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High-lysine corn generated by endosperm-specific suppression of lysine catabolism using RNAi

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Summary

Because of the limited lysine content in corn grain, synthetic lysine supplements are added to corn meal-based rations for animal feed. The development of biotechnology, combined with the understanding of plant lysine metabolism, provides an alternative solution for increasing corn lysine content through genetic engineering. Here, we report that by suppressing lysine catabolism, transgenic maize kernels accumulated a significant amount of lysine. This was achieved by RNA interference (RNAi) through the endosperm-specific expression of an inverted-repeat (IR) sequence targeting the maize bifunctional lysine degradation enzyme, lysine-ketoglutarate reductase/saccharopine dehydrogenase (ZLKR/SDH). Although plant-short interfering RNA (siRNA) were reported to lack tissue specificity due to systemic spreading, we confirmed that the suppression of *ZLKR/SDH* in developing transgenic kernels was restricted to endosperm tissue. Furthermore, results from our cloning and sequencing of siRNA suggested the absence of transitive RNAi. These results support the practical use of RNAi for plant genetic engineering to specifically target gene suppression in desired tissues without eliciting systemic spreading and the transitive nature of plant RNAi silencing.

Introduction

In plants, lysine is derived from aspartate in a number of enzymatic steps, with dihydrodipicolinate synthase (DHDPS) catalysing the first committed step for lysine production and lysine-ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH), a bifunctional enzyme, responsible for lysine catabolism (Galili, 2002). Lysine regulates its own accumulation by feedback inhibiting DHDPS and forward activating LKR/SDH (Galili, 2004). Since lysine is an important quality trait in crop plants, these two key steps in lysine metabolism have been the subject of studies for modifying seed lysine content. Seed-specific expression of a bacterial lysine feedback-insensitive DHDPS resulted in up to a 100-fold increase in free lysine accumulation in seeds of transgenic *Arabidopsis*, canola and soybean (Falco *et al*., 1995; Zhu and Galili, 2002). In contrast, significant accumulation of lysine in maize seed could only be achieved through deregulating lysine anabolism in the embryo (Mazur *et al*., 1999; Huang *et al*., 2005). This could be due to the presence of considerable amount of LKR/SDH activity in developing maize endosperm (Kemper *et al*., 1999) and it is likely that lysine catabolism rather than lysine biosynthesis modulates the lysine levels in developing maize endosperm. In this study we investigated whether the suppression of *LKR/SDH* alone in maize endosperm by RNA interference (RNAi) could lead to the accumulation of free lysine in mature kernels. Previously, the reduction of *AtLKR/SDH* either by T-DNA insertional mutagenesis or RNAi was shown to cause the rise of free lysine levels in *Arabidopsis* seeds (Zhu *et al*., 2001; Zhu and Galili, 2004).

RNAi results in gene-specific down-regulation triggered by double-stranded RNA (dsRNA) (Fire *et al*., 1998). While in *Caenorhabditis elegans*, *Drosophila melanogaster* and other animal organisms, RNAi may be achieved by the injection of dsRNA (Fire *et al*., 1998; Kennerdell and Carthew, 1998), expression of dsRNA using transgenes with inverted repeats (IR) of target sequences through *Agrobacterium*-mediated transient or stable transformation is usually how RNAi is

deployed in plants (Schob *et al*., 1997; Chuang and Meyerowitz, 2000). In plants, the transgenic dsRNA are thought to be processed by RNase III-type enzymes called Dicer-like (DCL) enzymes and produce small short-interfering RNA (siRNA) (Xie *et al*., 2004; Bordersen and Voinnet, 2006). The siRNA then serve as the sequence specific guides to cleave homologous mRNA and direct DNA or histone modification at homologous loci and cause gene silencing at the transcriptional or post-transcriptional level (Bordersen and Voinnet, 2006). Although gene knockout by antisense suppression (Oeller *et al*., 1991) and sense co-suppression (Napoli *et al*., 1990) has long been a useful tool to study gene function in plants, gene suppression by dsRNA is far more efficacious (Chuang and Meyerowitz, 2000). Since all of these approaches utilize similar RNA silencing pathways that require the dsRNA intermediate, this is likely due to prompt dsRNA formation by transcribing directly from the IR transgene. In contrast, it has been proposed that the formation of dsRNA in antisense suppression and sense co-suppression approaches depends on either the transcription of an unintentional IR T-DNA insertion structure or the conversion of a single-stranded transgenic RNA by an endogenous RNA-dependent RNA polymerase (RdRP) (Tang *et al*., 2003; Sanders and Hiatt, 2005).

The study of plant RNA silencing pathways also reveals phenomenon such as transitive RNAi and systemic spreading that are not common in animal systems other than *C. elegans* (Klahre *et al*., 2002; Vaistij *et al*., 2002). Transitive RNAi is caused by secondary siRNA promoted by primary siRNA originating from the dsRNA with the action of RdRP (Vaistij *et al*., 2002). These secondary siRNA sequences may include any unpaired region of the transgenic IR transcript (Brummell *et al*., 2003) and the target mRNA sequences that lie upstream or downstream of the initial IR targeted region (Van Houdt *et al*., 2003). Therefore, the presence of transitive RNAi may lead to inactivation of mRNA species that are not targeted by the initial trigger sequence derived from the IR. Non-cell-autonomous suppression is another plant RNAi characteristic that has to be considered when applying RNAi to genetic engineering. Plant siRNA can travel intercellularly through plasmodesmata or utilize the vascular system for long-distance translocation (Yoo *et al*., 2004; Kim, 2005). This could impair the use of RNAi in cases when tissue-specific suppression is desired.

