Rubisco Small Subunit, Chlorophyll *a*/*b*-Binding Protein and Sucrose:Fructan-6-Fructosyl Transferase Gene Expression and Sugar Status in Single Barley Leaf Cells in Situ. Cell Type Specificity and Induction by Light[1]

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We describe a highly efficient two-step single-cell reverse transcriptase-polymerase chain reaction technique for analyzing gene expression at the single-cell level. Good reproducibility and a linear dose response indicated that the technique has high specificity and sensitivity for detection and quantification of rare RNA. *Actin* could be used as an internal standard. The expression of message for Rubisco small subunit (*RbcS*), chlorophyll *a*/*b*-binding protein (*Cab*), sucrose (Suc):fructan-6-fructosyl transferase (*6-SFT*), and *Actin* were measured in individual photosynthetic cells of the barley (*Hordeum vulgare*) leaf. Only *Actin* was found in the non-photosynthetic epidermal cells. *Cab*, *RbcS*, and *6-SFT* genes were expressed at a low level in mesophyll and parenchymatous bundle sheath (BS) cells when sampled from plants held in dark for 40 h. Expression increased considerably after illumination. The amount of *6-SFT*, *Cab*, and *RbcS* transcript increased more in mesophyll cells than in the parenchymatous BS cells. The difference may be caused by different chloroplast structure and posttranscriptional control in mesophyll and BS cells. When similar single-cell samples were assayed for Suc, glucose, and fructan, there was high correlation between *6-SFT* gene expression and Suc and glucose concentrations. This is consistent with Suc concentration being the trigger for transcription. Together with earlier demonstrations that the mesophyll cells have a higher sugar threshold for fructan polymerization, our data may indicate separate control of transcription and enzyme activity. Values for the sugar concentrations of the individual cell types are reported.

Photosynthesis and the synthesis of temporary storage polysaccharides are regulated separately in leaves. This is true both at the level of gene expression, apparently in response to internal sugar status (Smeekens, 2000), and at the level of fine control of enzyme activity (Stitt, 1995). Control of photosynthetic capacity helps to maintain optimal coupling between the capacity of the light reactions to harvest light energy and the capacity of the dark reactions to fix carbon. By contrast, changes in carbohydrate accumulation are frequently in response to changes in the balance between supply of and demand for fixed carbon (Farrar et al., 2000). One of the problems in demonstrating unequivocally that sugar status in vivo is a signal for control of expression of genes central to these processes is that tissues are heterogeneous. We have shown previously that photosynthesis and carbohydrate metabolism are highly cell specific in barley (*Hordeum vulgare*) leaves: The epidermis is effectively sugar-free, and the mesophyll and parenchymatous bundle sheath (BS) have different patterns of sugar, fructan, and starch accumulation (Williams et al., 1989; Koroleva et al., 2001). We would therefore predict that the expression of key genes underlying these processes is similarly cell specific. This paper sets out to test that idea.

There is evidence from other workers that several of the genes encoding proteins for photosynthesis and carbohydrate metabolism may be sugar regulated (Sheen, 1990; Krapp et al., 1993). Their expression is also altered by prolonged light treatment, perhaps acting by modifying sugar status (Matsukura et al., 2000). Rubisco small subunit (*RbcS*) and Suc:fructan-6-fructosyl transferase (*6-SFT*) are among these genes (Giuliano et al., 1988; Koroleva et al., 2000). The evidence suggesting that these genes are sugar regulated comes mainly from experiments where either unrealistically high-sugar concentrations are applied exogenously or the whole-tissue sugar status and whole-tissue gene expression are measured (Smeekens, 2000). Such experiments ignore cell specialization (Tomos et al., 2000) and the possibility that the sugars are predominantly in one cell type while the genes are being expressed in another. In this paper, we ask whether the expression of specific genes within individual cells is related to the concentrations of sugars within them.

Single-cell sampling and analysis (Tomos and Leigh, 1999), in which cell sap is extracted using a

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fine glass microcapillary, is the tool of choice for the localization of plant metabolites and enzymes (Fricke et al., 1994; Koroleva et al., 1998, 2000). It is now being applied to the study of mRNA distribution. Techniques of measuring mRNA in individual cells or microdroplets, previously applied to animal cells, offer a new approach to the understanding of processes, such as signal transduction and the modulation of multiple genes in individual cells (Chelly et al., 1989; Kumazaki et al., 1994; Brail et al., 1999). These have been adapted to plants (Karrer et al., 1995; Brandt et al., 1999; Gallagher et al., 2001; Koroleva et al., 2001) where reverse transcriptase (RT)-PCR of extracts from single cells has proved useful in amplifying specific mRNA leading to the detection of even rare mRNA transcripts from individual cells. In this study, we have applied a significantly improved method to study the abundance of chlorophyll *a*/*b*-binding protein (*Cab*) and *RbcS* mRNA in individual cells in barley leaves in situ. We have also studied the change of expression of *6-SFT*, a gene that codes for a fructosyl transferase involved in the synthesis of storage fructan (Sprenger et al., 1995). For each of these proteins, the underlying physiology is well understood, and they constitute an ideal system in which to examine the regulation of gene expression at the single-cell level in response to environmental perturbations that alter internal sugar status. We also explored the use of *Actin* mRNA, a transcript expected to be present in low abundance (Karrer et al., 1995), as a constitutive internal standard to test the reproducibility and quantification of the method.

RESULTS

Use of an Internal Standard to Improve the Quantification of Differential Gene Expression

We performed three groups of experiments to assess our ability to quantify the abundance of specific mRNA species in individually sampled cell extracts and to determine the inherent variation associated with the experimental procedure (Fig. 1, a–c). Figure 1a presents the data for the expression of *Actin* and *Cab* in samples from one or pooled from five and 10 cells. Negative controls (no single-cell extracts in the RT-PCR mixture) did not show any signal. The expected bands of 204 (*Actin*) and 396 bp (*Cab*) were found from samples from single cells. The band intensity in samples from one and five mesophyll cells was some 10% and 50%, respectively, of the intensity from pooled samples from 10 mesophyll cells. Figure 1b illustrates a comparable relationship between signal intensity and different amounts of cDNA added to the PCR mixture. As expected, increasing amounts of cDNA template produced proportionally higher amounts of product. Figure 1c shows that the intensity of the band increased progressively with an increase in volume of PCR products loaded on the gel. We finally compared expression between replicate single-cell samples to determine the inherent variability of the technique. Five individual aliquots, obtained from a pool of 10 mesophyll cells, were each extracted separately for mRNA and were subjected to RT-PCR. There was very little variation between the samples (Fig. 1d). These results indicate that both the RT-PCR and the loading steps contribute minimally to any observed heterogeneity.

To quantify the level of expression of the genes of interest, we used the *Actin* gene as an internal standard, based on the hypothesis that it is a constitutively expressed message. Figures 2 through 4 show that *Actin* gene transcripts were detected in all three-cell types and under all treatments. Similar transcript levels were found in all of the different samples from the groups of individual cells under different light conditions.

Taken together, these data suggest that we are justified in taking the intensity of the *Actin* signal from the various samples as a measure of inherent variability in the sampling and analytical procedures.

Influence of Light Intensity on the Accumulation of Specific mRNA in Leaf Epidermal, Mesophyll, and Parenchymatous BS Cells

Northern-blot analysis clearly showed that light induces *Cab*, *RbcS*, and *6-SFT* transcriptions in whole barley leaves (Fig. 2b). Also, in each case, more transcripts were found in leaves exposed to moderate light than to low light. In contrast, *Actin* would appear to be constitutively expressed.

