Chapter 10

Flux Measurements of Cations Using Radioactive Tracers

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Abstract

Standard procedures for the tracing of ion fluxes into roots of plants are described here, with emphasis on cations, especially potassium (K^+). We focus in particular on the measurement of unidirectional influx by use of radiotracers and provide a brief introduction to compartmental analysis by tracer efflux (CATE).

Key words: Influx, Efflux, Potassium, Cations, Radiotracers

1. Introduction

Plant root transport systems are major portals through which the mineral substances that support life enter terrestrial food webs, usually in ionic form. Not surprisingly, the uptake and distribution of nutrient ions by plants has long been a major area of research in plant physiology, ecology, and biophysics. In particular, major strides have been made by means of radioactive and stable isotopes, with two of the most notable pioneers being G. de Hevesy (1), starting in the 1920s, and later E. Epstein (2), from the 1950s on.

The main advantage of isotope methodology over other approaches is that it enables the measurement of unidirectional fluxes, which are indispensable in the determination of kinetic flux parameters (e.g., K_M, V_{max} in a Michaelis-Menten sense), and therefore in the study of specific transporter capacities, energetics, mechanisms, and regulation. This information cannot be precisely determined by use of net flux measurement. The need to isolate a flux in one direction is particularly important under conditions where the flux in the opposite direction is high, and the turnover of intracellular pools is rapid (3). In addition, because fluxes of one isotope are usually observed against a background of a different isotope of the same element, tracer measurement can be conducted under fairly high nutrient conditions, relative to other methods.

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Isotopes can also be used in the measurement of net fluxes, but for this purpose a host of other well-established methods can also be applied, including: measurements of depletion in the external medium and/or accumulation in the tissue, use of ion-selective vibrating electrodes (e.g., MIFE, SIET), and the use of ion-selective fluorescent dyes.

In this article, we shall provide a basic procedure for the measurement of ion transport in plant roots, with special emphasis on cation uptake. Of all ions transported into plant roots, potassium (K^+) has probably been studied in the greatest detail historically and is one of the most rapidly absorbed and highly accumulated cations, along with calcium (Ca^{2+}), and (when present) ammonium (NH_4^+) and sodium (Na^+). For these reasons, we shall take the potassium ion as a prime example; by and large, however, the methodology presented here can be extended to uptake measurements for other cations, as well as anions, with few changes to protocol (see Note 1).

2. Materials

1. Plant material, typically intact, hydroponically grown seedlings, or excised roots (see Notes 2–4).
2. Glass or plastic vessels for pre-absorption, labeling, and desorption steps, typically 50–500 ml.
3. Equipment for aeration and/or stirring of solutions, often consisting of a compressed air source, a distribution system such as an aquarium-style manifold, and plastic tubing.
4. Dissecting tools (scalpel, forceps, scissors).
5. Labeling solutions (see Notes 5 and 6).
6. Pipettors and pipette tips (1 ml) for specific activity samples
7. Radiotracer, typically 5–20 millicuries for ^{24}Na and ^{42}K (see Notes 7–9).
8. Safety equipment, including protective clothing, eyewear, and shielding (see Note 7).
9. Radiometric equipment, usually a gamma or scintillation counter, plus a Geiger-Müller counter for safety purposes (see Note 9).
10. Low-speed (clinical-type) centrifuge and centrifuge tubes (see Note 10).
11. Drying oven (see Note 10).
12. Analytical balance.

3. Methods

1. Measure specific activity of uptake solution (see Note 11)
2. Pre-equilibrate plants for 5–10 min, if appropriate (see Note 12)
3. Immerse roots in radioactive solution (see Note 13)
4. Remove plants from radioactive solution after appropriate labeling period, typically 2–10 min (see Notes 14 and 15)
5. Desorb roots of extracellular tracer, typically for 5–10 min (see Note 16)
6. Detach roots from shoots, if appropriate (see Note 17)
7. Weigh plants, subsequent to centrifugation or drying (see Note 17)
8. Count radioactivity in plant samples, usually by gamma- or scintillation-counting (see Note 9)
9. Calculate the flux, using absorbed counts, specific activity of labeling solution, root mass, and labeling time (see Notes 18 and 19)