In this study, an IR sequence corresponding to part of the SDH coding region of maize *LKR/SDH* (*ZLKR/SDH*) cDNA (Kemper *et al*., 1999) was inserted into a transgene cassette driven by a maize endosperm-specific promoter. We investigated not only the efficacy of accumulating free lysine in mature kernels, but also whether the suppression of *ZLKR/SDH* was specific to the developing endosperm. The transitive nature of RNAi was also examined by analysing the expression of several endogenous genes that were potential targets of secondary siRNA generated from the nascent IR transcript and by sequencing siRNA cloned from transgenic endosperm tissue. Notably, this study represents the first transgenic RNAi study done on a major crop plant species such as maize and can provide important information for applying RNAi in plant biotechnology.

Results

Transgenic F_1 seeds showed reduced LKR/SDH protein accumulation

The T-DNA region of the binary vector used in the study, pMON79464, is shown in Figure 1(a). The maize endosperm-

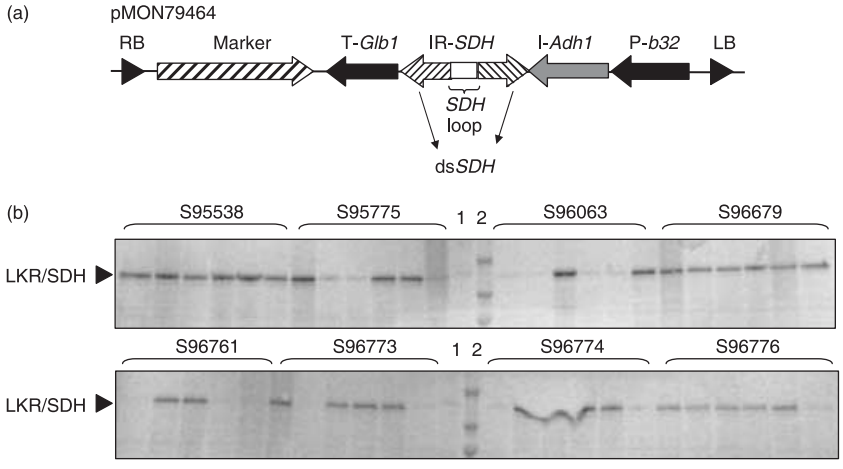

Figure 1 ZLKR/SDH Western blot analysis of transgenic F_1 kernels. (a) The binary vector used in the study, pMON79464, contained a transgenic cassette with an IR sequence, IR-*SDH*. It corresponded to part of the SDH coding region of *ZLKR/SDH* cDNA, driven by the endosperm-specific promoter, P-*B32*, along with the intron, I-*Adh1*, to enhance the expression of the transgene and terminated by *Glb1* terminator, T-*Glb1*. (b) Five to six F_1 kernels were examined per line. S95775, S96063, S96761, S96773, S96774 and S96776 represented lines showed reduced ZLKR/SDH in segregating kernels, while S95538 and S96679 had normal levels of LKR/SDH in all kernels. The *Escherichia coli.* expressed ZLKR/SDH control (Lane 1) showing a expected larger size band was due to lack of cleavage of its chloroplast-targeting leader peptide.

specific promoter, P-*b32* (Hartings *et al*., 1990), was used to drive an IR sequence. The IR region, IR-*SDH*, corresponded to part of the SDH coding region of *ZLKR/SDH* cDNA. More than 50 independent R0 transgenic plants were generated. Only those containing a single copy of the *epsps-cp4* marker gene as determined by an Invader™-based zygosity assay (Third Wave Technology, Madison, WI, USA) were pollinated with wild-type pollen to produce 21 F_1 transgenic lines. Among them, 15 lines were found to have reduced maize LKR/SDH protein (ZLKR/SDH) in segregating F_1 kernels as shown for representative lines S95775, S96063, S96761, S96773, S96774 and S96776. The remaining 6 lines had normal level of ZLKR/SDH as shown for S95538 and S96679 (Figure 1b).

Transgenic RNAi targeting ZLKR/SDH behaved as a dominant high lysine trait in F_3 kernels

All F_1 lines displaying the ZLKR/SDH reduction phenotype were planted and self-pollinated to obtain F_2 seeds. Two randomly selected F_2 lines, S95769 and S96100, were advanced to the F_3 generation by planting 50 F_2 seeds for each line. Since F_2 plants were derived from self-pollinated hemizygous F_1, they segregated homozygous, hemizygous and null and produced F_3 ears that seeds contain 100%, 75% and 0% transgenic seeds, respectively. As shown in Figure 2, homozygous ears had up to a 20-fold increase in free lysine content compared to the null control. The average lysine increase in hemizygous ears was 77.7% (63.4%, 92.0%; $\alpha = 0.05$) relative to homozygous ears. As expected, the transgenic RNAi targeting *ZLKR/SDH* appeared to behave as a dominant high lysine trait.

