962) containing 3.0 mg/L Bialapos for plant regeneration. Bialapos-resistant seedlings with well-developed roots were transferred to pots containing vermiculite and grown under greenhouse conditions with a temperature of 25°C and a light period of 14 hours. The plants were watered with half-strength MS nutrient solution every week. The presence of the

transgenes in primary transformants (T$_0$), was verified by PCR analysis and sequencing of the amplified fragments. The expression of the transgenes in the T$_1$ transformant seeds was verified by semi-quantitative PCR and invertase activity assay. The primary transformants were allowed to self-fertilize until T$_3$ generation. Trails were performed with 14 plants per line for two lines for every of T$_3$ transgenic event. And two repeats were set by the method of randomized blocks. T$_3$ transformants were subjected to determination of photosynthetic efficiency. The agronomic traits, including plant height, ear height, number of green leaves before harvesting, ear length, ear diameter, hundred-grain weight and grain weight per ear, were investigated for T$_3$ transformants compared with the wild type. T-test was used to analyze the significance of difference on the traits between the transformants and wild type.

RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated from the immature seeds at 12 days post pollination using Trizol (Invitrogen). The cDNA synthesis was carried out using M-MLV Transcriptase (Invitrogen). A pair of primers (5'-GGGATA-CAAACGGCACAGGC-3' and 5'-TCAGGGAC-GACTTGGTAGGG-3') were designed to amplify the 0.4 kb fragment and Actin1 (GRMZM2G126010) was used as the endogenous control. Amplification conditions were as follows: one cycle of 5 min at 94°C; 30-35 cycles of 40 s at 94°C, 30 s at 59°C, and 1 min at 72°C, and one final cycle of 8 min at 72°C.

Enzyme extraction

About 0.2 g maize kernels at 12 days after pollination were homogenized in 1 ml of extraction buffer with a mortar and pestle. Extraction buffer used in the isolation of soluble and particulate protein contained 50 mM Hepes, 2 mM EDTA, 2mM EGTA, 1mM MgCl$_2$ and 1mM MnCl$_2$ (Miller and Chourey, 1992). Homogenate suspensions were centrifuged at 15,000 rpm for 3 min, and the supernatant was removed. The pellet was washed three times in extraction buffer succeeded by final resuspension in HAc-NaAc. Bound enzyme was recovered in the supernatant subsequent to centrifugation at 15,000 rpm for 10 min. Enzyme suspensions were dialyzed against extraction buffer overnight at 4°C.

Invertase activity assay

For invertase (EC 3.2.1.26) assays, 50 µl of dialyzed extracts were incubated for 30 min at 37°C in a reaction mixture containing 200 µl of 0.2 M sodium acetate, and 50 µl of 0.04 M sucrose (Tsai et al, 1970). Samples incubated in the absence of sucrose served as controls. Enzyme reactions were terminated by the addition of copper-containing arsenomolybdate solution and quantitated by spectrophotometric absorbance at 660 nm. Specific activity of invertase is reported in micromoles per liter of glucose produced per gram kernel per minute.

Photosynthesis analysis

To determine the effects on photosynthesis of *lncw2* overexpression, leaf gas-exchange (Li-6400, Li-Cor Inc, Lincoln, NE, USA) was used to identify the relationship between light-saturated net CO$_2$ assimilation rate (*A*) and the intercellular CO2 concentration (ci), as described previously (Fuentes et al, 2001). These measurements were undertaken with leaf temperature, VPD and PPFD maintained at 25°C, <1.4kP and 1600 µmol m^{-2} s^{-1}, respectively. From this *A*/c_i relationship *in vivo* estimates of the maximum carboxylation velocity of Rubisco (*V$_{c,max}$*), the maximum rate of electron transport contributing to RuBP regeneration (*J$_{max}$*) and light-saturated *A* at ambient CO$_2$ (*A$_{sat}$*) were calculated as described previously. The linear initial slope of the *A*/PPFD response was used to determine the maximum quantum efficiency of CO$_2$ assimilation (Ø$_{CO2}$) on an absorbed light basis. Leaf PPFD absorption was measured by a Taylor integrating sphere (Li-1800-12, Li-Cor Inc, Lincoln, NE, USA).

