Chapter 26

Isotope Techniques to Study Kinetics of Na^+ and K^+ Transport Under Salinity Conditions

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Abstract

Radioisotopes (particularly ^{22}Na, ^{24}Na, ^{42}K, and ^{86}Rb) have been used for many decades to trace the fluxes and accumulation of sodium and potassium ions in plant tissues. In this article, standard procedures for the tracing of ion fluxes are described, with emphasis on special problems encountered when examining K^+ and Na^+ transport under salinity conditions. We focus in particular on unidirectional influx measurements, while also providing a brief introduction to compartmental analysis by tracer efflux.

Key words: Influx, Efflux, Salinity, Sodium, Potassium, Radiotracers

1. Introduction

Isotopic tracing of nutritional and toxicological processes in plant tissues has greatly enhanced our understanding of plant physiology, biophysics, and biochemistry in a wide range of contexts. Of these, the study of ion transport across the membrane barriers of the plant cell has particularly benefited from the tracer approach. Key advances in this area include the identification and characterization of the following:

- Nutritionally relevant high- and low-affinity ion transport systems (1–3).
- Uptake, release, and partitioning of toxicants in intact plants (4–6).
- Subcellular transport and accumulation of nutrients or toxic ions (6–9).
- Adaptations of plants to their edaphic environments (3, 9).

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The chief advantage of isotope methodology over other approaches is that it makes possible the measurement of unidirectional fluxes, which are more appropriate than net fluxes in determining kinetic flux parameters (e.g., K_M, V_{max}), and in enabling the study of specific transport capacities and their regulation. 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 (10). 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 (but see caveat below). Interestingly, both of these conditions may apply to the study of Na^+ fluxes in plants. 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 the following:

- Measurements of depletion in the external medium.
- Measurements of accumulation in the tissue.
- Use of ion-selective vibrating electrodes (e.g., MIFE, SIET).
- Use of ion-selective fluorescent dyes.

In the study of salt stress and tolerance in plants, the transport and accumulation of sodium and potassium are considered to be of crucial importance (11). Thus, measurements of both unidirectional and net fluxes of both ions are appropriate in this context. However, because of the unusual conditions imposed by salt stress (e.g., high sodium background, hyperosmolarity), special considerations need to be made when designing experimental protocols. In this article, we shall address general approaches to isotope-based measurements of influx, efflux, and net flux, and features that specifically affect the measurements of K^+ and Na^+ fluxes under salinity conditions. A note of caution is appropriate at the outset, regarding the interpretation of tracer fluxes in salt-stressed roots: one of the early impacts of salt stress is an osmotic one, which can result in cellular damage, particularly to membranes. This can have a significant effect on ion fluxes in plants suddenly exposed to high external [Na^+], often suppressing influx and enhancing efflux (12).

2. Materials

1. Plant material (see Notes 1–3).
2. Glass or plastic vessels for pre-absorption, labeling, and desorption.
3. Equipment for aeration and/or stirring of solutions.

4. Dissecting tools.
5. Pre-absorption, labeling, and desorption solutions (see Notes 4 and 5).
6. Pipettes for specific activity samples.
7. Radiotracer (see Notes 6–8).
8. Safety equipment, including protective clothing, eyewear, and shielding (see Note 6).
9. Radiometric equipment (see Note 9).
10. Low-speed (clinical-type) centrifuge (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, if appropriate (see Note 12).
3. Immerse roots in radioactive solution (see Note 13).
4. Remove plants from radioactive solution after appropriate labeling period (see Notes 14 and 15).
5. Desorb roots of extracellular tracer (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 (see Note 9).
9. Calculate the flux (see Notes 18–19).

4. Notes

1. Plants should be grown hydroponically, so that culture conditions can be precisely controlled, and roots are directly accessible for examination. Typically, seedlings are used once they have grown to a convenient size and age (e.g., 1 week for barley, 3 weeks for rice, 4–5 weeks for *Arabidopsis*), but it is important to consider the use of plants from a variety of developmental stages.
2. 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. 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.

