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Seed-specific expression of the wheat puroindoline genes improves maize wet milling yields

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Summary

The texture of maize (*Zea mays* L.) seeds is important to seed processing properties, and soft dent maize is preferred for both wet-milling and livestock feed applications. The puroindoline genes (*Pina* and *Pinb*) are the functional components of the wheat (*Triticum aestivum* L.) *Hardness* locus and together function to create soft grain texture in wheat. The PINs (PINA and PINB) are believed to act by binding to lipids on the surface of starch granules, preventing tight adhesion between starch granules and the surrounding protein matrix during seed maturation. Here, maize kernel structure and wet milling properties were successfully modified by the endosperm-specific expression of wheat *Pins* (*Pina* and *Pinb*). *Pins* were introduced into maize under the control of a maize γ-*Zein* promoter. Three *Pina/Pinb* expression positive transgenic lines were evaluated over two growing seasons. Textural analysis of the maize seeds indicated that the expression of PINs decreased adhesion between starch and protein matrix and reduced maize grain hardness significantly. Reduction in pressure required to fracture kernels ranged from 15.65% to 36.86% compared with control seeds. Further, the PINs transgenic maize seeds had increased levels of extractable starch as characterized by a small scale wet milling method. Starch yield was increased by 4.86% on average without negatively impacting starch purity. The development of softer maize hybrids with higher starch extractability would be of value to maize processors.

Introduction

Maize or corn (*Zea mays* L.) is the most important cereal in the world after wheat and rice and the most important crop in the United States. The texture of maize seeds, which is related to the proportions of hard (horny, flinty) and soft (floury, starchy) endosperm, is a critical trait that influences various end-uses of maize such as starch yield and the power required for both wet and dry milling (May, 1987; Mestres and Matencio, 1996). Maize seed texture also affects starch conversion rates in ethanol biofuel production along with the value of maize as feed because of its effects upon starch digestibility (Corona *et al.*, 2006; Torney *et al.*, 2007). Based on kernel characteristics, maize grain has been divided into five general classes: flint, popcorn, flour, dent, and sweet (Watson, 1987). Softer textured dent maize is preferred for wet-milling, which is the largest non-feed user of maize in the US. Maize starch makes up more than 80% of the world starch market, and most maize starch extraction is done in the US by a wet milling process (Jobling, 2004). There is considerable interest in maize having enhanced levels of extractable starch (reviewed in Bothast and Schlicher, 2005). Therefore, the development of maize hybrids with higher starch yields following wet milling would enhance starch production. While much effort has gone into

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studies of starch structure in maize and alterations in the amount and frequency of branching (Giroux *et al.*, 1996; Slattery *et al.*, 2000; reviewed in Hannah and James, 2008), little effort has gone into studies of the proteins surrounding the starch granules in maize that could potentially be manipulated to improve starch extractability.

While no studies affecting corn texture have focused on proteins surrounding corn starch granules, numerous studies have focused on the effect of variation in storage protein synthesis upon maize kernel development. Zeins are the major storage proteins in maize endosperm. The first maize mutant identified that affected both zein content and seed texture was *Opaque-2*, which had high lysine content, increased protein quality and an opaque, starchy phenotype (Mertz *et al.*, 1964). At least 18 mutations have now been described that affect storage protein synthesis in maize and cause a soft, starchy endosperm (reviewed in Coleman and Larkins, 1999; Hunter *et al.*, 2002). Only in *opaque-2* and *floury-2* are the molecular bases for the mutations known. *Opaque-2* mutations reduce α-zein synthesis and result in small unexpanded protein bodies (Geetha *et al.*, 1991), while *floury-2* is associated with irregularly shaped protein bodies (Coleman *et al.*, 1997). However, *opaque-2* and *floury-2* mutant kernels typically have an increased susceptibility to attacks by pest and diseases, increased mechanical damage and reduced yield (Lambert *et al.*, 1969; Salamini *et al.*, 1970). While *opaque-2* and *floury-2* mutations decrease the vitreousness of maize seeds, there is no single major maize gene that acts in a dominant fashion to increase grain softness, as in wheat and its relative species.

Wheat (*Triticum aestivum* L.) grain hardness is controlled by a single locus termed *Hardness* (*Ha*) (Law *et al.*, 1978). The *Ha* locus functionally consists of two genes, *Puroindoline a* and *b*, *Pina* and *Pinb*, respectively (Gautier *et al.*, 1994). Soft kernel texture results when both *Pina* and *Pinb* are functional, whereas hard-textured wheats contain a mutation in either *Pin* gene (Giroux and Morris, 1997, 1998). Both PINs (PINA and PINB) contain a backbone of 10 Cys residues and likely form a tertiary structure similar to that of nonspecific lipid-transfer proteins (ns-LTPs), comprised of four α-helices separated by loops and stabilized by five disulphide bridges (Sterk *et al.*, 1991; reviewed in Bhave and Morris, 2008). The unique tryptophan-rich domain found in both PINA and PINB is believed to be a non-stick agent enabling PINs to bind to starch granule surface lipids. Binding to starch granule surface lipids then prevents adhesion between starch granules and the surrounding protein matrix during seed maturation and reduces grain hardness (Giroux and Morris, 1998). This, in turn, reduces the amount of energy needed for milling, enhances milling yield, and reduces damage to starch granules (Martin *et al.*, 2001, 2007; Feiz *et al.*, 2008).

