An Analysis of Long-Distance Water Transport in the Soybean Stem Using H$_2$\textsubscript{15}O

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The lateral water movement in the intact stem of a transpiring soybean plant was analyzed quantitatively by a real-time measurement system utilizing labeled water, H$_2$\textsubscript{15}O and gamma ray detectors. A large volume of water escaping from xylem vessels during its transport was detected. The escape of water was not influenced by evaporation from the stem surface or mass flow in the sieve tubes. It was assumed that the total amount of water transported through xylem vessels was kept almost completely constant along the internode. As a result, most of the escaped water was found to re-enter the xylem vessels, i.e. water exchange occurred. The analysis of radiographs of tritiated water suggested that the self-diffusion effect of water was strong for lateral water movement, although another driving force besides thermal motion was included in the process, and that the process was also affected by the water permeability of the plasma membrane. An analysis based on a mathematical model showed that the net volume of water which escaped from xylem vessels was not dependent on the transpiration rate of the plant.

Keywords: H$_2$\textsubscript{15}O — Internode — Real-time measurement — Soybean — Water exchange — Water relations.

Abbreviations: AFS, apparent free space; MRI, magnetic resonance imaging; PETIS, positron-emitting tracer imaging system; RH, relative humidity; THO, tritiated water.

Introduction

With the development of the study of transport physiology, it has become clear that mass transport in plants is not just a simple flow through xylem vessels and sieve tubes, but also includes xylem–phloem exchange during transport (Van Bel 1990, Patrick et al. 2001). For the lateral transport, a quantitative, two-way model of xylem–phloem transport has been proposed. It supposes symplastic and apoplastic routes operating in parallel for the radial transport (a detailed explanation of this model was reviewed in Van Bel 1990). Along the symplastic xylem–phloem route, solutes escape from xylem vessels via the pits, are transported into the ray cells, and are finally loaded into the sieve tubes. Via the apoplastic xylem–phloem route, solutes are transported from the xylem vessels via a cell wall continuum towards the phloem due to a concentration gradient. These complex, lateral transport mechanisms have several physiological consequences. A part of the solute was found to be sequestered in the apoplastic or symplastic stores and was used to buffer variations between sources and sinks in daily and seasonal changes (Thorpe et al. 2005). The route from xylem to phloem was exploited for the transfer of nitrogen to the fruits of soybean plants (Layzell and LaRue 1982). It may be used to remove superfluous ions from plants (Levi 1970). Thus, the mechanism may serve for the selective withdrawal and transport of solutes. The mechanism is able to meet the requirements of various tissues and organs at specific time points (Van Bel 1990).

The question remains as to how the movement of water as a solvent is affected by lateral transport. It has been reported that the longitudinal transport velocity of water through phloem and xylem was much slower than that of phosphorus and sugar (Biddulph and Cory 1957, Gage and Aronoff 1962, Trip and Gorham 1968). Van Bel (1974, 1976) analyzed the uptake of a solution by tissues surrounding the xylem vessels by simultaneous perfusion of tritiated water (THO), sugar and amino acid through stem segments of tomato plants. The results showed that transport through xylem vessels may be considered as a chromatography-like process with different longitudinal transfer velocities for each component. Water escaped more easily from the xylem vessels, resulting in a different longitudinal displacement of solvent compared with solutes. Van Bel assumed that dilution of the labeled water during its transport, which was caused by exchange with innate non-labeled water, is driven by diffusion as based on the Horwitz theory (Horwitz 1958).

The present study also assumes that water was transported in a lateral direction along the two-way route

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described above. However, detailed analyses of the time dependence of water transport by intact stems of transpiring plants have never been accomplished. This is due to the technical difficulties associated with the use of THO as a water tracer in studies on water transport (Cline 1953). THO is a radioactive tracer with beta ray energy of 19 keV. Since this energy is too weak to penetrate plant tissues, samples have to be extracted to measure the amount of THO (Bull et al. 1972). Other isotope tracers, such as D_{2}O and H_{2}^{18}O, which have also been used to monitor water relations, also require destruction of plant tissue prior to measurement by mass spectrometry (DeKroon et al. 1996, Gan et al. 2002).

For a meaningful study of water transport, it is imperative to observe real-time water transport in intact stems of transpiring plants. It has been reported that the flow rate through xylem vessels depends on the solute concentration because the pits (the contact sites between vessels and between vessels and the surrounding parenchyma cells) control the mass flow rate, which is dependent on the hydrogel character of the pit membrane (Zwieniecki et al. 2001, Zwieniecki et al. 2003). It is thought that the change in water conductivity controlled by the pit membrane is also caused by the substrates unloaded from phloem as well as xylem vessels (Thompson and Zwieniecki 2005). These reports suggest that longitudinal and radial transport of solute via xylem and phloem have a large effect on water movement in the stem. Thus, it is important to keep the shoot in the natural state to analyze the longitudinal and lateral water movement in the stem. For more conclusive studies, a measuring system was developed that allows non-invasive detection of trace amounts of water in plants.