3. 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.
4. For steady-state investigations, labeling solutions should be identical to plant growth solutions, except for the addition of radiotracer. In addition, all other growth conditions, especially temperature, light, and humidity, should be maintained during experimentation. 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.
5. Particularly in older studies, the uptake of a labeled substance has often been 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 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 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., see ref. (13)).
6. 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. 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. Tracers for K^+ and Na^+ tend to have slightly longer half-lives, on the order of several hours (see below). 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.

7. While the short half-lives of some radiotracers may make them difficult to obtain, they 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. Nevertheless, longer-lived tracers are much more widely used due to their availability and longer handling period.
8. With respect to the tracing of plant K^+ and Na^+ fluxes under salinity conditions, radiotracers are much more frequently used than stable tracers. Perhaps surprisingly, the most widely used tracer for K^+ tracing is not potassium at all, but a radioisotope of its alkali-metal "analog," rubidium-86. However, there is substantial evidence that Rb is an imperfect substitute for K, for example with respect to its translocation to the shoot (14). ^{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., see ref. (15)). 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 (16). In the case of Na^+ tracing, only two isotopes 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 these isotopes typically involves bombardment of non-radioactive ^{39}K and ^{23}Na. 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.
9. Almost all radiotracing in biological systems involves the measurement of electromagnetic radiation associated with the decay of beta (plus or minus)-emitting nuclides. Liquid scintillation counters and gamma counters are used to measure most of these 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 used, 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-counter system

is uniform from one sample to the next (or corrections are 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 on a dry weight basis, centrifugation is not required (see Note 17). Tissue is typically dried in an oven at around 50°C, or air-dried, for several days, or until weight becomes stable.
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).
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.
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 bubbled, 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 below) by roots is an important issue 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 (17). 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 by reducing the duration of labeling and desorption (10). 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. Indeed, the rapid, futile cycling of Na^+ under salt stress has been reported many times (5, 18–20), and it has been recommended that the uptake period be as short as 2 min, to minimize the effects of simultaneous efflux (19). However, such reports and recommendations must be approached with caution, because the character and biological significance of Na^+ efflux from plant roots is still poorly understood. It is resistant, in many cases, to changes imposed by a wide range of experimental treatments (4), and appears not to have a commensurate respiratory burden associated with it (20, 21) despite its putatively active transport mechanism (11). This has led to speculation that there may be a large artifactual component to the excess tracer flux, which does not represent a cycling across cell membranes but may be apoplastic in nature (11, 18, 22). This flux, which has yet to be rigorously demonstrated, may be related to the well established (e.g., in rice) "apoplastic bypass" of sodium as it travels from root to shoot, independent of symplastic discrimination (22, 23). While apoplastic components of sodium transport in plants may nevertheless prove critical to the understanding of salinity stress, it is important not to conflate them with the measurement of membrane transport activity.

15. In practice, however, it can be very difficult to distinguish between extracellular events and 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? 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 (1), the requirement for unidirectional flux measurements is stringent, and thus the former approach would be recommended. However, veracity of the efflux component of the flux as a trans-membrane phenomenon must also be determined in the 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 (mostly negative). 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. The length of the desorption period is typically 5 min, but can often be more precisely determined for these phases by means of compartmental analysis by tracer efflux (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 7,000 rpm) is required to remove surface and interstitial water; however, this water can sometimes contain tracer that originated within cells, especially when substrate concentrations are high, and this should be accounted for (24). 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 $\varphi = \frac{Q^*}{S_o w t_L}$, 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, accounting for tracer efflux from root cells during labeling and desorption, and based on parameters obtained using CATE analysis (for details, see ref. (10); 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 (25), and (2) a lag phase that retards the appearance in the shoot of some labeled ions, particularly K^+, is often observed (e.g., see ref. (26)). 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 (23), which contributes to the non-cellular component of influx into the plant (see above).
19. CATE has been used extensively in biology and medicine (26) to quantify ion fluxes and metabolic pool sizes, including those of K^+ and Na^+ in plants examined under salinity conditions (5, 20, 27, 28). While its use in plant science has diminished somewhat in recent years, it remains an important methodology

in medical science, particularly pharmacokinetics (e.g., see ref. (29)). 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 non-radioactive 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. (7, 30–32) for rationale and procedures), a few points are worth mentioning. Exponential half-times of tracer release from intra- and extracellular 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. (10). 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 (31, 32), and should also be sufficiently distinct kinetically from one another to be resolved (33). 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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