Regulation of sulfate uptake in carrot cells: Properties of a hypercontrolled variant

(cystine/hypersuppression/sulfate efflux/haploid culture)

IAN J. FURNER AND Z. R. SUNG

Departments of Genetics and Plant Pathology, University of California, Berkeley, Berkeley, California 94720

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ABSTRACT Sulfate uptake in haploid carrot cultures can be experimentally controlled by the sulfur source provided for growth. The rate of sulfate uptake is low in cells grown on cystine or sulfate and high in sulfur-starved cells. A selenate-resistant variant cell line has been isolated from a haploid carrot line. The variant shows hypersuppression of sulfate uptake by cystine and essentially normal control by the other treatments. While both lines efflux intracellular sulfate in the presence of external sulfate, the rate of efflux from the variant is 4–6 times higher at comparable levels of initial intracellular sulfate. Further, properties of the efflux and uptake in both lines suggest that they are mediated by the same system. We propose that the variant possesses an altered uptake–efflux system that is more readily reversed and more subject to control by some metabolite derived from cystine.

Sulfate uptake has been studied in a variety of organisms, and permease-deficient mutants have been selected by resistance to sulfate analogs in *Escherichia coli* (1), yeast (2), *Aspergillus* (3), and *Neurospora* (4). In *Aspergillus*, a mutant showing hypersuppression of sulfate uptake by methionine has been isolated and was found to be allelic to other permease-less mutants (5). In general, the regulation of these permease activities has been attributed to repression by some reduced sulfur compound; however, there is also evidence for inhibition of uptake by some early intermediate in reduction (2).

Tobacco cells (6) and potato tuber discs (7) show suppression of sulfate uptake if incubated with sulfur-containing amino acids. Using tobacco cells, Smith (8, 9) found that sulfate uptake was suppressed by growth on cyst(e)ine or sulfate and, on the basis of correlative evidence, suggested that uptake was controlled by feedback inhibition by the intracellular sulfate pool.

In this paper, we describe a variant carrot cell line that has altered control of sulfate uptake by cystine. The results show that this line has an altered uptake–efflux system and is impaired in sulfate reduction. The relationships among these phenotypes are discussed.

MATERIALS AND METHODS

Culture Methods and Variant Isolation. The parental cell line has been described (10), and growth in suspension culture was monitored turbidimetrically (11). Media used in this work were based on Gamborg's B5 medium (12), modified by replacing $(\text{NH}_4)_2\text{SO}_4$ with NH_4NO_3 and other sulfates with equimolar chlorides. This medium, referred to as –S medium, was used unsupplemented or supplemented with cystine (Eastman). Suspension cultures of both lines were grown in 100 ml of medium in 500-ml flasks with continuous shaking at 120 rpm at 25°C in the light. Plants were produced from both lines on

B5 medium devoid of 2,4-dichlorophenoxyacetic acid and callus was reinitiated from root tips on B5 medium supplemented with 2,4-dichlorophenoxyacetic acid at 1 mg/liter. Chromosome counts were done as described (10).

The variant was isolated from the parental line, HA, by plating cells on –S medium/1 mM djenkolate/0.2 mM sodium selenate/1% agar. After 4 months, those cells that grew up to callus colonies were subcultured onto –S medium/1 mM cystine. The line described, S232, was isolated by this procedure from a culture that had been treated 1 month earlier with 0.3% ethyl methanesulfonic acid for 3 hr. It is not known if the variant was induced by the mutagen.

Labeling Cells. Suspension culture cells used in uptake and efflux experiments were filtered through 500- μm nylon mesh, washed three times, and suspended in culture medium at a fresh weight of 400 mg/100 ml. Washing and resuspension were done with the medium used for growth. Sulfate uptake and efflux studies were done in 1 mM MgCl_2 /1 mM CaCl_2 /1 mM 4-morpholineethanesulfonic acid/2% glucose, pH 5.0. Cells were washed three times in this medium, suspended at 20 mg fresh weight/ml and dispensed as indicated below.

