

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

Yongrui Wu and Joachim Messing¹

Waksman Institute of Microbiology, Rutgers University, Piscataway, New Jersey 08854

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

CORN seeds are used as food for humans and as 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 reported in which the transcriptional activator of the 22-kDa α -zein gene Opaque 2 (O2) is mutated (Schmidt *et al.* 1992) and non-zein proteins are proportionally increased, resulting in twice the lysine content than in normal maize (Mertz *et al.* 1964, 1965). However, this *o2* mutant has several drawbacks, such as 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, there are quantitative trait loci, referred to as *o2* modifiers (*Mo2s*), that convert soft into hard endosperm without losing 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 two- to threefold higher in QPM than in normal inbreds and in unmodified *o2* mutants (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 have to monitor a 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 RNA interference (RNAi) construct directed against both 22- and 19-kDa zeins, but linked to the visible green fluorescent protein (GFP) marker gene. Indeed, when such a 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 α -zeins with RNAi (Segal *et al.* 2003; Huang *et al.* 2006), with the latter showing a 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 cauliflower mosaic virus 35S promoter, immediately upstream of the double RNAi cassette, resulting in pTF102-P6z1RNAi (abbreviated as P6z1RNAi) (Figure 1A). The hybrid of B73 \times 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 were extracted. Indeed, the 22- and 19-kDa α -zeins were dramatically reduced in the six green kernels compared to the six normal

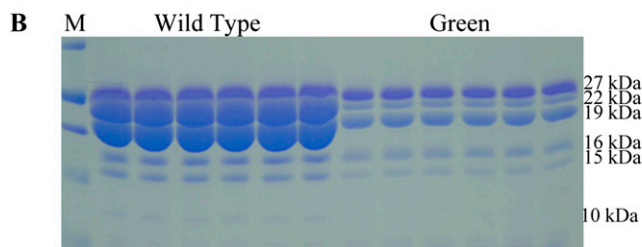
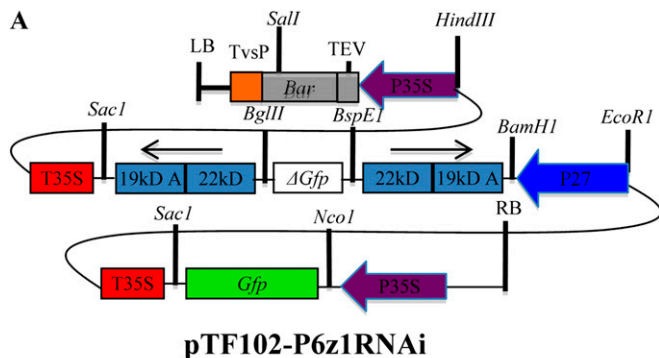


Figure 1 Silencing of the 22- and 19-kDa α -zeins linked with the GFP marker. (A) pTF102-P6z1RNAi construct structure. The *Zp22/6* gene was used for the 22-kDa and *z1A2* gene for the 19-kDa inverted repeat (Song and Messing 2002, 2003). The binary vector pTF102 was used for all intermediate construct building and *Agrobacterium*-mediated transformation (Frame *et al.* 2002). (B) SDS-PAGE analysis of zein accumulation in progeny from (B73 \times Mo17) \times P6z1RNAi/+. Six normal and "green" kernels at 18 days after pollination (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.

kernels (Figure 1B). At maturity, the vitreous and nongreen kernels were 216, while the opaque and green kernels were 222, exhibiting a ratio of 1:1 segregation (Figure 1C).

Dominance of Mo2s

A hybrid of B73 \times Mo17 with P6z1RNAi was used to pollinate the QPM lines pool 41 and 42. While half of the kernels from (B73 \times Mo17) \times P6z1RNAi/+ were opaque, most progeny from pool 41 \times P6z1RNAi/+ and pool 42 \times P6z1RNAi/+

Table 1 Amino acid composition analysis in ground cornmeal

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

Samples were ground cornmeal of bulked mature kernels from B73 \times Mo17 and mature green and vitreous kernels from pool 42 \times P6z1RNAi/+.

