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Techniques

Overcoming the Difficulties in Collecting Apoplastic Fluid from Rice Leaves by the Infiltration–Centrifugation method

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Physiological and biochemical studies on the leaf apoplast have been facilitated by the use of the infiltration–centrifugation technique to collect intercellular washing fluid (IWF). However, this technique has been difficult to implement in rice (*Oryza sativa* L.) for various reasons. We compared the collection efficiency of leaf IWF between two types of rice varieties (Indica and Japonica), as well as between rice and other species (spinach, snap bean and wheat). Although the extraction of IWF in most species took only 2–3 min, it took up to 35 min in rice. The difficulty in infiltration with rice was ascribed to the small stomatal aperture and hydrophobicity of the leaves. In this study, we have established an improved method for collecting IWF and determining the apoplastic air and water volumes in rice leaves. We have shortened the infiltration time to 8 min via the following improvements: (i) infiltration under outdoor shade in the daytime to prevent stomatal closure and a rise in temperature of the infiltration medium; (ii) soaking of leaves in a surfactant solution to decrease the leaf hydrophobicity; and (iii) continuous pressurization using a sealant injector to facilitate the infiltration. The rapid collection of IWF achieved using this technique will facilitate study of the leaf apoplast in rice.

Keywords: Intercellular washing fluid • Leaf apoplast • Rice.

Abbreviations: G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; IWF, intercellular washing fluid; LFW, leaf fresh weight; ΔP, the pressure difference at the interface between air and liquid phases in a rice stoma soaked in water; R, radius of the semi-cylinder approximating the gas–liquid interface in a rice stoma; σ, the surface tension of water; V_{air}, apoplastic air volume; V_i, infiltration volume; V_{wat}, apoplastic water volume; W_{INI}, initial weight.

Introduction

Cell walls in the apoplast serve as a passage for water, inorganic ions and low molecular weight substances, as well as a frame to retain the shape and size of each cell (Clarkson 2007). The cell walls in leaves, i.e. the first tissue suffering from infection by pathogenic microbes and from air pollutants, are the frontline of defense systems in plant leaves against physical and/or chemical stresses. Therefore, the physiological and biochemical functions in the apoplast have attracted attention (Sakurai 1998, Sattelmacher 2001, Sattelmacher and Horst 2007), and several methods have been developed to isolate apoplastic fluid: (i) a pressure technique (Jachetta et al. 1986); (ii) the elution method (Long and Widders 1990); and (iii) the infiltration–centrifugation method (Klement 1965). Besides the direct measurements of the collected apoplastic fluid, other approaches such as the use of ion-selective microelectrodes (Felle and Hanstein 2002) have also been applied.

Among the methods developed so far, the infiltration–centrifugation technique has been widely applied to many plant species (Klement 1965, Luwe et al. 1993, Lyons et al. 1999, Cheng et al. 2007) due to its efficiency and simplicity. In this method, the intercellular air spaces are replaced by infiltration medium with pressure by repetitions of depressurization and pressurization. The intercellular washing fluid (IWF), i.e. a mixture of the infiltration medium and apoplastic fluid, is then collected by centrifugation. Some concerns over this method have been raised (Dietz 1997), however. The infiltration–centrifugation process could damage the plasma membrane of the cells, and the stress by submersion during the infiltration could alter ion and metabolite compositions in apoplastic fluid. Furthermore, the dilution of apoplastic fluid by the infiltration medium could incur reactions by the cells, and, hence, the collected fluid may not reflect the real state of the apoplast.

Among these potential problems, the damage to the plasma membrane can be avoided in most cases by limiting the gravity force used in centrifugation. As for the alteration to the apoplastic fluid by infiltration, Lohaus et al. (2001) found few differences in concentrations of ions and metabolites of IWF between infiltration media (deionized water, KCl, $CaCl_2$ and MES), concentrations of an infiltration medium (0 and 180 mM) or the infiltration time (the time from the beginning

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of infiltration to the beginning of centrifugation in the range from 2 to 30 min). The concerns have thus been dealt with, and infiltration–centrifugation has been the most widely used method for apoplast research due to its quick, inexpensive and easy operation (Lohaus 2007).