Accumulation of *Cab*, *RbcS*, and *6-SFT* gene transcripts was measured in different individual cells sampled from leaves after 9 h of exposure to low and moderate irradiance and to 40 h of darkness. *Cab*, *RbcS*, and *6-SFT* mRNA were detected in samples from both the mesophyll and BS cells, but not from epidermal cells (Fig. 2a). In both mesophyll and BS cells, these genes were expressed at a lower level in the dark (where *6-SFT* was generally not detectable) and in low light than in extracts from plants grown in moderate light. Light-associated accumulation of *Cab*, *RbcS*, and *6-SFT* mRNA occurred in both mesophyll and BS cells. No change of *Actin* gene transcription was observed in any cells under the different light conditions.

If the *Actin* signal is proportional to cytoplasmic volume in each sample, then we can compare the relative concentrations of the other mRNA species in the different cell types. This approach suggests that *Cab*, *RbcS*, and *6-SFT* mRNA accumulation in mesophyll cells was generally higher than in BS cells. Applying a statistical sign test to the results of nine independent experiments (data not shown), the level of *Cab* and *6-SFT* transcription was significantly higher in mesophyll than in BS cells ($P = 0.004$).

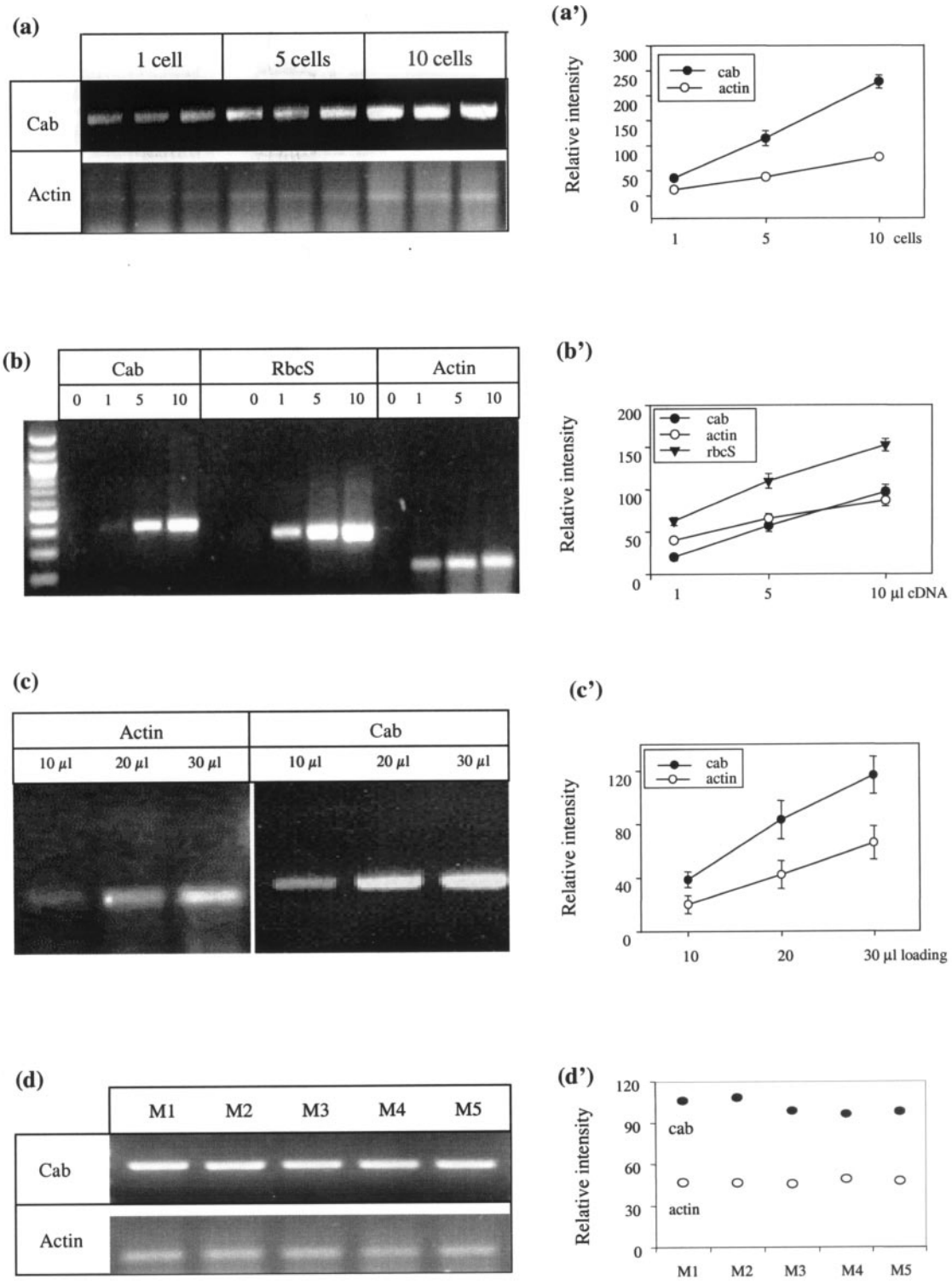

Figure 1. Signal reproducibility and quantification and the assessment of the use of *Actin* mRNA as a constitutive "internal" standard to normalize the spot intensities to sample cytoplasmic volume. a, Signal for *Cab* and *Actin* gene samples from one or pooled from five or 10 mesophyll cells extracted from three different plants. b, Signals obtained from 1, 5, or 10 μL of cDNA (RT product from three pooled cell extracts). c, Signals obtained from loading 10, 20, and 30 μL of RT-PCR products. d, Ten mesophyll cells were mixed in lysis/binding buffer and divided into five equal aliquots (M1, M2, M3, M4, and M5). Each sample was applied to RT-PCR separately and each RT-PCR product was loaded onto agarose gel. a' through d' illustrate relative intensities quantified using image analysis. Error bars = SD of the mean of the three samples (when not shown, they are smaller than the dimension of the symbol).

