

Changes in Photosynthate Unloading from Perfused Seed Coats of *Phaseolus vulgaris* L. Induced by Osmoticum and Ethylenediaminetetraacetate (EDTA)

ERLE C. ELLIS, ROBERT TURGEON and ROGER M. SPANSWICK¹

Section of Plant Biology, Plant Science Building, Cornell University, Ithaca, NY 14853-5908, USA

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ABSTRACT

Photosynthate unloading in *Phaseolus vulgaris* L. seed coats was studied by treating perfused seed coats with differing concentrations of an osmoticum and ethylenediaminetetraacetate (EDTA). Large changes in osmoticum concentration typically produced rapid changes in efflux of unlabelled sugar and steady-state-labelled ¹⁴C-photosynthate. Osmoticum-induced changes in photosynthate efflux were caused by phloem import stimulation at low cell turgor and net efflux stimulation by high cell turgor. Even though rapid changes in sugar and tracer efflux were often induced by osmoticum treatments, the specific activity of sugar released from seed coats was not greatly affected by these treatments and was similar to the specific activity of sugar remaining in the seed coat after perfusion. Thus, tracer was transported from the phloem throughout the seed coat sugar pool before it was released to the apoplast. This result is most consistent with symplastic phloem unloading throughout perfused seed coats, because apoplastic transport between cells within the seed coat was blocked by perfusion. Photosynthate efflux was stimulated by simultaneous treatment of seed coats with EDTA and different concentrations of an osmoticum; loss of photosynthate from seed coats did not appear to be tissue-specific.

Key words: *Phaseolus vulgaris*, seed coat, photosynthate unloading, turgor, EDTA.

INTRODUCTION

The seed coat and embryo of legume seeds are symplastically isolated and so photosynthates must be released to the seed coat apoplast before uptake by the embryo (Thorne, 1985). Phloem unloading to the seed coat apoplast probably involves the symplastic transport of photosynthates from the phloem to cells near the inner surface of the seed coat before significant efflux to the apoplast occurs (Patrick and McDonald, 1980; Offer and Patrick, 1984; Patrick, Jacobs, Offer, and Cram, 1986; Grusak and Minchin, 1988; Ellis, Turgeon, and Spanswick, 1992a, b). However, some photosynthate efflux directly from the phloem to the seed coat apoplast could occur in parallel to symplastic unloading (Wolswinkel, 1987b; Ellis *et al.*, 1992b).

¹⁴C-photosynthate efflux from excised seed coats of *Phaseolus vulgaris* was rapidly enhanced when the manni-

tol osmoticum concentration in the perfusion solution was changed from 100 mol m⁻³ to 0 mol m⁻³ (Patrick, 1984; Patrick *et al.*, 1986). This result, and those of later researchers, have been interpreted as the stimulation of a turgor-sensitive efflux control mechanism in cells near the inner surface of the seed coat by high cell turgor (Patrick *et al.*, 1986; Grusak and Minchin, 1988; Ellis *et al.*, 1992b).

In general, photosynthate efflux is also increased by perfusing seed coats with osmoticum concentrations greater than 300 mol m⁻³ (Wolswinkel and Ammerlaan, 1984; Wolswinkel *et al.*, 1986; Minchin and McNaughton, 1986; Ellis and Spanswick, 1987; Grusak and Minchin, 1988). This stimulation of photosynthate efflux has been attributed to the enhancement of phloem import by low cell turgor at the sink end of the mass flow pathway

¹ To whom correspondence should be addressed.

(Wolswinkel and Ammerlaan, 1984; Minchin and McNaughton, 1986; Ellis and Spanswick, 1987; Grusak and Minchin, 1988).