In this study, we applied the infiltration–centrifugation method to collect leaf apoplastic fluid from rice, on which few reports are available despite the species' primacy as a staple crop particularly in Asia. A previous study has shown that collection of apoplastic fluid from rice leaves with infiltration–centrifugation was much more difficult than from other plant species, such as spinach and snap bean (Hayashi et al. 2008). There were three problems with rice. First, the IWF seeping from rice leaves during centrifugation stuck to the leaf surfaces, making it time-consuming and laborious to collect it. Secondly, an excessively long time was needed to complete the infiltration. The long infiltration time could impose a severe stress on the leaf tissue, which might lead to altered physiological behavior. The long infiltration time might also allow cellular responses to the dilution of ions and metabolites in the apoplastic fluid. A shorter infiltration time is preferable, if not necessary, despite the experimental results of Lohaus et al. (2001). Thirdly, the solution of Indigo carmine to determine the dilution ratio of IWF (Husted and Schjoerring 1995) by infiltration risks overestimating the apoplastic water volume. A previous study (Hayashi et al. 2008) reported values of apoplastic water volume in rice leaves which were considerably larger than those reported in other studies, i.e. approximately 10% of the total leaf volume (Speer and Kaiser 1991, Husted and Schjoerring 1995, Luwe and Heber 1995). We have suspected that partial adsorption of Indigo carmine to the leaf tissues resulted in overestimation in the case of rice.

The main objectives of the present study were to characterize the above-mentioned problems in collecting the IWF from rice leaves, and to solve the problems specific to rice by improving the methods of collecting the IWF and estimating the apoplastic air and water volumes.

Results

Gateway of the infiltration medium and IWF

Cut edges of leaves, as well as stomata, were considered as a gateway for the infiltration medium and IWF during infiltration and centrifugation, respectively. We tested water infiltration only through stomata in rice, wheat and snap bean under laboratory room light. The leaf blades of rice, wheat and snap bean were soaked in water in a beaker in a vacuum desiccator with the cut edges of leaves exposed to air. The desiccator was depressurized using a vacuum pump, but no infiltration of water was observed in any of the leaves under depressurization, while many air bubbles appeared on the leaf surfaces. When the leaves were returned to normal atmospheric pressure, sudden and complete infiltration of water took place in the leaf blades of wheat and snap bean, whereas the rice leaves showed no water infiltration. With the cut edge of leaves not being in contact with water, stomata should be the only pathway for water infiltration into wheat and snap bean leaves.

Water infiltration for rice leaves required repeated depressurization and pressurization using syringes. Even when the cut edge was in water, water did not infiltrate beyond a range of about 5 mm from the cut edges. Thereafter, infiltration took place at random across the entire area of leaves and increased with the repeated depressurization and pressurization. It was therefore suggested that the major gateway of water infiltration into plants including rice was stomata, with a minor contribution of the cut edges.

Comparison between stomatal opening under outdoor shade and room light

Stomatal opening of rice leaves was compared between outdoor shade and room light with pot-grown rice plants at the middle ripening stage on a clear day in October. When the mean photosynthetic photon flux density in a sunny place was 1,230 μmol m^{-2} s^{-1}, that in outdoor shade and room light was 127 and 8 μmol m^{-2} s^{-1}, respectively. Under these conditions, stomatal conductance in a sunny place, outdoor shade and room light was 0.104 ± 0.026, 0.093 ± 0.024 and 0.038 ± 0.019 mol m^{-2} s^{-1}, respectively, which indicates the severe stomatal closure in room light.

Shortening infiltration time for rice leaves

To shorten the infiltration time for rice, we conducted the following experiments on clear days on July 15 and 16, 2009 (64 and 65 d after transplanting).

Infiltration under outdoor shade instead of laboratory room light. Leaf segments cut with scissors were immediately soaked in 100 mM KCl, and subjected to alternate repetitions of depressurization and pressurization by hand under laboratory room light or outdoor shade. The test in triplicate showed a significant acceleration: the filtration was completed in 15 min under outdoor shade, whereas it took 26 min under laboratory room light (**Table 1**).