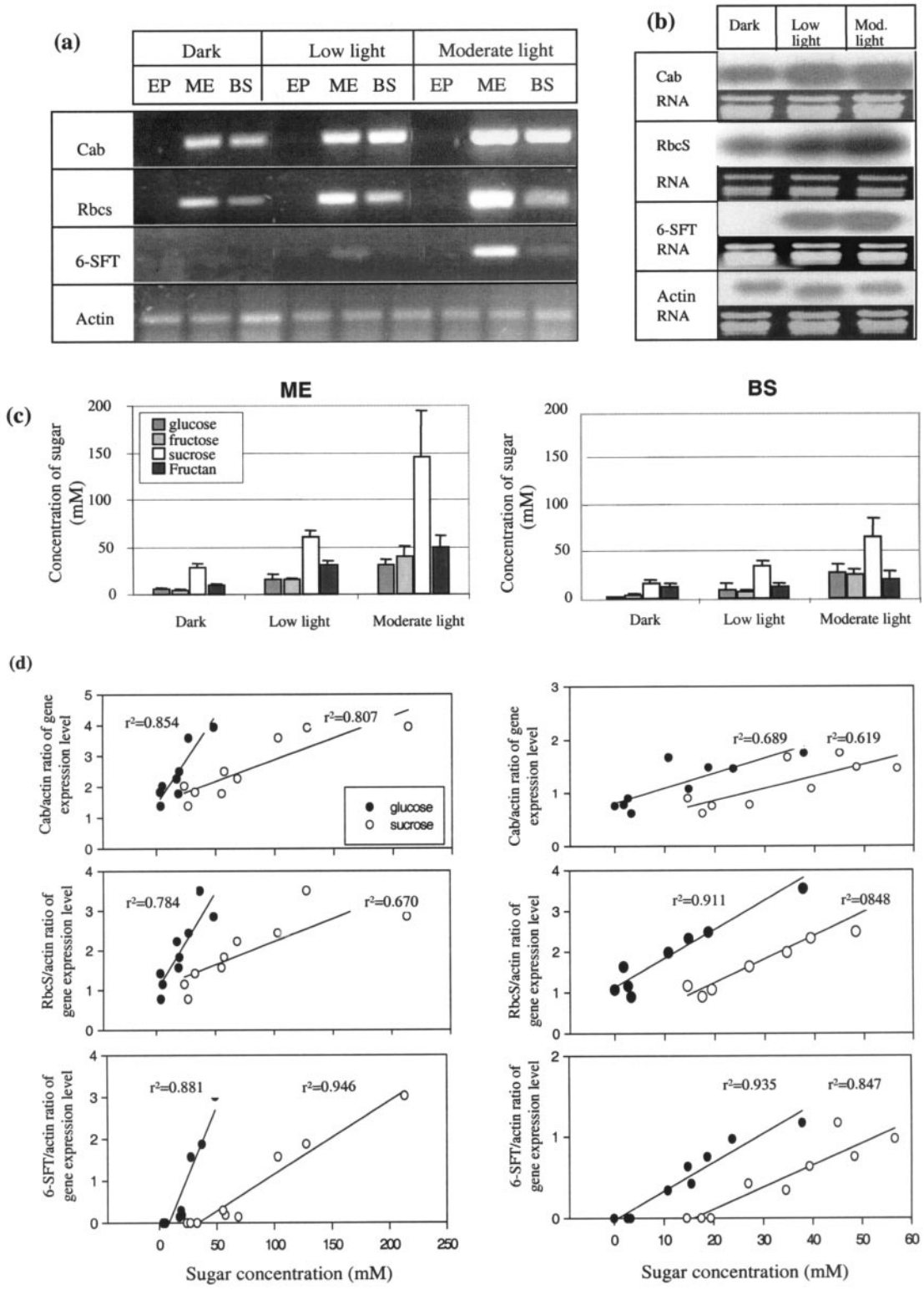

Figure 2. Expression of light-regulated genes (*Cab*, *RbcS*, and *6-SFT*) and sugar concentration in different cell types: epidermal (EP), mesophyll (ME), and parenchymatous BS of barley plants grown under different light conditions (dark, low light [100 μmol photons m^{-2} s^{-1}], and moderate light [500 μmol photons m^{-2} s^{-1}]). a, Ethidium bromide-stained gel. b, Northern blots labeled with sequence-specific cDNA probes of extracts of leaves treated with different light intensities: dark, low light, and moderate light. Total RNA 5 μg for *RbcS*; and 10 μg for *Cab*, *6-SFT*, and *Actin*. c, Concentration of sugars in mesophyll (ME) and parenchymatous BS cells. d, Correlation between amount of gene transcripts (expressed relative to actin) and concentration of Glc and Suc.

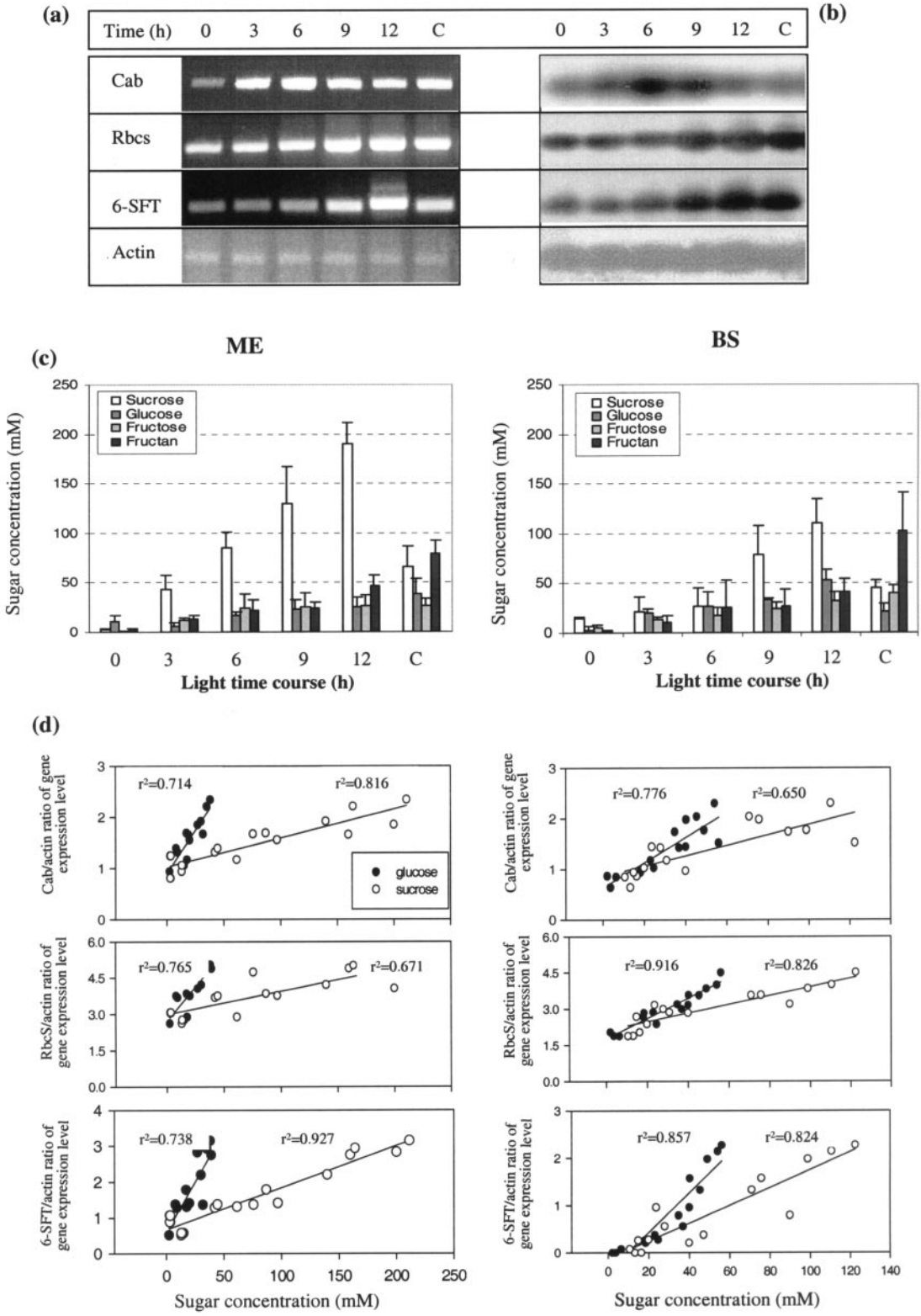

Figure 3. The time course of expression of light-dependent (*Cab*, *RbcS*, and *6-SFT*) and *Actin* genes and sugar concentration from groups of three mesophyll cells extracted from 19-d-old barley seedlings that had been kept in the dark for 40 h. Light exposure began at time 0 h. Control samples were taken after 5 d of continuous light (C). a, Ethidium bromide-stained gel. b, Southern blots of the same gel with probes of *Cab*, *RbcS*, *6-SFT*, and *Actin* cDNA. c, Concentration of sugar in mesophyll (ME) and parenchymatous BS cells. d, Correlation between gene expression (relative to actin) and sugar concentration (Glc and Suc) during time course of light.

