Note

Rescue of a Dominant Mutant With RNA Interference

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ABSTRACT

Maize *Mucronate1* is a dominant *floury* mutant based on a misfolded 16-kDa γ -zein protein. To prove its function, we applied RNA interference (RNAi) as a dominant suppressor of the mutant seed phenotype. A γ -zein RNAi transgene was able to rescue the mutation and restore normal seed phenotype. RNA interference prevents gene expression. In most cases, this is used to study gene function by creating a new phenotype. Here, we use it for the opposite purpose. We use it to reverse the creation of a mutant phenotype by restoring the normal phenotype. In the case of the maize *Mucronate1* (*Mc1*) phenotype, interaction of a misfolded protein with other proteins is believed to be the basis for the *Mc1* phenotype. If no misfolded protein is present, we can reverse the mutant to the normal phenotype. One can envision using this approach to study complex traits and in gene therapy.

TRANSLUCENT or vitreous maize kernels are harder and able to sustain stronger mechanical strength during harvesting, transportation, and storage. There is a direct link between a vitreous seed phenotype and the type of storage proteins in the seed, collectively called zeins in maize. Zeins, encoded by a multigene family, constitute >60% of all maize seed proteins. They are classified into four groups (α -, β -, γ -, and δ -zein) on the basis of their structures (ESEN 1987). Zeins are specifically synthesized in the endosperm ~10 days after pollination (DAP) and deposited into protein bodies (WOLF *et al.* 1967; BURR and BURR 1976; LENDING and LARKINS 1992). Irregularly shaped protein bodies are found in *floury* or *opaque* kernel phenotypes (COLEMAN *et al.* 1997; KIM *et al.* 2004, 2006; WU *et al.* 2010; WU and MESSING 2010). The terms “*floury*” and “*opaque*” were originally created on the basis of the genetic behaviors of the mutant allele causing the soft kernel texture. The *floury* mutants behave as semidominant or dominant mutants, as *floury1* and *floury2* do, while the *opaque* mutants are recessive, as *opaque1* and *opaque2* are (HAYES and EAST 1915; LINDSTROM 1923; EMERSON *et al.* 1935; MAIZE GENETICS COOPERATION 1939). Similar to *floury2* with a single mutation in the signal peptide of a 22-kDa α -zein resulting in an unprocessed protein (COLEMAN

et al. 1995), *De*-B30* produces an unprocessed 19-kDa α -zein (KIM *et al.* 2004). It was hypothesized that the two mutant proteins with an unprocessed signal peptide are misfolded and docked in the membranes of the rough endoplasmic reticulum (RER), blocking the deposition of other zein proteins (COLEMAN *et al.* 1995; KIM *et al.* 2004). In *Mucronate1* (*Mc1*), a 38-bp deletion in the C terminus of the 16-kDa γ -zein (γ 16-zein) gene resulted in a frameshift and a protein with a different amino-acid tail. This modified 16-kDa γ -zein ($\Delta\gamma$ 16-zein) has altered solubility properties, which would explain the formation of irregular protein bodies. Because *De*-B30* and *Mc1* are semidominant and dominant, respectively, they belong to the *floury* mutant class.

The γ -zein genes (γ 27-zein and γ 16-zein) are homologous copies because maize underwent allotetraploidization and both gene copies have been retained during diploidization (XU and MESSING 2008). The two γ -zeins and the 15-kDa β -zein have a redundant function in stabilizing protein-body formation (WU and MESSING 2010). Knockdown of both γ -zeins with a single RNA interference (RNAi) construct conditioned only partial opacity in the crown, the top of the kernel, as opposed to the remainder or gown area of the kernel. Consistent with its light kernel phenotype, protein bodies in such a γ -zein RNAi (γ RNAi) mutant exhibited a slight alteration in morphology. This phenotype is clearly distinguishable from the *Mc1* phenotype, which is far more severe. Therefore, if *Mc1* is caused by a misfolded chimeric 16-kDa γ -zein, preventing its expression should restore normal kernel phenotype. Indeed, a