MRI (magnetic resonance imaging) is a newly developed method, useful for the observation of water distribution in tissues (Ishida et al. 2000). It allows non-invasive measurements of mass flow rates in both xylem vessels and sieve tubes (Kuchenbrod et al. 1996, Rokitta et al. 1999, Scheenen et al. 2002). However, the dynamic interaction between innate water and newly absorbed water during their upward transport in living plants is unclear due to the limited sensitivity and time resolution of MRI.

Recently, the radioisotope tracer H_{2}^{15}O has been introduced to meet these shortcomings. In nuclear medicine, H_{2}^{15}O has been used for the analysis of regional cerebral blood flow by positron emission tomography (PET) (Raichle et al. 1983). ^{15}O is a positron nuclide, i.e. positrons are emitted from the decay of ^{15}O. A pair of gamma rays, which make an angle of 180° to each other, are emitted as the result of pair annihilation of the positron in question. The energy of the gamma rays is 511 keV. This allows measurement of H_{2}^{15}O within living tissues with a high signal-to-noise ratio by discriminating the energy and counting the annihilation gamma rays by using a pair of gamma ray detectors combined with a coincidence circuit. Moreover, since the half-life of ^{15}O is extremely short (122 s), the experiment can be repeated using the same plant after various treatments, which eliminates sample errors and corroborates the reproducibility of the results.

The positron-emitting tracer imaging system (PETIS) is a tool for visualizing the two-dimensional movement of positron nuclides in plant (Kume et al. 1997). Recently, H_{2}^{15}O movement recorded with PETIS has been used for the study of water transport in tomato and rice plants (Mori et al. 2000, Kiyomiya et al. 2001, Nakanishi et al. 2002). They investigated the effect of light conditions and reagents (including NaCl, ABA and methylmercury) on water movement in plants. PETIS, however, does not allow a quantitative analysis due to technical difficulties, such as the correction for relative detection efficiencies within the field of view and the positron escape from the samples (Levin and Hoffman 1999). Thus, the problem of quantitative analysis of long-distance transport of water was not yet solved. We designed a measuring system that does enable quantitative measurement. The positional information is maintained by downscaling the size of the gamma ray detectors. Furthermore, the effect of the positron escape from the samples is reduced by setting the detectors adjacent to either side of the target site. With this system, the volume of H_{2}^{15}O in 1 cm of an internode could be measured quantitatively and non-invasively, i.e. it enables us to analyze the longitudinal and lateral water movement in the intact stem of the transpiring plants.

Here we report the results obtained with this new design, including a mathematical model which describes how H_{2}^{15}O is translocated through a stem internode.

**Results and Discussion**

*Water which escaped from xylem vessels and the distribution of the water*

In the present study, we use the term ‘H_{2}^{15}O’ for the newly introduced water to distinguish this from the innate water. A precise description of the term is that 10 g of H_{2}^{16}O contained about 1 \times 10^{-11} g of H_{2}^{15}O.

H_{2}^{15}O was applied to the cut end of the stem, 10 cm below the cotyledon, of soybean plants which were about 3 weeks old (Fig. 1). The radioactivity of H_{2}^{15}O, i.e. the volume of H_{2}^{15}O, in the internode initially increased linearly. The slope of the curve then decreased gradually (Fig. 2). The profile of the radioactivity distribution was similar to the results of Mori et al. (2000) obtained by PETIS. The amount of H_{2}^{15}O taken up by the internode exceeded the aggregate capacity of the xylem (1.9 μl) within a few minutes of application of H_{2}^{15}O. The capacity of the vessels was estimated from the measurement of their transverse...
sectional area by microscopy \[ = 1.9 \pm 0.30 \times 10^{-7} \text{ m}^2 \] (total cross-sectional area) \( \times 1 \times 10^{-2} \text{ m} \) (the length of measuring region in stem). The increasing amount of \( \text{H}_2\text{H}^{15}\text{O} \) per 1 cm of the internode was \( 5.18 \pm 0.47 \times 10^{-2} \mu\text{l s}^{-1} \), which was calculated from the linear part of the slope in Fig. 2. The amount of \( \text{H}_2\text{H}^{15}\text{O} \) continued to increase and occupied a volume of 40 \( \mu\text{l} \) after 1.5 min, which was \( > 20 \) times greater than the vessel capacity (Fig. 2A). This observation indicates that large amounts of water continuously escaped from the xylem vessels to the surrounding tissues. Lateral water movement associated with its longitudinal transport has been reported for some other plant species (Raney and Vaadia 1965, Trip and Gorham 1968, Bull et al. 1972). Thus, the same phenomenon was observed in an independent sample in the same manner as \( \text{H}_2\text{H}^{15}\text{O} \). THO spread throughout the transection of the stem within 10 min. In contrast to our expectations, a high amount of THO was not observed in the vascular ring but in the pith. This indicates a rapid inward movement of water independent of the transpiration. The newly introduced water containing THO could not have moved by diffusion alone to 1 cm from the application point within 10 min, given the average diffusion distance of about 1.7 mm \( (i^2 = 2Dt) \), where \( i \) is the average diffusion distance, \( D \) is the diffusion coefficient, \( 2.4 \times 10^{-9} \text{ m}^2 \text{s}^{-1} \), and \( t \) is the measuring time). Thus, THO must have been translocated mainly through the xylem vessels followed by diffusional escape at the point of measurement (Fig. 2B).

