Novel Genetic Selection System for Quantitative Trait Loci of Quality Protein Maize

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Running Title: QPM conversion

Keywords: Dominance, Maize, RNAi, GFP, High-lysine
ABSTRACT

Quality Protein Maize combines a high-lysine trait with kernel hardness, for which a new simpler genetic selection was designed.

INTRODUCTION, RESULTS, AND DISCUSSION

Corn seeds are used as food for humans and feed for livestock. However, essential amino acids like lysine and tryptophan are deficient because of the abundance of major storage proteins, called zeins. As a consequence, in countries where corn is consumed as the primary or sole protein source, malnutrition is common (Osborne and Mendel 1914). In those countries, demand for high-lysine corn is on the rise. Actually, high-lysine corn has been known, where the transcriptional activator of the 22-kDa α-zein genes Opaque 2 (O2) is mutated (Schmidt et al. 1992) and non-zein proteins are proportionally increased, resulting in twice the lysine content than normal maize (Mertz et al. 1964; Mertz et al. 1965). However, this o2 mutant has several drawbacks, like reduced grain yield, soft chalky endosperm, greater susceptibility to pests and diseases, and higher moisture content at harvest time, which precluded its direct commercialization. Fortunately, quantitative trait loci (QTL), referred to as o2 modifiers (Mo2s), exist that convert soft into hard endosperm without loosing the high lysine trait; this combination is called quality protein maize (QPM) (Vasal et al. 1980). Interestingly, transcript and protein of 27-kDa γ-zein accumulate 2- to 3-fold higher in QPM than normal inbreds and unmodified o2 mutant (Geetha et al. 1991; Holding et al. 2008) and silencing 27- and 16-kDa γ-zein genes resulted in clumping of protein bodies and opacity of QPM seeds, demonstrating hypostasis of γ-zeins over o2 modifiers (Wu et al. 2010).
QPM has been introduced to many developing countries in South America, Africa, and Asia (Prasanna et al. 2001). However, conversion of QPM into local germplasm is a lengthy process that discourages the spread of the benefits of QPM, because breeders had to monitor high lysine level, the recessive o2 mutant allele and the Mo2s. Here, we present a simpler and accelerated QPM selection. Instead of using the recessive o2 mutation, we use an RNAi construct directed against both 22- and 19-kDa zeins, but linked to the visible green fluorescent protein (GFP) marker gene. Indeed, when such green and non-vitreous phenotype was crossed with QPM lines, the Mo2s produced a vitreous green kernel, demonstrating that high-lysine and kernel hardness can be selected in a dominant fashion.

**Visible marker for α-zein reduction:** The transgenic high-lysine corn was reported to be generated by endosperm-specific silencing of lysine catabolism (Houmard et al. 2007) or accumulation of alpha zeins with RNAi (Huang et al. 2006; Segal et al. 2003), with the latter showing similar phenotype to o2 mutant. We therefore chose inverted repeats of members of each α-zein class to assemble a double RNAi construct. However, if Mo2s restore vitreousness of the kernel, we could not use the opaque phenotype of the RNAi construct for selection. Therefore, we placed a chimeric GFP gene under the control of the CaMV 35S promoter, immediately upstream of the double RNAi cassette, resulting in pTF102-P6z1RNAi (abbreviated as P6z1RNAi) (Fig. 1A). The hybrid of B73 x Mo17 was pollinated with T0 transgenic pollen to produce T1 seeds for genetic analysis. Six normal and “green” immature kernels were sorted and zein proteins extracted. Indeed, the 22- and 19-kDa α-zeins were dramatically reduced in the six green kernels compared to the six normal kernels (Fig. 1B). At maturity, the vitreous
and non-green kernels were 216, while the *opaque* and green kernels were 222, exhibiting a ratio of 1:1 segregation (Fig. 1C).