Puroindolines, which are synthesized and accumulated only in the starchy endosperm cells of wheat seeds (Wiley *et al.*, 2007), have been used to effectively and consistently modify grain texture of wheat and rice (Krishnamurthy and Giroux, 2001; Beecher *et al.*, 2002; Martin *et al.*, 2006). The creation of grain hardness variation in other cereals such as maize could potentially improve end product qualities, food uses, and add value. Maize does not express PINs or even contain *Pin* homologues (Gautier *et al.*, 2000). Therefore, *Pina* and *Pinb* were introduced into maize under the control of a seed-specific promoter to determine whether expression of the PIN genes alters maize seed texture and wet milling yield.

Results

Analysis of transgenic plants

The transgenic plants were created in Hi-II via biolistic transformation at Iowa State University. Herbicide-resistant T_0 lines having both *Pina* and *Pinb* were pollinated with pollen from inbred line B73. Resistant T_1 plants were selfed to produce segregating T_2 progeny. The *Bar* marker gene segregated in a 3 : 1 ratio in each of the three independent lines presented here (Table 1). T_3 plants were tested using both PCR for the *Pin* genes as well as herbicide resistance to identify T_2-derived lines that were homozygous positive or negative for the transgene locus. There were 13 independent transgenic events generated, and three of them were selected for analysis on the basis of having good vigour, fertility and being expression positive for both PINA and PINB (results not shown) The three selected homozygous T_3 events carrying *pBAR184* and *pNB167* were designated ZP1, ZP5 and ZP7. All the seeds analyzed were taken from the central cob region of the ears, and all seeds were equilibrated to the same moisture content.

Transgenic line characterization

Southern blot analysis was used to confirm that each line resulted from an independent transformation event. Total genomic DNA was isolated from seedlings and restriction digested with *Hin*d III which cuts once within the *pNB167*

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Table 1 *Pina* and *Pinb* PCR tests and glufosinate herbicide reaction for three maize events transformed with *Pina*, *Pinb* and *Bar* and untransformed control

Event*	T_0 PCR (*Pina*/*Pinb*)†	T_2 segregation‡ (resistant/susceptible)	Chi-square (3 : 1)§	T_3 PCR for *Pins*¶ (+/−)	T_3 herbicide for *Bar*¶ (+/−)
Null	−/−	0/5	15	0/5	0/12
ZP1	+/+	10/3	0.026	18/0	11/0
ZP5	+/+	7/2	0.037	16/0	14/0
ZP7	+/+	8/1	0.926	16/0	14/0

*All PCR positive T_0 plants were crossed with untransformed plants to produce T_1 seeds; T_1 hemizygous lines were self-pollinated to produce T_2 seeds.

†PCR screening was performed using *Pina*- or *Pinb*-specific primer pairs on samples of genomic DNA from each T_0 plant.

‡T_2 progenies were leaf-painted with 0.1% glufosinate. Seedlings which showed minimal adverse effects were scored as resistant.

§Chi-square values test the fit of resistant/susceptible progeny of T_2 plants to a 3 : 1 ratio.

¶T_3 seedlings were tested with *Pina* and *Pinb* PCR screening and herbicide tests to obtain T_2-derived T_3 seed pools co-segregation homozygous positive or negative for the transgene locus and *Bar* gene. PCR screening was tested on *Bar* positive and negative plants.

construct. The transgenic events had multiple copies of the transgene cassette, ranging from one to more than 10 copies (Figure 1). The banding pattern was unique for each transgenic line, indicating that each arose from an independent integration event.

Total RNA was extracted from immature seeds of transgenic lines at 21 DAP and analysed by probing RNA gel blots with *Pina*- and *Pinb*-specific probes (Figure 2). RNA extracted from developing seeds of a soft wheat control variety Heron was used for comparison where the loading of Heron was equivalent to 0.25×, 0.5×, 1× or 2× that used for each maize sample. No transcripts were detected

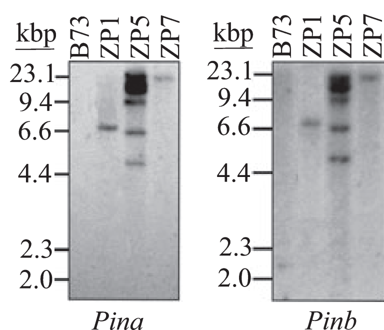

Figure 1 Southern blot analysis for three transgenic lines. Genomic DNA isolated from homozygous T_3 seedlings was digested with *Hin*d III. Untransformed B73 was used as a control. The ^{32}P-labelled DNA coding sequence of *Pina* or *Pinb* was used as hybridization probe. The position of DNA molecular size markers is indicated.