Photosynthate efflux from seed coats may be stimulated by rapidly changing the osmoticum concentration of the perfusion solution either from high to low (Patrick, 1984; Patrick *et al.*, 1986; Ellis and Spanswick, 1987), or from low to high (Minchin and McNaughton, 1986; Ellis and Spanswick, 1987; Grusak and Minchin, 1988). Sugar efflux from soybean seed coats was stimulated for a longer period of time when the osmoticum concentration was changed from low to high (10 to 500 mol m⁻³) than from high to low (Ellis and Spanswick, 1987). This is because the stimulation of photosynthate release by a change in osmoticum concentration from low to high is a secondary effect of increased phloem import, while a change from high to low produces a rapid and transient increase in efflux by the direct stimulation of a turgor-sensitive efflux control mechanism (Ellis and Spanswick, 1987; Grusak and Minchin, 1988).

The stimulation of photosynthate release from perfused seed coats by chelating agents such as EDTA and EGTA has been attributed to their ability to increase 'phloem exudation' and/or 'sink strength' (Thorne and Rainbird, 1983; Hanson, 1986; Wolswinkel, 1987a). Although Thorne and Rainbird (1983) demonstrated that EGTA (and EDTA) concentrations greater than 5 mol m⁻³ increased tracer import into agar-filled soybean seed coats when leaves were pulse-labelled with ¹⁴CO₂, this effect was not observed when ¹⁴C-sucrose was used for labelling (see Thorne and Rainbird, 1983, Table 2). The most consistent and clearly demonstrated effect of EDTA (and EGTA) in the experiments described above and in those of Wolswinkel (1987a) was the induction of a large net loss of photosynthate from the seed coat. While direct effects of EDTA on phloem import and unloading in seed coats have never been clearly demonstrated, EDTA certainly stimulates photosynthate efflux, and may provide a useful treatment for studying photosynthate compartmentation in seed coats.

The source of photosynthates released from attached, perfused seed coats is not easily determined because photosynthate efflux may occur directly from the phloem, from the seed coat symplast, or by symplastic movement from the phloem throughout the seed coat symplast before efflux to the perfusion solution. The purpose of this study is to determine whether the efflux of photosynthates from different sources within perfused seed coats may be differentially stimulated by rapid changes of osmoticum concentration or by treatment with EDTA. Steady-state labelling with ¹⁴CO₂ was used to generate a steadily-increasing sugar specific activity in the phloem (Ellis *et al.*, 1992b), so that rapid changes in the specific activity of seed coat efflux would provide evidence for changes in the source of photosynthates for efflux.

MATERIALS AND METHODS

Plant preparation, steady-state labelling and seed coat perfusion

Phaseolus vulgaris L. (cv. Redcloud) plants were grown in the greenhouse and were prepared for steady-state labelling and perfusion experiments 15 to 25 d after flowering as described in Ellis *et al.* (1992a) and summarized below. Steady-state labelling was initiated by enclosing the top leaf in a leaf chamber about 9 min after beginning the light period (photosynthetic flux density was 1000 μmol photons m⁻² s⁻¹). Approximately 20 min after beginning steady-state labelling, 'empty' seed coats were prepared by the protocol described in Ellis *et al.* (1992b). Within 5 min of empty seed coat preparation, seed coats were perfused with a solution containing 0.5 mol m⁻³ CaCl₂, 1.0 mol m⁻³ KCl, 5.0 mol m⁻³ Mes, pH 6.0 (NaOH). Various concentrations of mannitol (as an osmoticum) and 15 mol m⁻³ Na₂-EDTA were also present in the perfusion solution where indicated. A constant level of solution was maintained in the seed coat cup (Ellis *et al.* 1992b). The typical rate of perfusion was 100 mm³ min⁻¹, and varied no more than 25% during an individual experiment. Perfusate was collected as 10 min samples using a fraction collector, except that perfusate collected during the first 20 min of perfusion was discarded due to the initial difficulty in establishing uniform flow rates. To study the effects of changes in mannitol concentration and the presence of EDTA, the perfusion solution was changed at the input to the peristaltic pump. The lag time between a change of solution at the pump input and the appearance of new perfusion solution at the fraction collector was approximately 7 min. At the end of perfusion, seed coats were checked for attachment at the funiculus by pulling on them with forceps; data from detached seed coats are not reported.