^aAmino acid levels were expressed as the percentage of total cornmeal.

had a vitreous phenotype (Figure 2A) because the Mo2s overcame the action of the RNAi, which was monitored with GFP (Figure 2B). Control kernels of B73 \times Mo17 were vitreous and nonfluorescent. As expected, the green and vitreous seeds from pool 42 \times P6z1RNAi/+ with reduced accumulation of α -zeins at maturity (Figure 2C) contained an increased lysine level of >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 wild type (LG+) by traditional means. First, one crosses LG+ with the *o2* mutant and then backcrosses with LG+ for five generations and self-crosses for three 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 progeny are backcrossed again with LGo2 for five generations and then self-crossed for at least three generations to obtain a modified LG *o2* mutant (LGMo2) (Figure 3). This lengthy and laborious method would take at least 5 years and 8 months. In contrast, the QPM-RNAi (Figure 2B) can be directly pollinated with LG+, simply selecting green and vitreous progeny, which can easily be followed during recurring backcrosses with LG+. After five backcross generations, resultant progeny are self-crossed for three 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 (Figure 3). Even if one would like to create LG

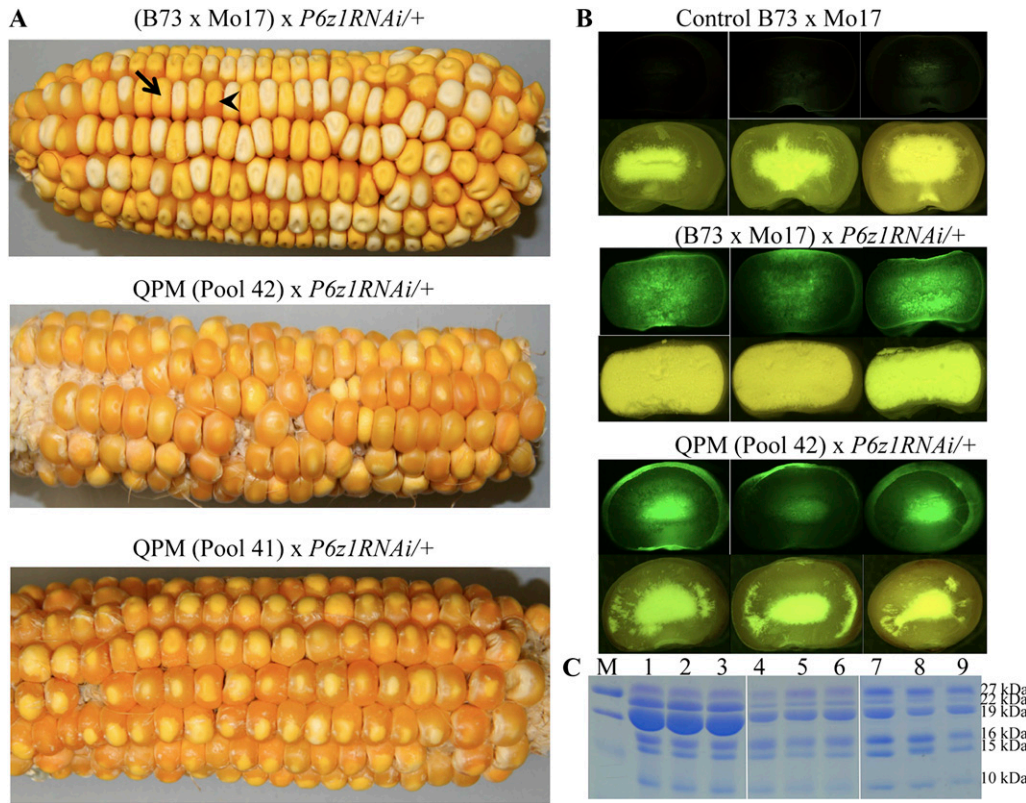


Figure 2 Modification of RNAi mutant with *Mo2s*. (A) Ear phenotypes. The opaque and vitreous kernels from the cross of (B73 × Mo17) × *P6z1RNAi/+* (Top) showed a ratio of 1:1 segregation. Two representative kernels with opaque and vitreous phenotypes are indicated by arrow and arrow-head, respectively;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

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 progeny with genotype *Mo2s/+; RNAi/+; o2/+* are selected and self-crossed for three generations to generate LG*Mo2* without the *P6z1RNAi* transgene, still saving >2 years.

Conclusion

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

Acknowledgments

The research described in this article was supported by the Selman A. Waksman Chair in Molecular Genetics at Rutgers University.