To corroborate this conclusion, lateral water movement was examined in detail. Cut internode ends, 1 cm below the cotyledon, were placed into non-labeled water after THO was applied for 5 s. Fig. 3 shows the radiograph of THO by pulse application. A high accumulation of THO was observed in xylem vessels just 5 s after

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Fig. 1 A diagram of the measurement system using \( \text{H}_2\text{H}^{15}\text{O} \). \( \text{H}_2\text{H}^{15}\text{O} \) in the vial was applied to the cut shoot. A pair of gamma ray detectors was adjusted to the position for measurement, 2 cm above the cotyledon. Sample plants are about 25 cm in height. In the experiment, a long cable, about 4 m, was used to connect the gamma ray detector with the coincidence circuit. The vial and detectors are shielded by lead blocks. The illustrated parts, except for the coincidence circuit, are set in the growth chamber. S.C.A, single channel analyzer; PMT, photo multiplier tube.

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Fig. 2 (A) The volume occupied by \( \text{H}_2\text{H}^{15}\text{O} \) water in the internode at the position 2 cm above the cotyledon. The vertical axis indicates the \( \text{H}_2\text{H}^{15}\text{O} \) volume in the stem per cm. \( \text{H}_2\text{H}^{15}\text{O} \) was supplied at time point 0. Each symbol represents the data of individual cut shoots (\( N = 5 \)). The dotted and solid lines show the total volume of the water and the maximum capacity of the vessels in the sample seedlings per 1 cm, respectively. The total water volume of that section was about 49–56 \( \mu\text{l} \), based on the diameter of the sample stem (2.9 ± 0.10 mm), and a water content of 83%, estimated from the dry and fresh weights of the sections. (B) Radiograph of THO at the stem transection. The upper radiograph shows the THO distribution in the transection of the stem (lower image) at the measuring position after perfusion of THO. THO was applied to the samples for 10 min in the same manner as \( \text{H}_2\text{H}^{15}\text{O} \). The density of the radiograph is presumed to be proportional to the amount of radioactivity. Bar = 1 mm. The average of the density of the radiograph of the control, stem without THO, was the same level as that of no sample, i.e. background level.
its application. The region with a high accumulation of THO expanded to the whole xylem area after 10 s. After a further 10 s, a high accumulation of THO had progressed in a horizontal direction. One minute after the pulse treatment, the radiograph of THO was similar to that after 10 s, i.e., the accumulation in a horizontal direction observed at 20 s had disappeared. Two minutes after the pulse treatment, the distribution of THO had expanded to the whole transection. On the radiographic image, the intensity of the dark image was less at 10 min after treatment as compared with 0, 10 and 20 s after treatment. It was therefore concluded that the total amount of THO in the transection decreased. This shows that xylem vessels were used for longitudinal water transport. The water escape must have been 'flushed' away quite rapidly since the radioactivity disappeared with time (Fig. 3). Unfortunately, the route of lateral water movement (apoplastic or symplastic) could not be identified due to the lack of resolution. Moreover, it was not clear whether the blurring of the radiographic images was caused by technical or biological factors, because movement of the THO for 1 min by self-diffusion alone is about 550 μm.

Next, we analyzed in detail to which tissues the water was transported after escaping from the xylem vessels. Below four models describing these possible destinations for the escaped water are discussed: (a) the water flows towards the stem surface and evaporates; (b) the water is collected and transported downward by phloem; (c) the water flows through tissue other than xylem vessels; (d) water re-enters the xylem vessels.

Water in stem tissues easily evaporates through the surface (Yamamoto 1995). The evaporation from the stem surface may act as a driving force in the outward radial water transport. However, the model (a) cannot be valid, because no change in H$_2^{15}$O escape was observed after the surface of the internode was covered with Vaseline to prevent evaporation (Fig. 5A).

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The second model (Fig. 4b) finds support in the fact that water is exchanged between xylem and phloem (Köckenberger et al. 1997, Patrick et al. 2001). In addition, the high osmolarity of the sieve tube contents may drive radial flow of water (Van Bel 1990). However, the second potential route is discounted here because no change in H$_2^{15}$O escape was measured when the tissue outside of the cambium was removed to knock out phloem transport (Fig. 5B).