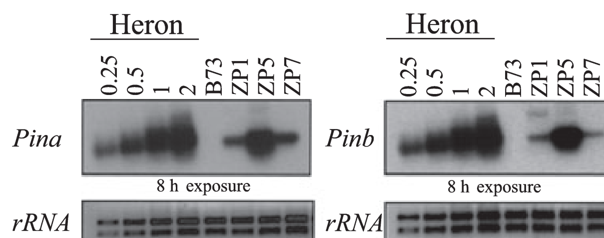

Figure 2 Northern analysis of *Pina* and *Pinb* expression in transgenic maize. B73 is a negative control. Heron RNA was loaded in a series of increasing amounts to account for varying signal intensities among transformed lines. Both control and transgenic lines were exposed for 8 h. A duplicate ethidium bromide-stained agarose gel shows discrete bands of rRNA fractionated, indicating a similar loading from lane to lane and a lack of rRNA degradation.

in control maize inbred B73. The *Pina* RNA level in ZP1 was less than half that of Heron, the level of *Pina* expression in ZP5 was approximately equal to that of Heron, and *Pina* expression in ZP7 was intermediate between ZP1 and ZP5. Similar results were seen with *Pinb* levels for each of these events.

Analysis of total and starch bound puroindolines

Triton X-114 (TX-114) specifically extracts hydrophobic proteins such as PINA and PINB. We extracted PINs from whole seed meal of each event (Figure 3). Soft wheat control Heron has a functional PINA and PINB, together yielding one visible band, and negative control B73 lacks the 13 kDa PIN band. Total content of PIN was greater in ZP5 than in an equivalently loaded wheat lane (Heron 1×), and ZP1 and ZP7 PIN levels were less than half that of Heron.

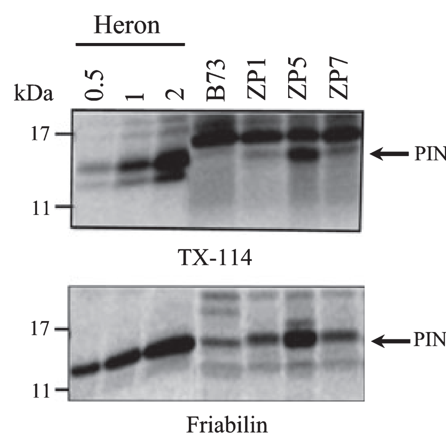

Figure 3 Total extractable (TX-114) and bound starch (Friabilin) PIN levels on stained SDS-PAGE gels. The 13 kDa band labelled PIN consists of both PINA and PINB. Expression was compared with the soft wheat control variety Heron which was loaded at half (0.5), same (1), or double (2) that of each of the maize samples.

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Friabilin is the classic term for total PINA and PINB bound to the surface of starch granules and can be used to measure the proportion of PIN that is present in functional form. The levels of starch bound PIN observed were consistent with the TX-114 result (Figure 3). PIN starch surface levels were highest in ZP5 and lower in ZP1 and ZP7.

Puroindoline transformants have decreased grain hardness

Data for grain hardness, protein content and starch content are presented in Table 2. The null controls included the untransformed control inbred line B73 and homozygous negative lines of each event. Kernel weight and protein content of transgene positive lines and their corresponding negative control lines were similar ($P > 0.05$). Significant grain hardness decreases were observed in all three transgenic lines compared to their negative control lines. The inbred B73 seeds required 20.94 kilogram-force (kgf) and were similar in hardness to the negative controls of each event (19.92 kgf). ZP5 had the greatest reduction in grain hardness (11.80 vs 18.69 kgf). Reduction in pressure required to fracture kernels ranged from 15.65% (ZP7) to 36.86% (ZP5) compared with the negative control seeds. The starch content of all three transgenic lines was slightly increased in both years, but only was significantly increased ($P < 0.05$) in ZP1 (74.2% vs 69.9%).

Table 2 Means for grain hardness, kernel weight, protein content and starch content of transgene positive and transgene negative for three T_3 events transformed with *Pina* and *Pinb* on an average of 2 years

Event	Source*	Kernel weight (g)†	Grain hardness (kgf)‡	Protein content (%)§	Total starch (%)¶
ZP1	+/+	0.249(ns)	14.34***	10.76(ns)	74.2*
	−/−	0.244	20.42	11.37	69.9
ZP5	+/+	0.240(ns)	11.80***	10.83(ns)	71.5(ns)
	−/−	0.248	18.69	11.10	68.5
ZP7	+/+	0.240(ns)	17.41**	11.60(ns)	69.9(ns)
	−/−	0.254	20.64	10.71	69.3
B73	wt	0.269	20.94	10.35	68.2

*T_2-derived T_3 seeds with co-segregation homozygous positive(+/+) or homozygous negative(−/−) for the transgene locus and *Bar* gene.
†The values presented are the average of three lines of each event over 2 years.
‡Hardness in kilogram-force (kgf) required to crack the seed determined by FORCE ONE™ Fdix force gauge. Each event was tested on three homozygous positive lines, and each line was analysed using about 25 individual seeds. The total number of seeds of each event analysed was about 70.
§Determined by a Leco FP-2000 (N × 6.25).
¶Determined by a Megazyme total starch assay kit.
*, **, *** denote significance at $P < 0.05$, 0.01, and 0.001, respectively, in comparisons of homozygous positive (+/+) transgenic lines and the corresponding homozygous negative (−/−) lines.
ns, not significant.

Transmitted light images of kernels

Maize kernels were viewed with transmitted light to assess vitreousness. The approximate amount of light transmitted is noted by a percent figure following the name of each event or the negative control line B73 (Figure 4). Transmitted light was highest for B73, indicating a hard endosperm. Transmitted light for the ZP events were all lower than for B73, indicating a softer endosperm compared to the control. Among the ZP events, ZP1 was the darkest. These results were similar to the results of the grain hardness test.