Uptake Experiments. In uptake experiments, 0.5-ml portions of the cell suspension were placed in 10-ml disposable culture tubes (Scientific Products). Two hours later, uptake was initiated by addition of [^{35}S]sulfate (Amersham) and an appropriate amount of carrier K_2SO_4 in 0.5 ml of medium. The final sulfate concentration was 0.1 mM. Incubation was done at 27°C with continuous shaking at 200 rpm. After a fast uptake in the first 15 min, uptake was linear up to 5 hr. Thus, a 2-hour labeling period was used as an index of the rate of uptake. At the end of the uptake period (2 hr), the reaction was terminated by the addition of 5 ml of 10 mM K_2SO_4 in the same medium. The cells were collected by vacuum filtration and washed five times. Dried filter discs (Whatman GFC) were assayed in scintillation fluid. At least two tubes were used for each treatment and the data presented are the mean of at least two experiments. A control for nonspecific adsorption, zero incubation time, was subtracted from each result.

Labeling Specific Metabolites. In experiments to examine the labeling of specific metabolites, a modified method of Hodson (13) was used. Tubes of labeled cells were prepared as above except that 1 ml of 10 mM iodoacetamide was added at the time indicated. After a further 10 min of incubation, cells were washed as described above. Cells from four tubes were collected for each sample and frozen. The next day each sample was suspended in 4 vol of water, boiled for 2 min, and the debris was spun down. Portions of the clear supernatant were spotted on thin-layer chromatography plates (Kontes LK6DF). The compounds were separated by ascending chromatography with 88% phenol/concentrated $\text{NH}_4\text{OH}/\text{H}_2\text{O}$ (174:1:26). The dry plate was exposed to x-ray film and the located compounds were cut out and assayed in scintillation fluid.

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Efflux Experiments. In efflux experiments, cells starved for sulfur for 6 days were used. First, 15–150 ml of cell suspension was loaded with [35 S]sulfate in Erlenmeyer flasks. At the end of the labeling period (1–5 hr), cells were collected by gentle filtration, washed four times in sulfur-free incubation medium, scraped from the filter, and suspended in the same medium at 10 mg fresh weight/ml. Typically, 15–20 ml of cell suspension were incubated in a 25-ml Erlenmeyer flask.

Efflux was measured by removing 1-ml samples of cell suspension, pelleting the cells by centrifugation, and assaying the radioactivity in 0.5 ml of supernatant. Sulfate-stimulated efflux was initiated by the addition of K_2SO_4 to a final concentration of 2 mM unless otherwise stated. Sulfate-stimulated efflux was initiated 2 hr after washing the cells. All incubations were carried out at 25°C with shaking at 120 rpm.

Estimates of intracellular sulfate were made by comparing sulfur soluble in acidic $BaCl_2$ with total sulfur in boiled cell extracts collected 2 hr after washing. Specifically, 40 mg of cells was collected by vacuum filtration and boiled for 2 min in 0.76 ml of 20 mM K_2SO_4 . The debris was spun down. Two 0.2-ml samples were taken, to one was added 0.2 ml of 100 mM $BaCl_2$ in 1 M HCl and to the other was added HCl alone. The samples were spun down again to remove the precipitate and 0.2-ml samples of supernatant were assayed for radioactivity. Sulfate was estimated from the difference between the two treatments.

All assays were done by using 4 ml of 3a70B counting cocktail (Research Products International) and a Beckman LS 250 counter. Specific activities of [35 S]sulfate were varied during the experiments but were generally 0.5–2 mCi/mmol (1 Ci = 3.7×10^{10} becquerels).