The model (c) (Fig. 4c) was excluded for the following reasons. The average total cross-sectional areas of xylem vessels (± SD), $\frac{1}{N} \sum_{i=1}^{N} S_{ij}(x)$, at 2 cm (position...
The average of the ratios of the samples other indicators were calculated as follows. If the water which escaped from the pipe did not return to it, the amount of water transported should decrease with height along the pipe. Furthermore, if the capacity of the pipe to transport water (such as the diameter of the pipe and the proportion of water escaping) were constant, the decrease in the amount of water transported through it, i.e. the flow rate ($m$) were 1.03 ± 0.08 and 1.07 ± 0.11, respectively ($N = 6$). The value of $\Sigma S_{vi}^2$ is proportional to the sum of the biquadrate of the diameter of each xylem vessel; namely, it corresponds to the capacity of water transport. Both values were almost equal to 1. Therefore, it is plausible to assume that the capacity of water transport remains equal between the three measuring points, although the architecture of xylem vessels usually changes along the internode. For simplicity, we regarded xylem vessels as pipes with many surface pores, and imagined that water was transported through them. If the water which escaped from the pipe did not return to it, the amount of water transported should decrease with height along the pipe. Furthermore, if the capacity of the pipe to transport water (such as the diameter of the pipe and the proportion of water escaping) were constant, the decrease in the amount of water transported through it, i.e. the flow rate ($\mu l s^{-1}$), results in a decrease in the flow velocity ($mm s^{-1}$) through it. Conversely, if the flow velocity was kept constant in a region, the flow rate would be constant, too. The flow velocity of water through xylem vessels could be approximately estimated from the time delay of the $H_2^{15}O$ to reach each measuring points. In addition, it was confirmed that the $H_2^{15}O$ initially detected would have passed through xylem vessels, because a high level of accumulation of THO was observed at the xylem vessels (Fig. 11).

The profiles of $H_2^{15}O$ distribution along the internode (Fig. 6) were similar, although the regression line decreased with the distance between the measuring point and the site of application. However, the periods needed to transport detectable amounts of $H_2^{15}O$ to the respective measuring points (intersections between d and e, as well as e and f) suggest that the flow velocity of $H_2^{15}O$ through the xylem vessel was almost constant (4 mm $s^{-1}$) (Fig. 6).

There is a second reason to discount the third model. The ratio of $H_2^{15}O$ escape could be calculated (= the amount of water which escaped from the xylem vessels/the total amount of water transported through the xylem vessels). If we regard the total flow rate as approximately equal to the transpiration rate, the escape rate of $H_2^{15}O$ from the xylem vessels was 0.052 $\mu l s^{-1}$ (Fig. 2A). The transpiration rate of the sample under the same conditions was 0.91 ± 0.13 $l m s^{-1}$. Therefore, the percentage of water that escaped from the vessel flow was $5.7 \pm 0.82% cm^{-1}$. Provided that this ratio (about 6%) is maintained along the entire internode length of 8–9 cm, the total amount of water escaping from xylem vessels is >40% ($5.7 \times 8.5 = 48\%$) of the vessel water transported. If the other part of the internode, 8 cm below the cotyledons, had also been taken into consideration, the estimate would be >90% ($5.7 \times 16.5 = 94\%$).
In addition, tissues other than xylem vessels are indirectly involved in mass flow. In tomato plants, the volume of the AFS (apparent free space), which was associated with mass transport, was 2.5 times as large as the volume of the xylem vessels (Van Bel 1978). However, the mass flow of water through xylem vessels could be described by the Hagen–Poiseuille law (DeBoer and Volkov 2003); the flow rate is proportional to the fourth power of the tube diameter. The water path other than through xylem vessels has comparatively low water conductivity. Therefore, about 40% of the total volume of water transported through xylem vessels could not possibly be transported through compartments other than the xylem vessels. In addition, upward water flow observed by MRI was only reported to follow the xylem vessels (Kuchenbrod et al. 1996, Rokitta et al. 1999, Scheenen et al. 2002). In conclusion, model 3 is ruled out to explain the behavior of water, although evidence for its rejection is indirect (Fig. 4c).

In accordance with the rejection of the above three models, most of the water which escaped from xylem vessels must re-enter the xylem vessels along the internode (Fig. 4d). To construct a mathematical model based on these experimental results, i.e. the fourth model, is designed to analyze the water exchange in detail.

The model of water exchange in a stem

The results showed that water was mainly transported through the xylem vessels, and was mixed with water from the surrounding tissues (Fig. 4d). To construct a mathematical model, we made the following plausible assumptions: (i) H$_2^{15}$O in the xylem vessels was diluted with H$_2^{16}$O (innate water) from the adjacent tissues during upward transport; (ii) the diameter of the internode at the measuring positions was constant during the measurement, within 20 min; and (iii) the $^{15}$O was only present as H$_2^{15}$O. To examine the third assumption, the exchange effect of the isotope, $^{15}$O, was studied by mixing it with cellulose powder. The exchange rate was $0.97 \pm 0.021$ (N = 5); therefore, the effect in the plant must be negligible in the experiment because the effect of the surface of cellulose powder on the isotope exchange is much greater than that for the plant.