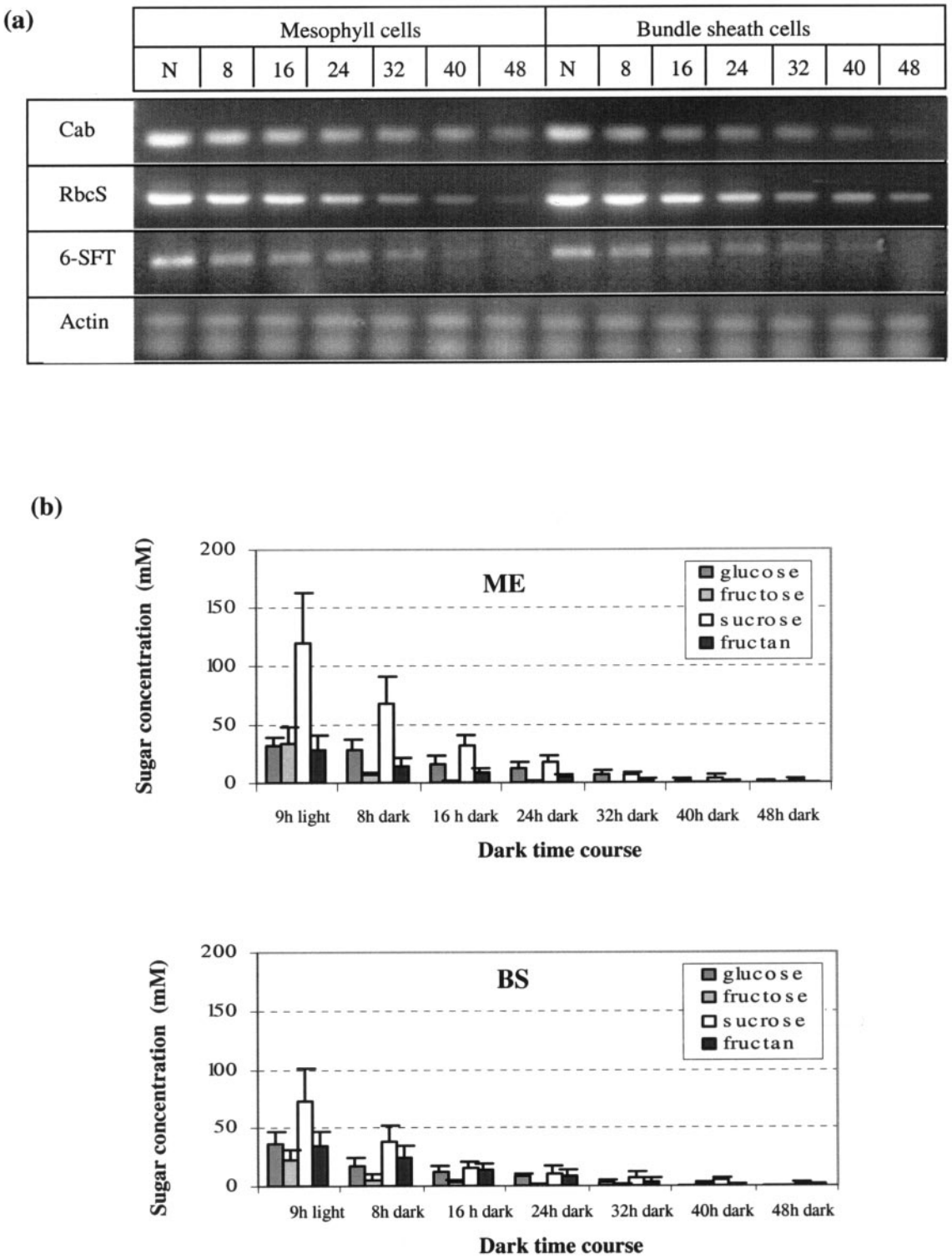

Figure 4. *Cab*, *RbcS*, *6-SFT*, and *Actin* gene expression and sugar concentration in mesophyll and parenchymatous BS cells during a dark time course. N represents normal light regime plants (9 h of light). a, Ethidium bromide-stained gel. b, Sugar concentration in mesophyll and parenchymatous BS cells.

The Time Course of Light-Dependent Accumulation of mRNA in Leaf Mesophyll and Parenchymatous BS Cells

To investigate the time course of the light-dependent accumulation of *Cab*, *RbcS*, and *6-SFT* mRNA in mesophyll cells, we exposed 18- to 20-d-old plants that had been kept in the dark for 40 h to 0, 3, 6, 9, and 12 h light. Control plants were kept in continuous light for 5 d before sampling. The genes studied were transcribed at a lower level in plants grown in the dark for 40 h (Fig. 3a). For example, *RbcS* was present at 45% of its maximum level at 12 h. Upon illumination, the amount of mRNA increased for about 6 and 9 h for *Cab* and *RbcS*, respectively. The increase continued for the entire 12-h period for *6-SFT*, although there was only a small increase during the first 6 h. Plants held in continuous light generally showed lower transcript levels than the maxima for the induced plants. No change was observed in the level of *Actin* transcript.

The disappearance of *RbcS*, *Cab*, and *6-SFT* mRNA accumulation in the absence of light was studied by transferring plants to continuous darkness and measured mRNA levels during a dark time course (Fig. 4a). The results showed that *RbcS*, *Cab*, and *6-SFT* mRNA levels decline continuously over period of 48 h. Very little mRNA of *6-SFT* was found after 40 h of darkness. *Cab* and *RbcS* mRNA were at very low levels after 48 h of darkness. No significant difference was found between mesophyll and BS cells on the pattern of gene expression.

To detect changes in gene expression during development, we analyzed the time course of light-dependent mRNA accumulation at three different leaf ages. Because of its ease of measurement as the result of its high expression, we chose to use the *Cab* gene (Fig. 5, a and b). For younger plants (14 d old, generally before full expansion of the third leaf), the level of *Cab* expression was highest after 12 h of light. For 19- and 24-d-old plants (when the third leaf was fully expanded), the maximum expression occurred after 9 and 6 h of light, respectively. Normalized against the *Actin* signal of the cell samples, similar amounts of *Cab* mRNA were detected in mesophyll cells as the leaves grew older, although the difference in the day of maximum expression remained (Fig. 5c). There appears to be a change in the response to light as the leaf aged.