Analysis of data from perfused seed coats

Methods for analysis of ¹⁴C and sugar in seed coats and perfusates have been described (Ellis *et al.*, 1992b). Sucrose + glucose measurements are presented as a consequence of the sugar assay used in this study (Ellis *et al.*, 1992a); most of the sugar present was sucrose (Ellis *et al.*, 1992b). Tracer data were normalized to a specific activity of 1.0 GBq (mol C)⁻¹ (Ellis *et al.*, 1992a). A seed size correction factor based on the sugar content of the basal portion of cut seed coats (approximately 70% of intact seed coat sugar content) was used to adjust perfused seed data for the fraction of the seed that was lost due to excision (Ellis *et al.*, 1992b).

Experimental design

Experiments were designed to explore the possibility that osmoticum concentration changes and EDTA treatments might change the tissue-specificity of photosynthate release from perfused seed coats. Osmoticum treatment sequences were intended to maximize the number of combinations of osmoticum concentration changes and to allow some interpretation of effects of previous treatments on later treatments. EDTA was never applied at the beginning of perfusion because the treatments with EDTA would certainly have influenced all later treatments.

RESULTS AND DISCUSSION

General characteristics

The effects of mannitol concentration changes and EDTA treatments sometimes varied considerably between replications (Figs 1 to 4); the extent of this variability is illustrated by the size of the error bars. Although some

of this variability may have been introduced by the use of seeds at different developmental stages, variability seems to be a characteristic of osmoticum concentration change effects. For this reason, presentation of the results of single 'representative' experiments was not a reliable method for demonstrating the effects of treatments, and the mean \pm s.e. are used for data presentation in the current study.

Osmoticum-induced changes in photosynthate efflux

The stimulation of sugar- and ^{14}C -efflux (Figs 1, 3, 4) by rapid changes in osmoticum concentration demonstrates that rapid changes in cell turgor can immediately influence photosynthate unloading in perfused seed coats (Patrick, 1984; Minchin and McNaughton, 1986; Patrick *et al.*, 1986; Ellis and Spanswick, 1987; Grusak and Minchin, 1988). Previous studies showed that efflux of unlabelled sugar and ^{14}C were increased when the osmoticum concentration in the perfusion solution was changed from 100 to 10 mol m^{-3} (Patrick, 1984; Patrick *et al.*, 1986; Ellis and Spanswick, 1987). The reason for the absence of this effect in the current study (Fig. 2) is not known.

Significant increases in sugar- and ^{14}C -efflux were usually observed in response to large changes in osmoticum concentration (from 500 mol m^{-3} to 10 or 100 mol m^{-3} mannitol, or vice versa; Figs 1, 3, 4); small changes in osmoticum concentration (from 10 to 100 mol m^{-3} mannitol or vice versa; Figs 1, 2) had no observable effect. The stimulation of photosynthate efflux by changes in osmoticum concentration in either direction (from high to low or from low to high) is consistent with the stimulation of seed coat unloading by high cell turgor and the stimulation of phloem import by low cell turgor (Patrick *et al.*, 1986; Ellis and Spanswick, 1987; Grusak and Minchin, 1988).

When osmoticum concentrations were changed from high to low, the magnitude of sugar- and ^{14}C -efflux was greater when the change in osmoticum concentration was greater. For example, efflux was increased to a greater extent by a change from 500 to 10 mol m^{-3} (Fig. 3) than by a change from 500 to 100 mol m^{-3} mannitol (Fig. 4). However, efflux responded to low-to-high osmoticum concentration changes in the opposite way; larger changes in osmoticum concentration produced smaller effluxes. This can be observed by comparing the change from 100 to 500 mol m^{-3} (Fig. 1) with the change from 10 to 500 mol m^{-3} (Fig. 2). Photosynthate effluxes following low-to-high osmoticum concentration changes were also much smaller than those following high-to-low changes (500 to 10 mol m^{-3} , Fig. 3; 500 to 100 mol m^{-3} , Fig. 4). The different efflux responses to high-to-low versus low-to-high osmoticum concentration changes may be explained by the different activation characteristics of