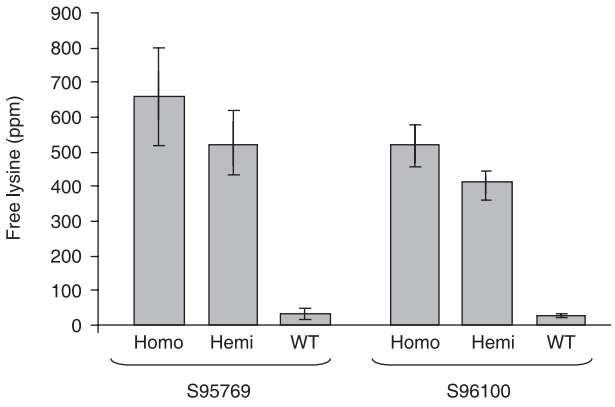

Figure 2 Lysine content in transgenic F_3 kernels. The F_2 transgenic plants which segregated 1 : 2 : 1 for homozygous, hemizygous and null, were self-pollinated to produce F_3 ears. The lysine content was measured by LC/MS/MS from the ground mill samples of individual ear. Each bar represented the average lysine content of more than 8 ears ± confidence interval ($\alpha = 0.05$).

Endosperm-specific reduction of ZLKR/SDH in developing F_4 transgenic kernels

Tissue from homozygous F_3 plants and F_4 developing kernels of lines S95769 and S96100, and wild-type were collected for ZLKR/SDH Western blot analysis. The ZLKR/SDH in transgenic lines was almost undetectable in the endosperm as early as 18 days after pollination (DAP) (Figure 3a). In the embryo, a faint ZLKR/SDH band was observed and appeared to be comparable to wild type. This was consistent with the results shown in F_1 seeds where whole kernel tissue was used for analysis. Residual ZLKR/SDH protein observed in kernels that showed ZLKR/SDH suppression may be from the embryo (Figure 1b). Reduction of ZLKR/SDH was confirmed by assessing LKR activity. In comparison to wild type, a significant reduction in LKR activity was found only in the developing endosperm of transgenic kernels (Figure 3b). In Figure 3(c), Western blot data showed the ZLKR/SDH suppression lines and wild type had identical ZLKR/SDH expression patterns in other vegetative and reproductive tissues. ZLKR/SDH was present in shoots and immature ears, but absent in leaf and pollen/tassel tissues.

The endosperm-specific reduction of ZLKR/SDH in transgenic lines was also supported by Northern blot analysis. *ZLKR/SDH* mRNA is known to be relatively abundant in developing endosperm (Kemper *et al*., 1999). The *ZLKR/SDH* mRNA in transgenic endosperm was significantly reduced throughout the kernel developmental stages examined (Figure 4a). In the embryo, a longer exposure of the blot revealed that there was a similar *ZLKR/SDH* expression pattern between transgenic and wild-type embryos. The extra bands observed below the predicted *ZLKR/SDH* mRNA band in the blot are likely derived from the transgene. When the blot was probed with a DNA fragment corresponding to the LKR coding region which was not used in construction of the transgenic cassette, these bands were not observed (data not shown).

To verify that the transgenic suppression of *ZLKR/SDH* was mediated by RNA silencing, small RNA Northern blot analysis was performed. The typical 21- and 24-nt siRNA involved in RNA silencing were detected only in the developing endosperm of transgenic lines (Figure 4b). Even after prolonged exposure, the siRNA were not evident in the transgenic embryo. Although low transgene expression was detected in the embryo (Figure 4a), it apparently was not enough to induce the production of siRNA.

