rgf1, a mutation reducing grain filling in maize through effects on basal endosperm and pedicel development†

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Received 7 December 1999; revised 3 March 2000; accepted 14 March 2000.
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‡Dedicated to Professor Jeff Schell on his retirement, with thanks for his guidance and inspiration.

Summary

The maize cob presents an excellent opportunity to screen visually for mutations affecting assimilate partitioning in the developing kernel. We have identified a defective kernel mutant termed rgf1, reduced grain filling, with a final grain weight 30% of the wild type. In contrast with most defective endosperm mutants, rgf1 shows gene dosage-dependent expression in the endosperm. rgf1 kernels possess a small endosperm incompletely filling the papery pericarp, but embryo development is unaffected and the seeds are viable. The mutation conditions defective pedicel development and greatly reduces expression of endosperm transfer layer-specific markers. rgf1 exhibits striking morphological similarities to the mn1 mutant, but maps to a locus approximately 4 cM away from mn1 on chromosome 2 of maize. Despite reduced starch accumulation in the mutant, no obvious lesion in starch biosynthesis has been detected. Free sugar levels are unaltered in rgf1 endosperm. Rates of sugar uptake, measured over short (8 h) periods in cultured kernels, are increased in rgf1 compared to the wild type. rgf1 and wild-type kernels, excised at 5 DAP and cultured in vitro also develop differently in response to variations in sugar regime: glucose concentrations above 1% arrest placentalchalazal development of rgf1 kernels, but have no effect on cultured wild-type kernels. These findings suggest that either uptake or perception of sugar(s) in endosperm cells at 5–10 DAP determines the rgf1 kernel phenotype.

Keywords: endosperm, sugar, starch, assimilation, transfer layer, seed.

Introduction

The main storage carbohydrate of the cereal grain, starch, is synthesized from sugar transported from source leaves. Sugar transport takes place in phloem vessels, driven by a pressure drop generated by sugar accumulation in leaf phloem and sugar unloading in sink tissues. Before entering developing maize kernels, solutes are discharged from the sieve elements into companion cells at the phloem terminals. The subsequent route of sugar molecules can be divided into three stages: first, the sugars cross the various layers of the pedicel; then they are discharged into the apoplast of the endosperm cavity; and finally they are taken up via transporters located on the plasmalemma of endosperm cells. The contribution of each of these steps to carbohydrate assimilation has been studied.

Felker (1992) evaluated the contribution of cob (pedicel) tissue to sugar uptake by isolated kernels cultured in vitro. Similar rates of uptake were observed from the culture medium into the pedicel for [14C]sucrose as for [14C]fluorosucrose, which is not hydrolysed by invertases, suggesting that the rate of uptake by the pedicel is not dependent on invertase activity there. This observation is strengthened by the finding that the major kernel invertase activity, the In CW2 gene product, is restricted to the basal endosperm and is not expressed in the pedicel (Carlson and Chourey, 1999). On the other hand, sugar uptake by the pedicel
required active transporters as it could be inhibited by PCMBs, a non-cell-permeable sulphhydryl group modifier. PTS (3-hydroxy, 5,8,10-pyrene trisulphonate), an apoplastic tracer, was unable to penetrate the pedicel, and thus the endosperm, within 24 h; whereas fluorescein, a symplastic tracer, permeated all tissues in the explant within 24 h, suggesting that passive sugar transport through pedicel tissues is at least partly symplastic. Earlier experiments (Felker and Shannon, 1980) had shown that sugar transfer into the pedicel was much more rapid than movement between pedicel and endosperm cavity. This may be due to resistance to symplastic movement within the pedicel, which depends on the density of plasmodesmatal connections.

Sugar efflux from the pedicel of developing maize kernels was studied by Porter et al. (1985) by replacing the endosperm with an agar solute trap. The rate of unloading into the endosperm cavity was comparable to unloading into the endosperm of intact kernels for up to 6 h. Sucrose unloading took place without the requirement for an energy source. When invertase was inhibited, up to 60% of the sugar unloaded from the pedicel was sucrose. The results suggested a passive efflux of sucrose from the maize pedicel symplast into the endosperm compartment, followed by its extracellular hydrolysis to hexoses, and their uptake by membrane-bound H+/symporters.

Downloaded sugars enter the endosperm apoplastically, as no plasmodesmatal connections exist between the pedicel and the basal endosperm. To promote solute uptake into the endosperm, the basal aleurone layer is modified to transfer cells (Pate and Gunning, 1972). Adjacent basal endosperm cells also possess cell wall projections similar to those in transfer cells, and presumably also contribute to solute transfer. Basal endosperm transfer cells have been characterized cytologically (Davis et al., 1989; Felker and Shannon, 1980) and molecular markers specific for this cell layer have been isolated. Hueros et al. (1995, 1999) identified a group of small, secreted polypeptides which are basal cell-specific and transiently expressed during the period of metabolic activity of the transfer cells.

Sugar transport into the developing kernel is promoted by the presence of apoplastic invertase activity in the basal endosperm layer. Miller and Chourey (1992) and Cheng et al. (1996) showed that the mutant miniature1 (mn1), which reduces grain weight to <30% of the wild type, has only traces of invertase activity in the basal endosperm. They provide evidence that the Inw2 locus, encoding a cell-wall invertase which cosegregates with mn1 on chromosome 2, is mutated in mn1 lines (Talierco et al., 1999), and is responsible for the reductions in both cell wall-bound and soluble invertase activities observed (Carlson and Chourey, 1999).