4. Notes

1. A few interesting differences between cations and anions, with respect to plant roots, might be mentioned here. The uptake of cations can substantially exceed that of anions when ammonium is the main nitrogen source, while the reverse is true when nitrate is the source (4, 5). In either case, due to the metabolism of anions, the accumulation of cations often exceeds that of anions (cations other than NH_4^+ are rarely metabolized), with charge balance being achieved via the synthesis of organic acids and the transport of protons (4, 6). Interestingly, the uptake of both anions and cations is at least partially driven by the inwardly negative charge on the plasma membrane of root cells; in the case of anions, this is typically facilitated by the cotransport of protons (7).
2. Plants should be grown hydroponically, so that culture conditions can be precisely controlled, and roots are directly accessible for examination. It is important to consider the use of plants from a variety of developmental stages, as their nutrient requirements will change with age. It should also be considered that seed reserves play an important role in the nutrition of a young seedling.
3. Excised roots are frequently used in tracer-flux experiments, as they reduce complications arising from transpiration and

translocation to the shoot. However, their use also entails tissue damage at the point of excision, which can be propagated to the remainder of the root segment. Therefore, excised tissue should be aged for several hours prior to measurement, ideally in conjunction with an indicator of recovery (8). It may also be necessary to supplement this heterotrophic tissue with a source of energy (e.g., sucrose). Even when such precautions are taken, however, valuable information is inevitably lost when excised roots are used—e.g., information about the partitioning of a substance between organs of the plant, or the influence of transpiration as a potential driver of the flux. Thus, we recommend that, when possible, intact plants be used for tracer flux measurements.

4. Often, several plants are bundled together at the shoot base and treated as a single replicate. This can improve statistics, and measuring accuracy when seedlings of low root mass are used, or when specific activity is low.

5. For steady-state investigations, labeling solutions should be identical to plant growth solutions, except for the addition of radiotracer. Volumes of solution should be chosen to ensure that depletion of nutrients by plant roots is not significant over the duration of the protocol, especially when working with low concentrations of rapidly accumulated substrate (200 ml of solution is typically sufficient for a 5-min uptake procedure). In addition, all other growth conditions, especially temperature, light, and humidity, should be maintained during experimentation and, therefore, simple bench experimentation, in marginally controlled lab spaces, should be avoided. For non-steady-state conditions, the uptake solutions and/or ambient conditions are often modified, e.g., to include variations in substrate concentrations (as in the development of flux isotherms), or the provision of metabolic inhibitors (see Note 12).

6. Particularly in older studies, the uptake of a labeled substance is often monitored against a background of only that substance, plus a small amount of Ca^{2+} (usually as $CaSO_4$), typically at about 100–200 μM, to maintain membrane integrity and basic membrane function. While this simplified approach may reduce complications due to interactions between the traced substance and other materials in solution, it also may change the nature of the experimental system. For example, removing the K^+ provided during plant growth (or the NH_4^+, if present) will likely result in electrical hyperpolarization of the plasma membrane, thus changing the driving force for ions across the membrane and therefore, quite possibly, their fluxes. In general, we recommend using complete nutrient solutions, unless the hypothesis guiding a particular study requires the removal or addition of solution components (e.g., ref. 9).

7. Often the choice between stable and radioactive isotope is a matter of convenience, and will depend upon the availability of appropriate isotopic material or instrumentation (see Note 9). In general, the processing time for radioactive counting is much shorter than that for measurement of stable isotopes by use of mass spectrometry. On the other hand, radioisotopes of some elements (e.g., N, O) are very short-lived and are only available to researchers working in close proximity to a production facility such as a cyclotron. While less widely available and affording less handling time, however, such tracers are advantageous from the perspective of radioactive waste; for example, a sample of ^{13}N will have more or less completely decayed into stable ^{13}C within a few hours of its production. Still, longer-lived tracers are much more commonly used; these include tracers for K^+ and Na^+ that have slightly longer half-lives, on the order of several hours (see Note 8). In all cases involving radiotracers, appropriate safety measures must be taken. This often involves a combination of shielding types, such as Plexiglas and/or lead, depending on the nature of the isotopic decay.
8. Perhaps surprisingly, the most widely used isotope for K^+ tracing is not potassium at all, but ^{86}Rb, a radioisotope of rubidium, an alkali-metal "analog" of potassium. This is due in large part to its long half-life (18.65 days) relative to isotopes of K (see below). However, there is substantial evidence that Rb is an imperfect substitute for K, for example with respect to its translocation to the shoot (10). ^{40}K, with its extremely long half-life (1.25 billion years), is rarely used as a tracer but rather as an environmental indicator (e.g., ref. 11). The other radioisotopes of K are all too short-lived to be of significant practical use, except for ^{42}K and ^{43}K (half-lives, respectively, of 12.36 and 22.3 h). Of these, ^{42}K is almost exclusively used as a tracer in plant systems, although ^{43}K has been successfully used, for example, in the microautoradiographic tracing of K^+ fluxes in stomatal cells (12). In the case of Na^+ tracing, only two radioisotopes of sodium are sufficiently long-lived to be useful: ^{22}Na and ^{24}Na (half-lives of 2.6 years and 14.96 h, respectively); both have been used extensively in plant systems. It is worth noting that the production of ^{42}K and ^{24}Na typically involves bombardment of nonradioactive ^{39}K and ^{23}Na, respectively. Because these nuclear transformations are generally incomplete, researchers must consider the presence of (sometimes substantial) residual amounts of "cold" isotope in their preparation of experimental solutions. This can be contrasted with the production of ^{13}N, via the proton bombardment of the oxygen atom of water; in such a case, there is no background ^{14}N to contend with.
9. Almost all radiotracing in biological systems involves the measurement of electromagnetic radiation associated with the