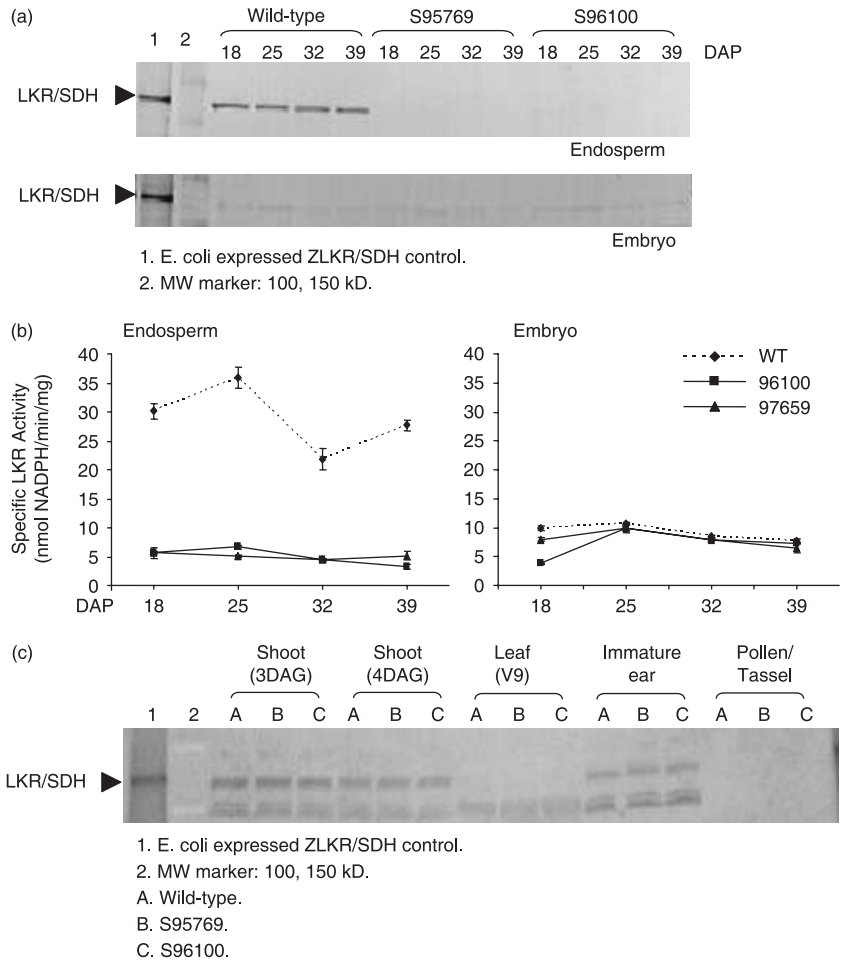

Figure 3 ZLKR/SDH Western blot and activity analysis of homozygous transgenic F_4 developing kernels and F_3 plants. (a) F_4 homozygous developing kernels were collected from the representative transgenic lines, S95769 and 96100. In the endosperm, the transgenic kernels showed reduction in ZLKR/SDH as early as 18 DAP (days after pollination). Although low in the embryo, there is no apparent difference in the ZLKR/SDH level among wild-type and transgenic kernels. (b) The transgenic lines showed reduced LKR activity in the developing endosperm, while in the embryo they remained relatively unchanged when compared to wild-type. Each time point represented the average of three measurements ± standard deviation. (c) The F_3 transgenic plants showed a similar ZLKR/SDH expression pattern to wild-type in 3 and 4 days after germination (DAG) shoots, leaves (V9), immature ears and pollen/tassel tissues. The additional bands appeared below the expected ZLKR/SDH were likely due to non-specific interaction with the ZLKR/SDH antibody in various maize tissues.

The expression of endogenous *b32*, *Adh1* and *Glb1* was unchanged in transgenic lines

The maize endogenous genetic elements *b32* promoter, *Adh1* intron, and *Glb1* terminator were used along with the IR sequence targeting *ZLKR/SDH* to construct the transgenic cassette. It is known that secondary siRNA can be produced from unpaired RNA sequences linked to dsRNA and effect RNA silencing of their corresponding targets (Brummell *et al.*, 2003). In addition to the *Adh1* intron sequence, the transgenic transcript contained approximately 50 and 200 nt of 5′ and 3′ untranslated sequences from *b32* and *Glb1*, respectively. As shown in Figure 5, the expression patterns of *b32*, *Adh1* and *Glb1* in developing kernels were similar between transgenic and wild-type plants. Based on the band intensity and the relative exposure time, *b32* had higher expression in the endosperm and *Glb1* had higher expression in the embryo as expected. The low level of *b32* mRNA detected in the embryo was consistent with previous Northern blot analysis that showed a low level of *B32*-driven transgenic transcript in the embryo after 24 h of exposure (Figure 3a).

Cloning of small RNA from the developing endosperm of transgenic kernels

The presence of secondary siRNA can also be determined by the cloning of siRNA. Small RNA isolated from the 25 or 32 DAP developing endosperm of transgenic line S95769 for was used to construct a small RNA library for large-scale sequencing. Among approximately 200 000 sequences analysed, the most abundant ones were miRNA as expected and only 4568 sequences (2.29%) were found to be transgenic related siRNA (Figure 6a). The expected transgenic transcript formed a dsRNA and an unpaired loop region that spanned 2001–2459 and 1487–2000 bp of *ZLKR/SDH* cDNA, respectively. All but 14 transgenic siRNA matched the sequence of the dsRNA region and 12 matched the loop region. The last two transgenic related siRNA were mapped to the T-Glb1 sequence. More importantly, no secondary siRNA were generated from the endogenous *ZLKR/SDH* mRNA target. These results were consistent with the RNA gel blot analysis and suggested that transitive RNA silencing did not occur in the maize endosperm. As expected, these cloned transgenic