Morphology

Maize seed fracture planes and purified starch of event ZP5 and B73 were imaged using field emission scanning electron microscopy (FE-SEM) and scanning electron microscopy (SEM) and are shown in Figure 5. The maize starch granules were polihedric in shape with variable diameters. Figure 5a,b show seed cross fracture planes in the transgenic lines and untransformed control B73 at different levels of magnification. The starch granules in the transgenic lines had a rounder and smoother surface compared to the control line and less pronounced protein bodies. The percent of tightly packed starch granules was much less in ZP5 than in B73. Figure 5c shows starch granules after purification. The purified starch granules

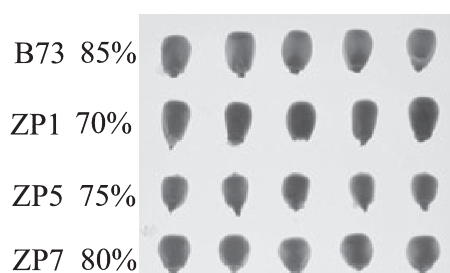

Figure 4 Transmission of light by mature maize kernels. Darker kernels are non-vitreous and the lighter kernels are vitreous kernels. The approximate amount of light transmitted is noted by per cent values of horneous endosperm.

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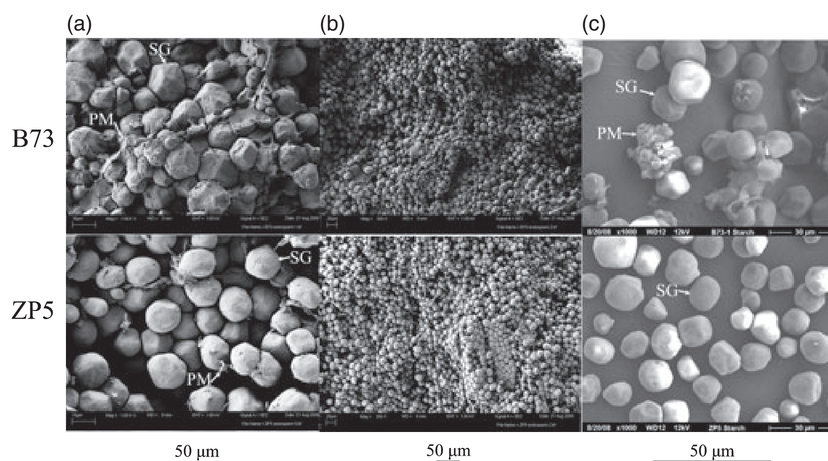

Figure 5 Field emission scanning electron microscopy (FE-SEM) analysis of endosperm cross section (a, b) and scanning electron microscopy (SEM) analysis of purified starch (c) of transgenic lines and negative controls. Line ZP5 was used as representative of the three PIN transgenic lines. (a) FE-SEM image of endosperm fracture at 1000× magnification. The granules are rough and clumped with adhering protein matrix materials in B73, while in ZP5 the granules have a more round and smooth surface and the protein matrix is less apparent. (b) FE-SEM image of endosperm fracture at 200× magnification. (c) SEM image of purified starch. The granules are rough and occur as clumps with protein matrix materials in B73, while in ZP5 the granules are clean and with no clumping or adhering material. Arrows indicate the location of starch granules (SG) and protein matrix (PM).

from ZP5 were clean and with no clumping or adhering material, while starch granules purified from B73 were rough in appearance and occurred as clumps with attached protein matrix materials. The observed differences are similar to that observed by Robutti *et al.* (1974) in comparisons of floury and flint maize.

Wet milling and starch extractability

The wet milling yield of three transgenic lines and their corresponding negative controls was measured (Table 3). Starch yields were significantly higher in all three transgenic lines ($P < 0.01$ for ZP1 and ZP5, $P < 0.05$ for ZP7). The starch yield of B73 was 58.7% and similar to the average of the negative controls of these events (58.9%). Increased starch yield for transgenic positive versus their transgenic negative controls ranged from 2.5% for ZP7 (61.6% vs 59.1%) to 5.2% for ZP5 (63.7% vs 58.5%). The average starch yield increase across all three events was 4.1%, with the highest yield increase seen in ZP5. The relative increase in starch yield ranged from 4.2% (ZP7) to 8.9% (ZP5). The sieve recovered tailings consisted

Table 3 Means for yields (dry basis) and purities for wet milling fractions of transgene positive and transgene negative for three T_3 events transformed with *Pina* and *Pinb* on an average of 2 years

Event*	Source†	Germ yield (%)	Tailing yield (%)	Starch yield (%)	Starch recovery‡ (%)	Starch content of starch§ (%)	Protein content of starch¶ (%)	Starch content of tailing§ (%)	Protein content of tailing¶ (%)
ZP1	+/+	6.97	5.21*	63.8**	86.0	93.0	1.99	39.1	18.60
	−/−	6.90	6.69	59.0	84.4	92.7	2.52	44.5	21.91
ZP5	+/+	7.07	5.48*	63.7**	89.1*	92.1	2.61	36.4*	18.35
	−/−	7.26	6.55	58.5	85.4	93.8	3.30	45.2	20.83
ZP7	+/+	7.53	5.52	61.6*	88.1*	92.3	3.24	46.8	19.60
	−/−	7.10	5.67	59.1	85.3	93.2	2.86	44.6	17.15
B73	WT	7.03	5.73	58.7	86.1	91.0	3.52	50.4	19.18

*Three replicates of each event were conducted for the 10-g scale wet milling test.