Increasing the leaf wettability by soaking in surfactant solution. To test if the infiltration can be accelerated by eliminating the air layers, we compared the infiltration time between the following three treatments: (i) the leaf segments were soaked in the infiltration solution in the syringe, and subjected to repeated manual depressurization and pressurization; (ii) the leaf segments were soaked in deionized water for 20 min to increase the leaf wettability, blotted dry with paper towels, soaked in the infiltration solution in the syringe, and then subjected to repeated manual depressurization and pressurization; and (iii) the leaf segments were soaked in a surfactant solution, i.e. a 0.05% (v/v) Triton X-100 solution (Raskin and Kende 1983), for 2 min to eliminate the surface air layers, thoroughly rinsed with deionized water, blotted dry with paper towels, soaked in the infiltration solution in the syringe, and

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Table 1 Infiltration time for rice leaves as affected by light condition, pre-treatment by surfactant and pressurization

Experimental conditions and treatments			Infiltration time[a] (min)
Light condition	**Pre-treatment to remove hydrophobicity of leaf surface**	**Pressurization**	
Room light in laboratory	No treatment	By hand	25.6 ± 3.4
Outdoor shade	No treatment	By hand	15.3 ± 0.7
Outdoor shade	20 min soaking in water	By hand	7.4 ± 0.9
Outdoor shade	2 min soaking in 0.05% Triton X-100 surfactant solution	By hand	7.4 ± 0.5
Outdoor shade	2 min soaking in 0.05% Triton X-100 surfactant solution	Using a sealant injector	6.4 ± 0.3

[a] Data are the mean ± SD (n = 3–6).

then subjected to repeated manual depressurization and pressurization.

We found that the second treatment had the same effect as the third treatment, and that these treatments effectively prevented the formation of air layers. Thus, a remarkable reduction in the infiltration time, i.e. 8 min, was achieved by the elimination of air layers on the leaf surfaces by soaking the leaves in water for 20 min or in a surfactant solution for 2 min prior to the infiltration.

Use of a sealant injector for prolonged retention of pressure. First, rice leaf segments were treated with the Triton X-100 solution, and placed in a 60 ml syringe. Air in the leaf intercellular spaces was expelled by repeated manual depressurization and pressurization under outdoor shade for 3 min. About 50% of the whole leaf area was infiltrated at this point. Next, the syringe was set in the sealant injector to retain the pressurization until the completion of infiltration. The infiltration time was shortened by 1 min in this way, but an extra time of 30–40 s was required to set the syringe in the sealant injector. Whereas shortening of the infiltration time for a sample was thus quite small, the use of sealant injector raised the efficiency of pressurizing a series of samples by omitting the manual labor required to apply the pressurization and enabling multiple samples to be pressurized in parallel at a time.

Infiltration time of rice leaves by the improved method

The improved procedure for rice leaves to accelerate the infiltration on the basis of the experiments mentioned above is summarized in **Fig. 1**. The infiltration time for rice leaves by this procedure was approximately 4–6 min on days 45–58 and 7–8 min on days 60–120 after transplanting. With leaves of young rice plants on and before day 44 after transplanting, the infiltration was completed within 4 min without a sealant injector.

Centrifugal force for IWF collection without cytoplasmic contamination

Cytoplasmic contamination of the IWF for wheat and rice leaves was examined because the centrifugal forces for these leaves were higher than those observed in previous studies (Kollist et al. 2000, Hayashi et al. 2008). Kollist et al. (2000) had reported the collection of the wheat IWF at 160 $\times g$ for 10 min of centrifugation; however, we could not collect the IWF at all under such conditions. We then tested the centrifugation at accelerations of 1,000, 2,000 and 3,000 $\times g$ for 10 min for the IWF collection from wheat leaves. Glucose-6-phosphate dehydrogenase (G6PDH) activity was occasionally detected in the IWF at 2,000–3,000 $\times g$ [0.0017–0.0046 nkat g^{-1} leaf fresh weight (LFW)] but not at 1,000 $\times g$, where the G6PDH activity in the leaf tissue extract was 19.8 ± 1.07 nkat g^{-1} LFW. We therefore adopted centrifugation at 1,000 $\times g$ for 10 min when collecting the IWF from wheat.