Results

Sequence characteristics of lncw2 gene

A specific 2,956 bp fragment was amplified with the specific primers, concluding 6 introns and 7 extrons. The resultant fragment shared homology of 96% in nucleotide sequence with the reference *lncw2* gene. Two main domains (48 to 373, 413 to 535) were comprised in the deduced amino acid sequence. So far, most of *lncw2* registered in Genbank were identified from maize, sorghum, *Oryza sativa*, *Oryza rufipogon* and *Brachypodium distachyon*, whereas a few members were identified from other species (Figure 2). Homology analysis indicated that the amino acid sequence shared homology ranging from 67-99% among these species. Phylogenetic analysis of *lncw2* from the seven plant species revealed several members that tend to be more closely related to those from other plant species than from the same plant species (Figure 2). For instance, the *lncw2* gene (BAB908855.1) from *Oryza sativa* showed higher homology with the one (ABB77250.1) from *Bambusa oldhamii* than that (ADE60596.1) from *Oryza sativa*. This implicates that evolution of *lncw2* occurred before the separation of these species.
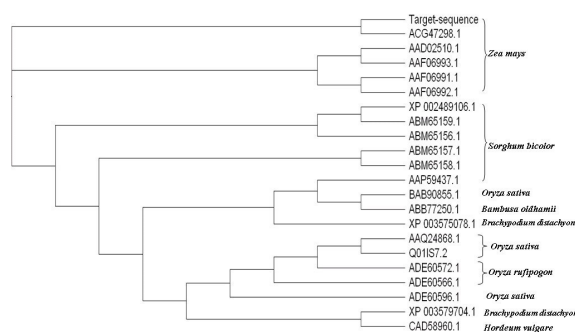

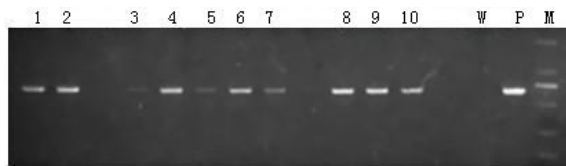Figure 2 - Phylogenetic analysis of *lncw2* from different plant species using ClustalX.

Figure 3 -Specific amplified fragments by PCR from T$_0$ trans-transformants. M: DNA marker (DL2000); P: Positive control (plasmid p3300-27kD-lncw2-bar); W: Wild type of 18-599R; 1-10: transformants.

Generation and PCR identification of T$_0$ transformants

The plasmid p3300-27kD-Incw2-bar was introduced into immature embryos by Agrobacterium-mediated transformation. Thirteen Bialapos-resistant transformants were gained. Because there was also endogenous *Incw2* gene in the genome of the wild type, PCR primers (5'-GACACGCAGAAGTA-CAGAATG-3', 5'-CGTTGGGTGGTGGATTAGT-TAG-3') was designed to specifically amplify the fragments containing partial sequences both of 27kD promoter and *Incw2* gene. As a result, ten of the transformants appeared to contain the 680 bp target gene (Figure 3). The sequences of the amplified fragments were completely consistent with the expected sequences.

Over-expression of *Incw2* in T$_1$ transgenic kernels

The above 10 transformants, as well as the wild type, were subjected to analysis of *Incw2* transcriptional level by semi-quantitative RT-PCR. The results displayed that five of them showed significantly higher expression of *Incw2* compared to the wild-type line, namely line 1, line 2, line 6, line 9 and line 10. While no distinct difference of the transcript was detected between the other transformants and the wild type. Among them line 1 stood out because it possessed the highest expression of *Incw2* and its expression level was at least 10 fold higher than that of wild type, then followed by line 10 and line 6. The plant with the lowest increase was line 9 (Figure 4).

Invertase activity was also determined in the kernels of the five T$_1$ lines. All of the lines showed higher invertase activity, ranging from 1.3-1.7 fold, as compared with the wild type. The overexpression of Incw2 observed at the RNA level correlated with elevated invertase activity (Figure 5). For instance, line 1 was observed with both the highest RNA level of Incw2 and invertase activity among the transformants. As for line 9, the lowest up-regulation in Incw2 transcript also led to the corresponding lowest enhancement in invertase activity in maize kernels. On the other
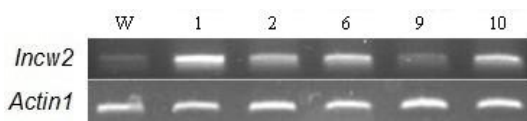

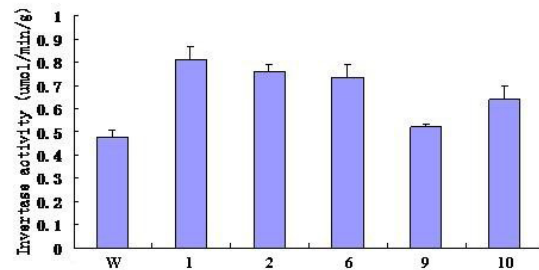Figure 4 -Over-expression of Incw2 transcripts in T1 kernels.