phloem import, which is stimulated by low cell turgor, and photosynthate release from the seed coat, which is stimulated by high cell turgor (Patrick *et al.*, 1986; Ellis and Spanswick, 1987; Grusak and Minchin, 1988).

The kinetics of efflux in response to different types of osmoticum concentration changes (low-to-high, high-to-low) were somewhat obscured in the current study as a result of averaging multiple repetitions of the same experiment because treatment effects in different repetitions were not precisely synchronized (individual experiments were presented by Ellis and Spanswick, 1987). However, the results of the current study are compatible with the efflux kinetics observed by Ellis and Spanswick (1987); high-to-low osmoticum concentration changes tended to increase photosynthate efflux more rapidly than low-to-high changes.

Osmoticum-induced changes in the specific activity of eluted sugar

Even though changes in osmoticum concentration often caused sudden releases of sugar and tracer, the specific activity of eluted sugar did not change dramatically following a change in osmoticum concentration (the apparent decrease in eluted sugar specific activity in Fig. 2 was present in only one out of three replications). The constancy of eluted sugar specific activity demonstrates that labelled and unlabelled sugar were fairly well mixed within the seed coat, because the bulk of seed coat sugar is outside the phloem in seed coats (Ellis *et al.*, 1992a), and a sudden release from the phloem or the bulk symplast of the seed coat would lead to a rapid change in the specific activity of eluted sugar if tracer imported by the phloem were not well distributed in the seed coat. Tracer movement from the phloem throughout the seed coat could only occur by symplastic transport, because the rapid loss of solutes to the perfusion solution prevented apoplastic transport within perfused seed coats. Another possibility is that different sugar pools within the seed coat (which may have had different specific activities) reacted identically to changes in osmoticum concentration. However, this is unlikely because changes in turgor affect phloem import and efflux from the bulk symplast of seed coats in opposite ways (Patrick *et al.*, 1986; Grusak and Minchin, 1988; Ellis *et al.*, 1992a). Thus, the constancy of eluted sugar specific activity is best explained by the symplastic transport of tracer from the phloem to the inner surface of the seed coat before unloading to the apoplast (Patrick *et al.*, 1986; Grusak and Minchin, 1988; Ellis *et al.*, 1992b).

Osmoticum changes and cellular damage

Large and rapid changes in osmoticum concentration could have caused plasmolysis and the damage of plas-