**Dominance of Mo2s:** A hybrid of B73 x Mo17 with *P6z1RNAi* was used to pollinate QPM lines Pool 41 and 42. While half of the kernels from (B73 x Mo17) x *P6z1RNAi/+* were *opaque*, most progenies from Pool 41 x *P6z1RNAi/+* and Pool 42 x *P6z1RNAi/+* had a vitreous phenotype (Fig. 2A) because the Mo2s overcame the action of the RNAi, which was monitored with GFP (Fig. 2B). Control kernels of B73 x Mo17 were vitreous and non-fluorescent. As expected, the green and vitreous seeds from Pool 42 x *P6z1RNAi/+* with reduced accumulation of α-zeins at maturity (Fig. 2C) contained an increased lysine level of more than 25% over a control (Table 1), exceeding the 18.5% achieved previously with RNAi against the 22-kDa zeins only (Segal et al. 2003).

**QPM conversion:** There are two main steps in conversion of QPM into local germplasm wildtype (LG+) by traditional means. First, one crosses LG+ with the o2 mutant, then backcrosses with LG+ for 5 generations and selfcrosses for 3 generations to obtain a homozygous LG o2 mutant (LGo2). Because the o2 allele is recessive, one cannot use the *opaque* phenotype as a selective marker during backcrosses and has to follow the allele by selfing or with a molecular marker. After introgression of o2, the LGo2 is used to pollinate a QPM donor line. The resultant progenies are backcrossed again with LGo2 for 5 generations and then selfcrossed for at least 3 generations to obtain a modified LG o2 mutant (LGMo2) (Fig. 3). This lengthy and laborious method would take at least 5 years and 8 months. In contrast, the QPM-RNAi (Fig. 2B) can be directly pollinated with LG+, simply selecting green and vitreous progenies, which can easily be followed during recurring backcrosses with LG+. After five backcrosses
generations, resultant progenies are selfcrossed for 3 generations to generate LG(RNAi)-QPM. During the entire process, green and vitreous kernels are selected in each generation, which could save 2 years and 8 months (Fig. 3). Even, if one would like to create LG without any transgene by replacing it with the o2 mutation, introgression of Mo2s would still be faster. The P6z1RNAi transgene could be segregated and the o2 allele introduced. By crossing QPM-RNAi/+-BC5 (Mo2s/+; RNAi/+; +/++) and o2/+/-BC5 (+/+; +/+; o2/+), half of the green and vitreous kernels should carry the o2 allele, which could be screened by the specific o2 allele markers; then the vitreous and green progenies with genotype Mo2s/+; RNAi/+; o2/+ are selected and selfcrossed for 3 generations to generate LGmo2 without the P6z1RNAi transgene, still saving more than 2 years.

**Conclusions:** The dominance of Mo2s over an RNAi phenotype has been used to accelerate backcrossing a multigenic trait into maize cultivars for enhanced nutrition.

The research described in this manuscript was supported by the Selman A. Waksman Chair in Molecular Genetics.

**Figure Legends:**

Figure 1: Silencing of the 22- and 19-kDa α-zeins linked with GFP marker. (A) pTF102-P6z1RNAi construct structure. The Z22/6 gene was used for the 22-kDa and z1A2 gene for the 19-kDa inverted repeat (SONG and MESSING 2002; SONG and MESSING 2003). The binary vector pTF102 was used for all intermediate construct building and Agrobacterium-mediated transformation (FRAME et al. 2002). (B) SDS-PAGE analysis of zein accumulation in progenies from (B73 x Mo17) x P6z1RNAi. Six normal and “green”
kernels at 18 DAP were selected with a fluorescence dissection microscope (Leica MZ16 F) for zein extraction. Total zein loaded in each lane was equal to 500 mg of fresh endosperm at 18 DAP. Protein markers (M) from top to bottom are 37, 25, 20, 15, and 10 kDa. (C) Segregation of vitreous and opaque kernels scored with a light box.