By using a non-permeable sulphhydryl group inhibitor, PCMBs, Felker and Goodwin (1988) showed for endosperm suspension culture cells, and Thomas et al. (1992) for TsTu mutant kernels, that symporters are involved in sugar uptake into the endosperm, as reported for other sink tissues of bean (Weber et al., 1997) and wheat (Wang et al., 1999). Aoki et al. (1999) have isolated a sucrose H+/symporter gene from maize, which was shown by RNA blot analysis to be expressed in the developing pedicel and other sink tissues, and monosaccharide H+/symporter sequences from maize are represented in the ZmDB EST database (Gai et al., 2000; http://zmdb.iastate.edu). In situ hybridization will be required to establish whether these genes are expressed in the basal endosperm transfer layer.

To contribute to understanding the steps involved in solute assimilation in the developing maize kernel, we have analysed a newly identified mutant, reduced grain filling, rgf1. Like mn1, rgf1 is severely impaired in its rate of starch and fresh weight accumulation after 13 DAP. We show that the lesion conditioned by the rgf1 mutation is associated with alterations in both pedicel and transfer layer development and that, despite phenotypic similarities, rgf1 is distinct from previously characterized mutants of the Mn class.

Results

Isolation of the rgf1 mutant

A cob bearing several partially filled kernels was identified in the selfed F1 generation from the cross A69Y × glossy2-En (Tacke et al., 1995). To confirm the presence of a genetic lesion, defective kernels from a homozygous progeny plant were sown and crossed reciprocally with wild-type A69Y plants. The F1 cobs from these crosses exhibited reduced but uniform kernel size irrespective of the direction of the cross. Selfing of the heterozygous kernels gave rise to F2 cobs on which kernel size was segregating (Figure 1a). Four size classes could be distinguished, these occurring in the ratios listed in Table 1, which indicated gene dosage-dependent expression of the rgf1 phenotype. The Rgf1:rgf1 allelic composition of individual kernels was confirmed by sowing and selfing them. Plants from wild-type kernels (Rgf1/Rgf1) and extreme mutant phenotype (rgf1/rgf1) produced ears with uniform kernel phenotypes, whereas plants from the two classes of intermediate kernel types (Rgf1/rgf1) produced ears with segregating kernels. The homozygous rgf1 genotype was recovered from kernels obtained from segregating ears using embryo rescue. The rgf1 kernels derived from selfing an rgf1/rgf1 plant were plumper than homozygous rgf1 kernels on a segregating cob, and germinated indistinguishably from wild-type kernels. We attribute the difference in rgf1 kernel
development to a competition effect between kernels for available nutrients on the segregating cob.

Relationship of rgf1 to the miniature class of mutants

Both rgf1 and miniature mutants are characterized by a much reduced rate of endosperm development which, in the case of the homozygous mutant kernels, results in the formation of a gap between the aleurone layer and the overlying pericarp, giving the latter a papery appearance (Figure 1b). The rgf1 embryo is unaffected, and apart from a slightly delayed germination its vegetative growth is indistinguishable from the wild type, as is also the case for mn1. The rate of FW accumulation following self-pollination was determined for homozygous rgf1 and wild-type kernels. An FW increase was observed for rgf1 kernels only up to 16 DAP, with a 30 DAP FW of only 0.1 g, less than 1/3 that of the wild type. A deviation in the rate of FW accumulation between wild type and rgf1 was seen by 13 DAP (Figure 2), indicating a role for Rgf1 in early endosperm development.

As the rgf1 phenotype resembles that of the miniature class of mutants, an allelism test was carried out with the three available mutants, mn-1, 2 and 3. Given the semidominant nature of the rgf1 mutation, all heterozygous rgf1/mn kernels should exhibit a dosage-dependent reduced size compared to the wild type, but provided rgf1 and the mn locus are not allelic, the kernels should be larger than either of the homozygous parents. This was always observed, and shrivelled F1 kernels with papery pericarps were not seen, as seen in Figure 1(b) for the rgf1/mn1 allelism test. Therefore rgf1 is a lesion at a different locus to mn1, mn2 or mn3. Given the similar chromosomal locations of rgf1 and mn1, further confirmation was needed that recombination occurred between the two loci. This was achieved by selfing F1 kernels from the mn1 x rgf1 crosses; these gave rise to cobs bearing wild-type kernels at a frequency of 1.2% (341 cases out of 27450 F2 kernels), indicating a genetic distance of 2.3 cM between mn1 and rgf1.

By mapping the Rgf1 locus using AFLP markers, Rgf1 was identified in a genetic background related to an Argentinean inbred, A69Y. To maximize the polymorphisms in a mapping population, it was crossed to the North American line B37 (Gerdes et al., 1993). F1 plants were pollinated by A69Y (rgf1/rgf1) to give a BC1 generation segregating the easily distinguishable (rgf1/rgf1/rgf1) and (Rgf1/Rgf1/rgf1) endosperm phenotypes, and marker fragments coupled to Rgf1 were sought using AFLP. In an initial screen of 19 primer combinations carried out on 48 rgf1/rgf1 and 42 Rgf1/rgf1 DNAs, five polymorphic bands linked to Rgf1 were identified (Table 2).

![Figure 1. Morphology of mature cobs segregating the rgf1 allele, and derived from rgf1 x mn1 crosses.](image)

(a) Phenotype of an F2 cob from (rgf1/+) (b) Phenotypes of mature cobs arising from mn1/rgf1 allelism test From left to right: mn1 homozygote, rgf1 x mn1; mn1 x rgf1; rgf1 homozygote; wild-type cob.

<table>
<thead>
<tr>
<th>Table 1. Frequencies of kernel size classes on a segregating cob of (rgf1/+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kernel size (large → small)</td>
</tr>
<tr>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Predicted genotype</td>
</tr>
<tr>
<td>Number of kernels in this size class</td>
</tr>
</tbody>
</table>

The five linked AFLP markers were tested for segregation in the Co159 × T232 RIL population (Burr and Burr, 1991; Burr et al., 1994). One marker, E35/M34, was segregating and mapped on chromosome 2 between markers npi 242C and acc A.