decay of beta (plus or minus) -emitting nuclides (i.e., positrons or electrons). Liquid scintillation counters and gamma counters are used to measure most biologically important radioisotopes. Scintillation is the most widely used counting method, despite the need to use a scintillation "cocktail" in which the sample is dissolved or suspended; gamma counting has no such requirement. Regardless of counting device, it is essential that it correct for radioactive decay, particularly when using short-lived tracers. As well, care must be taken to ensure that the geometry of the sample-detector system is uniform from one sample to the next (or corrections be made to account for differences), since the position of the detector relative to the sample can influence the quantity of radiation measured. This phenomenon is not always fully acknowledged by providers of detection instrumentation and usually must be worked out by the individual researcher. Similarly, manufacturer claims about the effectiveness of detector shielding against ambient radiation (e.g., from nearby samples within the counter) are at times exaggerated, and such issues must also be worked out for individual measuring systems.

10. When fluxes are to be expressed as dry weight, centrifugation is not required; conversely, when expressed as fresh weight, oven drying is not required (also see Note 17).

11. Prior to placing plants into an uptake solution, a small sample of solution (typically, 1 ml or less) must be removed to determine its specific radioactivity. Specific activity is usually expressed as cpm/μmol (cpm = counts per minute, which is related, by the counting efficiency of the detecting instrument, to the dpm, or disintegrations per minute), and is often denoted "SA," or S_o (the subscript "o" indicating the outside solution, bathing the roots). Typical values for SA are in the range of $1–2 \times 10^5$ cpm. See Note 18 for details of flux calculation.

12. When plants are to be measured under non-steady-state conditions, their roots are often first exposed to modified solution without radiotracer, for an equilibration period of 5–10 min, prior to their exposure to modified solution containing tracer. If inhibitors or other additives are to be used, sufficient time must be allowed for their effects to take place.

13. When roots of intact plants are immersed in radioactive uptake solution, care must be taken to ensure that the aerial parts of the plant do not come into contact with the solution. This is particularly important when solutions are vigorously stirred or aerated, or with plants having a short stem, such as *Arabidopsis*. A shielding collar is sometimes used to isolate stems and leaves from solution. If they are not isolated from radioactive solution, substantial translocation artifacts may be obtained (sometimes identifiable due to their high variability).

14. The duration of tracer absorption (and desorption; see Note 16 below) by roots is an important aspect to consider when tracing influx at a high substrate concentration, because such "low-affinity" influxes can be extremely high and are often associated with very high efflux rates and rapid turnover times (7). Under such conditions, a failure to consider the simultaneous efflux of a substrate over the course of influx measurement will result in an underestimate of the flux. Such errors can be minimized, however, by reducing the duration of labeling and desorption (3), although this courts the danger of adding increased proportions of background counts from the apoplast. In general, when such high efflux rates are observed, it is particularly important to identify the source(s) of tracer release, by using, for example, compartmental analysis by tracer efflux (CATE; refs. 13, 14; see Note 19). While such a situation can occur when tracing low-affinity fluxes of K^+ (or fluxes of Ca^{2+} in any range, due to its very rapid cytosolic turnover; see ref. (15)), the measurement of sodium influx under salinity conditions may be the most pronounced example of this situation, since external concentrations of Na^+ can be 100 mM or more, greatly exceeding the naturally occurring, and experimentally provided, levels of other ions (16). In the case of K^+, recent evidence (17) suggests that above 1 mM external K^+, the efflux component (which can become very pronounced; see ref. (18)) may be apoplastic in nature, since inhibitors such as Cs^+ and Ba^{2+} (which have powerful effects on K^+ efflux in the high-affinity range) fail to affect this component.
15. In practice, it can be very difficult to distinguish extracellular events from those occurring across cell membranes. This presents a dilemma to the researcher: should one attempt to prevent underestimates of influx across the membrane caused by tracer efflux from the cell during measurement, by opting for very short labeling and desorption times (see Note 16)? Alternatively, should one minimize artifacts associated with extracellular accumulation of tracer, by opting for the opposite? The answer to this depends at least partly on the intention of the study. Because the accumulation of sodium in plant tissues is central to the toxicology of salinity stress, the latter approach (basically a net-flux measurement) can be quite appropriate and satisfactory in many situations. On the other hand, if the study is to focus on a classical enzyme-kinetics evaluation of membrane transport systems (19), or an analysis of the energetics of unidirectional transport processes (20), the requirement for unidirectional flux measurements is stringent, and thus the former approach would be recommended. However, the veracity of the efflux component of the flux as a transmembrane phenomenon must also be determined in such

cases, for the particular experimental system, e.g., by performing CATE analyses (see Note 19).