RESULTS

Isolation, Selenate Resistance, and Chromosome Counts of the Variant Line. The variant line, S232, was isolated from the parental line, HA, by selecting for resistance to the sulfate analog, selenate, during growth on djenkolate, a poor sulfur source. Reuveny has shown that tobacco cells are extremely selenate sensitive when grown on this sulfur source and suggested that the sensitivity was due to derepression of the enzymes of sulfate assimilation (14). We used djenkolate as the sulfur source because it does not suppress sulfate, and presumably selenate, uptake in carrot cells (see below). The S232 line is resistant to selenate when grown on djenkolate and has remained stable for 2.5 years in the absence of selection. The LD_{95} (dose of drug reducing the callus plating efficiency by 95%) values are ≈ 30 and $300 \mu M$ for the HA and S232 lines, respectively. Callus reinitiated from plants derived from the two lines have the same resistance as the original lines. HA and S232 are of comparable ploidy, consisting of 70% haploid cells with the remainder being largely diploid cells. The data are consistent with S232 resulting from either a mutation or a stable shift in gene expression that has occurred in haploid tissue. Until this question can be resolved by sexual or somatic crosses, we feel such lines should be referred to as variants, a term that is neutral regarding the nature of the alteration.

Control of Sulfate Uptake. Sulfate uptake was measured in samples of the two lines grown under a variety of conditions of sulfur nutrition. Sulfate uptake is clearly conditioned by the sulfur source supplied for growth (Fig. 1). In HA cells, prior sulfur starvation results in high levels of uptake while previous growth on sulfate or cystine suppresses it. Although cells supplied with a sulfur source grow for at least 2 weeks without subculture, sulfur-starved cells typically cease growth after day 6; however, on supplementation with sulfate or cystine, the latter will grow without a lag period. Cystine is more effective than

FIG. 1. Sulfate uptake capacity in the HA (A) and S232 (B) lines grown under a variety of conditions. Curves: 1, sulfur-starved; 2, 2 mM sulfate; 3, 1 mM cystine. Capacity was determined as (nmol/10 mg fresh weight of cells) in 2 hr. Each point is the mean of three replicates from separate cultures; bars represent 1 SD.

sulfate in suppressing uptake early in the culture cycle; it is less effective than sulfate beyond day 4.

In the S232 variants, sulfur starvation results in high levels of uptake, usually higher than in HA (Fig. 1). Growth on sulfate suppresses it; sulfate is less effective in S232 compared with HA at early times (day 2) but is considerably more effective than in HA beyond day 4. The most striking difference between the two cell lines is in the suppression of uptake by cystine. The variant cells have little or no capacity to accumulate sulfate when grown on this sulfur source. Uptake in S232 is 1/5 to 1/10 that in HA grown under the same conditions. Cells grown on D-cystine or djenkolate give patterns similar to sulfur starvation in both lines.

Sulfate Efflux in the Two Lines. Preliminary experiments with cells uniformly labeled with sulfate (long-term labeling for 1 month) showed that the S232 line accumulated small pools of sulfate compared with HA. This result was surprising because the uptake capacity as is similar to that of HA in cells grown on sulfate. In an attempt to reconcile these two observations, we examine the efflux of sulfate from sulfate-loaded cells.

The loss of sulfate from sulfate-loaded cells of both lines is shown in Fig. 2. The effluxed material is sulfate, not other sulfur compounds, because it cochromatographs with sulfate and is precipitated by acidic $BaCl_2$. HA cells leak slightly after washing; in the absence of external sulfate, some of this loss can be reabsorbed (Fig. 2A, curve 4). If sulfate is added at 2 hr, a slight sulfate-stimulated efflux is observed (curve 1). Since the sulfate-stimulated efflux can be inhibited by agents that inhibit sulfate uptake [cystine (curve 2) and cycloheximide (curve 3)], efflux is presumed to occur via the sulfate uptake system. Both cystine and cycloheximide cause progressive loss of uptake capacity; >60% is lost in 2 hr of incubation.