The model includes the following variables: the water volume exchanged per unit time, $V$ (m$^3$/s$^{-1}$); the volume of H$_2^{15}$O outside xylem vessels, i.e. newly applied water, $A_{15}$ (m$^3$); and the volume of H$_2^{16}$O outside xylem vessels, i.e. innate water, $A_{16}$ (m$^3$).

When the volume of the internode is constant during the measurement, then

$$A_{16} + A_{15} = A \text{ (m}^3\text{)}$$

(1)
where $A$ indicates the total volume outside the xylem vessels (m$^3$).

In the same manner as equation (1), if the volumes of $H_2^{15}O$ (m$^3$) and $H_2^{16}O$ (m$^3$) inside the xylem vessels are set as $B_{15}$ and $B_{16}$, respectively, then

$$B_{15} + B_{16} = B (m^3)$$  (2)

where $B$ represents the total volume inside the xylem vessels (m$^3$).

The exchange rate of $A_{16}$ for $B_{16}$ is proportional to the concentrations of both $A_{16}$ and $B_{16}$.

Therefore

$$\frac{dA_{16}}{dt} = V \left( \frac{B_{16}}{B} - \frac{A_{16}}{A} \right)$$  (3)

To simplify equation (3), we introduced another assumption: (iv) the concentration of $H_2^{15}O$ in the xylem vessels was kept constant for a while after the innate water of the xylem vessels was flushed out due to the following reason. The content of $H_2^{15}O$ in the re-entered water should be low, and may be regarded as negligible, at an early stage after $H_2^{15}O$ application, because the amount of $H_2^{15}O$ in the surrounding tissues was much lower than that of $H_2^{16}O$. Thus, the concentration of $H_2^{15}O$ in the water transported to the point of the measurement would be stable while the condition was maintained. Consequently, we regarded the concentration of $H_2^{15}O (B_{15}/B)$ as constant, indicating that $B_{16}/B$ was also constant. The relationship can be described by a constant $C (B_{16}/B = C/A = \text{const})$. Equation (3) is then as follows:

$$\frac{dA_{16}}{dt} = - \frac{V}{A} \left( A_{16} - C \right)$$  (4)

Equation (4) can be solved analytically, then

$$A_{16} = M_0 e^{-t/\tau} + C$$  (5)

where $M_0$ and $\tau = A/V$ indicate the initial value and time constant, respectively.

The measurable value by the detector, $M$, is only the volume of $H_2^{15}O$, i.e. $A_{15} + B_{15}$. By substituting equation (5) in equation (1), and by adding $B_{15}$, $M$ becomes

$$M = M_1 - M_0 e^{-t/\tau}$$  (6)

where $M_1 = B_{15} + A - C$.

The values measured are consistent with the curve described in equation (6) (Fig. 7), which shows that the model describes the phenomena adequately.

**Calculation of the dilution and exchange rate**

If the time course of the volume of the $H_2^{15}O$ at the measuring point could be described by equation (6), a linear relationship could be established when the elapsed time, $t$, was small compared with the time constant $\tau$, 900 ± 140 s. In fact, a linear increase was observed in the initial part of the $H_2^{15}O$ uptake curve (Figs. 2, 5, 6, 9).

As described above (referred to the text in Fig. 4c), the capacity of water transport along the internode was approximately the same; therefore, the escape rate of $H_2^{15}O$ from xylem vessels must be the same as well along the internode.

In the $H_2^{15}O$ uptake curve, it becomes clear that when the gradient of the curve, $\Delta$, is proportional to the concentration of $H_2^{15}O (C)$, transported through xylem vessels, the equation

$$\Delta = a C$$  (7)

is applicable, in which $a$ represents the proportional coefficient. In addition, the amount of escaped water showed linearity in the early stage of water exchange (Figs. 2, 5, 6, 9), indicating that the concentration of $H_2^{15}O$ in xylem vessels could be regarded as constant in this stage.

$H_2^{15}O$ was diluted due to exchange with the innate water around the xylem vessels. Thus, the dilution rate per unit-distance transport, $\varphi$, could be described as follows, when $H_2^{15}O$ was transported from $P_1$ to $P_2$.

$$\varphi = \left( \frac{C(P_2)}{C(P_1)} \right)^\frac{1}{d} = \left( \frac{\Delta_2}{\Delta_1} \right)^\frac{1}{d}$$  (8)

in which $P_1$ and $P_2$ represent the position of the detectors, and $d$ is the distance between $P_1$ and $P_2$, and the flow rates of $H_2^{15}O$ escape at the positions are $\Delta_1$ and $\Delta_2$, respectively (Fig. 8). Therefore, the dilution rate, $\varphi$ could be calculated from the value of the gradients, $\Delta$ at $P_1$ and $P_2$. The exchange rate $\eta$, which indicates the rate of water escape per unit-distance of longitudinal transport, is also calculated by

$$\eta = 1 - \varphi$$  (9)