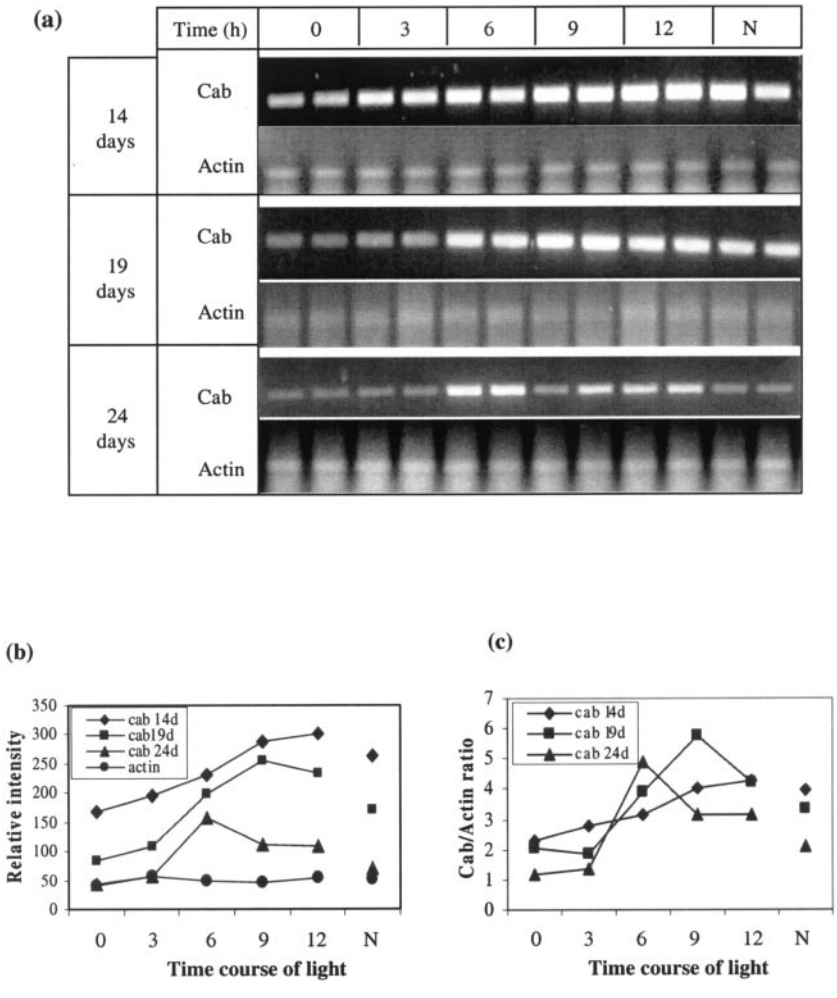

Figure 5. Time course of *Cab* and *Actin* gene expression upon illumination in barley leaf mesophyll samples from the third leaf of different aged plants (14, 19, and 24 d old). Full expansion of the leaf was around 18 d. Each plant had been maintained in continuous darkness for 40 h before illumination and analysis, except for those labeled N, which were sampled 9 h into the light period of normal diurnal plants. Each time point represents three pooled single-cell samples replicated from two different plants. a, Ethidium bromide-stained gel. b, The mRNA abundance was quantified as in Figure 2 (the data from the two plants were averaged). c, *Cab* to *Actin* ratio of gene relative expression level.

Influence of Light Conditions on Sugar Concentrations of Leaf Epidermal, Mesophyll, and Parenchymatous BS Cells

Light Intensity

After 40 h in darkness the concentrations of sugars are very low in both mesophyll and BS cells (Fig. 2c). In epidermal cells under all conditions, these concentrations are below the detection limit of the technique (approximately 5 mM; data not shown). Light treatment increases Fru, Glc, and Suc in both mesophyll and BS cells with their concentrations under moderate light being approximately double those at low light. Meanwhile, fructan increases with light intensity in the mesophyll but not in the BS cells. In general, Suc accumulation in mesophyll was much higher than that in BS cells under all light conditions.

Light Time Course

Nearly all of the sugars increased during illumination. Suc especially had increased considerably and reached a maximum at 12 h of treatment in both mesophyll and BS cells. Suc was always higher in mesophyll cells than in BS cells. In plants exposed to 5 d of continuous light, Suc had decreased significantly in both mesophyll and BS cells. However, continuous light stimulated fructan synthesis. Glc and Fru remained relatively constant over 5 d of continuous light (Fig. 3c).

Dark Time Course

The concentration of all kinds of sugar decreased gradually in continuous darkness. Very low-sugar contents were detected after 32 h of darkness (Fig. 4b).

The fructan to Suc ratio was generally higher for BS cells than for the mesophyll under different light conditions. For example, for the cells after 9 h of illumination, the fructan to Suc ratio was 0.406 in BS cells, but only 0.251 in mesophyll.

Correlation between Gene Expression and Concentration of Sugar in Mesophyll and BS Cells

To investigate whether there is a relationships at the level of the individual cells between gene transcripts and sugar status, measurements of the transcript levels (ratio of specific gene/actin) of each of the light-induced genes were compared with the concentrations of the sugars and fructan in adjacent cells of the same leaves. In one series, the leaves were exposed to the same range of light regimes as described above (40 h of darkness, 9 h of low light, and 9 h of moderate light; Fig. 2). In the second, the cells were sampled over a time course of 12 h of illumination in moderate light (Fig. 3). In both experiments, a clear correlation between *Cab*, *RbcS*, and *6-SFT* gene transcript levels and cell concentrations of both Glc and Suc were observed for both mesophyll and BS cells (Figs. 2d and 3d).

The relationship between the *6-SFT* transcripts, Suc and fructan, is illustrated in Figure 6. In this case, the plants had been maintained previously in the dark for 40 h. A difference was observed in the behavior of the mesophyll and BS cells of the same leaves (Fig. 6). In both cell types, both fructan and Suc had fallen to very low levels before illumination. The 6-SFT transcript has dropped to levels below the detection limit in the BS cells, but was still detectable in this experiment at a moderate level in the mesophyll cells.

In the experiment illustrated in Figure 6, both Suc and fructan were significantly elevated in these cells by 3 h, suggesting that their synthesis began immediately when light became available. Suc concentrations increased monotonically and coordinately with 6-SFT transcripts in both cell types. In contrast, fructan concentration appeared to "stall" between 6 and 9 h before recovering its increase by 12 h, after which a gentle increase was maintained to 5 d. In epidermal cells, under all conditions, sugar concentrations are below the detection limit of the technique (approximately 5 mM; data not shown).

The Nature of the Transcript Induction by Light

Because it was not expected that light per se is the direct inducer of *6-SFT*, an experiment was performed during which the sugar content of the cells was elevated in the absence of illumination. This was achieved by feeding Suc and Glc to excised leaves via the exposed xylem. Under these conditions *6-SFT* transcript was induced but that of *Cab* and *RbcS* was not (Fig. 7).

As discussed below, it is thought that although the *6-SFT* correlation may be a direct effect of sugar on transcription, that of *Cab* and *RbcS* was not. The data provide circumstantial evidence for this.

DISCUSSION

Our aim was to test the hypothesis that the expression of the key genes *RbcS*, *Cab*, and *6-SFT* underlying photosynthesis and fructan synthesis is cell type specific, matching the underlying physiology of these processes. We also asked whether the concentrations of key carbohydrate found related to the expression of these specific genes within individual cells. Before examining the answers to these questions, however, we will assess the technique we have used.

Technical Advances

The key questions are whether our RT-PCR technique is adequate for the small copy numbers sampled from individual cells and how quantitative is the technique. Being able to measure the abundance of individual mRNA species in specific cells without