†T_2-derived T_3 seeds with co-segregation homozygous positive(+/+) or homozygous negative(−/−) for the transgene locus and *Bar* gene.

‡Starch recovery was defined as the ratio of total weight of starch recovered to total starch content.

§Determined by a Megazyme total starch assay kit.

¶Determined by a Leco FP-2000 (N × 6.25).

*, ** denote significance at $P < 0.05$, and 0.01, respectively, in comparisons of homozygous positive transgenic lines and the corresponding homozygous negative lines.

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primarily of fine fibre with some adhering starch and protein since the pericarp was removed by hand before blending. All transgenic lines exhibited lowered tailing yield with a significant difference observed for ZP1 and ZP5 ($P < 0.05$), with a decrease of 1.48% and 1.07%, respectively. There was no significant difference in germ yield among the transgenic lines and their controls ($P > 0.05$). Starch recovery was corrected for seed starch concentration and showed a trend similar to starch yield. Starch recovery was not significant between the positive and negative lines of ZP1 but was significant for both the ZP5 and ZP7 events in which the transgenic lines had higher starch recovery than the negative controls (89.1% vs 85.4%, and 88.1% vs 85.3%, respectively). Starch and protein content of wet-milled starch and tailing fractions are also given in Table 3. Starch content of the recovered starch fraction was not significantly different between transgenic lines and their controls ($P > 0.05$). Although the differences in starch content of the tailing fraction were not significant ($P > 0.05$), the tailing fraction obtained from the softest transgenic lines, ZP1 and ZP5, had lower starch content than their corresponding controls and B73. The mean protein content of purified starch and tailing had a similar trend for the starch content of purified starch and tailing. Protein content of starch separated from the transgenic lines was not significantly different although ZP1 and ZP5 starch trended lower in protein content. Similarly, protein content of tailing fractions from ZP1 and ZP5 were lower than their controls.

Discussion

The texture of maize seeds is critically important in determining commercial end uses. Development of high extractable starch maize hybrids is a major goal (Paulsen *et al.*, 2003). The puroindoline genes play a critical role in creating soft wheat grain texture. Here, we show that expression of these genes in the maize endosperm also creates a softer endosperm. While the exact mechanism underlying this cause remains to be elucidated, the data presented here show that the underlying biochemistry allowing the PINs to bind to starch and modify endosperm texture has been maintained in maize over evolutionary time. PINs expression under the control of the maize *Zein* promoter significantly reduced the grain hardness in each of three transgenic events examined. Seed size, protein and starch content were relatively unaltered in each event (Table 2). However, starch content was increased in ZP1 and trended higher for all three events (Table 2). Since, PIN expression in wheat is not linked to differences in total starch content (Feiz *et al.*, 2008), it seems likely that the increased starch content seen in ZP1 may simply reflect that the ZP whole meals had smaller particle size after milling and were easier to convert to glucose. ZP5 had the softest seeds (Table 2) and it also had the highest PIN expression levels at both the RNA and protein level (Figures 2 and 3). Pressure required to fracture kernels was reduced 36.9% and correlated with the percent light transmitted with all three transgenic events being less vitreous than the negative control B73 (Figure 4). Adhesion between the starch granule and protein matrix was reduced by PIN expression as evidenced by SEM and FE-SEM (Figure 5). These images revealed that the starch granules in ZP5 were smoother, and with less surrounding protein matrix (Figure 5). These data suggest that PINA and PINB may function as a non-stick agent and reduce the close adherence between starch granules and maize proteins, and in turn, a floury endosperm. The appearance of starch granules within the transgenic lines was altered (Figure 5) and the relative difference between starch granules from hard and soft endosperm was similar to that described by Robutti *et al.* (1974) in comparisons of opaque-2 maize and normal maize. A possible explanation for our observed differences is that during the natural drying process of grain, the protein matrix loses water and shrinks. If adhesion within the protein matrix remains strong, starch granules are pulled closer and closer together, and if the starch granules still contain enough water to be flexible at this stage, the results would be polygonal, tightly packed granules. If the adhesion between the starch and protein is not strong, the starch granules would be round (Robutti *et al.*, 1974). The Pins reduced the tight adhesion between the starch and the surrounding protein matrix, which then gave an altered shape of starch granules.

Two biochemical components in maize, alcohol-soluble prolamins (zeins) and amylose in starch have been shown to affect maize endosperm texture (Mestres and Matencio, 1996). In vitreous maize endosperm, starch granules are surrounded by protein bodies and are embedded in a dense matrix. In contrast, starch granules in floury endosperm are less compact and the protein matrix is discontinuous (Kotarski *et al.*, 1992). Maize starchy endosperm mutants, for example, *opaque-2* and *floury-2*, have high corn protein quality and alter the structure of the endosperm, but are typically associated with susceptible to mechanical damage and insect and fungi attacks (Lambert *et al.*, 1969). Wheats that vary naturally or contain

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transgenically overexpress PINs have softer seeds and increased starch yield but do not have altered agronomic traits such as seed size or yield (Martin *et al.*, 2001; Feiz *et al.*, 2008). Therefore, we anticipate that none of the undesirable agronomic problems found for opaque-2 and floury-2 would apply to PIN transgenic maize since both opaque-2 and floury-2 modify endosperm texture via affecting the accumulation of various endosperm storage proteins (Fontes *et al.*, 1991; Schmidt *et al.*, 1992), not via binding directly to starch granules.