Figure 5 - Invertase activity of transformants.

hand, the increase ranges of invertase activity were revealed to be far less than that of Incw2 RNA. There was one main reason for this. Several kinds of invertase are present in maize kernels, and Incw2 is only one of them. The increase of Incw2 transcripts just enhanced the Incw2 activity specifically, which might contribute a little to increase of the total invertase activity.

Effect of Incw2 overexpression on photosynthesis and chlorophyll content

T$_3$ transgenic plants with the wild type were grown in the normal fertilization level. All of the five transformants showed a similar phenotype to the control plants, with no significant difference on plant height and ear height. Photosynthesis efficiency and chlorophyll content were respectively determined for the functional leaves at 11:00 in the morning at 12 days after pollination (Jain et al, 2008). The results displayed both the photosynthesis efficiency and chlorophyll content of all transformants except line 9 were higher than those of the wild type line, and line 1 showed the highest values among the transformants which was in accordance with our expectation. It is suggested that *Incw2* overexpression probably made a feedback up-regulation on leaf photosynthesis by promoting the synthesis of chlorophyll during the grain filling stage. The number of green leaves was investigated at four hours before the ears were harvested. Compared to the wild type, the line 1 and line 10 showed increased number of green leaves to different extents (Table 1). This demonstrated *Incw2* gene may also function on delaying leaf senescence.

Increased Incw2 activity resulted in improved

Figure 6 - Comparison of kernel size between transgenic line 1 and wild type.

Table 1 - Physiological indexes and agronomic traits of T_3 transformants.

Transformant or wild type	Chlorophyll content (SPAD)	Photosynthesis efficiency (μmol CO_2 m^{-2} s^{-1})	Plant height (cm)	Ear height (cm)	Green leaf harvesting number before harvesting
1	28.69±2.80*	28.38±1.09*	212±5.6	100.1±2.0	10.0±0.7*
2	27.88±2.89*	21.14±3.28*	197±6.0	96.5±3.8	8.6±0.3
6	25.66±1.36*	22.52±1.79*	201±3.5	94.7±2.5	7.8±0.4
9	24.63±2.33	17.46±2.24	207±3.9	93.2±4.5	8.3±0.5
10	25.84±1.45*	20.84±2.23*	208±5.1	101.6±5.9	9.5±0.5*
Wild type	21.44±2.01	15.19±1.35	198±4.1	96.5±2.2	7.2±0.4

* significant at the 0.05 probability levels.

yield-related traits

Because grain weight per ear and hundred-grain weight, as well as ear length, ear diameter and ear weight are all the direct factors influencing maize total yield. We determined whether the changes could be observed in field trials. The dried ears were used to investigate these traits (Table 2). There were significant increases in 100-kernel weight from 30.6 g in wild type to 34.4 g in line 1 and 32.7 g in line 2. The increases in 100-kernel weight were observed to be closely related to the increase in kernel size (Figure 6). It is interesting that the increases in ear diameter were also be detected in the two lines. The ear length of line 1 and 6 were observed to be increased by about 10% compared to that of the wild type respectively (Figure 7). As far as ear weight and grain weight per ear were concerned, only line 1 was significantly improved on this trait. It's worth noting that all of the five yield-related traits were obviously improved in transgenic line 1 (Table 2). Grain weight per ear has a direct contribution to crop yield for maize. While under unchanged densities, the improved grain weight per ear signify the increased yield per unit. As to line 1, the increase by about 9.5 % in grain weight per ear may contribute to a substantial increase in yield per unit.

Discussion

Both the increased range of *Incw2* transcripts and invertase activity were various among different transformants. It may be due to the main reason that the target gene was inserted to different loci of maize genome, which led to position effect for the expression of *Incw2* gene (Matzke et al, 1988). Another possible reason was there were different copy numbers of the target gene among different lines. The presence of a multi-copy transgene locus is considered to be a cRNA-mediated RNA degradation process and lead to transgene silencing (Stam et al, 1997). It is suggested that the transgene was introduced to a gene silencing region and failed to transcribe to mRNA for the other lines in our research. We propose that only a little increase of the mRNA in line 9 and 10 just accounted for no improvement of the five yield-related traits for the line. As for line 1, it possessed the most Incw2 transcripts and the highest invertase activity, as well as the most excellent ear-related traits. This is consistent with our expectation. However the reasons that there were not perfect increasing correlations among the mRNA transcript, invertase activity, photosynthesis efficiency and yield for most of the lines are still unclear at present.