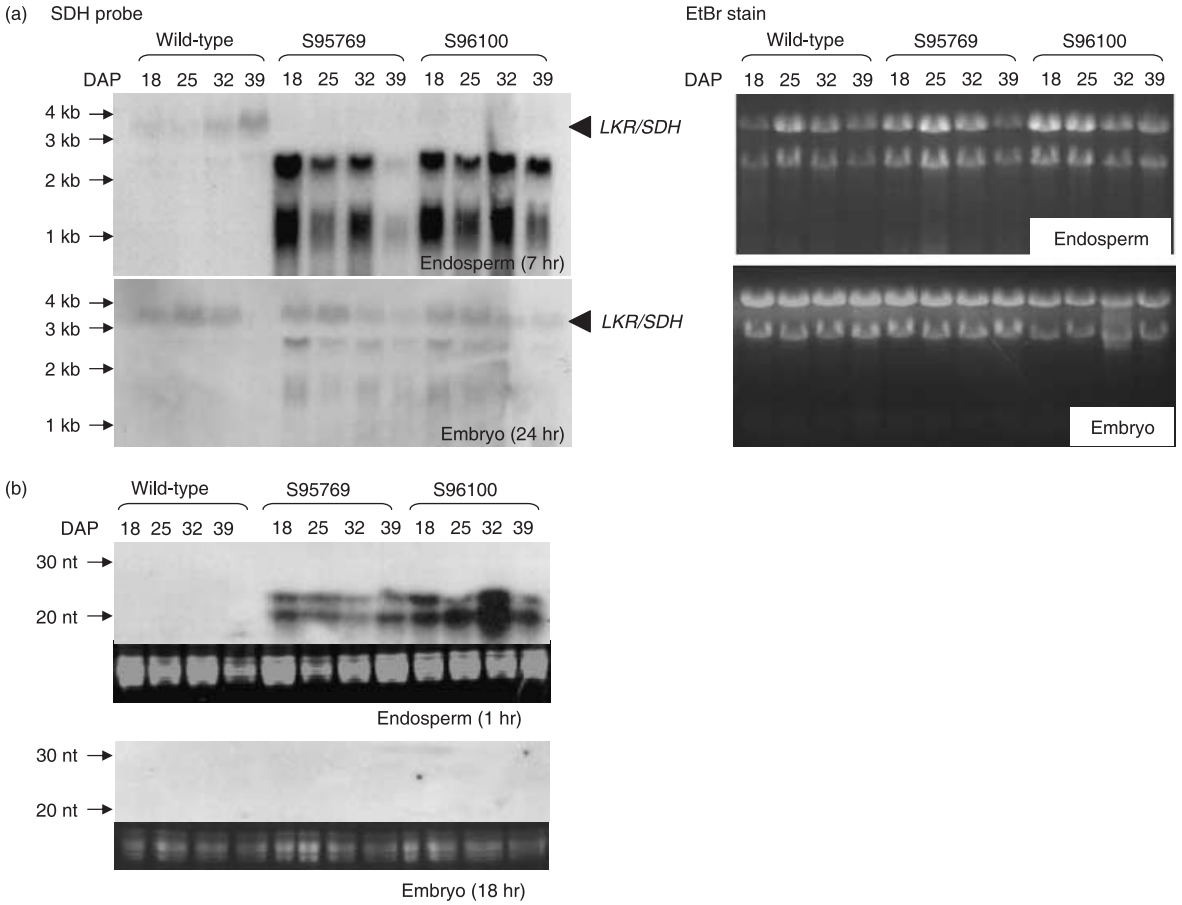

Figure 4 *ZLKR/SDH* Northern blot analysis of homozygous transgenic F_4 kernels. (a) Transgenic kernels showed endosperm-specific reduction of *ZLKR/SDH* mRNA (left panel). In the endosperm, the transgenic kernels showed a reduced Z*LKR/SDH* mRNA (~3.4 kb) level as early as 18 days after pollination (marked by solid triangles). The embryo *ZLKR/SDH* mRNA bands were similar between wild-type and transgenic kernels. The additional lower bands observed only in the transgenic lines were from the transgenic transcript, since they do not hybridize to the LKR probe (data not shown). (b) The small RNA Northern blot of transgenic kernels exhibited a typical banding pattern consisting of 21 and 24 nt siRNA in the endosperm, but not in the embryo. Images of EtBr strained SDS-PAGE gels before being transferred to a nylon membrane for hybridization were included.

siRNA were mostly 21 and 24 nt in length and consisted of both sense and antisense strands (Figure 6b).

Discussion

In this study, we report the production of high lysine corn by suppression of the lysine catabolic enzyme ZLKR/SDH in the endosperm. This was achieved by RNA silencing through the endosperm-specific expression of an IR sequence that targeted part of the SDH coding region of *ZLKR/SDH* mRNA. Although details of RNA silencing pathways have been proposed in model plant systems, relatively little is known about crop plants. Using high lysine corn lines generated by RNAi, we carried out a detailed molecular characterization. These results not only provide some insight into how RNAi works in crop plants, but also provide an assessment of the potential use of RNAi for commercial purposes.

Lysine catabolism, not biosynthesis, in developing endosperm impacts lysine accumulation in corn grain

Previously it was found that in maize, the expression of a feedback-insensitive DHDPS in embryo tissue, but not in endosperm tissue resulted in free lysine accumulation in mature seeds (Mazur *et al*., 1999; Huang *et al*., 2005). It is generally thought that DHDPS is the rate limiting step in lysine metabolism. However, in the maize endosperm, the inability to accumulate high levels of lysine seems to be due to high rates of lysine catabolism (Kemper *et al*., 1999). The ability of transgenic maize kernels to accumulate free lysine by RNAi suppression of *ZLKR/SDH* alone supports this hypothesis. Also, maize DHDPS has been shown to lose over 90% of its activity at 200 μM of lysine *in vitro* (~30 p.p.m.) (Shaver *et al*., 1996) which approximates the lysine level in wild-type maize seed and is 1/20 the level of lysine in transgenic RNAi seed (Figure 2).