Vignaux *et al.* (2006) compared a 10-g laboratory wet milling procedure for maize with a larger scale 100-g procedure and found that hybrid effects on the starch and gluten separation were more apparent with the 10-g procedure. Accordingly the 10-g test procedure was used in our analysis. The softer transgenic kernels exhibited a greater separation of starch granules from the surrounding protein matrix and had increased starch extraction yield (Table 3). The tailing yield was lower in transgenic lines. In ZP1, the tailing yield was 5.21% with 39.1% starch, which amounted to 2.75% of total starch lost in the tailing fraction, which was mainly fibre. The tailing yield of the ZP1 negative control line was 6.69% and contained 44.5% starch, which amounted to 4.26% of the total starch lost to the tailing fraction. Therefore, 1.51% less starch was lost to tailing in the transgenic line, which would translate into higher starch yield. A similar result was observed in ZP5. The protein content of the tailing fraction was also lower in the ZP transgenic lines, which suggested that the fibre retained more starch and protein in the negative line and indicated that the softer seeds gave greater initial starch recovery. The effect of the transgenic addition of puroindoline to maize will likely be easier germ removal, finer ground particles and easier starch–protein separation. That would reduce total milling energy to mill and increase total starch yield. While no small scale test accurately measures ease of germ removal, we observed that germ and pericarp removal were easier in each of the transgenic lines (data not shown). That observation is consistent with a previous report where a close positive correlation at the cereal species level between presence or absence of puroindolines and the ease with which embryos could be detached from their caryopsis was observed (Morris, 1993).

Increasingly, maize starch is also used as a renewable raw material in the form of bioenergy after conversion to ethanol (Torney *et al.*, 2007). In maize ethanol production, a batch method is generally used in which the amount of enzymes required as well as the time required for total conversion of starch to ethanol are two of the most important factors (Westby and Gibbons, 1982). We anticipate that ethanol production from dry milling processing could also be improved due to the use of softer textured PIN maize. The softer textured maize would require less enzymes and time to achieve total conversion to ethanol for two reasons. First, PINs on the surface of starch granules would result in greater ease of separation of starch granules from the protein matrix. Second, smaller particle size after milling will result in greater surface area for enzyme digestion.

In conclusion, this study demonstrates that the maize kernel structure and wet milling quality were successfully modified and improved by the endosperm-specific expression of wheat *Pins*. Expression of wheat *Pins* reduced the adhesion between the starch and the surrounding protein matrix. The endosperm-specific expression lines had softer kernels and likely would require less energy during milling. The transgenic lines also had a significantly higher starch extractability and yield. Therefore, the endosperm-specific expression of *Pins* in maize could improve the economic value of maize destined for use in wet milling.

Experimental procedures

Plasmid constructs

The *Pina* and *Pinb* endosperm-specific vector, *pNB167*, is shown in Figure 6. To create this vector, the puroindoline genes were amplified from genomic DNA of the soft wheat cultivar Chinese Spring using *Taq* DNA polymerase (Promega, Madison, WI, USA) with the following primers:

Pina: Forward: 5′- GGTGTGGCCTCATCTCATCT;
Reverse: 5′-TCACCAGTAATAGCCAATAGTG.
Pinb: Forward: 5′- AATAAAGGGGAGCCTCAACC;
Reverse: 5′- TCACCAGTAATAGCCACTAGGGAA.

Cycling parameters were 94 °C for 3 min, 40 temperature cycles of 94 °C 30 s, 55 °C 30 s, 72 °C 1 min, followed by a 5-min final extension at 72 °C. The PCR products were digested with *Bam*HI and *Xba*I, and then ligated between the maize

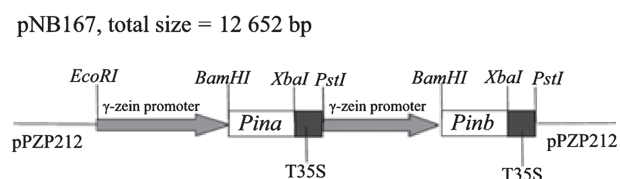

Figure 6 *Pina* and *Pinb* endosperm-specific expression vector *pNB167*. Both *Pina* and *Pinb* are under the control of the maize 27 kDa γ-*Zein* promoter and the CaMV 35S polyadenylation region terminator (T35S) with the vector backbone being *pPZP212*.

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27 kDa γ-*Zein* promoter (Marks *et al.*, 1985) and the cauliflower mosaic virus (CaMV) 35S polyadenylation region terminator. The promoter sequence was amplified from maize genomic DNA. The CaMV 35S terminator was amplified from the pPZP212 vector (Hajdukiewicz *et al.*, 1994). The *Pina* and *Pinb* coding sequence and its flanking sequence were then placed into a modified pPZP212 backbone. All resultant plasmids were sequenced to ensure correct orientation and fidelity of ligation junctions. The construct *pBAR184* used for selection (Frame *et al.*, 2000) contains the maize *Ubiquitin* promoter-*Bar* gene cassette as a selectable marker conferring resistance to the herbicides bialaphos (4-[hydroxy(methyl-)phosphinoyl]-L-homoalanyl-L-alanyl-L-alanine) and glufosinate (DL-homoalanin-4-yl(methyl) phosphinic acid).