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FIGURE 1.—Segregation analysis of the accumulations of mRNAs and proteins from the cross of the *Mc1* mutant and the γ RNAi line by RT-PCR and SDS-PAGE. (A) γ RNAi gene segregation from progeny ($\Delta\gamma 16/\Delta\gamma 16 \times \gamma$ RNAi/+) by PCR amplification with a specific primer set (GFPE, ACAACCACTACCTGAGCAC and T35SHindIII, ATTAAGCTTTGCA GGTCAGTGGATTTTGG). Kernels 3, 8, 9, 10, and 12 are positive for the γ RNAi gene and the rest of them are negative. M, DNA markers from top to bottom band are 3, 2, 1.5, 1.4, and 1 kb. (B) RT-PCR analysis of mRNA accumulation from the normal $\gamma 16$ and mutant $\Delta\gamma 16$ alleles in the endosperms with the genotypes corresponding to the embryos analyzed above. Total RNA was extracted by using TRIzol reagent (Invitrogen). Two micrograms of RNA was digested with DNase I (Invitrogen) and then reverse-transcribed. Twenty-five nanograms of cDNA from each of the twelve endosperms was applied for PCR (25 cycles of 30 sec, 94 °C; 30 sec, 58 °C; and 1 min, 72 °C). A specific primer set ($\gamma 16$ F, ATGAAGGTGCTGATCGT TGC and $\gamma 16$ R, TCAGTAGTAGACACCG CCG) was designed for amplification of the full-length $\gamma 16$ -zein coding sequence (552 bp). The lower band (514 bp) from the mutant $\Delta\gamma 16$ allele is 38 bp shorter than that from

the normal allele (552 bp). Kernels 3, 8, 9, 10, and 12 with the γ RNAi gene accumulated significantly less mRNA compared to those without the γ RNAi gene (kernels 1, 2, 4, 5, 6, 7, and 11). BA, hybrid of B $\times$ A lines. M, DNA markers from top to bottom are 1 kb, 750 bp, and 500 bp. (C) Profile of zein accumulations of 20 kernels from the progeny as described in the text. The zein extraction method has been described elsewhere (Wu *et al.* 2009). The $\Delta\gamma 16$ -zein from *Mc1* was not extracted by traditional total-zein extraction protocol (70% ethanol and 2% 2-mercaptoethanol). The $\gamma 27$ - and $\gamma 16$ -zeins were knocked down to a nondetectable level in kernels 1, 2, 3, 5, 7, 10, 12, 13, 16, and 20. In γ RNAi-gene segregating progeny (kernels 4, 6, 8, 9, 11, 14, 15, 17, 18, and 19), the $\gamma 16$ -zein from the normal $\gamma 16$ allele is marked by arrowheads. Protein loaded in each lane was equal to 500 μ g fresh endosperm at 18 DAP. The size for each band is indicated by the numbers in the "kDa" columns. BA, hybrid of B $\times$ A lines; 1–20, kernels from the progeny described above; M, protein markers from top to bottom are 50, 25, 20, and 15 kDa.

simple cross of *Mc1* with a maize line carrying the γ RNAi transgene produced a non-floury phenotype, providing an example of RNAi as a dominant suppressor of a dominant phenotype and as a general tool in marker rescue.

Analysis of the progeny from the cross of *Mc1* and γ RNAi mutants: *Mc1* seeds (Stock ID U840I) were requested from the Maize Genetics Cooperation Stock Center. The γ RNAi transgenic lines have been reported in previous work (Wu *et al.* 2010; Wu and MESSING 2010). Twelve progeny kernels from the cross of the *Mc1* mutant [homozygous for the dominant-negative mutant 16-kDa γ -zein alleles ($\Delta\gamma 16/\Delta\gamma 16$) and heterozygous for the γ RNAi line (γ RNAi/+)] were dissected at 18 DAP for segregation and mRNA accumulation analyses. For each kernel, the embryo and endosperm were separated for DNA and RNA extraction, respectively. As shown in Figure 1A, five and seven kernels were positive and negative for the amplification of the γ RNAi gene with a specific primer set, exemplifying a 1:1 segregation of the γ RNAi gene.