In addition to the $H_2^{15}O$, we investigated the uptake of fluorine ($^{18}F$), which has often been used as a tracer for
apoplastic transport (Mckay et al. 1988, Keutgen et al. 2002), to compare both the uptake profiles. A 1/5 Steinberg solution containing 18F was applied to the sample in the same manner about 1 h after of the H2 15O application. Fig. 9 shows the rates of water and fluorine (18F) escape from the xylem vessels in the same internode. The profile of the escape rate of 18F was clearly different from that of water. An early phase of linear escape was not observed and the curves at P1 and P2 were almost the same. 18F also must have been translocated mainly through the xylem vessels followed by diffusional escape at the point of measurement, like water, because the diffusional distance for this measurement period is so short. The fluorine which escaped from xylem vessels may move via the apoplastic route described above, because the uptake of fluorine by living cells is very slight, in the same manner as inulin (Mckay et al. 1988). The uptake profile of 18F was shown to be independent of the position of measurement, indicating that the dilution effect of 18F during its transport through the xylem vessels was slight, unlike water. In addition, the dilution of water due to exchange was not negligible; the dilution rate and the rate of water exchange of this sample were 85.4 and 14.6%, respectively, in this case (Fig. 9a).

The effect of the transpiration rate on the net volume of water exchange

To study the effect of the transpiration rate on the net volume of water exchange, the relationship between the transpiration rate and relative humidity (RH) was examined (Fig. 10). The relationship could be described by the second degree curve based on the equation,

\[ V(x) = -1.46 \times 10^{-6}x^2 - 1.66 \times 10^{-5}x + 0.0194(R^2 = 0.9) \]

in which V and x indicate the transpiration rate and the RH, respectively. To compare the net volumes of water exchange in plants under different humidity conditions, one should pay attention to the fact that the flow rate through xylem vessels is strongly affected by the RH conditions at the time of measurement. Therefore, the transpiration rate of plants under a certain RH regime could be estimated by equation (10). The net volume of water exchange, \( \lambda(x) \), under a certain RH, x, could be estimated by the rate of the exchange, \( \eta(x) \), and the transpiration rate, \( V(x) \), as follows:

\[ \lambda(x) = \eta(x) \cdot V(x) \]
The rate of \( \lambda(x) \), \( r \),

\[ r = \frac{\lambda(x_1)}{\lambda(x_2)} \]  

(12)

was calculated from the measurement of water exchange under various RH conditions (Table 1). The value of \( r \) was more or less constant, 1.05 \( \pm \) 0.21 (\( N = 11 \), \( \pm \)SD). In conclusion, the net volume of water exchanged with that in the surrounding tissue was independent of the transpiration rate.

Van Bel (1974, 1976) claimed that water escape can be explained by the Horwitz model based on the diffusion theory (Horwitz 1958). THO was allowed to perfuse through stem segments of tomato plants under gravity, and the rates of THO re-collected in the outflow and THO remaining in the respective stem sections were measured (Van Bel 1974). He concluded that the higher the apparent flow velocity, the larger the amount of the THO molecules not available for lateral diffusional escape (Biddulph et al. 1963, Van Bel 1974), because the rate of re-collected THO appeared to be highly dependent on the flow rate of the perfusion. Some of his graphs suggested a reversibility of the escape, i.e. re-entrance of THO into the xylem vessels. In the present experiments, the net volume of exchanged water was almost constant in spite of the changes in the mass flow rate. The interpretation is in agreement with previous reports that the outflow constant in the Horwitz theory was close to the water diffusion coefficient (Van Bel 1974).

To establish diffusional escape as the driving force for lateral water movement, samples were immersed in water at 0 or 27°C for 5 and 10 min after the pulse application of THO. In this treatment, the whole shoot was frozen before excising the internode segment to avoid the drastic change in the pressure in xylem vessels due to cutting near the target point. Fig. 11 shows the radiograph of THO at the transection of the stem of the samples. A large amount of THO was observed in xylem vessels after 5 s (Fig. 11a). THO could reach the whole area of the transection within 5 min

### Table 1

<table>
<thead>
<tr>
<th>Sample number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>RH L</td>
<td>52.0</td>
<td>29.5</td>
<td>35.8</td>
<td>55.0</td>
<td>53.0</td>
<td>51.8</td>
<td>56.1</td>
<td>54.0</td>
<td>33.5</td>
<td>24.0</td>
<td>31.3</td>
</tr>
<tr>
<td>( \varphi )</td>
<td>0.939</td>
<td>0.852</td>
<td>0.973</td>
<td>0.953</td>
<td>0.965</td>
<td>0.930</td>
<td>0.847</td>
<td>0.910</td>
<td>0.948</td>
<td>0.960</td>
<td>0.865</td>
</tr>
<tr>
<td>RH H</td>
<td>78.0</td>
<td>61.0</td>
<td>51.5</td>
<td>77.1</td>
<td>82.7</td>
<td>82.7</td>
<td>74.5</td>
<td>80.0</td>
<td>75.6</td>
<td>50.6</td>
<td>48.2</td>
</tr>
<tr>
<td>( \varphi )</td>
<td>0.892</td>
<td>0.810</td>
<td>0.977</td>
<td>0.928</td>
<td>0.944</td>
<td>0.915</td>
<td>0.807</td>
<td>0.840</td>
<td>0.880</td>
<td>0.950</td>
<td>0.869</td>
</tr>
<tr>
<td>( r )</td>
<td>0.895</td>
<td>1.06</td>
<td>1.35</td>
<td>0.972</td>
<td>1.05</td>
<td>1.48</td>
<td>0.918</td>
<td>0.917</td>
<td>0.756</td>
<td>0.975</td>
<td>1.19</td>
</tr>
</tbody>
</table>