Maize transformation

Maize transformation was conducted in the Iowa State University Plant Transformation Facility according to their standard protocols (Frame *et al.*, 2000). Highly embryogenic maize line Hi-II was used in biolistic transformation. Embryogenic calli generated from immature embryos of the Hi-II genotype were co-bombarded with the plasmids *pBAR184* and *pNB167*. Bialaphos-resistant calli were screened by PCR for the *Pina* and *Pinb* coding sequences. Calli of *Pina/Pinb* PCR positive transgenic events were regenerated and brought to maturity in the greenhouse. The greenhouse conditions consisted of a 16 : 8 photoperiod with a daytime temperature of 28 °C and a nighttime target temperature of 21 °C. Herbicide-resistant *Pina/Pinb* PCR positive T_0 lines were pollinated using inbred line B73. Herbicide resistance of T_0 and all subsequent progeny plants was tested by painting an individual leaf with 0.1% glufosinate ammonium (AgrEvo USA Company, Wilmington, DE, USA) when plants were at the two-leaf stage. The plants were scored as being resistant or susceptible 7 days after glufosinate application. Resistant plants maintained normal green coloration while susceptible plants were substantially yellow in colour. Herbicide-resistant T_1 plants were heterozygous for the transgene locus and were self-pollinated to produce T_2 progeny. Individual T_2 plants from each transformation event were tested for both *Pin* genes as well as for the presence of *Bar*. T_2 plants giving progeny (T_3 plants) in which more than 12 consecutive sprayed plants were herbicide resistant and *Pina/Pinb* PCR positive were considered homozygous positive for *Bar* and *Pina/Pinb*, and T_2 plants having progeny where more than five consecutive sprayed plants were herbicide susceptible and *Pina/Pinb* PCR negative were considered homozygous negative for *Bar* and *Pina/Pinb*. T_0 and T_1 plants were planted in a Montana State University greenhouse. T_2 and T_3 plants were planted both in the greenhouse at Montana State University and in the field at the University of Florida in 2007. T_4 plants were planted in the field at the University of Florida in 2008. Each line was planted in two rows and each row had 15 plants with between row spacing of 90 cm and within row spacing of 30 cm. Ears from individual selfed plants were harvested, dried for 3 days at 37 °C in a forced air incubator and then maintained at room temperature and ambient humidity (30%). The moisture content of all seeds at the time analyses were performed was ∼10%. The same seed source was used for all the seed quality analysis including kernel weight, starch and protein content, germ yield and oil content. All values represent three independently derived homozygous positive or negative lines for each event of both years. Seeds from three ears within the same line were pooled.

Southern analysis

Southern blot analysis was performed using DNA extracted from young leaf tissue of homozygous T_3 transgenic lines. Genomic DNA was isolated from young leaves according to Riede and Anderson (1996), and digested with *Hin*d III for 5 h at 37 °C. Fifteen μg of the digested DNA was fractionated on a 0.8% agarose gel for 8 h at 40 V, and blotted to a nylon membrane (Osmonics Inc., Minnetonka, MN, USA). Blots were hybridized to ^{32}P-labelled probes prepared by a random primer method (MP Biomedicals Inc., Irvine, CA, USA). Following hybridization, the membranes were washed three times at low stringency [2× SSPE (standard saline phosphate/EDTA), 0.1% SDS (sodium dodecyl sulphate)] and then twice at high stringency (0.2× SSPE, 0.1% SDS). All washes were shaken 15 min at 65 °C. Washed membranes were exposed to x-ray film (Research Products International Corp, Prospect, IL) at −80 °C using an intensifying screen. Probes were made from the coding sequence of wheat *Pina* or *Pinb*, amplified as previously described (Gautier *et al.*, 1994).

Northern analysis

Northern blot analysis was performed by standard methods as described previously (Giroux and Morris, 1997). RNA was prepared from 21 days after pollination (DAP) developing maize seeds using a Trizol protocol (Invitrogen, Carlsbad, CA, USA) as described in detail in Hogg *et al.* (2004) with the following changes. First, three seeds of each event were ground with a mortar and pestle in liquid N_2. Approximately 0.2 cm^3 of seed powder was placed in a 2-mL tube and then 0.5 mL of RNA extraction buffer was added, and the sample was vortexed until homogenous, then the following steps were as described previously. For blotting, 2 μg of RNA were separated on a 1% formaldehyde agarose gel and blotted to a nylon membrane. Blots were hybridized and washed as described above. To quantify total *Pina* and *Pinb* expression, an expression scale was used including 0.25×, 0.5×, 1×, and 2× total RNA concentration levels, where 1× equalled 2 μg of total RNA. The RNA used to make this scale was taken from the soft wheat Heron (Giroux and Morris, 1998), which expresses the *Pina-D1a* and *Pinb-D1a* alleles.