The assigned map position was first confirmed by checking the map position of the E35/M34 AFLP marker with a second RIL population (T232 × Cm37). The assignment was further confirmed by checking in a segregating rfg1/rgf1 backcross population of 179 individuals the segregation of a closely linked SSR marker, bnlg 1613. The putative positions of rfg1 and n1 relative to markers in the vicinity are given in Table 2.

The rfg1 mutant possesses an aberrant pedicel but near-normal transfer layer morphology

The phenotype of rfg1 kernels suggested that solute transport through the basal part of the endosperm might

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**Figure 2.** Fresh weight accumulation in wild-type and rfg1 kernels. Fresh weights were determined for five to 10 wild-type and five to 10 rfg1 kernel pools from three cobs each at 3-day intervals after 10DAP. Error bars, SD between different cobs.

**Table 2.** Summary of rfg1 mapping data

<table>
<thead>
<tr>
<th>Population considered</th>
<th>No. of plants</th>
<th>No. of recombinants</th>
<th>Marker</th>
<th>Distance (cM)&lt;sup&gt;b&lt;/sup&gt; from rfg1</th>
<th>from E35/M34</th>
</tr>
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<tbody>
<tr>
<td>BC</td>
<td>89</td>
<td>2</td>
<td>AFLPE35-M32</td>
<td>2.2</td>
<td>-</td>
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<tr>
<td></td>
<td>2</td>
<td>2</td>
<td>AFLPE35-M34</td>
<td>2.3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1</td>
<td>AFLPE35-M37</td>
<td>1.1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6</td>
<td>AFLPE36-M33</td>
<td>6.7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4</td>
<td>AFLPE39-M45</td>
<td>4.5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>npi248&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>10.0</td>
<td>-</td>
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<td>npi242&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>6.1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>accA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>4.5</td>
<td>-</td>
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<tr>
<td></td>
<td>3</td>
<td>pbs15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>9.4</td>
<td>-</td>
</tr>
<tr>
<td>RIL (Co159 × T232)</td>
<td>39</td>
<td>4</td>
<td>npi242A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>- 10.9</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>npi297&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>16.0</td>
<td>-</td>
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<tr>
<td></td>
<td>3</td>
<td>bnlg1613&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>2.2</td>
<td>-</td>
</tr>
<tr>
<td>RIL (T232 × Cm37)</td>
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<td>4</td>
<td>accA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>- 7.8</td>
<td>-</td>
</tr>
<tr>
<td></td>
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<td>-</td>
<td>7.6</td>
<td>-</td>
</tr>
<tr>
<td>BC</td>
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<td>4</td>
<td>bnlg1613&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.2 2.3</td>
<td>-</td>
</tr>
<tr>
<td>F2</td>
<td>27450</td>
<td>341</td>
<td>m1</td>
<td>2.3</td>
<td>-</td>
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Order of markers on chromosome 2 (Genetic distance between adjacent markers in cM in brackets):

<table>
<thead>
<tr>
<th>npi297</th>
<th>npi242A</th>
<th>pbs15</th>
<th>accA</th>
<th>E35-M34</th>
<th>npi242C</th>
<th>npi240</th>
</tr>
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<tbody>
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<td>5.1</td>
<td>1.5</td>
<td>4.9</td>
<td>4.5</td>
<td>6.1</td>
<td>3.9</td>
<td></td>
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</tbody>
</table>


<sup>b</sup>cM distances from E35–M34 are from our analysis of the Co159 × T232 RIL population.
be defective. The possibility of an alteration in morphology of the pedicel or the transfer cells was investigated by comparing 10 μm sections of 15 DAP wild-type and mutant kernels (Figure 3). The pedicel of wild-type kernels contains one to two cell layers of loose tissue adjacent to the basal endosperm. These border a layer of dense cells with thick cell walls, which stain strongly with toluidine blue. This thick-walled cell layer (TWCL), which comprises most of the placentochalaza, is oriented perpendicular to the predominant direction of solute transport through the pedicel. By 15 DAP the layer is enucleate. The TWCL is almost absent in rgf1 mutant kernels, resulting in a gap eight to 10 cell layers wide which contains only the loose tissue, as described for the wild type. Cells in this layer have thin walls and are not organized into parallel files as in the TWCL. This disparity in TWCL development is already apparent by 10 DAP, supporting a role for the rgf1 gene product early in endosperm development. In contrast, the transfer cells of rgf1 endosperms are not obviously different at this level of resolution to those of wild-type kernels (Figure 3), and wall ingrowths are still visible.

**Transfer layer gene expression is reduced in rgf1 kernels**

The rgf1 mutation shows a clear dosage effect indicating an effect on endosperm gene expression, and yet embryo development is not affected. Therefore it must be the manifestation of an endosperm-expressed gene which indirectly affects both endosperm and pedicel development. A comparison by 2-D SDS-PAGE-IEF and SDS-PAGE-NEPHGE gel fractionations of total proteins from wild-type and mutant endosperm collected at 19 DAP (not shown) failed to yield any reproducible differences. This rules out the effect of abundant structural or storage proteins in the determination of the rgf1 phenotype.