RH, relative humidity; \( \varphi \), dilution rate; \( r \), rate of \( \lambda(x) \); \( \lambda(x) \), net volume of water exchange; \( x \), relative humidity. The rate, \( r \), can be estimated by measuring \( \varphi \) for the same sample under different relative humidities.
under both conditions (0 and 27°C) after the THO pulse treatment (Fig. 11b, c). The profile of the radiographs of THO under 27°C conditions was similar to the previous results (Fig. 2B), i.e. local accumulation of THO at pith was detected, supporting the proposal that rapid inward movement of water was independent of the transpiration. On the other hand, the profile under 0°C conditions showed uniform THO distribution (Fig. 11c). In addition to the self-diffusion (thermal motion), some driving force may be involved in the lateral water movement in this case, because an average distance of water movement by self-diffusion for 5 min at 0°C is 0.8 mm ($l^2 = 2Dt$, $D = 1.1 \times 10^{-9}$, $2.4 \times 10^{-9}$ (m$^2$ s$^{-1}$) referred to in Fig. 2), i.e. the distance is too short to explain the profile of the radiograph (Fig. 11c). The average distances of water movement by self-diffusion for 10 min at 0 and 27°C are 1.1 and 1.7 mm, respectively, suggesting that the period is enough for THO to reach the whole area in an internode transection. However, the radiograph at 27°C was clearly different from that at 0°C. The water movement within a stem may be strongly affected by plasma membrane partitioning, because the water permeability of the plasma membrane is dependent on the temperature (Lee et al. 2005).

Materials and Methods

**Sample preparation**

Soybean seedlings [Glycine max (L.) Merr. cv. Enrei] were germinated in vermiculite at 27°C in the dark. After 3 d, the seedlings were transferred to a 1/5 Steinberg solution and were grown in 80% RH at 27°C with a 16 h light (150 μmol m$^{-2}$ s$^{-1}$) and 8 h dark cycle (Biotron LPH300; NK System Co., Osaka, Japan). The culture solution was renewed every week. Samples aged from 18 to 20 d old were used for this experiment.

**Measurement of the vessel area**

To measure the vessel area, a 0.05% safranine solution was applied to the plant for 30 min with 50% humidity at 28°C, with light. Stem sections of 150 μm thickness were cut with a vibratome (VT1000S; Leica Microsystems Co., Tokyo, Japan) at 2 and 6 cm above the cotyledon. The section was photographed under a microscope and the image transferred to a computer, by which the xylem vessel area was measured using an image-analyzing software program (Image-Pro, Scanalytics, Inc., Fairfax, VA, USA).

**Generation of $H_2^{15}O$**

The gas $^{15}O_2$ was generated by the reaction of $^{14}$N (d, n)$^{15}$O; the $H_2^{15}O$ vapor was then synthesized by the reaction of the oxygen ($^{15}$O) and hydrogen gases under high temperature in the presence of a platinum catalyst. The $H_2^{15}O$ was captured by bubbling the vapor through a bottle filled with 10 ml of distilled water. The $H_2^{15}O$ was used in the present experiment after the radioactivity of the $H_2^{15}O$ was measured using a dose calibrator (Atomlab 100A; Biodex Medical Systems Co., Shirley, NY, USA). The specific activity of $H_2^{15}O$ was 2 GBq per 10 g of water.