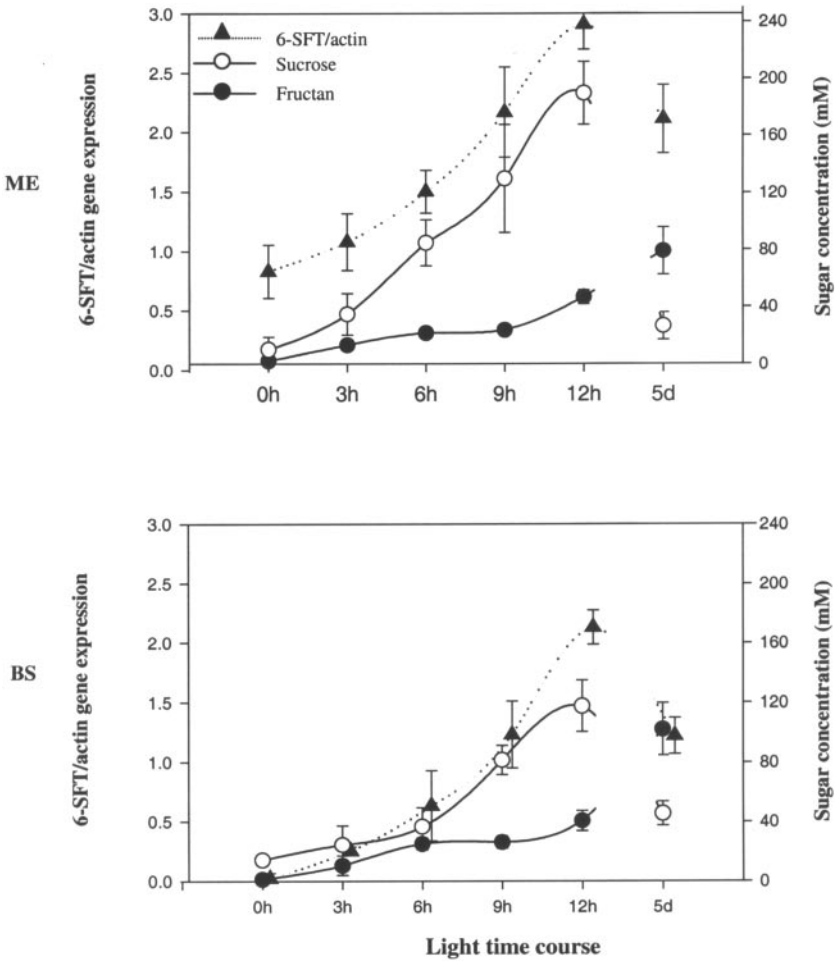

Figure 6. A relationship between *6-SFT* expression (relative to actin) and sugar concentration (Suc and fructan) in mesophyll (ME) and parenchymatous BS cells during time course of light and 5 d (5d) continuous light. Each data point is an average ± SD for three to four samples from different leaves.

resorting to reporter gene technology is of considerable value in the study of tissue-level compartmentation.

Although RT-PCR techniques have been widely used to measure gene expression in tissues, resolution is limited by the amount of mRNA within the sample (typically 0.1–1 pg). We have previously reported a method for detecting a small range of genes from single cells using RT-PCR, however, the method was only able to detect high-expression genes (Gallagher et al., 2001). Therefore, RT-PCR procedures with higher sensitivity are needed for the reliable analysis of gene expression at the single-cell level. In this study, we describe a novel, highly efficient two-step single-cell RT-PCR method based on the use of Sensiscript RT and HotStar *Taq* DNA polymerase (Qiagen USA, Valencia, CA). Bosch et al. (2000) compared the Qiagen Sensiscript RT with the commonly used Moloney murine leukemia virus or Moloney murine leukemia virus H enzyme. They found the use of Sensiscript resulted in an approximately 100-fold improvement in the cDNA detection level. The highly specific PCR with HotStar *Taq* DNA polymerase provided for a high-temperature activation step in the PCR technique that eliminated the formation of nonspecific amplification products. Together, this combination ensured highly specific and reproducible RT-PCR. Reproducibility was maintained at all steps from mRNA extraction, through RT and PCR, to gel loading (Fig. 1, b–d).

We used two-step RT-PCR where cDNA representative of the total mRNA pool was initially synthesized by RT. The second-step PCR using this pool could then be optimized independently for each gene of interest without constraining the RT step. Furthermore, the ability to subsample aliquots of the products of the first RT reaction for second-stage PCR allowed the investigation of the spatial and temporal expression of several target genes from the same original sample. Several figures show the response to illumination of expression for four different genes (*Actin*, *Cab*, *RbcS*, and *6-SFT*) from the same cell.

In general, a quantitative RT-PCR method consists of four steps: generation of internal standards, RT-PCR, detection of products, and data analysis. To quantify the level of expression of the genes of interest, we investigated the use of the *Actin* gene expression as an internal standard. Two lines of evidence suggest that *Actin* message is suitable for this role. First, it produced a linear standard curve, and second, it provided a remarkably uniform signal in replicate samples taken during several light-dependent

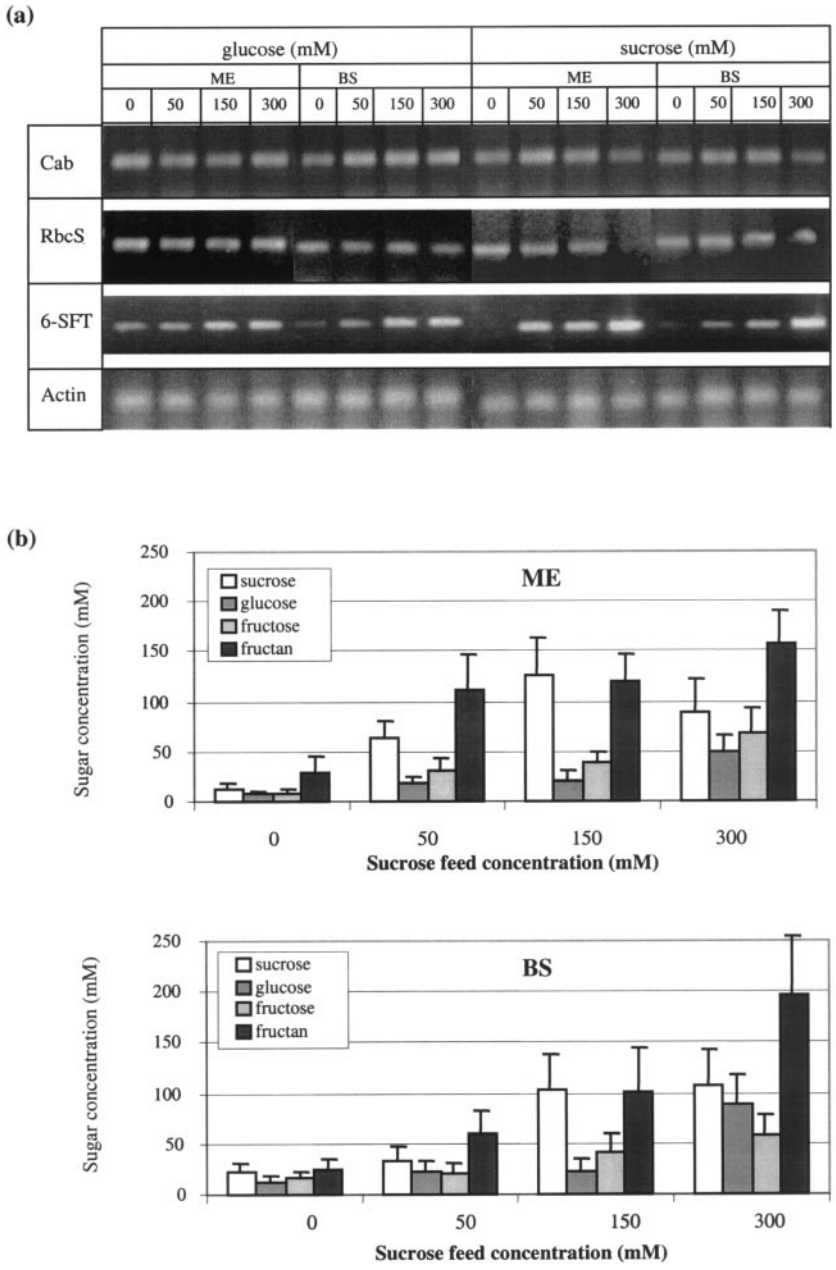

Figure 7. Expression of light-dependent genes (*Cab*, *RbcS*, and *6-SFT*) and sugar concentration in mesophyll (ME) and parenchymatous BS cells sampled from excised leaves incubated in various solutions (0–300 mM) Glc or Suc for 40 h in the dark. Each data point is an average ± SD for three to four samples from different leaves.

time courses (Figs. 1 and 3). We took two approaches to test the quantification of our measurements, because such small quantities are involved and it was not possible to use the conventional technique of loading known aliquots of total mRNA on gels. The most direct was the construction of "standard curves" for the superabundant *Cab* and the less abundant *Actin* message. Better reproducibility was predictably obtained from the pooled samples, but even single-cell samples provided acceptable data. This suggests that reproducible cytoplasmic volumes were obtained and that the RT-PCR procedure did not enter the saturation phase of the reaction. Evidence of the absence of saturation can also be obtained from estimating the quantity of the expressed message by comparison with the quantitative standard DNA ladder run with each sample.