As the rgf1 phenotype is suggestive of a defect in assimilate transfer, it was of interest to examine gene expression in basal transfer cells during kernel development. The availability of antibodies for two transfer cell-specific proteins, BETL1 and BETL2 (Hueros et al., 1995; Hueros et al., 1999) was exploited to examine protein concentrations of these markers in wild-type and rgf1 endosperm. BETL1 is a cell wall-bound protein, whereas BETL2 is found both in the cell wall and intracellularly. Both are novel, low molecular-weight polypeptides of unknown function which are transiently expressed and turn over by 25 DAP. Total protein extracts from kernels harvested at various stages during endosperm development were probed by immunoblotting to detect BETL1 and BETL2 (Figure 4a). BETL1 was scarcely detectable in the rgf1 mutant, whereas BETL2, although readily detected, was significantly reduced in concentration. Mature BETL1 and BETL2 polypeptides are both subject to post-transla-
Figure 4. Expression of BETL2 in rgl1 and other mutants affected in starch synthesis and grain filling.

(a) Total protein extracts from immature wild-type and rgl1 endosperm harvested at indicated DAP were fractionated by 15% SDS-PAGE and electroblotted onto PVDF membranes. Upper panels in (a) and (b) incubated with BETL-1 and BETL-2 antibody, respectively; lower panel, filter-loading control incubated with antibody raised to cytosolic GAPDH.

(b) In situ hybridizations using BETL2 cDNA probe to the following genotypes: rgl1 (A,B); mn1 (D,E); mn2 (F,G); bz1 (H,I); bz2 (K,L); sh2 (N,O); wild type (C,J,M,P). Underlined sections hybridized with anti-sense probe, remainder with sense probe. Bar = 200 μm.
Table 3. Sugar and starch accumulation during kernel development of WT and rgf1

<table>
<thead>
<tr>
<th>Carbohydrate/DAP</th>
<th>13</th>
<th>16</th>
<th>19</th>
<th>22</th>
<th>25</th>
<th>28</th>
<th>32</th>
<th>40</th>
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<tbody>
<tr>
<td>Glucose in mg/g DW</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>WT</td>
<td>62 ± 14</td>
<td>46 ± 9.4</td>
<td>24 ± 8.1</td>
<td>14 ± 2.3</td>
<td>9 ± 1.5</td>
<td>15 ± 5.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rgf1</td>
<td>56 ± 7.7</td>
<td>48 ± 11.4</td>
<td>34 ± 4.3</td>
<td>21 ± 5.7</td>
<td>18 ± 9.</td>
<td>8 ± 0.3</td>
<td></td>
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</tr>
<tr>
<td>Fructose in mg/g DW</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>WT</td>
<td>52 ± 7.6</td>
<td>41 ± 8.6</td>
<td>22 ± 7</td>
<td>12 ± 2.3</td>
<td>9 ± 2.1</td>
<td>12 ± 3.5</td>
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<tr>
<td>rgf1</td>
<td>50 ± 7.2</td>
<td>38 ± 1.7</td>
<td>32 ± 6.5</td>
<td>17 ± 1.1</td>
<td>16 ± 2.2</td>
<td>11 ± 0.3</td>
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<tr>
<td>Sucrose in mg/g DW</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>WT</td>
<td>69 ± 6.6</td>
<td>90 ± 12.7</td>
<td>47 ± 12.7</td>
<td>30 ± 6.2</td>
<td>32 ± 7.6</td>
<td>16 ± 3.5</td>
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<tr>
<td>rgf1</td>
<td>75 ± 19.8</td>
<td>77 ± 2.4</td>
<td>41 ± 10.1</td>
<td>38 ± 10.1</td>
<td>46 ± 8.8</td>
<td>26 ± 1.1</td>
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<td></td>
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<tr>
<td>Starch in mg/g DW</td>
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<td></td>
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<tr>
<td>WT</td>
<td>41 ± 7.8</td>
<td>79 ± 18.4</td>
<td>300 ± 29</td>
<td>277 ± 22</td>
<td>255 ± 29</td>
<td>339 ± 29</td>
<td>272 ± 22</td>
<td>262 ± 33</td>
</tr>
<tr>
<td>rgf1</td>
<td>7.3 ± 0.8</td>
<td>17 ± 1.5</td>
<td>46 ± 2.6</td>
<td>41 ± 5.9</td>
<td>n.d.</td>
<td>n.d.</td>
<td>39 ± 7.8</td>
<td>41.6 ± 8.6</td>
</tr>
<tr>
<td>rgf1-field grown</td>
<td>16 ± 2.5</td>
<td>26 ± 4.4</td>
<td>37 ± 3.4</td>
<td>39 ± 6.1</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Sugars were extracted with 80% ethanol from 50 mg maize meal, and assayed using a Boehringer Mannheim test kit. Error values given for sugar measurements represent the standard deviation of three different extracts, and or starch measurements represent the standard deviation of two different extracts.

Rfg1 mutant effects on grain filling in maize

Tional propeptide cleavages (G. Hueros and M. Maitz, unpublished results); we note that these processes were, at least qualitatively, unaffected by the rgf1 mutant, as the sizes of the polypeptides detected were the same as in wild-type extracts. Transfer-layer gene expression is nevertheless significantly reduced in rgf1. To investigate whether the reduction in BETL gene expression was specific for rgf1, or if it might occur in other mutants reducing carbohydrate assimilation, BETL2 expression was examined in other mutant backgrounds. We compared rgf1, mn1 (miniature1), mn3 (miniature3), bt1 (brittle1), bt2 (brittle2) and sh2 (shrunken2) endosperm sections by in situ hybridization (Figure 4b). Brittle1 encodes an ADPG translocator responsible for ADPG uptake into amyloplasts. Brittle2 and Shrunken2 encode two subunits of ADP-glucose pyrophosphorylase. In all cases except for rgf1, BETL2 expression was unaffected, indicating the effect of rgf1 on BETL gene expression is specific for this mutant, and that BETL2 expression is a useful marker for effects on transfer-layer gene expression.