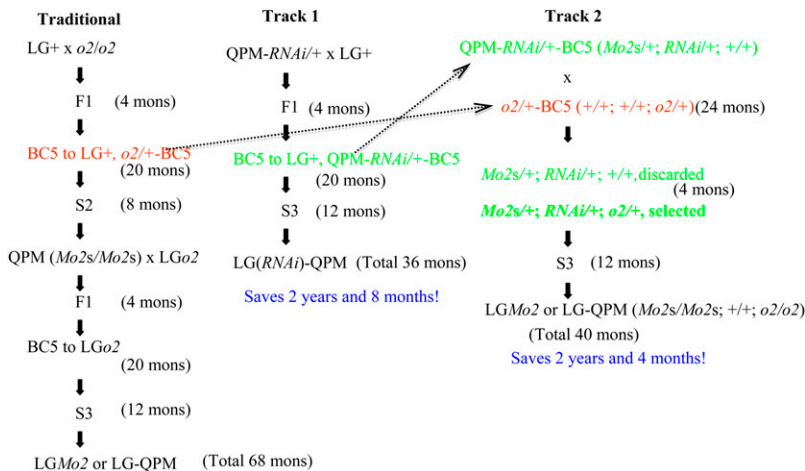


Figure 3 Comparison of traditional and accelerated methods for QPM conversion. LG+, local germplasm wild type; LGo2, LG *o2* mutant; *Mo2s*, *o2* modifiers; LG*Mo2*, modified LGo2 or LG-QPM; and LG(*RNAi*)-QPM, modified LG with *P6z1RNAi* transgene;

Literature Cited

- Frame, B. R., H. Shou, R. K. Chikwamba, Z. Zhang, C. Xiang, *et al.*, 2002 *Agrobacterium tumefaciens*-mediated transformation of maize embryos using a standard binary vector system. *Plant Physiol.* 129: 13–22.
- Geetha, K. B., C. R. Lending, M. A. Lopes, J. C. Wallace, and B. A. Larkins, 1991 *opaque-2* modifiers increase γ -zein synthesis and alter its spatial distribution in maize endosperm. *Plant Cell* 3: 1207–1219.
- Holding, D. R., B. G. Hunter, T. Chung, B. C. Gibbon, C. F. Ford, *et al.*, 2008 Genetic analysis of *opaque2* modifier loci in quality protein maize. *Theor. Appl. Genet.* 117: 157–170.
- Houmard, N. M., J. L. Mainville, C. P. Bonin, S. Huang, M. H. Luethy, *et al.*, 2007 High-lysine corn generated by endosperm-specific suppression of lysine catabolism using RNAi. *Plant Biotechnol. J.* 5: 605–614.
- Huang, S., A. Frizzi, C. A. Florida, D. E. Kruger, and M. H. Luethy, 2006 High lysine and high tryptophan transgenic maize resulting from the reduction of both 19- and 22-kD alpha-zeins. *Plant Mol. Biol.* 61: 525–535.
- Mertz, E. T., L. S. Bates, and O. E. Nelson, 1964 Mutant gene that changes protein composition and increases lysine content of maize endosperm. *Science* 145: 279–280.
- Mertz, E. T., O. A. Veron, L. S. Bates, and O. E. Nelson, 1965 Growth of rats fed on opaque-2 maize. *Science* 148: 1741–1742.
- Osborne, T. B., and L. B. Mendel, 1914 Nutritional properties of proteins of the maize kernel. *J. Biol. Chem.* 18: 1–16.
- Prasanna, B. M., S. K. Vasal, B. Kassahun, and N. N. Singh, 2001 Quality protein maize. *Curr. Sci.* 81(10): 1308–1319.
- Schmidt, R. J., M. Ketudat, M. J. Aukerman, and G. Hoschek, 1992 Opaque-2 is a transcriptional activator that recognizes a specific target site in 22-kD zein genes. *Plant Cell* 4: 689–700.
- Segal, G., R. Song, and J. Messing, 2003 A new opaque variant of maize by a single dominant RNA-interference-inducing transgene. *Genetics* 165: 387–397.
- Song, R., and J. Messing, 2002 Contiguous genomic DNA sequence comprising the 19-kD zein gene family from maize. *Plant Physiol.* 130: 1626–1635.
- Song, R., and J. Messing, 2003 Gene expression of a gene family in maize based on noncollinear haplotypes. *Proc. Natl. Acad. Sci. USA* 100: 9055–9060.
- Vasal, S. K., E. Villegas, M. Bjarnason, B. Gelaw, and P. Goertz, 1980 Genetic Modifiers and Breeding Strategies in Developing Hard Endosperm opaque-2 Materials, pp. 37–73 in *Improvement of Quality Traits of Maize for Grain and Silage Use*, edited by W. G. Pollmer and R. H. Phipps. Martinus Nijhoff, London.
- Wu, Y., D. R. Holding, and J. Messing, 2010 γ -Zeins are essential for endosperm modification in quality protein maize. *Proc. Natl. Acad. Sci. USA* 107: 12810–12815.

Communicating editor: M. Kirst