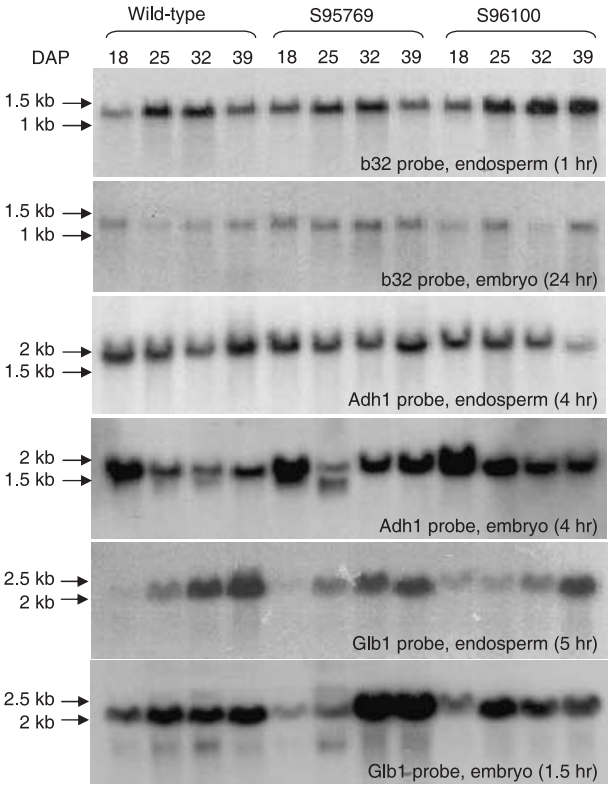

Figure 5 Northern blot analysis of endogenous *b32*, *Adh1* and *Glb1*. As described in Figure 1(a), genetic elements derived from endogenous maize genes, such as *b32* promoter, *Adh1* intron and *Glb1* terminator were used in the construction of the *ZLKR/SDH* suppression cassette. These Northern blot analysis results showed that the expression patterns of these genes in developing kernels were similar between transgenic and wild-type plants. The exposure time for each blot was indicated in parenthesis.

This implies that the flux of lysine into the endosperm from the phloem probably occurred during endosperm development since the lysine biosynthesis may be inhibited by the high level of lysine. However, we cannot rule out the possibility that lysine biosynthesis and catabolism are partitioned in different compartments of the maize endosperm. We are currently investigating the possibility of further enhancing maize seed lysine content by coupling deregulated lysine biosynthesis with *ZLKR/SDH* reduction.

Maize endosperm-specific RNAi

Although RNAi induced by expression of dsRNA is effective and efficient in plants, it has been used mostly as a tool to study gene function in model plant systems. Only a few studies have examined the possibility of using RNAi to modify the expression of endogenous genes for agricultural applications in crop plants. For example, transgenic dsRNA were used to target seed storage proteins to change the grain quality of corn (Huang *et al*., 2006) and rice (Kusaba *et al*., 2003), or a regulatory protein to increase beneficial metabolites in tomato (Davuluri *et al*., 2005). In the first two instances, the tissue specificity of RNAi was not a concern since the target proteins were seed specific and therefore the suppression only occurred in seed regardless of whether the transgenic siRNA were systemically spreading or not. Specificity of RNAi becomes more important when the application involves modifying an endogenous pathway that is active in multiple tissues in a tissue-specific manner. Constitutive RNAi suppression may cause side-effects and render the technology undesirable. Such is the case in tomato where Davuluri *et al*. reported fruit-specific RNAi-mediated suppression (Davuluri *et al*., 2005). The authors observed that gene silencing signals were not passed beyond the seed stage. In each generation, the transgenic seeds gave rise to wild-type plants up until fruit development when the silencing was triggered again. It has been hypothesized that perhaps there is no transmission of silencing signals in and out of developing seeds (Kusaba, 2004). It could be the absence of plasmodesmata between the seed and its surrounding tissues, or the existence of a surveillance system that excludes silencing signals from passing into seed, as described for the shoot apex (Foster *et al*., 2002).

Similarly, our results indicated that the transgenic suppression of ZLKR/SDH was restricted to endosperm and suppression was not observed in embryo, seedlings, and tissues later in plant development. With the exception of the endosperm, ZLKR/SDH expression in transgenic plants was indistinguishable from wild-type plants. This lack of RNA silencing spreading between endosperm and embryo suggests the presence/absence of a silencing signal barrier/channel between these two tissues.