rgf1 has only minor effects on enzymes involved in starch biosynthesis

Starch accumulates in rgf1 kernels at approximately 20% of wild-type concentrations, a ratio that is maintained from 13 to 40 DAP (Table 3). Despite this, the concentrations of sucrose, glucose and fructose remain remarkably similar to those in wild-type kernels during kernel development (Table 3), suggesting that the primary defect is likely to be in the rate of sugar flux. Maize mutations affecting starch synthesis result in alterations in sugar flux and/or in free sugar concentrations (Creech, 1965; Pan and Nelson, 1995). Although free sugar concentrations in rgf1 are not elevated, we have considered the possibility that rgf1 might be caused by a defect in starch synthesis. Possible effects on the expression of four key enzymes of starch biosynthesis were checked using cDNA probes for Sh1 (Shrunken1, encodes sucrose synthase) and Bt1, Sh2 and Bt2 (Cao and Cao, 1997; Sullivan et al., 1991; Figure 5). In no case was a major difference in gene expression in rgf1 seen, although Bt1 and Bt2 transcripts increased slightly in abundance relative to the wild type, which may reflect positive regulation through effects on sugar flux (Koch, 1996), sugar concentrations being unaltered (Table 3). Similarly, transcript abundance of the major cell-wall invertase gene lnw2 (Taliero et al., 1999) was unaffected (Figure 5). Further, using specific antisera we have shown that the Bt1, Bt2, Sh1 and Sh2 protein concentrations, as well as that of sucrose phosphate synthase, were not markedly altered by rgf1 (not shown). A formal possibility remains that one of the gene products estimated here was produced in wild-type amounts, but was defective. However, even if this was the case the lack of effect on gene expression in the starch biosynthetic pathway as a whole, and lack of free sugar accumulation seen in starch synthesis mutants (Creech, 1965), makes this unlikely to be a cause of the rgf1 phenotype.

Starch accumulation is reduced in rgf1 kernels without major effects on sugar pools

The morphological similarity between rgf1 and mn1 suggests that a defect in either sugar transport or metabolism may condition the rgf1 phenotype. As a first step, the glucose, fructose and sucrose contents of wild-type and rgf1 kernels were measured by extracting whole kernels (endosperm and pedicel) at six stages during

endosperm development (Table 3). No significant differences were seen. To rule out the possibility that an effect on sugar partitioning into mutant endosperm might be masked by accumulation of high sugar concentrations in the pedicel, as reported by others (Felker and Shannon, 1980), the sugar concentration in dissected pedicel and endosperm was also compared between the wild type and *rgf1*, but again no differences were found (not shown).

Despite the lack of a major effect on steady-state sugar concentrations in the kernel, the rate and extent of starch accumulation was severely reduced in *rgf1* (Table 3). Mutant kernels failed to accumulate starch after approximately 20 DAP, irrespective of culture in greenhouse or field conditions.

In *mn1* mutant kernels, both cell wall-bound and soluble invertase activities are greatly reduced, and reduced starch accumulation is attributed to a reduction in sugar flux as a consequence of the lack of basal cell invertase (Cheng et al., 1996). *mn1* cosegregates with *Incw2*, and the *Incw2* gene product is specifically expressed in the transfer layer. Linkage evidence suggests that *Mn1* may correspond to the *Incw2* locus. These findings prompted us to measure invertase activity in developing kernels of the *rgf1* mutant. Measurement of cell wall-bound and soluble invertase fractions (Figure 6) showed soluble invertase activity was unaffected in *rgf1*, but cell wall-bound enzyme activity was reduced to 50–70% of the wild-type value. This contrasts with total invertase activities of <6% of the wild type in *mn1* homozygotes (Cheng et al., 1996).

**Figure 6.** Invertase-specific activity in wild-type and *rgf1* kernel extracts. Error bars, SD of two different extracts.

**Sugar flux in cultured kernels is not reduced in *rgf1***

*rgf1* kernels do not appear to lack major enzymes needed for sugar assimilation and starch synthesis, nor do they have elevated concentrations of free sugars. A further possibility might be an effect on the rate of sugar transport. In order to investigate this, developing kernels were excised and cultured *in vitro* with 14C-labelled sucrose (or glucose). At regular intervals over a 8 h period, radioactivity incorporated into ethanol-soluble sugars and insoluble polysaccharides was measured (Figure 7a). Irrespective of whether [14C]glucose or [14C]sucrose was supplied, the rate of incorporation in polysaccharides was similar for *rgf1* and wild-type kernels, whereas higher concentrations of free radioactive sugars accumulated in the mutant kernels. The distribution of sugar taken up was monitored by autoradiography of hand-dissected kernels and quantified with a phosphorimager (Figure 7b). The results demonstrated a highly significant increase in the fraction of sucrose taken up into the endosperm in the *rgf1* mutant compared to the wild type. Wild-type kernels incorporated a mean of $1.38 \times 10^4 \pm 1.08 \times 10^3$ cpm, whereas *rgf1* kernels
incorporated a mean of $3.11 \times 10^4 \pm 8.8 \times 10^3$ cpm (from counting 20 samples of each genotype, result significant with Student’s $t$-test at 95%, $P<0.05$). However, the difference in uptake into the endosperm was abolished if the pedicel was removed before incubation of the kernel.