The S232 line has larger faster effluxes under all conditions. In the absence of external sulfur sources, a large efflux is observed that is not dependent on added sulfate (Fig. 2B, curve 4). Much of this loss can be reabsorbed after 2 hr. If sulfate is added at 2 hr, a second large and fast efflux is observed (curve 1). In this treatment, nearly half the initial load was lost by the end of the experiment. The sulfate-stimulated efflux is inhibited by cystine and cycloheximide, suggesting that it is mediated by the same mechanisms as in HA, presumably via the uptake system (curves 2 and 3).

Metabolite Pools in Sulfate-Fed Cells. In short-term experiments, S232 cells take up sulfate slightly faster than HA; smaller pools of reduced compound are found in these cells (Fig. 3). Consequently, the sulfate pool in S232 represents a larger proportion of the sulfur recovered. In both lines, cysteine rap-

FIG. 2. Efflux of sulfate from loaded HA (A) and S232 (B) cells under a variety of conditions. Curves: 1, Addition of K_2SO_4 at 2 hr (final concentration, 2 mM); 2 and 3, cells were suspended in the presence of 1 mM cystine or 10 ppm cycloheximide, respectively, and treated 2 hr later with K_2SO_4 (final concentration, 2 mM); 4, no additions. Total loaded sulfur determined at the start of the experiment was 8.3 and 8.5 nmol/10 mg fresh weight for HA and S232, respectively. Efflux was determined as nmol/10 mg fresh weight of cells.

idly reaches a small stable pool that serves as a precursor to an expanding glutathione pool. Both pools are smaller in S232. (Cysteine, glutathione, and sulfate account for >98% of the sulfur-containing compounds recovered by this method. Early intermediates would probably be destroyed by the extraction. In some experiments, traces of methionine have been observed.) The apparent inconsistency between short- and long-term experiments, S232 possessing more sulfate than HA in short-term experiments but less in long-term ones, is probably due to the significant net efflux in S232 cells in long-term experiments.

Patterns of Efflux and Compartmentation. We reasoned that the high efflux rates observed in S232 might be a consequence of possessing a larger sulfate pool at comparable loading levels. To test this hypothesis, cells of both lines were loaded

FIG. 3. Sulfur-containing compounds found in sulfate-fed HA (A) and S232 (B) cells after various periods of incubation with $^{35}SO_4^{2-}$. Compounds were separated as their acetamidated derivatives. Curves: 1, sulfate; 2, reduced glutathione; 3, cysteine. Compound pools were determined as nmol/10 mg fresh weight of cells. Each point is the mean of two independent experiments; bars represent 1 SD and are omitted where smaller than the symbol representing the point.

to different extents, washed, and incubated for 2 hr in the absence of external sulfate. A sample was taken and the initial sulfate pool was determined. Efflux was then initiated by the addition of sulfate. The amount lost at each time point was subtracted from the initial pool and the data were plotted semi-logarithmically (Fig. 4).

When examined in this way, effluxes from both lines can be resolved into two components of different slopes, an initial fast efflux during the first hour and a second slower efflux during the first to fifth hours (and beyond). In higher plant tissues, effluxes having two or more distinct phases are frequently found and have been attributed to subcellular compartmentation (15). The fast component would result from efflux from the cytoplasm and the second, slow, efflux, would result from loss from the vacuole. The two phases of efflux will be referred to as cytoplasmic and vacuolar, in accordance with the above model, although other explanations are possible. Examination of Fig. 4 reveals that in S232 both effluxes are faster and, further, the cytoplasmic efflux contains a larger proportion of the cellular sulfate than it does in HA. The latter result implies that S232 has a larger proportion of the cellular sulfate in the cytoplasm and hence less in the vacuole. Since the second efflux is also faster, what is presumed to be the vacuolar sulfate must be more available to efflux in this line. The differences in efflux rates cannot be fully explained by the differences in reduction (see Discussion).