Absence of secondary siRNA production in maize endosperm

Transitive RNAi caused by secondary siRNA has to be considered to avoid adverse effects of unintended gene suppression. Secondary siRNA are promoted by primary siRNA and can be generated in two ways. In plants, a transgenic dsRNA is processed by DCLs to produce primary siRNA. The primary siRNA can then initiate 'cis' secondary siRNA production from the single-stranded region of the original transgenic transcript or 'trans' secondary siRNA production from their endogenous target mRNA, including sequences upstream and downstream of the initial trigger site. Using a transgene cassette containing a polygalacturonase (PG) cDNA fused with a heterologous

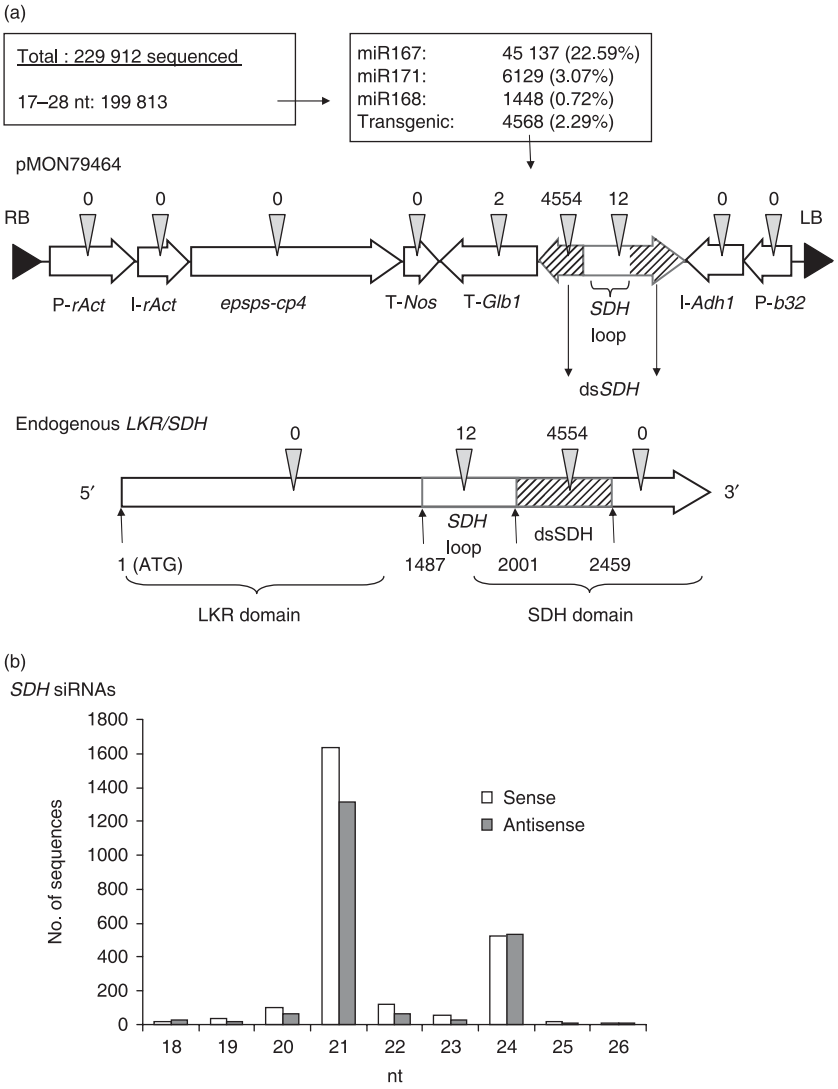

Figure 6 The sequence distribution of cloned siRNA. (a) The T-DNA region of pMON79464 included a marker gene cassette (P-*rActin*/I-*rActin*/*epsps-cp4*/T-*Nos*) and the *LKR/SDH* suppression cassette. The suppression cassette contained an IR sequence corresponding to part of the *SDH* coding region of *ZLKR/SDH* cDNA, IR-*SDH* (Figure 1a), which resulted in a transcript containing an *SDH* dsRNA region (strip box) and an unpaired loop (open box). The illustration summarizes the distribution of small RNA clones. (b) The histogram of cloned *SDH* siRNA shows that they were derived from both strands and the majority of their sizes were 21 and 24 nt.

IR sequence, Brummell and colleagues (2003) successfully suppressed endogenous *PG* and delayed the ripening of transgenic tomato. Such gene silencing by 'cis' secondary siRNA, however, was not detected in our transgenic lines. Through Northern bolt analysis and the cloning of small RNA, we concluded that the endogenous genes, whose genetic elements were used in the construction of the IR transgene cassette, were not subjected to RNA silencing. Moreover, we did not recover any 'trans' secondary siRNA from cloned small RNA. Interestingly, these transgene-derived siRNA, effective in silencing *ZLKR/SDH*, were found to represent only 2.29% of the endogenous small RNA population. Although plant small RNA pathways are known to be involved in defence and development, it is unlikely that transgenic siRNA at this level would impact these endogenous processes. Together, these findings provide important data regarding the commercial regulatory aspect of RNAi technology.

Application of RNAi for crop improvement

Although loss-of-function mutants can be recovered for the genetic improvement of crop plants through conventional breeding, they are usually recessive and lack tissue specificity which may limit their commercial utilization. Genetic engineering using RNAi offers a more useful technology to down-regulate endogenous genes in a dominant and tissue specific manner. As exemplified in the present study, a maize high lysine trait was engineered through endosperm suppression of *ZLKR/SDH* by RNAi. This would not be possible by isolating *ZLKR/SDH* loss-of-function mutants since high concentrations of lysine in vegetative tissues are known to cause agronomic abnormalities (Negrutiu *et al*., 1984; Frankard *et al*., 1992). We are now assessing the trait stability over successive generations and performance under different environmental conditions.