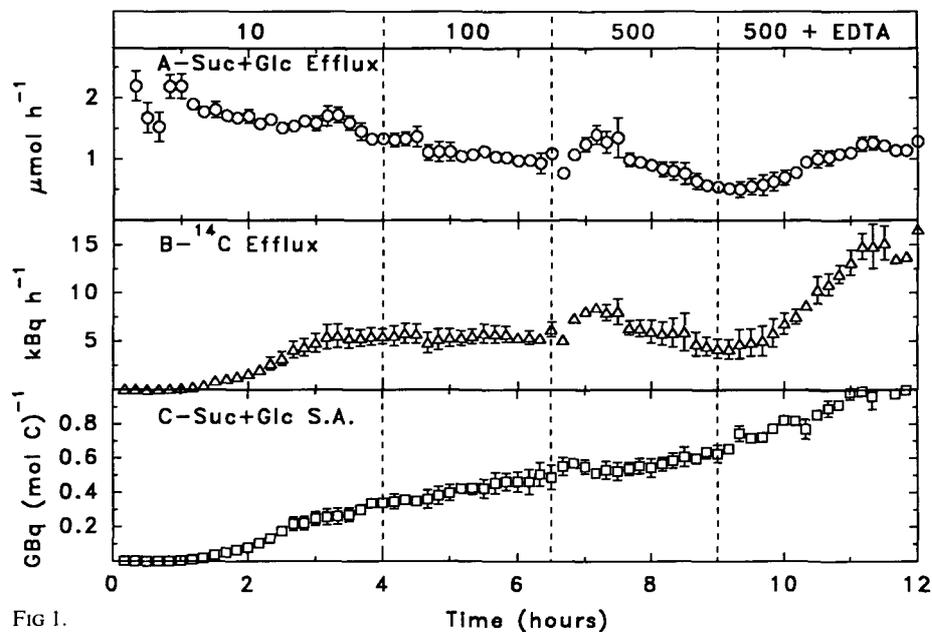


FIG 1.

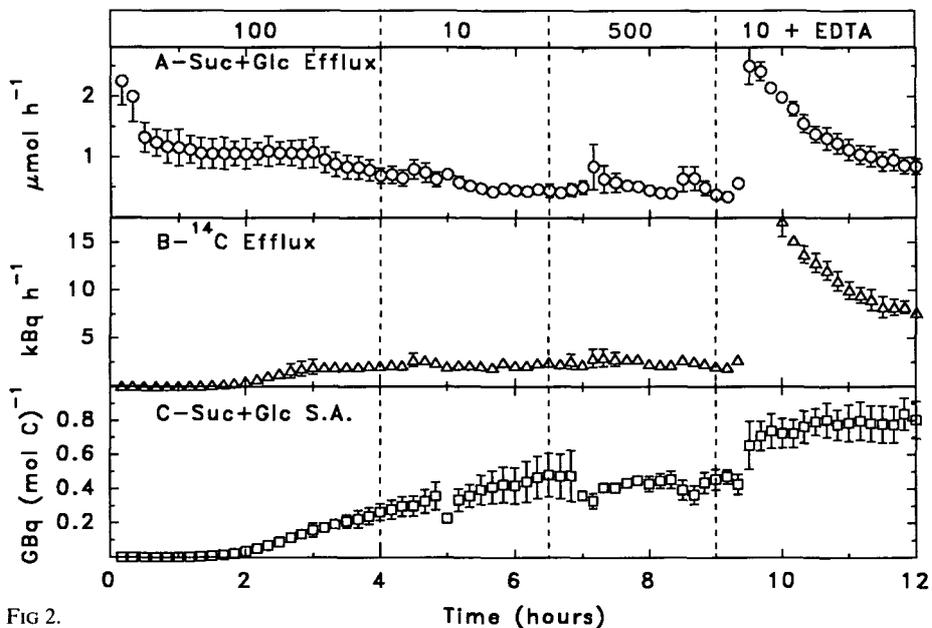


FIG 2.

modesmatial connections between cells in the seed coat. Large changes in osmoticum concentration near the beginning of the perfusion period (500 to 10 or 100 mol m⁻³, Figs 3, 4) were associated with low eluted sugar specific activities. This may have resulted from a blockage of symplastic tracer flux from the phloem to other seed coat cells. However, rapid stimulations of sugar- and ¹⁴C-efflux by osmoticum treatments did not lead to rapid changes in the specific activity of eluted sugar and this suggests that osmoticum concentration change treatments did not severely disrupt the movement of sugars within the seed coat.