Sucrose is the principal and preferred form of photosynthate for long-distance transport to terminal storage sink tissue, such as the developing seed. Invertase is the first enzyme in the metabolic pathway that mobilizes photoassimilated sucrose into numerous reactions of the developing maize seed (Jain et al, 2008). Incw2 protein, localized in the apoplast between basal endosperm and the pedicel, is necessary for normal development of the endosperm (Hirose et al, 2002). A maize mutant, *miniature-1*, lacking Incw2 activity in the pedicel and endosperm, showed a marked inhibition of seed growth during early stages of development (Miller and Chourey, 1992). In the

Table 2 - Grain yield-related traits of T3 transformants.

Transformant or wild type	Ear length (mm)	Ear diameter (mm)	Ear weight (g)	Grain weight per ear (g)	Hundred kernel weight (g)
1	119.4±0.9*	50.3±0.7*	112.0±1.5*	75.0±0.6*	34.4±0.4*
2	105.1±1.2	50.2±0.5*	101.5±2.7	69.0±0.9	32.7±0.9*
6	118.8±0.8*	47.6±0.9	108.7±2.4	70.3±1.2	32.5±1.0
9	108.9±0.6	48.3±0.8	103.4±4.8	68.8±0.5	30.8±1.0
10	109.1±1.5	48.9±1.2	103.6±3.3	67.7±1.4	30.1±0.5
Wild type	108.4±1.1	48.0±0.9	104.5±3.8	68.5±1.8	30.6±0.2

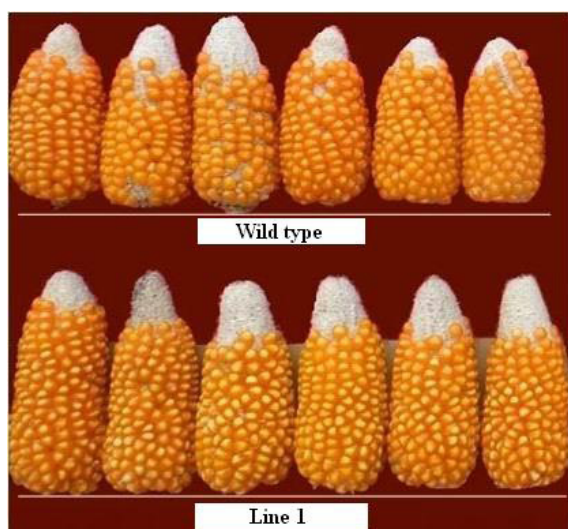* significant at the 0.05 probability levels.

Figure 7 - Comparison of ear length and ear diameter between line 1 and wild type.

present research, an *Incw2* gene driven by a 27 kD zein promoter was over-expressed in maize endosperm cell specifically. The results displayed over-expression of the gene improved grain weight and kernel size to a certain degree, which indicated the abundant invertase promoted the transport of more photosynthate to maize kernel at seed filling stage. On the other hand, we observed improved photosynthesis efficiency at 12 days after pollination as well as the increased green leaf number before harvesting, indicating that the enhanced export of photosynthate from vegetative organ can promote its photosynthesis at the reproductive stage and delayed leaf senescence. The finding is consistent with the conclusion drown by Jin et al in the research on tomato (Jin et al, 2009). While it is suggested that the significant increases in ear length and ear diameter were possibly resulted from the improved photosynthesis.

As a C4 plant, maize possesses higher photosynthetic rates than many other crops (Brown, 1977), so promoting the transport of photosynthate is believed to be an efficient approach for increasing grain yield of maize. Previous researches only demonstrated mutation of *Incw2* gene would lead to the decrease of grain weight and starch content of maize (Miller and Chourey, 1992). However no reports showed expression of the gene in wild type could result in the increase of maize kernel yield. Here, we provided a new thread to raise the grain yield of maize, and created three transgenic lines with fair application value, for which at least one yield-related trait was improved. Among them line 1 stood out because all of the traits were significantly improved in it. We will aim the next study at conducting yield evolution over years and sites, and introducing *Incw2* gene to other elite maize inbred lines by back cross breeding subsequently, identifying the effects on improving yield with more certainty.

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