Effect of Extracellular Sulfate on Efflux. To further characterize the effluxes of the two lines, the effect of various levels of extracellular sulfate was examined. The rate of the initial efflux shows the same pattern of dependence on extracellular sulfate despite the difference in rate (Fig. 5A). The requirement for external sulfate is high; efflux rates are of the order of a few nanomoles per milliliter per hour, but require 2 μ mol of external sulfate per ml to be maximal. If the role of the external sulfate were merely to prevent reabsorption of leaked sulfate, the level required might be expected to be lower. The requirement for external sulfate can be approximated to Michaelis-Menten kinetics; a Lineweaver-Burk plot of the same data is shown in Fig. 5B. K_m values are 3.6 ± 0.1 and $3.1 \pm 1 \times 10^{-4}$ M for HA and S232, respectively. This phenomenon again suggests that this efflux is carrier mediated, with the external sulfate behaving as a substrate in the process. For purposes of comparison, K_m values for uptake in short time periods are $\approx 3 \times 10^{-5}$ M in both lines (i.e., $\approx 1/10$ as much).

Effect of Intracellular Sulfate Level on Efflux. In an attempt to measure the affinity of internal sites involved in efflux, the relationship between the initial rates of the cytoplasmic and the

FIG. 4. Sulfate remaining in loaded HA (A) and S232 (B) tissue after various periods of sulfate-stimulated efflux. Values were calculated from the initial pool and the amount lost at each time point. Each curve represents a flask of cells loaded to a different initial level by a different loading concentration. SO_4^{2-} was determined as nmol/10 mg fresh weight of cells.

FIG. 5. (A) Dependence of initial rate of cytoplasmic efflux on the external sulfate concentration in HA (●) and S232 (○) cells determined by logarithmic-linear regression of efflux curves during first half hour after addition of sulfate. Efflux was determined as (nmol/10 mg fresh weight of cells)/hr. Initial total sulfate pools, 5.1 and 4.8 nmol/10 mg fresh weight. (B) Double reciprocal plot of the data shown in A. The apparent K_m values for efflux with respect to extracellular sulfate are 3.6 ± 0.1 and $3.1 \pm 1 \times 10^{-4}$ M for HA and S232, respectively. (Mean $\pm$ SD of two and three determinations, respectively.)

presumed vacuolar effluxes to the initial intracellular sulfate pool was examined (Fig. 6). As noted above, both effluxes are faster in the S232 line but the cytoplasmic efflux is increased more than the vacuolar efflux. The rate of vacuolar efflux shows a linear relationship to the total pool in both cell lines. The increase in cytoplasmic efflux with respect to the total pool is a saturating curve in both cell lines. Probably, the development of a plateau in the cytoplasmic efflux curve represents not the saturation of sites involved in efflux but rather a shift in the subcellular distribution of sulfate. For example, in the S232 line at low loading levels (<3 nmol/10 mg), $\approx 25\%$ of the pool is recovered in the cytoplasmic efflux. In contrast, at high loading levels (≈ 10 nmol/10 mg), only 12–14% of the pool is recovered in this efflux. This effect can be seen in Fig. 4; a similar shift is observed in the HA line but cannot be accurately quantified because the cytoplasmic efflux is absolutely small, constituting $<2\%$ of the total pool.

DISCUSSION

The control of sulfate uptake by the supplied sulfur source in the HA line is similar to that in tobacco cells (7–9). The sulfate-

FIG. 6. Relationship of initial rates of cytoplasmic efflux (●) and presumed vacuolar efflux (○) to initial sulfate pool in HA (A) and S232 (B) cells. Rates were deduced from logarithmic-linear regressions of remaining sulfate solved at time zero. The initial pool was varied by loading with different concentrations of sulfate. The data presented are from several experiments performed on different days. Efflux rate was determined as (nmol/10 mg fresh weight of cells)/hr; initial cell SO_4^{2-} pool was determined as nmol/10 mg fresh weight of cells.