Osmoticum and EDTA-induced changes in photosynthate unloading

Sugar- and ¹⁴C-efflux were always stimulated when EDTA was first added to the perfusion solution. The stimulation of sugar- and ¹⁴C-efflux by combined osmoticum and EDTA treatments usually paralleled the effects observed without EDTA. For example, the change from high to low osmoticum concentration (500 mol m⁻³ mannitol to 10 mol m⁻³ mannitol + EDTA, Fig. 2) produced a larger stimulation of efflux than did the change from low to high (10 mol m⁻³ mannitol to 500 mol m⁻³ mannitol + EDTA, Fig. 3). The fairly constant increase in

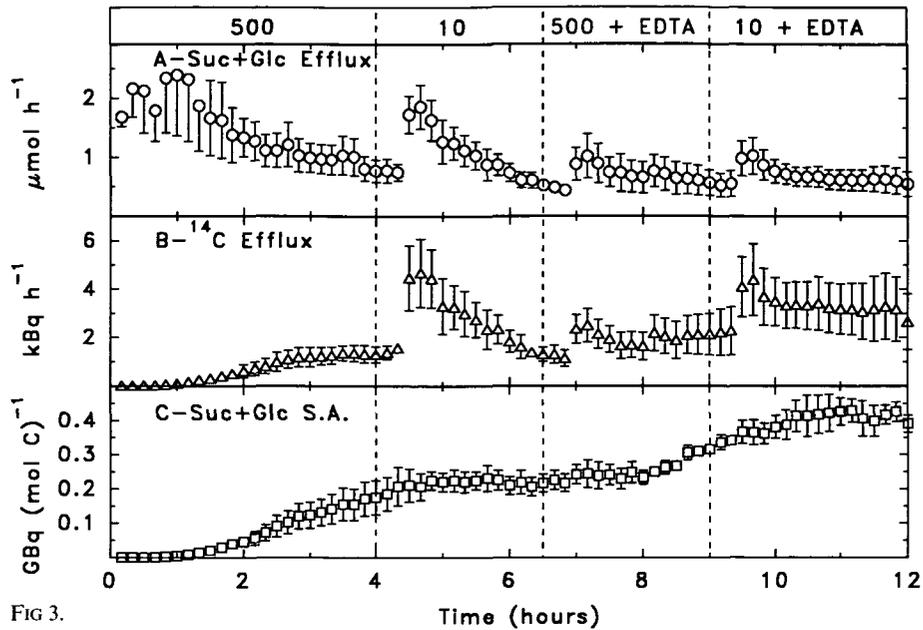


FIG 3.

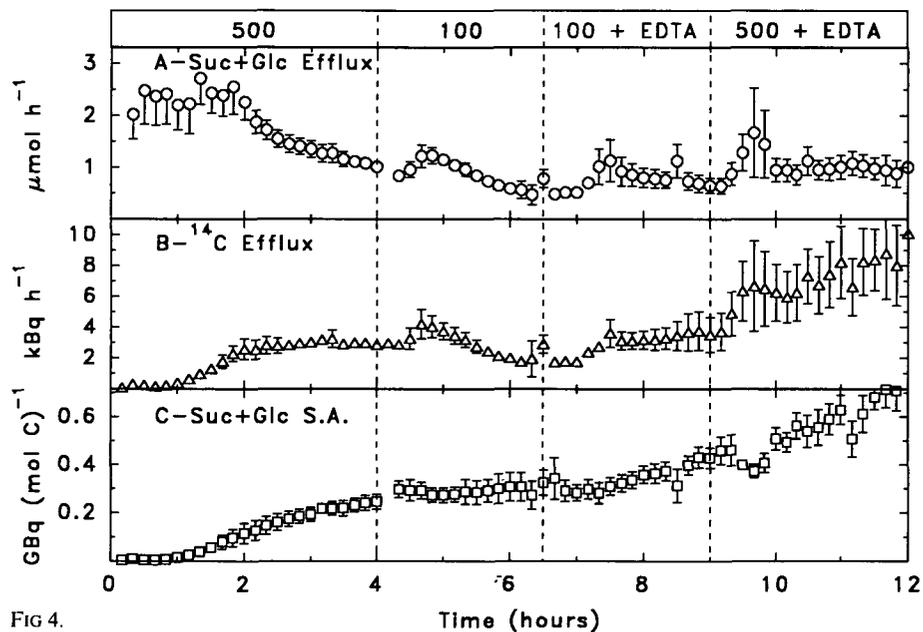


FIG 4.