Finally, the northern blots obtained from whole-leaf extracts of plants treated under different light intensity showed clear qualitative similarity with the information gained from the RT-PCR products of the individual cells. The results were as would be expected if the whole leaf represented the averaged pool of the different cell types.

The Effect of Light and Sugar on Gene Expression

Measurements of expression after illumination of leaves showed significant changes in message abundance. *RbcS* and *Cab* transcript increase continuously

during induction in bulk pea (*Pisum sativum*) leaf for up to 24 h (Khanna et al., 1999), whereas Brandt et al. (1992) showed that *Cab* increases in barley leaves for at least 8 h. A circadian rhythm in transcription entrained by light has also been demonstrated for *Cab* in Arabidopsis (Thain et al., 2000). We found a similar time course in individual mesophyll cells, although there is little increase in transcript after 6 to 9 h for *Cab* and 9 to 12 h for *RbcS*. In both cases, continuous light results in lower (or similar in the case of Southern blot) values, possibly because of the repression of these genes (Dijkwel et al., 1997) by the Suc accumulation that occurs under continuous light (Housley and Pollock, 1985).

Sprenger et al. (1995) demonstrated an increase in *6-SFT* transcript in bulk extracts from leaves illuminated for 24 h. Müller et al. (2000) subsequently showed that it is also induced in the dark when Suc is supplied to excised leaves.

In this study, quantitative correlation of transcript levels of each of the studied genes with Suc, Glc, and fructan concentrations have provided novel information regarding their interrelationship (Fig. 7). There are good linear correlations between both cell Glc and Suc concentrations and the (actin-normalized) transcript levels for *Cab*, *RbcS*, and *6-SFT* in both cell types under the various light regimes. This could be superficially interpreted to mean that all three genes are induced by both sugars. Furthermore, this induction would appear to be more sensitive to Glc than to Suc. However, when Glc and Suc were fed to the excised leaf bases in the dark, although *6-SFT* transcript levels displayed the same correlation to the sugars, no increase in *Cab* and *RbcS* transcript was observed. This was not surprising because their induction is a direct effect of light (Quail, 1991; Kloppstech, 1997), which also (coincidentally) increases sugar content. *Cab* and *RbcS* transcription is not induced by sugar.

The *6-SFT* transcript is induced in the absence of light. From our experiments, however, we are unable to determine whether Suc or Glc or both are involved. Under the conditions used here, we did not inhibit the metabolic exchange between the Glc and Suc pools. The apparent increased sensitivity to Glc probably has no significance and may merely illustrate the poise of the Suc-Glc balance in these cells.

Is Gene Expression Specific to Different Cell Types?

The genes coding for chloroplast protein, *Cab* and *RbcS*, were generally only expressed in the photosynthetic cell types of mesophyll and BS. The transcripts cannot be detected in samples from epidermal cells harvested in the light. Dark or low-light intensity grown plants exhibited that *RbcS* gene was transcribed at similar level in both mesophyll and BS cells; however, under moderate light, *RbcS* transcripts were higher in mesophyll than that in BS cells. The difference is likely regulated by light-induced developmental signals or conditions. A mutation, BS defective2, that involved maintenance of BS cell-specific photosynthetic enzymes and chloroplast structure was found in Roth's group (Roth et al., 1996). They suggested that differential accumulation of *RbcS* is controlled posttranscriptionally and that such mechanisms contribute to the suppression of *RbcS* mRNA levels in BSl cells. A much stronger differential between the two cell types is observed for *Cab* (Fig. 2). The differential *Cab* gene transcript pattern may be caused by differences in their chloroplast development. BS cells represent only about 15% of chloroplast-containing cells in the leaf (Kinsman and Pyke, 1998). Also, some evidence suggests that reduced *Cab* gene expression in BS cells might be caused directly by a phytochrome-pathway-specific regulator (Li et al., 1995). A *CUE1* (for *Cab* underexpressed) gene of Arabidopsis has been isolated that is a cell-specific positive regulator linking light and intrinsic development in leaf mesophyll cells. The mutant showed defects in expression of several light-dependent genes and in chloroplast development, specifically in mesophyll cells.

Light-dependent transcript levels of *6-SFT* in mesophyll cells, when normalized to *Actin*, are considerably higher than that in BS cells. This higher level of *6-SFT* mRNA is not immediately consistent with the observation that BS cells appear to have a lower Suc threshold for the induction of fructan biosynthesis than do mesophyll cells (Koroleva et al., 1998). It suggests, however, that transcription and enzyme activity may be controlled in different ways in different cell types.

6-SFT Transcript and Fructan Synthesis in the Individual Cells

In this work, the sugar concentrations of individual epidermal, mesophyll, and BS cells were taken from cells adjacent to those sampled for gene expression. One statistically significant experiment provided further insight into the interrelationships illustrated in Figure 6. In this experiment, after 40 h of darkness, the level of *6-SFT* transcripts had dropped below the detection limit in the BS cells but had not done so in the mesophyll cells. Upon illumination, the transcript level began to increase immediately with a similar time course in both cell types (Fig. 8). Upon illumination, the levels of fructan, the 6-SFT enzyme product, also immediately began to rise—reaching 20 mM (hexose equivalents) by 6 h. Such a rapid synthesis of fructan shows that significant quantities of the active enzyme must have been present in both cell types. In the case of the BS cell, this was in the absence of gene transcript and indicates the persistence of protein in the absence of message. The rate of initial fructan synthesis in these BS cells is presumably regulated by the availability of Suc substrate rather than either protein or transcript availability.

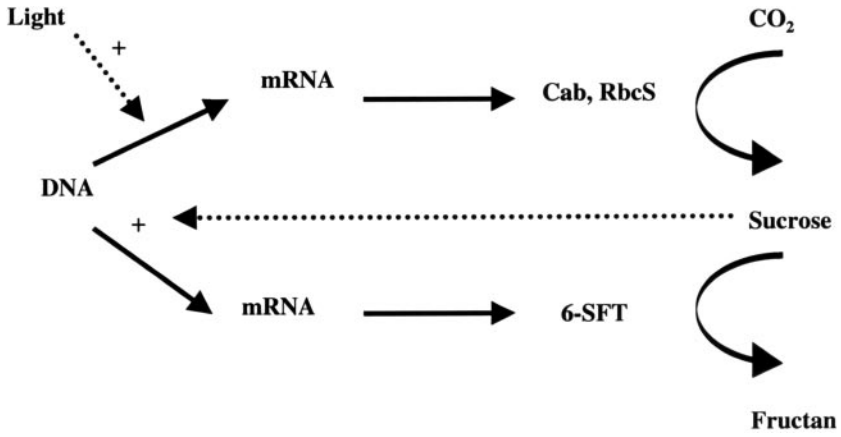

Figure 8. Diagram illustrates the direct and indirect effect of light on the expression of 6-SFT and the photosynthetic genes *Cab* and *RbcS*.

A reproducible feature of Figure 8 is the "stall" in fructan accumulation between 6 and 9 h. We speculate that this may be attributable to the accumulation in these (starved) cells of two classes of fructan that we cannot distinguish with our enzymatic assay. The first form, synthesized by the persistent protein, saturates at about 20 mM; whereas the second form, synthesized by protein translated from freshly transcribed message, accumulates after 9 h (reaching 80 and 100 mM in the mesophyll and BS cells, respectively, by 5 d of continuous illumination). That this second phase of fructan synthesis is a direct consequence of *6-SFT* transcription is however unlikely, because we show that in the mesophyll cells, the transcript also persists at significant levels after 40 h in the dark. The actual transcript levels after 5 d in continuous light are lower than those measured 12 h into the photoperiod. It would appear that if *6-SFT* does increase for 24 h during a single light period (Müller et al., 2000), additional levels of regulation prevent such accumulation in continuous light.