Although much has been learned about plant RNAi in recent years, its machinery and biological functions are still not fully understood. Nevertheless, there are examples of commercial cultivars produced by RNAi. Identified in the 1970s, LGC-1 (Low Glutelin Content-1) is low protein rice used by patients on a restricted protein diet. The cloning of this dominant mutant locus revealed a gene structure harbouring an IR of the *glutelin* gene and *glutelin* siRNA were found in the mutant (Kusaba *et al*., 2003). In soybean, pigmented seed coats are common in all wild accessions, but cultivated varieties have been selected for a yellow seed coat. This is due to an endogenous duplicated IR allele in cultivated varieties that drives silencing of CHS (chalcone synthase) genes, thereby inhibiting pigmentation of the seed coats (Tuteja *et al*., 2004). Another example is the Flavr Savr tomato, the first commercialized genetically modified food developed by Calgene (Davis, CA, USA; now part of Monsanto Company, St. Louis, MO, USA) in the early 1990s. Initially, antisense constructs against polygalacturonase (PG) cDNA were used to transform tomato to delay fruit ripening. However, molecular characterization of all the lines with the commercial efficacy had T-DNA inserts containing IR elements (Sanders and Hiatt, 2005). Recently, small RNA Northern blot analysis has confirmed the presence of siRNA corresponding to the PG cDNA sequence in these transgenic tomato lines (Allen and Krieger, pers. commun.).

Experimental procedures

Vector construction and plant transformation

The binary vector, pMON79464, contained the maize endosperm *b32* promoter (Hartings *et al*., 1990), P-*b32*, maize *Adh1* intron (Dennis *et al*., 1984), I-*Adh1*, an IR sequence corresponding to part of the SDH region of the maize *LKR/SDH* (*ZLKR/SDH*) cDNA (Kemper *et al*., 1999), IR-*SDH*, and maize *Glb1* 3′UTR (Belanger and Kriz, 1991), T-*Glb1* (Figure 1a). The IR sequence, *IR-SDH*, consisted of a fusion of two segments corresponding to bases 2459-1487 and 2001-2459 of *ZLKR/SDH* cDNA, GenBank accession number AF003551 (start codon = 1). The selectable marker gene (*epsps-cp4*) and the rest of the genetic elements in the binary vector were similar to those previously described (Huang *et al*., 2004). The binary plasmid was electroporated into *Agrobacterium tumefaciens* ABI strain and introduced into maize embryos by *Agrobacterium*-mediated transformation (Armstrong and Rout, 2001).

LKR/SDH Western blot analysis

The Western blot procedures used were as previously described (Huang *et al*., 2005). The rabbit anti-ZLKR/SDH antibody was produced by immunizing rabbits against *Escherichia coli* expressing recombinant ZLKR/SDH (Cocalico Biologicals, Reamstown, PA, USA).

Plant RNA isolation and Northern blot analysis

Total RNA was isolated from dissected endosperm and embryo collected at desired developmental time points using Tri Reagent (Molecular Research Center, Cincinnati, OH, USA), and further separated to high molecular weight (HMW) RNA (> 200 nt) and low molecular weight (LMW) RNA (< 200 nt) by mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) following the manufacturers' instructions. Fifteen μg of HMW or LMW RNA of each sample was loaded on a 1% (w/v) agarose/formaldehyde gel or a 15% TBE-Urea Criterion gel (Bio-Rad, Hercules, CA, USA), respectively, and transferred to Nytran SuperCharge (Schleicher and Schuell, Keene, NH, USA) or Zeta-Probe (Bio-Rad) nylon membranes, respectively. Prehybridization and hybridization were performed in the hybridization buffer PerfectHyb™ Plus (Sigma, St. Louis, MO, USA). The washing and detection of the membranes were conducted following the manufacturer's procedures, except for the LMW RNA blots where a lower hybridization (38 °C) and washing (43 °C) temperatures was used. The PCR fragments corresponding to the cDNAs of various genes examined were radioactively labelled and used for probes.

Small RNA cloning and sequencing

Small RNA library construction was done as previously described with minor modifications (Lau *et al*., 2001; Llave *et al*., 2002). LMW RNA was separated on a 15% TBE-Urea Criterion gel (Bio-Rad) and small RNA eluted with Probe Elution Buffer (Ambion) according to the manufacturer's instructions. The library was sequenced commercially by 454 Life Sciences (Branford, CT, USA).

LKR activity assay

The protein extraction and LKR activity assay were performed as previously described (Huang *et al*., 2005).

Free lysine analysis by LC/MS/MS

Ground samples (50 mg) were extracted with extraction buffer (50% methanol, 50% water and 1% formic acid) with a 30 : 1 w/v ratio and vortexed for 20 min, and then kept at 4 °C overnight to complete the extraction. After centrifugation to pellet debris, the supernatant was transferred and necessary dilutions were made for LC/MS/MS analysis. A C18 column, 4.6 mm × 50 mm (Alltech Associates, Deerfield, IL, USA) and an API 3000 Triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) was used to separate and detect the lysine content in the sample extracts.

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