The G6PDH activities in the IWF and the leaf tissue extract were measured for rice leaves at the second leaf position counted from the top leaf on days 72–79 after transplanting. The G6PDH activity in the leaf tissue extract was 6.23 ± 0.87 nkat g^{-1} LFW. The IWF was collected by centrifugation in the range of 5,000–30,000 $\times g$ for 15 min at 4°C. The G6PDH activities in the IWF were hardly detected at $\leq$14,000 $\times g$ (**Fig. 2**). However, a clear increase in the G6PDH activity, 0.0025 nkat g^{-1} LFW, was detected at 15,000 $\times g$. A remarkable increase in the G6PDH activity, 0.0558 nkat g^{-1} LFW, was found at 30,000 $\times g$. Consequently, a centrifugal force $<$14,000 $\times g$ may be applied to rice leaves with a negligible amount of cytoplasmic contamination.

Collection method of IWF

The recovery rates of the IWF were 75% with spinach leaves centrifuged at 400 $\times g$ for 10 min, 18% for snap bean leaves at 556 $\times g$ for 5 min, 60% for wheat leaves at 1,000 $\times g$ for 10 min, and 55% for rice leaves at 6,000 $\times g$ for 15 min. The former three plant species provided the IWF under mild centrifugation, although the recovery rate was low in snap bean. With bean, raising the centrifugal gravity for a higher recovery rate resulted in increased cytoplasmic contamination. In comparison, with rice, centrifugation at 3,000 $\times g$ for 15 min provided little IWF, and a centrifugal force $>$5,000 $\times g$ was required to collect a sufficient amount of IWF.

Apoplastic air volume

Table 2 shows the apoplastic air volumes of spinach, snap bean, wheat and rice, in which the values of rice cultivar Koshihikari were obtained from top leaves at the early growth stage. The apoplastic air volumes were large in the two dicotyledonous

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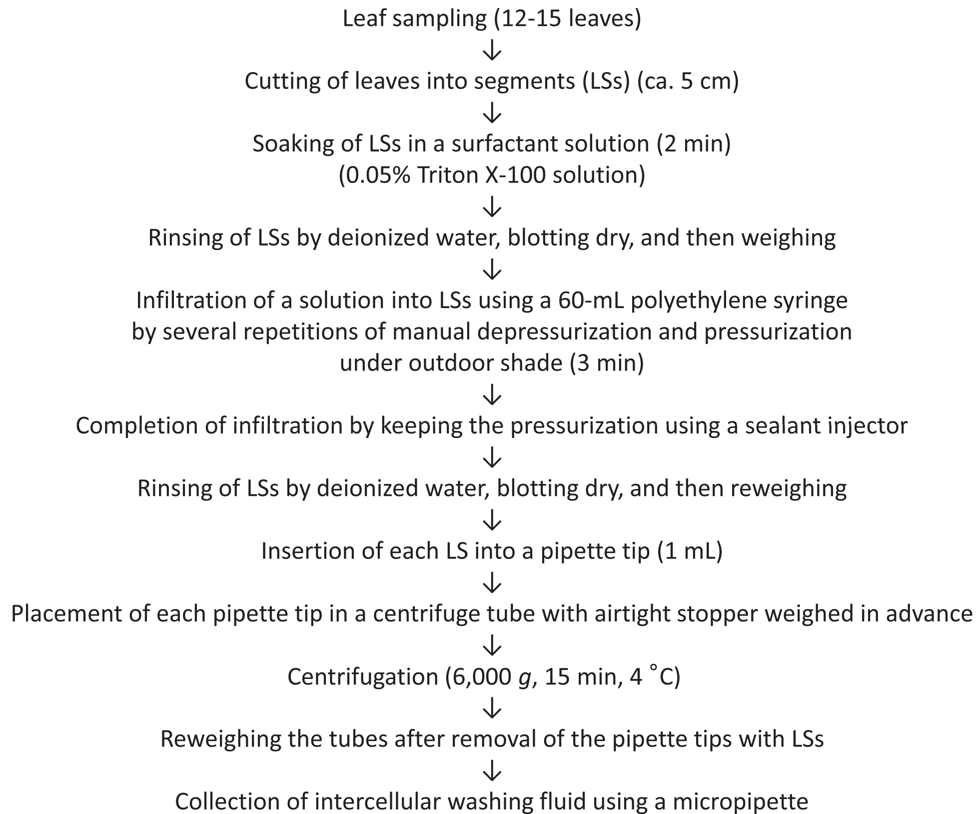

Fig. 1 Operationial procedure of a modified infiltration–centrifugation method to collect the intercellular washing fluid from rice leaves.