FIGS 1–4. Time-course of seed coat perfusion with changing mannitol concentrations with or without 15 mol m^{-3} EDTA. Mannitol concentrations (in mol m^{-3}) and the presence of EDTA is indicated at the top of the figure. Data are the mean \pm s.e. of three experiments (except Fig. 4, which represents data of four experiments). (A) Sucrose + glucose efflux; (B) ^{14}C -efflux; (C) specific activity of sucrose + glucose. Tracer data were normalized to a $^{14}\text{CO}_2$ specific activity of $1.0 \text{ GBq (mol C)}^{-1}$. Zero time refers to beginning of seed coat perfusion.

eluted sugar specific activity typically observed following EDTA treatments indicates that EDTA did not inhibit phloem import to the seed coat.

EDTA treatments sometimes produced changes in photosynthate efflux that were quite different from the effects of osmoticum concentration changes in these experiments and in those of Ellis and Spanswick (1987). For example, the change from 500 mol m^{-3} mannitol to a solution containing both 10 mol m^{-3} mannitol and EDTA (Fig. 2) increased photosynthate efflux above the

rate present during the first hour of perfusion. The tracer efflux stimulated by the above treatment was greater than any other observed in these experiments. It declined rapidly following the initial surge, probably due to the net loss of photosynthate from the seed coat. When EDTA was not present, changing from 500 to 10 mol m^{-3} mannitol also produced a large stimulation of photosynthate efflux (Fig. 3). In contrast, when EDTA was present both before and after the change from 500 to 10 mol m^{-3} mannitol (Fig. 3), sugar- and ^{14}C -efflux were

stimulated only slightly. The early loss of sugar and tracer during the previous treatments of Fig. 3 may have reduced the amount available for later release. However, EDTA may have induced a high degree of membrane leakiness which reduced the alteration of cell turgor by the later treatment in Fig. 3.

The specific activity of eluted sugar increased rapidly following the change from 500 mol m⁻³ mannitol to 10 mol m⁻³ mannitol + EDTA (Fig. 2), but, following the initial increase, the specific activity of eluted sugar did not appear to increase further over time. This rapid stimulation of eluted sugar specific activity most likely represents an enhanced release of photosynthate from the phloem and/or cells close to the phloem. These cells probably contain sugar of a higher specific activity than cells near the inner surface of the seed coat because symplastic mixing of tracer is not instantaneous. The large efflux stimulated by this treatment suggests a widespread release of photosynthates from all cells of the seed coat, including the phloem. This is in contrast to efflux under constant or changing osmoticum concentrations which appears to occur primarily from cells near the inner surface of the seed coat (Patrick *et al.*, 1986; Grusak and Minchin, 1988; Ellis *et al.*, 1992b). The absence of a significant increase in eluted sugar specific activity following the initial stimulation indicates that phloem import was not enhanced, perhaps due to the low concentration of osmoticum in the apoplast. Therefore, it appears that the treatment described above generally stimulated photosynthate efflux from seed coat cells, including the phloem and/or cells neighbouring the phloem, without significantly increasing phloem import to the seed coat.

Another unusual effect of EDTA was the slow increase in sugar and tracer efflux caused by EDTA treatment when the osmoticum concentration remained at 500 mol m⁻³ (Fig. 1). This effect was not observed when EDTA was introduced to a perfusion solution remaining at 100 mol m⁻³ mannitol (Fig. 4) and was the only example of a slow but large increase in photosynthate efflux in these experiments and in those of Ellis and Spanswick (1987). This result is comparable to the stimulation of sugar efflux from *Vicia faba* seed coats induced by treatment with 400 mol m⁻³ mannitol + 15 mol m⁻³ EDTA (Wolswinkel, 1987a). Eluted sugar specific activity continued to increase following EDTA treatment (Fig. 1), which indicates that phloem import of high specific activity sugar continued unabated. High osmoticum concentrations enhance phloem import (Minchin and McNaughton, 1986; Wolswinkel, Kraus, and Ammerlaan, 1986; Grusak and Minchin, 1988; Ellis *et al.*, 1992b), and thus it is likely that the stimulation of photosynthate release from the seed coat is not tissue-specific.