Figure 2: Modification of RNAi mutant with Mo2s. (A) Ear phenotypes. The opaque and vitreous kernels from the cross of (B73 x Mo17) x P6z1RNAi/+ (Up) showed a ratio of 1:1 segregation. Two representative kernels with opaque and vitreous phenotypes were indicated by arrow and arrowhead, respectively; Most progenies from Pool 42 x P6z1RNAi/+ (Middle) and Pool 41 x P6z1RNAi/+ (Bottom) showed vitreous phenotype; (B) Kernels were truncated for observation under a fluorescence dissection microscope (Upper) and natural light (Lower) in each panel. (Up panel) B73 x Mo17 control; (Middle panel) Green and opaque kernels from (B73 x Mo17) x P6z1RNAi/++; (Bottom panel) Green and vitreous kernels from Pool42 x P6z1RNAi/++; (C) α-zein accumulation pattern in mature seeds. Lanes 1-3, B73 x Mo17; lanes 4-6, green and opaque kernels from (B73 x Mo17) x P6z1RNAi/++; lane 7-9, green and vitreous kernels from Pool42 x P6z1RNAi/+. Protein markers (M) from top to bottom are 25, 20 and 15 kDa.

Figure 3: Comparison of traditional and accelerated methods for QPM conversion. LG+, local germplasm wildtype; LGo2, LG o2 mutant; Mo2s, o2 modifiers; LGMo2, modified LGo2, or LG-QPM; LG(RNAi)-QPM, modified LG with P6z1RNAi transgene;

Table Legends

Table 1. Amino acid composition analysis in ground corn meal


<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>B73 x Mo17</th>
<th>Pool 42 x P6z1RNAi/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>0.30%</td>
<td>0.38%</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.73%</td>
<td>0.48%</td>
</tr>
<tr>
<td>Leucine</td>
<td>2.01%</td>
<td>0.71%</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.41%</td>
<td>0.27%</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.36%</td>
<td>0.32%</td>
</tr>
<tr>
<td>Valine</td>
<td>0.44%</td>
<td>0.40%</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.35%</td>
<td>0.29%</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.55%</td>
<td>0.49%</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.43%</td>
<td>0.40%</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>0.63%</td>
<td>0.79%</td>
</tr>
<tr>
<td>Serine</td>
<td>0.56%</td>
<td>0.37%</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>2.68%</td>
<td>1.56%</td>
</tr>
<tr>
<td>Proline</td>
<td>1.61%</td>
<td>0.85%</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>0.04%</td>
<td>0.03%</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.88%</td>
<td>0.55%</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.59%</td>
<td>0.31%</td>
</tr>
<tr>
<td>Total ground kernels (g)</td>
<td>25.28</td>
<td>25.77</td>
</tr>
</tbody>
</table>

Samples were ground meal of bulked mature kernels from B73 x Mo17, and mature green and vitreous kernels from Pool 42 x P6z1RNAi/+.

*Amino acid levels were expressed as the percentage of total corn meal.*
Figure 1
Figure 2

A) (B73 x Mo17) x P6z1RNAi/+  
B) Control B73 x Mo17  
C) QPM (Pool 42) x P6z1RNAi/+
**Figure 3**

**Traditional**
- LG+ x o2/o2
  - F1 (4 mons)
  - BC5 to LG+, o2/+ - BC5 (20 mons)
  - S2 (8 mons)
- QPM (Mo2s/Mo2s) x LGo2
  - F1 (4 mons)
  - BC5 to LGo2 (20 mons)
  - S3 (12 mons)
- LGMo2 or LG-QPM (Total 68 mons)

**Track 1**
- QPM-RNAi/+ - x LG+
  - F1 (4 mons)
  - BC5 to LG+, QPM-RNAi/+ - BC5 (20 mons)
  - S3 (12 mons)
  - LG(RNAi)-QPM (Total 36 mons)

Saves 2 years and 8 months!

**Track 2**
- QPM-RNAi/+ - BC5 (Mo2s/+; RNAi/+; +/+)
  - o2/+ - BC5 (+/+; +/+; o2/+)(24 mons)
  - Mo2s/+; RNAi/+; +/+, discarded (4 mons)
  - Mo2s/+; RNAi/+; o2/+, selected
- S3 (12 mons)
- LGMo2 or LG-QPM (Mo2s/Mo2s; +/+; o2/o2)
  - (Total 40 mons)
  - Saves 2 years and 4 months!

Saves 2 years and 8 months!