**Development of rgf1 kernels is more susceptible to elevated sugar concentrations than in the wild type**

Biochemical analysis of the *rgf1* mutant suggests that the concentrations of free sugars are not significantly altered relative to wild-type kernels, although it is possible that the ratio of extracellular to intracellular sugar concentrations may be affected. Shannon *et al.* (1993) showed an elevation of sucrose in the pedicel of *mn1* kernels compared to the wild type in flux experiments with $^{14}$C-sucrose. In order to see whether the morphological alterations seen in *rgf1* might reflect changes in sugar concentrations not detected in our kinetic measurements, kernels were cultured in vitro on media of varying sugar concentrations. 5-DAP kernels were harvested in strips with attached hilar tissue and placed for 10 days on media containing 0.3–5% sucrose or glucose. After incubation the kernels were fixed and wax-embedded for sectioning. Examination of longitudinal sections of wild-type kernels showed no deviations from normal development in the pedicel or basal endosperm with varying sugar concentrations (Figure 8). In contrast, *rgf1* kernels failed to develop the placentalchala layer when cultured on glucose or sucrose concentrations higher than 1%. Although the placentalchala layer was still visible in *rgf1* kernels if incubated on glucose or sucrose concentrations of 1% or less, BETL1 and BETL2 protein did not accumulate, i.e. the treatment did not revert this aspect of the *rgf1* phenotype.

**Discussion**

**Genetic analysis of rgf1**

Neuffer and Sheridan (1980) identified four viable, completely dominant endosperm mutants out of a population of 2477 EMS-induced lines. Scanlon *et al.* (1994) isolated 63 kernel mutants out of a Mutator-tagging population. In neither population were mutant alleles reported which exhibited a gene dosage effect as seen for *rgf1*. In the classification of kernel mutants established by Neuffer and Sheridan (1980), the *rgf1* phenotype is most similar to the *miniature* class of mutants: the pericarp is partially unfilled, whereas the embryo and somatic tissues are viable and indistinguishable from the wild type. However, the *rgf1* mutation is semidominant, while all *mn* mutants described are recessive. As the *rgf1* allele conditions an endosperm defect, four kernel sizes could be distinguished on a segregating cob. These differences in kernel size were less evident when comparing cobs bearing kernels of a single mutant genotype. This indicates that the *rgf1* phenotype is influenced by competition between kernels for solute uptake on the cob. The unusual phenotype of *rgf1* appears to reflect a rate limiting gene product for kernel development, raising the possibility that by overexpressing *Rgf1*, an increase in kernel size might be achieved. However, the mutant *rgf1* allele might itself be overexpressing the gene product encoded at the locus, in which case overexpression would be detrimental.

**Relationship of rgf1 to mn1**

Co-segregation of markers linked to both *rgf1* and *mn1* on the short arm of chromosome 2 suggests the two mutations might be closely linked, or even in the same gene. The complementation of phenotype seen in the allelism test with *mn1*, however, demonstrates that the two mutations map at different loci. Selfing the *mn1/rgf1* F$_1$ plants yielded cobs bearing 1.24% of wild-type kernels, placing the two loci 2.3 cm apart. This finding, and the observation that invertase activities in *rgf1* endosperm were 10-fold higher than those seen for strong *mn1* alleles (Cheng *et al.*, 1996), supports the hypothesis that, despite their morphological similarities, *rgf1* and *mn1* affect different steps in endosperm sugar assimilation.

**rgf1 kernels are unaffected in steady-state sugar concentrations and in expression of starch synthesis enzymes**

Free sugar concentrations, starch content and sugar uptake were measured for wild-type and *rgf1* kernels during endosperm development. Surprisingly, free sugar concentrations were similar in wild-type and mutant kernels, and the rate of sugar uptake in cultured 15-DAP kernels was similar in *rgf1* and wild type. *rgf1* kernels accumulate 70% less starch than wild-type kernels. Despite this, in the in vitro flux measurements the rate of incorporation of $^{14}$C into polyglucans in cultured kernels was similar for *rgf1* and wild-type kernels.

A number of starch biosynthetic enzymes are regulated by sugar concentration or sugar flux. Koch (1996) termed these ‘feast’ or ‘famine’ genes, according to their responses. In general, starch biosynthetic enzymes correspond to feast genes and are activated by elevated sugar concentration. Giroux *et al.* (1994), for example, reported a positive regulation of Bt2 and Sh2 mRNA by sugar concentration. In *rgf1*, we have observed modest increases in Bt1 and Bt2 transcripts, and also elevated Bt2 and Sh2 protein concentrations, compared to the wild type. However, there were no hints of major enzyme deficiencies that might have accounted for the *rgf1* phenotype, and no
accumulation of free sugars, as generally seen for mutants reducing starch accumulation (Creceh, 1965).

Both soluble and cell wall-bound invertase activities were measured during endosperm development. A reduction in cell wall-bound invertase activity was seen in rglf extracts, whereas the much lower fraction of soluble invertase activity was unaltered in the mutant. Incw2 transcript abundance was unaffected by the rglf mutation, pointing to a post-transcriptional effect on invertase accumulation.

rgf1 has major effects on pedicel and endosperm development

Light microscopic analysis of transfer cells of rglf endosperms failed to reveal any significant differences to those of the wild type. Despite this, expression of the two transfer cell-specific genes tested, BETL1 and BETL2, was greatly reduced in rglf, indicating the Rglf gene product does affect gene expression in transfer cells. A further striking feature of rglf kernels is the effect on pedicel development, where the maternal placentochalaza fails to develop. This may be due to a local change in sugar flux, or to a diffusible signal arising from this change. The loss of intercellular adhesion which occurs is a consequence of alteration in cell turgor (Jarvis, 1998).

Effect of rglf on sugar assimilation

Free sugar concentrations, starch content and sugar uptake were compared for wild-type and rglf kernels during endosperm development (Table 3 and Figure 7) Despite a reduction in starch accumulation of 70%, free sugar concentrations were similar between wild type and rglf throughout development. In vitro uptake experiments showed a significant increase in sugar uptake into mutant endosperm compared to the wild type, although these studies were only followed for 8 h, and began at a stage when the placentochalaza defect was already evident. The differential rate of sugar uptake was only seen in kernels attached to the pedicel (Figure 7b), suggesting the placentochalaza in wild-type kernels may limit the rate of sugar transfer. The short-term increased rate of sugar uptake into rglf kernels observed in culture contrasts with decreased starch deposition in the mutant. The reduction in starch accumulation may be a consequence of reduced cell number, which determines kernel sink size early in development in maize (Jones et al., 1996).