The specific activity of sugar in seed coats and perfusates

The specific activity of eluted sugar was overestimated in these experiments because sugar was not purified before

specific activity determinations were made (Ellis *et al.*, 1992b; Materials and Methods). For this reason, specific activity measurements of eluted and seed coat sugar cannot be directly compared. However, higher specific activities of eluted sugar (near the end of the perfusion period) were associated with a higher specific activity of sugar remaining in the seed coat after perfusion. This can be seen by ranking the specific activity of seed coat sugar in Figs 1 to 4 (expressed as a percentage of intact seed coat specific activity; Fig. 1: 139.3%; Fig. 2: 95.6%; Fig. 3: 42.3%; Fig. 4: 60.6%) and comparing this with a ranking of the specific activity of eluted sugar at the end of the perfusion period. The correlation between eluted and seed coat sugar specific activity indicates that unlabelled and ¹⁴C-labelled sugar mixed before release to the perfusion solution; this effect was also observed by Ellis *et al.* (1992b). During perfusion, the mixing of phloem-imported tracer with seed coat sugar requires symplastic transport, because perfusion curtails apoplastic transport within seed coats (Ellis *et al.*, 1992b). Thus, the direct relationship between eluted and seed coat sugar specific activity is evidence for symplastic phloem unloading in perfused seed coats.

The specific activity of seed coat sugar after perfusion was sometimes higher than the value for the intact control (see data for Fig. 1 seed coat above). This may be explained by the stimulation of phloem import above the levels present in intact seed coats (perhaps by EDTA treatment), but the high variability of this observation (s.e. of seed coat sugar specific activity for Fig. 1 = ± 32%; mean s.e. for Figs 2–4 = ± 8.6%) suggests some degree of experimental error.

CONCLUSIONS

Osmoticum concentration changes stimulated sugar and ¹⁴C-photosynthate release from perfused seed coats. The effects of osmoticum concentration change treatments were consistent with phloem import stimulation by low cell turgor (Wolswinkel and Ammerlaan, 1984; Minchin and McNaughton, 1986; Wolswinkel *et al.*, 1986; Grusak and Minchin, 1988; Ellis *et al.*, 1992b) and photosynthate efflux stimulation by high cell turgor in the seed coat (Patrick, 1984; Patrick *et al.*, 1986; Wolswinkel *et al.*, 1986; Grusak and Minchin, 1988; Ellis *et al.*, 1992b). Rapid changes in sugar- or ¹⁴C-efflux induced by osmotic treatments were not accompanied by changes in the specific activity of eluted sugar, and the specific activity of sugar eluted near the end of the perfusion period paralleled the specific activity of sugar in the seed coat (see discussion above). These results demonstrate that unlabelled and ¹⁴C-labelled sugar were well mixed within perfused seed coats. The homogeneity of labelled and unlabelled sugar within perfused seed coats is evidence for the symplastic transport of sugar from the phloem to the inner surface of the seed coat before efflux to the

apoplast (Patrick *et al.*, 1986; Grusak and Minchin, 1988; Ellis *et al.*, 1992b).

EDTA treatments stimulated sugar- and ^{14}C -efflux from seed coats. When EDTA treatments were combined with osmoticum concentration changes, the characteristics of efflux stimulation paralleled those of osmoticum concentration changes without EDTA. The very large photosynthate releases sometimes induced by combined EDTA/osmoticum treatments appeared to result from an increase in photosynthate release from all cells of the seed coat, including the phloem. There was no evidence for specific EDTA stimulation of phloem import, unloading, or 'sink strength'.

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