stimulated effluxes from both lines are inhibited by cystine and by cycloheximide, treatments that strongly inhibit uptake. The result suggests that uptake and efflux are mediated by the same system and, further, that both agents result in the loss, inactivation, or unavailability of sites needed for both uptake and efflux. The effect of cycloheximide is general in the sense that it also inhibits amino acid uptake in these cells (data not shown) and a variety of uptake systems in tobacco cells (16). It is not clear whether this is a result of high turnover rates for proteins involved in higher plant uptake systems or some less specific effect of the drug. The loss of efflux capacity in cystine-treated cells results from either the loss of sites involved or competition for such sites by sulfate generated intracellularly from the cystine.

The S232 line is a stable variant that has pleiotropic alterations in sulfate uptake and assimilation. These alterations include (i) hypersuppression of uptake by cystine, (ii) deficiency in sulfate reduction, and (iii) higher rates of sulfate efflux in the presence and absence of extracellular sulfate. Sustained effluxes from both lines are highly dependent on extracellular sulfate; the levels required are high. The observation that effluxes from both lines can be approximated to Michaelis-Menten kinetics with extracellular sulfate plotted as substrate suggests that the effluxes represent a carrier-mediated reaction rather than non-specific leakage of cellular sulfate. The apparent K_m , with respect to external sulfate, is the same in the two lines despite the difference in rates.

Attempts to measure the affinity of sites involved in efflux with respect to intracellular sulfate in the two lines were unsuccessful. The fastest phase of efflux in both lines gave a saturating curve with respect to total cell sulfate but this appears to reflect a shift in compartmentalization of the sulfate. The second, presumably vacuolar, efflux showed no saturation in the range examined. This may reflect either that sites involved in efflux cannot be saturated or that their apparent K_m is >1 mM. The latter may be the case, as studies on sulfate effluxes from *Neurospora* gave a K_m of 20 mM for efflux with respect to total intracellular pool of sulfate (17).

The difference in compartmentalization, high cytoplasmic sulfate in S232 and low in HA, could potentially explain the difference in efflux rates. One might propose the cytoplasmic efflux is faster in S232 merely because there is more sulfate in the cytoplasm; however, this does not explain why the vacuolar flux is also faster.

The kinetic and control properties of the effluxes between the two cell lines are the same; the only differences appear to be in the rates and magnitudes of the fluxes. We propose that uptake and efflux occur in carrot cells by the same system, as in *Neurospora* (17). If this is accepted, it must be proposed that S232 is altered such that the uptake-efflux system is more reversible. The hypersuppression of uptake by cystine might be the result of increased inactivation or inhibition of the altered sulfate permease by some metabolite derived from cystine.

This explanation leaves two aspects of the phenotype of this line unexplained: the deficiency in sulfate reduction and the abnormal compartmentalization. A single explanation of the S232 variant that could account for its diverse phenotypes would be that the same permease is used throughout the endomembrane system. This would explain the high cytoplasmic sulfate as a backflux from the vacuole and the deficiency in reduction as due to inability of the plastids to accumulate enough sulfate for reduction to proceed faster. This model is supported by the observation that vacuolar efflux is faster in S232 than in HA. An alternative explanation would be that S232 has a second lesion impairing sulfate reduction in addition to the altered permease. The observed high cytoplasmic sulfate would be the

result of failure to deplete the sulfate in this compartment by reduction.

Intermediate models are, of course, possible. A partial block in sulfate reduction and the same uptake-efflux system at plasmalemma and tonoplast would explain the observations.

The central question of how cystine and sulfate control sulfate uptake remains unsolved. None of the observations presented in this paper is incompatible with feedback regulation by the intracellular sulfate pool as proposed by Smith (8, 9). On the other hand, no evidence supporting the suggestion was found. Resolution of the nature of the control of sulfate uptake in higher plants will have to await some means of quantifying the sites involved irrespective of their activity. This can only be done by identifying the protein(s) involved.

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