Due to the 38-bp deletion in the C terminus of the coding region, the $\Delta\gamma 16$ allele is shorter than the

normal one (Figure 1B). Therefore, most of $\Delta\gamma 16$ -zein was in the non-zein fraction. In progeny endosperms of another 20 kernels from the same cross described above segregating for the γ RNAi gene, two types of $\gamma 16$ -zeins were synthesized: the normal $\gamma 16$ -zein in the ethanol-soluble zein fraction and the $\Delta\gamma 16$ -zein in the non-zein fraction. In progeny inheriting the γ RNAi gene, the $\gamma 27$ - and $\gamma 16$ -zeins were reduced to nondetectable levels (Figure 1C). Although the $\Delta\gamma 16$ -zein is not in the ethanol-soluble zein fraction, the level of normal $\gamma 16$ -zein is a good indicator of the accumulation of the $\Delta\gamma 16$ -zein.

Rescue of protein-body morphologies in the *Mc1* mutant: Regular protein bodies are round with distinct membrane boundaries (Figure 2A) and 1–2 μ m in diameter at maturity. In homozygous and heterozygous *Mc1* mutants ($\Delta\gamma 16/\Delta\gamma 16$ and $\Delta\gamma 16/+$), protein bodies were irregularly shaped, some without discrete boundaries (Figure 2, C and D), which is quite different from the absence of normal $\gamma 27$ - or $\gamma 16$ -zeins in maize endosperm (Figure 2B). Indeed, protein bodies of the *Mc1* mutant, blocked in the accumulation of $\Delta\gamma 16$ -zein, showed morphologies with no discernible difference from those in the γ RNAi/+ line (Figure 2, B and E).

FIGURE 2.—Transmission electron micrographs of protein bodies. The method has been described elsewhere (Wu and MESSING 2010). (A) Nontransgenic BA. (B) $\gamma RNAi$ transgenic line ($\gamma RNAi/+$). (C) Mcl ($\Delta\gamma 16/\Delta\gamma 16$). (D) Cross of Mcl mutant and nontransgenic hybrid of B $\times$ A lines ($\Delta\gamma 16/+$). (E) Cross of Mcl mutant ($\Delta\gamma 16/\Delta\gamma 16$) and heterologous $\gamma RNAi$ transgenic line ($\gamma RNAi/+$). PB, protein body; RER, rough endoplasmic reticulum; CW, cell wall; Mt, mitochondria; SG, starch granule. Bars, 500 nm.

Recovery of *floury* phenotype in progeny: On the basis of these observations, it is reasoned that irregularly shaped protein bodies (Figure 2, C and D) in the Mcl mutant cause the *floury* phenotype (Figure 3, A and B).

Because knockdown of γ -zeins caused opacity only in the crown area (Figure 3C), one could envision that once the irregular protein bodies are restored, the kernel would become vitreous in the crown area of the

FIGURE 3.—Segregation of vitreous and *floury* kernels from a progeny ear. (A) Mcl mutant with $\Delta\gamma 16/\Delta\gamma 16$ genotype. (B) The cross of the Mcl mutant and the nontransgenic hybrid of B $\times$ A lines, showing *floury* phenotype as in A. (C) $\gamma RNAi$ transgenic line with partial opacity only in the crown area. (D) The cross of the Mcl mutant ($\Delta\gamma 16/\Delta\gamma 16$) and the heterologous $\gamma RNAi$ transgenic line ($\gamma RNAi/+$), showing a 1:1 ratio of vitreous and *floury* kernels. A row in the ear is marked with arrowheads and crosses to indicate vitreous and *floury* gowns of kernels. (E) Cross of the Mcl mutant ($\Delta\gamma 16/\Delta\gamma 16$) and the $\gamma RNAi$ homozygous transgenic line ($\gamma RNAi/\gamma RNAi$), showing all vitreous kernels. (F) Truncated kernel phenotype. (Top) Mcl , cross of $Mcl \times BA$, and $\gamma RNAi$ transgenic line. (Bottom) Three vitreous and *floury* kernels from D.

kernel. Indeed, the progeny ear from the cross of $\Delta\gamma 16/\Delta\gamma 16$ and $\gamma RNAi/+$ showed a 1:1 ratio of *floury* and vitreous kernels (Figure 3, D and F), and all kernels were vitreous when the *Mc1* mutant was pollinated by a homozygous $\gamma RNAi$ line (Figure 3E).

Conclusions: RNAi can be used to rescue mutations that are dominant negative with a single cross, providing a useful tool in genetic analysis, plant breeding, and potentially in gene therapy in general.

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