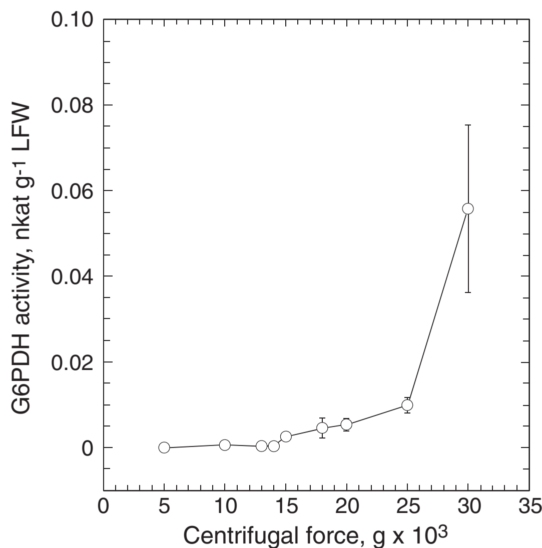

Fig. 2 G6PDH activity, an indicator of cytoplasmic contamination, of intercellular washing fluid from leaves of rice cultivar Koshihikari at centrifugal forces between 5,000 and 30,000×*g*. Data are the means ± SD (*n* = 3).

species: spinach ($0.372\,cm^3\,g^{-1}$ LFW) and snap bean ($0.271\,cm^3\,g^{-1}$ LFW), and were small in the two monocotyledonous species: wheat ($0.218\,cm^3\,g^{-1}$ LFW) and rice ($0.100–0.227\,cm^3\,g^{-1}$ LFW). **Table 3** shows the apoplastic air volumes in leaves at different positions for rice cultivar Koshihikari at the flowering stage. The fifth leaves, which are the oldest among the leaves tested here and had become yellowish, had the highest apoplastic air volume of $0.227\,cm^3\,g^{-1}$ LFW. The apoplastic air volumes increased with the leaf position from the top; the youngest leaves had the smallest value of $0.100\,cm^3\,g^{-1}$ LFW (**Table 3**).

Apoplastic water volume

The infiltration of spinach, snap bean and wheat leaves with 0.20% (w/v) Blue dextran 2000 took almost the same time as with colorless 100 mM KCl. With rice, in comparison, the infiltration time of the Blue dextran 2000 solution was 2–3 times that of 100 mM KCl, which might be ascribed to stomatal closure in response to the Blue dextran 2000 solution. **Table 2** shows the leaf apoplastic water volumes for spinach, snap bean, wheat and rice cultivars IR72 and Koshihikari at the early growth stage. The apoplastic water volume ranged from 0.109 to $0.170\,cm^3\,g^{-1}$ LFW among the species and cultivars. Assuming that a LFW of 1 g has a volume of $1\,cm^3$, $0.109\,cm^3\,g^{-1}$ LFW of apoplastic water volume corresponds to 10.9% of leaf volume. As shown in **Table 3**, the apoplastic water volumes of leaves at five different leaf positions of the rice cultivar Koshihikari decreased from the leaves at the top ($0.216\,cm^3\,g^{-1}$ LFW) to the fifth leaf position ($0.132\,cm^3\,g^{-1}$ LFW), in contrast to the apoplastic air volumes.