Culture of kernels excised before establishment of the mutant phenotype in the placentochalaza (at 5DAP) reveals another interesting aspect of sugar regulation. In rglf mutant kernels, the formation of this tissue depends on the sugar concentration in the medium (Figure 8). At low sugar concentrations, rglf pedicel development is normal. Higher sugar concentrations prevented these cells from developing in rglf but not wild-type kernels (Figure 8). We hypothesize that a sensing mechanism, which keeps sugar levels constant and allows wild-type kernel tissues to develop normally even at 5% sucrose, is defective in the rglf mutant, and that this results in alterations in pedicel structure. Although the major cytopathic lesion is in the pedicel, this must reflect a response to sugar flux in rglf endosperm. The Rglf locus may therefore participate in, or sense, sugar transport. Sugar flux is known to be sensed by specialized transporters in yeast (Lalonde et al., 1999); alternatively, intracellular sugar concentrations may be monitored by hexokinases (Jang et al., 1997). Defective sugar transport might be expected to affect sensing by either of these routes. The reductions

Figure 7. Distribution of 14C-labelled sugars after in vitro incorporation in developing kernels. (a) Incorporation of 14C-labelled glucose and sucrose into 15-DAP wild-type and rglf kernels after 1, 2, 3, 5 and 8 h incubation. Error bars, SD of two different extracts. (b) Autoradiography of kernel hand sections after 12 h incubation in [14C]sucrose. A, Longitudinal sections of intact 15-DAP wild-type and rglf kernels; -P, longitudinal sections of kernels incubated lacking the pedicel, from wild type and rglf.
Figure 8. Aberrant rgf1 kernel development is determined by an increased susceptibility to elevated sugar concentration. 

In vitro culture of 5-DAP rgf1 and wild-type kernels after 10 days’ incubation on media containing varying glucose concentrations. (a) rgf1, 1%; (b) wild type, 1%; (c) rgf1 2%; (d) wild type, 2%; (e) rgf1 3%; (f) wild type, 3%. Kernels were fixed and embedded in Fibrowax before sectioning and staining with DAPI and calciofluor. Observations were made under UV illumination.

in BETL1 and -2 protein accumulation in rfg1 endosperms are not reverted by incubation in low-sugar media, and are in general insensitive to changes in sugar concentration (M. Maitz and R.D. Thompson, unpublished results).

The phenotype of the rfg1 mutation is detectable by 10 DAP: the gene product is required during the ‘differentiation’ phase of endosperm development. Cell divisions cease, and final cell number is predetermined by about 12 DAP in maize. Borisjuk et al. (1998) reported that in developing Vicia faba cotyledons, rate of cell division and final cell number are controlled by glucose concentration. The correlation between cell number and sink capacity is well established in maize (Jones et al., 1996). Therefore one scenario would be that the rfg1 mutation causes a reduction in sugar flux at an early stage that determines cell number, and the reduced extent of endosperm development reflects a reduced sink size. Cheng and Chourey (1999) have also attributed to the Incw2 (Mtn1-encoded) invertase a critical role in providing hexoses early in endosperm development, based on analysis of mn1 mutants. Our data indicate the Rgf1 gene product may act downstream of Mn1 in hexose signalling. This hypothesis could be tested by measurements of cell numbers at different developmental stages in rfg1 and wild-type kernels on segregating ears.

**Experimental procedures**

**Reagents**

Speciality chemicals were obtained from the following sources: Peroxidase-coupled anti-rabbit IgG, glucose oxidase, paraformaldehyde (Sigma, Deisenhofen, Germany); Anti-digoxigenin Fab-fragment (Roche, Mannheim, Germany).

**Plant material and genetic mapping**

The mutation rfg1 was isolated in a cross of the A69Y inbred with a glossy2-En line (Tack et al., 1995). Plants were field-grown for genetic testing at the Instituto Sperimentale per la Cerealicolture, Bergamo, Italy. All biochemical and physiological analyses were carried out on plants grown with 16 h daylight in a greenhouse. Mutants mn1, mn2 and mn3 were provided by the Maize Seed Stock Center, Urbana, IL, USA (stocks 215D, 710B and 603H, respectively). For mapping purposes, rfg1 was crossed to the inbred B37, and the F1 was back-crossed to rfg1, giving rise to a BC population of Rgf1/rgf1 and rfg1/rgf1 plants. AFLP marker mapping was carried out with recombinant inbred lines CO159 × T232 and T232 × CM37 (Burr and Burr, 1991; Burr et al., 1994). The evaluation of the genetic distance between rfg1 and mn1 was obtained by scoring an F2 population from the cross rfg1 × mn1. The frequency of recombination (r) was calculated using the formula: percentage of wild-type seeds = (r/2r2) + (1 - r/2r2), and solving for r. As all mutant and marker loci concerned are tightly linked, genetic distance in cm = recombination frequency × 100.

**Molecular biological methods**

Standard DNA and RNA handling methods were carried out as described by Sambrook et al. (1989). RNA extractions were carried out as in Bartels and Thompson (1983). Mini-DNA-preparations from leaves for PCR were prepared using the procedure of Edwards et al. (1991).