16. In all cases, care must be taken to clear (desorb) tracer from known apoplastic phases, once labeling is complete. These phases include the surface water-film of roots, and the electrostatically binding "Donnan" phase of cell walls, which consists of fixed extracellular charges. Since these charges are mostly negative, this is a particularly important issue when measuring cation fluxes, especially in the case of the divalent cation Ca^{2+}. Desorption is generally done by immersing roots in a solution identical to the uptake solution, except that it contains no radiotracer; this solution is sometimes chilled to 4 °C to minimize loss of tracer from the symplast. Multiple desorption steps, each in fresh solution, are often used. The length of the desorption period is often 5–10 min, but can often be more precisely determined for extracellular phases by means of CATE (see Note 19).
17. Once desorption is complete, roots are typically detached from shoots (if intact plants are used) for separate counting, to estimate translocation rates. At this stage, roots can be weighed prior to counting if fluxes are to be normalized to root fresh weight. If so, a brief, low-speed centrifugation of root tissue (e.g., in a clinical centrifuge at $5000 \times g$) is required to remove surface and interstitial water. If dry weight is the standard, radioactivity of samples may be counted before or after drying and weighing.
18. The influx or net flux into the plant can be calculated quite straightforwardly using the formula $\phi = \frac{Q^*}{S_o w t_L}$, where ϕ is the flux (e.g., μmol/g h), Q^* (cpm) is the quantity of tracer accumulated in tissue (usually root and shoot combined), S_o is the specific activity of the uptake solution (cpm/μmol), w is the root weight (g), and t_L is the labeling time (h). More sophisticated calculations can also be made that account for simultaneous tracer efflux from root cells during labeling and desorption, based on parameters obtained using CATE analysis (for details, see ref. 3; also see Note 19, and caveats above). The absolute quantification of transport to the shoot is more problematic, because (1) the specific activity of the translocating pool is difficult to estimate, and (2) a lag phase may retard the appearance in the shoot of some labeled ions, particularly K^+ (e.g., ref. 21). Thus, investigations of root-to-shoot transport may require longer labeling times and is sometimes expressed as % of total tracer absorbed that is found in the shoot. One additional issue is the possibility of apoplastic bypass flow of Na^+ to the shoot, well documented in species such as rice (22), which contributes to the noncellular component of influx into the plant (see Note 14).

19. Compartmental Analysis by Tracer Efflux (CATE) has been used extensively in biology and medicine (23) to quantify ion fluxes and metabolic pool sizes, including those of K^+ in plants and algae (16, 24, 25). While its use in plant science has diminished somewhat in recent years, it remains an important methodology in medical science, particularly pharmacokinetics (e.g., ref. 26). This method uses long labeling periods (typically one to several hours) followed by a partial washing out of tracer from roots by means of a timed series of nonradioactive eluates. When done correctly, CATE can provide a more comprehensive view of unidirectional fluxes than the procedure outlined above, as well as compartmentation data on both cellular and whole-plant scales. However, it is more labor-intensive, generally limited to steady-state conditions, and prone to its own set of heuristic problems. While a detailed exposition of CATE is beyond the scope of this chapter (but *see* refs. 13, 14, 27, 28 for rationale and procedures), a few points are worth mentioning. Exponential half-times of tracer exchange in intra- and extra-cellular phases of plant roots, as well as the ratio of unidirectional efflux to influx, can be estimated using CATE. This information is pertinent to the design of "direct influx" protocols, because it facilitates the choice of: (1) labeling time, during which tracer efflux from an absorbing and releasing root can be minimized (hence reducing underestimates of the flux); and (2) desorption time, to maximize the release of tracer from extracellular spaces. For details on this approach, see ref. (3). CATE can also be useful as an independent line of investigation against which direct-influx results may be compared. When discrepancies appear between the two systems of measurement, a comparison can yield useful insights, both biological and methodological. Requirements for the correct interpretation of CATE data, however, are stringent. Phases of tracer release (e.g., surface film, cell wall, cytosol, vacuole) must be correctly identified (13, 14) and should also be sufficiently distinct, kinetically speaking, from one another to be resolved (29). In addition, steady state must be ensured, limiting the use of CATE for some investigations, such as those involving flux isotherms.

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