The fructan to Suc ratio is higher in the BS cells than that in mesophyll cells, suggesting that lower threshold for the initiation of fructan synthesis for BS cells is needed to preserve downhill gradient between mesophyll and vasculature as suggested by Koroleva et al. (1998). This is important for efficient transport of Suc toward the phloem.

The evidence in this paper supports the views that (a) different cell types in cereal leaves that differ in photosynthetic and carbohydrate metabolism show parallel differences in expression of specific genes and (b) that the expression of these genes after illumination is consistent with sugar being a signal which directly or indirectly regulates gene expression (Fig. 8).

MATERIALS AND METHODS

Plant Material and Growth Conditions

Barley (*Hordeum vulgare*) was grown as described previously (Koroleva et al., 1998). All samples were taken from the upper surface of the middle parts of the third leaf. For each experiment, leaves of similar developmental age (as judged by their state of full expansion) were chosen as replicates. For experiments on leaves in darkness, groups of three 18- to 20-d-old plants (14–16 d after transfer to Long-Ashton solution) were transferred to opaque 250-mL containers at 20°C, wrapped in aluminum foil, and grown on for 40 h (the nutrient solution was topped up each day). Samples were taken from plants that were illuminated for 0, 3, 5, 9, and 12 h after the 40-h dark period (at either 100 or 500 μmol photons m^{-2} s^{-1}; see text). Eighteen- to 20-d-old plants were exposed to continuous light (500 μmol photons m^{-2} s^{-1}) for 5 d (referred to as continuous).

mRNA Extraction from Single Cells

The cell contents were extracted from individual epidermal, mesophyll, and BS cells using a fine silanized glass microcapillary (Fricke et al., 1994; Tomos et al., 1994; Koroleva at al., 1998), except that an air-filled capillary (rather than one back-filled with buffer or oil) was used for RNA extraction. This made it much easier to see cell sap after single-cell aspiration and considerably increased the success rate. The cell contents were forced into the microcapillary by cell turgor pressure. In most cases, samples analyzed by RT-PCR were pooled from three adjacent cells sampled in sequence over approximately 1 min for epidermal and 3 min for subsurface cells. This reduced between-sample variation without losing the fine resolution of single-cell sampling and analysis. Individual epidermal and BS cell samples had a volume of approximately 100 pL, whereas those from individual mesophyll cells were smaller (20–50 pL). Samples (25 mg) of whole-leaf tissue were used as control. The glass tip was inserted through a stomatal pore to reach mesophyll and BS cell while avoiding epidermal contamination (Fricke et al., 1994; Koroleva et al., 1998). Each sample was then immediately expressed, using the air pressure from a syringe, into a 0.5-mL microcentrifuge tube containing 80 μL of lysis/binding buffer (100 mM Tris-HCl, pH 7.5, 500 mM LiCl, 10 mM EDTA, 1% [w/v] lithium dodecyl sulfate, 5 mM dithiothreitol, and 10 units of RNase inhibitor [RNAsin, Promega, Madison, WI]; Dynal Biotech, Oslo). This lysate was transferred to a new tube containing 20 μL of oligo$(dT)_{25}$ (Dynabeads, Dynal Biotech) prewashed with lysis/binding buffer. The mixture was incubated at room temperature with gentle rolling for 5 min. The mRNA was then purified using a magnetic particle concentrator (Dynal Biotech) and washed twice with 100 μL of washing buffer Dynal A (10 mM Tris-HCl, pH 7.5, 0.15 M LiCl, 1 mM EDTA, and 0.1% [w/v] lithium dodecyl sulfate) and washing buffer Dynal B (10 mM Tris-HCl, pH 7.5, 0.15 M LiCl, and 1 mM EDTA).

RT-PCR

RT was performed using a Sensiscript RT kit (Qiagen USA). For each reaction, first-strand cDNA was synthesized in 20 μL containing 2 μL of 10× RT buffer, 2 μL of dNTP (final concentration of 0.5 mM each), 1 μL of RNase inhibitor (10 units μL^{-1}; Promega), 1 μL of Sensiscript RT, and 14 μL of diethylpyrocarbonate-treated water. To start the reaction, the mix was added to the extracted mRNA bound to the magnetic beads. The reaction was incubated at 37°C for 60 min. For the PCR reaction, 4 or 5 μL of cDNA from the RT reaction was added to HotStar *Taq* Master Mix (Qiagen USA; 2.5

units of HotStar *Taq* DNA polymerase), PCR buffer (1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.4, 50 mM KCl, and 5 mM dithiothreitol), 0.2 mM dNTP, and specific primers (0.5 μM each primer) in a total volume of 25 μL. Primer sequences were as follows: *Cab* sense primer, 5′-GACCAACGGCAGAATC GACCA; *Cab* antisense primer, 5′-ATGAGAAGGA CCTGGAAGCC (396 bp; accession no. X63197); *Actin* sense primer, 5′-CCCAG CATTGTAG-GAAGGC; *Actin* antisense primer, 5′-CCTCGGTGCGACACGGAGC (204 bp; accession no. U21907); *RbcS* sense primer, 5′-AGGTGTGGCCGATTG AGGGT; *RbcS* antisense primer, 5′-CGATGA AGCTGACGCACTGC (342 bp; accession no. AB020943); *6-SFT* sense primer, 5′-GCAGCGCAGCG GTT ACCATT; and *6-SFT* antisense primer, 5′-CATGAT GACCGTCCCGTTGG (271 bp; accession no. X83233). These gene sequences were obtained from the GenBank database, and the primers were designed using Vector NTI Suite (InforMax, Bethesda, MD). The PCR program consisted of 15 min at 95°C, followed by 35 cycles of 40 s at 94°C, 40 s at 54°C to 59°C, and 60 s at 72°C. The final extension step was for 10 min at 72°C.

Quantification of PCR Products

PCR products (10 μL) were separated by electrophoresis in 1.8% (w/v) agarose, and the DNA was visualized by ethidium bromide using an UV transilluminator and then photographed. To quantify band intensities, we used a quantified 100-bp DNA ladder (New England BioLabs, Beverly, MA) as a standard. The signal intensities were estimated by ID Image Analysis Software (Kodak Digital Science, Rochester, NY). The gel was then used for Southern blotting on Hybond-N^+ nylon membrane (Amersham Biosciences AB, Uppsala) and fixed using an UV cross-linker light (254 nm) of 120,000 $\mu J\ cm^{-2}$ (Hoefer UVC 500, Amersham Biosciences AB). Probes were made using an enhanced chemiluminescence labeling kit (Amersham Biosciences AB) and hybridized to the target DNA on the membrane. After hybridization, the filters were washed to remove unlabeled probe and then analyzed using an ECL detection system to make an image on x-ray film (Heslop-Harrison et al., 1990).

RNA Isolation and Northern-Blot Analysis

Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen USA) according to the manufacturer's instructions. Total RNA (5–10 μg) was separated by denaturing formaldehyde agarose gel electrophoresis and transferred onto a Hybond-N+ membrane (Amersham Biosciences AB). Equal loading of RNA in each lane was verified by ethidium bromide staining of RNA in the gel. The blots were hybridized with [α-^{32}P]dCTP gene-specific cDNA probes using Rediprime (Amersham Biosciences AB). Prehybridization, hybridization, and membrane washes were performed as described previously (Lu et al., 2001).

Sugar Assay in Individual Cell Saps

The concentration of sugar (Glc, Fru, Suc, and fructan) in single-cell samples were measured by a microfluorometric assay (Koroleva et al., 1998).

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