The quantitative and real-time measurement of $H_2^{15}O$ in the stem internode

A soybean plant, from which the cotyledons and the root 8 cm below the cotyledons had been removed, was fixed between lead blocks to shield radiation, and placed in the Biotron about 1 h before the measurement. A pair of gamma ray detectors was placed immediately adjacent to either side of the internode of the plant, 2 cm above the cotyledons. The detector modules (Photosensor Modules; Hamamatsu Photonics K. K., Hamamatsu, Japan) were composed of a BGO scintillator ($\text{Bi}_4\text{Ge}_3\text{O}_{12}$ crystal size 10 x 10 x 20 mm), photomultiplier tube and pre-amplifier. The gamma rays radiated from the sample were converted to the weak current by the modules. The weak current was amplified by a linear amplifier (704-4B; Oken Co., Tokyo, Japan), and discriminated by a timing single channel analyzer (706-2B, Oken Co.) based on its energy, corresponding to 511 keV. The signals passed through the timing single channel analyzer were counted with a coincidence circuit (Fast and slow coincidence 708-1B and Ratemeter S-2293B; Oken Co.) and recorded on a computer (OptiPlex GX1; Dell Co., Kawasaki, Japan) (Fig. 1). The timing of coincidence and the data export interval were set at 110 ns and 1 s, respectively. The counting efficiency of the gamma ray detector was calculated to be 0.12%, by comparing the count of the gamma ray detector with that of a gamma counter (Auto-gamma scintillation spectrometer 5230; GMI Inc. Ramsey, MN, USA) for the plant sample supplied with $H_2^{15}O$ (200 MBq ml$^{-1}$). The measuring system was placed in a Biotron, which was maintained at 27°C with an RH of 50% and constant light. The background noise was reduced to <0.01 c.p.s. by the coincidence circuit and the shielding of the detection unit with lead blocks.

The measurement of $H_2^{15}O$ volume was performed on plants with two treatments: (i) a plant with Vaseline spread on the surface of the internode to be measured between the cotyledon and the first leaf; and (ii) a plant with a 5 mm portion of the tissue outside of the cambium removed by a razor blade, from 3 cm above to 1 cm below the measuring position. After the measurement, the sample of tissue removed from outside of the cambium was treated with 0.05% safranine solution for 30 min. Both sections of the removed tissue, 150 μm in thickness, were observed under the microscope to verify the condition of the remaining phloem and the associated vessels.

**Quantitative measurement of water volume by two pairs of gamma ray detectors**

Two pairs of gamma ray detectors were placed close to either side of the internode of a soybean plant, 2 cm above the cotyledon (P1) and 3.5 cm above P1 (P2), respectively. The $H_2^{15}O$ was applied and measurements were carried out under a range of RHs from 24 to 83%, as described above.

A supplementary experiment was executed using a double-label approach: a 1/5 Steinberg solution containing $^{18}$F was applied to the same sample about 1 h after the 1/5 Steinberg solution containing $H_2^{15}O$ was applied.

**Radiography of THO distribution over the transection of the internode**

To examine the distribution of water within an internode transection, THO (Moravek Biochemicals Inc., Brea, CA, USA), 3.2 MBq g$^{-1}$, was supplied in the same manner as $H_2^{15}O$. After 10 min, an approximately 1.5 cm long internode segment was excised below the measurement site, 2 cm above the cotyledons, and immediately frozen by immersion in liquid nitrogen.
Following embedding in Tissue-Tek at −22°C, the sample was shaved to flatten the transection using a cryostat (Microm HM505N; Carl Zeiss Co., Tokyo, Japan) and exposed to an imaging plate (BAS-TR2025; Fujifilm Co., Tokyo, Japan) for 2 weeks at −80°C. The image from the imaging plate was scanned with an image analyzer (FLA-5000, Fujifilm Co. Japan) with a 16-bit data depth.

To examine lateral water movement in the internode, the cut shoots were placed into non-labeled water for various times (10 s, 20 s, 1 min and 2 min) after the cut-ends were dipped for 5 s into a THO solution. Then an approximately 1.5 cm internode segment was excised above the measurement site and frozen by immersion in liquid nitrogen. The following procedures were as described above. THO (37 MBq g−1) was applied to the cut end of the stem, 1 cm below the cotyledon. The exposure time to an imaging plate was 1 d at −80°C.

To examine diffusional lateral water movement in the internode, THO (37 MBq g−1) was applied to the cut end of the stem, 1 cm below the cotyledon, for 5 s. Three treatments were then carried out: (i) the sample was immediately frozen then exposed to an imaging plate; (ii) the sample was immersed in water at 0°C for 5 or 10 min followed by freezing and exposure to an imaging plate; and (iii) the sample was immersed in water at 27°C (room temperature) for 5 or 10 min followed by freezing and exposure to an imaging plate.

Measurement of the transpiration rate

Cotyledons and roots, 8 cm below the cotyledons, of soybean plants were removed. The cut shoot was placed in a plastic bottle containing 300 ml of distilled water. The bottle was set in the Biotron, which was kept in the growth conditions described above. Transpiration rates were measured by monitoring weight loss. The leaves of cut shoots were removed and their surface area was measured using a scanner (N676-U; Canon Inc. Tokyo, Japan).

The measurement of the effect of the exchange of the isotope 15O for 16O

To measure the effect of the exchange of the isotope 15O for 16O, 8 ml of H16O was poured into a vial filled with 2 g of cellulose powder (Fibrous cellulose powder CF11; Whatman Japan K.K., Tokyo, Japan). They were mixed homogeneously by a shaker (Vortex genius3; IKA Japan K.K., Yamatokoriyama, Japan) for about 5 min. Then, 50 μl of supernatant water was sampled after centrifuging for 1 min. The activity of the supernatant water/the activity of the initial H216O water) was calculated. The experiment was repeated four times.

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