Extraction and analysis of total and starch-bound puroindolines

Seeds from three transgenic maize lines, and the soft wheat control (Heron) were ground using a Perten 3303 Laboratory Mill (Perten Instruments, Stockholm, Sweden). Total puroindoline was extracted from the 100 mg of fine powder using Triton X-114 (TX-114) detergent by modifying the previously described method (Giroux *et al.*, 2003) to include an additional round of TX-114 fractionation. Protein pellets were suspended in SDS sample buffer and loaded onto 10%–20% Tris-glycine gels (Lonza Rockland, ME, USA). Puroindoline content was quantified using a scale of

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0.5×, 1× and 2× constructed using a Heron TX-114 protein extract where 1× equalled 4 μL loading (60 μL SDS-PAGE sample buffer per 100 mg powder).

Friabilin (starch bound PINA and PINB) was isolated from the surface of starch granules using the same genotypes as for the TX-114 protein extraction, by modifying the method of Bettge *et al.* (1995). 300 mg of finely ground mature seed flour was placed into 2-mL tubes, 1 mL of 0.1 M NaCl was added to each tube, and samples were vortexed and incubated 30 min. After a brief mixing and 5 s sitting, the starch containing supernatant was aspirated and transferred into a new preweighed 2-mL tube. Then 500 μL of 0.1 M NaCl was used to wash the sediment two additional times, placing the supernatant containing starch in the same preweighed tube. Then the starch was washed, extracted and fractionated as described previously (Bettge *et al.*, 1995). Total friabilin was quantified relative to friabilin extracted from the soft wheat Heron, using a scale of 0.5×, 1× and 2×, where 1× equalled 2.5 mg of starch recovered after the $ZnSO_4$ precipitation.

Measurement of grain hardness

Grain hardness was measured using a FORCE ONE™ Fdix force gauge (Wagner Instruments, Greenwich, CT, USA) by recording the maximum compression force in kilogram (kgf) required to crack individual seeds. Each transgenic event was represented by three T_1-derived homozygous transgene positive and three T_1-derived transgene negative lines, with 25 individual seeds analysed for each line from seeds grown in both the 2007 and 2008 growing seasons.

Transmitted light images of kernels

The transmitted light test provides a quick, effective method to measure the grain endosperm vitreousness (Wayne *et al.*, 2004). The light source was an Acculight light box (Model 6002; Knox Manufacturing Co., Wooddale, IL, USA). The proportion of light transmitted was determined visually using the same lines as for grain hardness. Hard texture or vitreous endosperm transmits more light than soft-textured or chalky endosperm.

Wet milling of maize

The maize wet milling method was adopted from previously published procedures (Ji *et al.*, 2004; Vignaux *et al.*, 2006). Ten g of kernels of each of the three transgene positive and negative lines for each event grown in 2007 and 2008 were steeped in 20 mL 0.2% SO_2 and 0.5% lactic acid, pH 3.2, 50 °C for 48 h. Then the pericarp and germ were manually removed with forceps. Germ was dried for 2 days at 37 °C using a forced air incubator and weighed. The separated endosperms were placed in a blending beaker with 100 mL ddH_2O and blended at highest speed for 10 min with a KITCHENAID® blender (Model KHB100; KitchenAid, St. Joseph, MI, USA). The homogenized slurry was filtered with a 3-in diameter 170 mesh (0.090-mm aperture) screen. The material on the screen was then washed several times, with a total ddH_2O volume of 500 mL. The material on the screen was dried for 2 days at 37 °C and weighed as tailing yield. The starch slurry was settled overnight at 4 °C and then the supernatant was drained off. The recovered starch was rinsed with 500 mL ddH_2O and allowed to settle 60 min and the supernatant was decanted. This step was repeated twice. The starch and protein were further separated by transferring the suspension to 250-mL Sorvall centrifuge bottles and centrifuging at 2500 ***g*** at 25 °C for 15 min using a Sorvall super T21 centrifuge (Kendro Laboratory Products, Newtown, CT, USA). The whole pellet was taken as starch after pouring off the supernatant. The starch was then dried for 2 days at 37 °C and weighed. Starch yields were calculated as the ratio of the weight of starch recovered to the total weight of the sample. The starch recovery was calculated as a ratio of the starch recovered to the total weight of starch content in the maize.

Starch content of whole seed meal and the dried wet milling starch product were measured via the total starch assay method (American Association of Cereal Chemists, 2003) using a Megazyme kit (Megazyme International, Bray, Co. Wicklow, Ireland). Protein content of whole seed meal and the dried wet milling starch product was measured using a Leco FP-2000 (Leco Corp., St. Joseph, MI, USA). Protein was calculated using the general factor N × 6.25.

SEM and FE-SEM imaging

Mature maize seeds of equivalent size and moisture content were fractured by the FORCE ONE™ Fdix force gauge (Wagner Instruments, Greenwich, CT, USA). Central endosperm fracture faces were vacuum-coated with gold and analysed using FE-SEM (Zeiss Supra 55VP; Carl Zeiss AG, Oberkochen, Germany). Maize starch obtained by the small-scale wet milling procedure described above was mounted directly onto an aluminium electron microscope stub and coated to 10Å thickness with gold. Starch granules were viewed via SEM (Model JSM 6100; JEOL, Tokyo, Japan) at 1000 magnification (12 kV).

Statistical analysis

All mean values represent testing of three independently derived T_1 homozygous positive or negative lines for each event. The means of a transgene positive event was compared with its respective transgene negative events using a *t* test. All statistical tests were conducted using GraphPad InStat software (v. 3.06; San Diego, CA, USA)

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