**Amplified fragment length polymorphism**

AFLP reactions were performed as described by (Vos et al., 1995), using Msel and EcoRI adapters and the following 32P-labelled primers:

E35 5'-GACTGCGTACCAATTCACA-3', E36 5'-GACTGCGTA- CCAATTCC-3'

E39 5'-GACTGCGTACCAATTCAGA-3', M31 5'-GATGAGTC- TGAATTAAA-3'

M32 5'-GATGAGTCCTAGTAAAAT-3', M33 5'-GATGAGTC- TGAATTAAA-3'

M34 5'-GATGAGTCCTAGTAAAAT-3', M35 5'-GATGAGTC- TGAATTAAA-3'

M36 5'-GATGAGTCCTAGTAAAAT-3', M37 5'-GATGAGTC- TGAATTAAA-3'

M38 5'-GATGAGTCCTAGTAAAAT-3', M39 5'-GATGAGTC- TGAATTAAA-3'

M40 5'-GATGAGTCCTAGTAAAAT-3', M41 5'-GATGAGTC- TGAATTAAA-3'

M42 5'-GATGAGTCCTAGTAAAAT-3', M43 5'-GATGAGTC- TGAATTAAA-3'

M44 5'-GATGAGTCCTAGTAAAAT-3', M45 5'-GATGAGTC- TGAATTAAA-3'

M46 5'-GATGAGTCCTAGTAAAAT-3', M55 5'-GATGAGTC- TGAATTAAA-3'

M57 5'-GATGAGTCCTAGTAAAAT-3', M58 5'-GATGAGTC- TGAATTAAA-3'

**Microsatellite analysis**

To analyse the segregation of microsatellite marker bnlg1613 (maize map, http://burr.bnl.gov), the following primers were used: 5'-GGGGTGATCCGATAGGC-3' and 5'-GGCGTCTGTTTCCC-TTCT-3'.

**Western blotting and immunoblot detection**

Proteins were fractionated on SDS–PAGE electrophoresis according to Laemmli (1970), and blotted onto polyvinylidene difluoride (PVDF) membranes in a tank blotter. Proteins were detected using enhanced chemiluminescence (ECL, Amersham-Pharmacia Biotech, Freiburg, Germany) and either exposure to NAR-5 X-ray film (Kodak, Suppliers Sigma-Aldrich, Taufkirchen, Germany) or, for quantitation, image capture using the Lumi-imager (Boehringer).

**Invertase extraction and enzyme assays**

See Doehlert and Felker (1987). Maize kernels were ground under liquid N2 in a pestle and mortar, taken up in 5 ml extraction buffer (50 mM Tris–maleate pH 7.1, 1 mM DTT) g−1 tissue, and shaken for 30 min at 4°C. The suspension was pelleted at 14,000 g for 10 min. The supernatant contains the soluble invertase. The pellet was washed 3× with extraction buffer, then the cell wall-bound invertase was extracted with extraction buffer supplemented with 1 M NaCl. After clearing both eluates by centrifugation, the extracts were both dialysed overnight. Enzyme activity was determined by adding sucrose according to Tsai et al. (1970). The resulting glucose was measured spectrophotometrically.
using the coupled glucose oxidase-catalysed reduction of NAD⁺ (Boehringer test kit, Roche Diagnostics, Mannheim Germany).

Cytological methods

Embedding of maize kernels in Fibrowax was carried out as described by Hueros et al. (1995). Mounts were examined by light microscopy with a Nikon Microphot-FXA.

Measurement of carbohydrate content

Sugar extraction. Maize kernels were freeze-dried for 48 h and ground to a fine powder. 50 mg maize meal was extracted with 500 μl 80% ethanol for 1 h at 80°C in a shaking water bath. The samples were centrifuged (pellet saved for starch measurement) and the supernatants dried under vacuum. When the sample volume had been reduced to 20 μl, 500 μl water was added and the sugars were dissolved by shaking for 1 h.

Starch extraction. The pellet remaining after sugar extraction was washed twice with 2 ml water, then taken up in 10 ml 50 mM NaOAc pH 4.5, and the starch was gelatinized by autoclaving. Both starch and sugar extracts were measured spectrophotometrically using the coupled glucose oxidase-catalysed reduction of NAD⁺ (Boehringer test kit).

Sugar-uptake measurements

15-DAP kernels were excised either to include the pedicel, or through the placentochalazal region to remove the pedicel, as indicated in figure legends. Fifteen kernels were incubated in 2 ml of a solution containing 186 mM sorbitol, 32 mM KCl, 5 mM morpholinoethane-sulphonic acid–Tris pH 6.5 and the 14C sugar as specified, at room temperature for 1–8 h. Labelling was terminated by removing the kernel from the labelling solution, washing it 3 × with water, and freezing it in liquid N2. To measure labelled sugars, the kernel was ground to a fine powder, 200 μl of 80% ethanol was added, and the sample heated at 80°C for 1 h. After briefly centrifuging, 100 μl of the supernatant was added to 4 ml scintillation fluid (Beckman, Palo Alto, CA, USA), and the sample was counted for 5 min. For autoradiography, 1-mm-thick hand-cut sections were dried between blotting paper by applying a hot electric iron. The dried sections were wrapped in cling film, exposed first to Kodak NAR-5 film, and quantified by phosphor-imager recording (Molecular Dynamics, Düsseldorf, Germany).

In vitro culture of maize kernels

Immature maize kernels were excised at 5 DAP, surface sterilized and incubated on nutrient medium as described by Müller et al. (1997), with sugar concentrations between 0.3 and 5%, as specified in the Results. After 10 days’ incubation in the dark, kernels were fixed and embedded in Fibrowax (Hueros et al., 1995).

Acknowledgements

We thank Prof. Prem Choure (PMCB, University of Florida) for the lnxw2 probe. Prof. Mario Motto and ISC Bergamo staff are thanked for help with mutant screens, and we acknowledge the technical assistance of Ursula Seul and Brigitte Piegeler. The project was supported by the Deutsche Forschungsgemeinschaft (SPP322-1005, SFB274), and the European Union (EU-FP4 project B104CT-962158).

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