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Table 2 Apoplastic air and water volumes in mature leaves

Species	Cultivar	Growth stage	Leaf position[a]	Apoplastic air volume[b] ($cm^3 g^{-1}$ LFW)	Apoplastic water volume[b] ($cm^3 g^{-1}$ LFW)
Spinach (*Spinacia oleracea* L.)	Active	Before harvesting	5–6	0.372 ± 0.068	0.162 ± 0.038
Snap bean (*Phaseolus vulgaris* L.)	Aron	Flowering	3	0.271 ± 0.014	0.157 ± 0.024
Wheat (*Triticum aestivum* L).	Ayahikari	Ripening	1 (flag leaf)	0.218 ± 0.013	0.170 ± 0.056
Rice (*Oryza sativa* L.)	IR72	Tillering[c]	2	0.224 ± 0.010	0.143 ± 0.024
Rice (*Oryza sativa* L.)	Koshihikari	Tillering[d]	1	0.232 ± 0.010	0.109 ± 0.014

[a] Leaf position was counted from the top.
[b] Data are the mean ± SD (n = 3–6).
[c] Fifty-six days after transplanting.
[d] Thirty days after transplanting.

Table 3 Apoplastic air and water volumes in leaves at five different positions of rice cultivar Koshihikari at the flowering stage

Leaf position[a]	Apoplastic air volume[b] ($cm^3 g^{-1}$ LFW)	Apoplastic water volume[b] ($cm^3 g^{-1}$ LFW)
1 (flag leaf)	0.100 ± 0.082	0.216 ± 0.035
2	0.118 ± 0.010	0.155 ± 0.024
3	0.137 ± 0.005	0.149 ± 0.013
4[c]	0.166 ± 0.004	0.139 ± 0.048
5[c]	0.227 ± 0.014	0.132 ± 0.013

[a] Leaf position was counted from the top.
[b] Data are the mean ± SD (n = 3–6).
[c] The leaves at positions 4 and 5 were senescent and had become yellowish.

Potassium ion concentration in apoplast and leaf tissue

K^+ concentrations were determined in apoplastic fluid and leaf tissue of Koshihikari rice at the tillering stage. The K^+ concentration in apoplastic fluid was 3.83 ± 1.06 mM (n = 16), whereas that in leaf tissue was 193 ± 21 mM (n = 3).

Discussion

Difficulty in infiltration to rice leaves

In the experiments using the vacuum desiccator, water infiltration occurred in wheat and snap bean leaves by depressurization, which was not the case with rice leaves. Since stomata are the gateway of water infiltration under pressurization (Terashima 1992, Lohaus et al. 2001), their opening should govern the water infiltration. The size of the stomatal opening has been reported to be: 18.8 × 1.18 µm for rice (mean value across four Indian cultivars, Uprety et al. 2002); 38×7 µm for wheat (Eckerson 1908); 15×5 µm for spinach (Iwabuchi and Kurata 2003); and 7×3 µm for snap bean (Eckerson 1908). Rice thus has the smallest stomatal opening among these plant species.

The water infiltration through a stoma takes place against the surface tension of the gas–liquid interface formed in the small pore of the stoma. Without the pressurization, the shape of the gas–liquid interface is almost flat. As the pressure increases, the interface becomes convex with a greater curvature, which is related to the pressure difference across the gas–liquid interface by a Laplace equation, i.e.

$$\Delta P = \sigma\left(\frac{1}{R_1} + \frac{1}{R_2}\right) \quad (1)$$

where ΔP is the pressure difference (Pa), σ is the surface tension ($N\,m^{-1}$), and R_1 and R_2 are the curvature parameters (m) of the gas–liquid interface.

In rice, the shape of a stoma resembles a slit and, hence, the shape of the gas–liquid interface can be approximated by a semi-cylinder with the axis being along the slit. For the semi-cylinder, the curvature parameters R_1 and R_2 can be approximated by R and ∞, respectively, where R is the radius (m) of the semi-cylinder and ∞ indicates a very long slit. The pressure difference ΔP can then be simplified, i.e.

$$\Delta P = \frac{\sigma}{R} \quad (2)$$

As ΔP increases, R is reduced, and the gas–liquid interface becomes more convex. When the curvature of the interface reaches the maximum, R becomes equal to a half of the slit width, and the gas–liquid interface is pushed into the stomatal pore.

In our experiment, when the syringe containing leaf segments in water was first depressurized by P_1, the pressure both inside and outside the leaf dropped to $(0.1013 - P_1)$ MPa. The three-way stopcock was then opened, and the pressure outside the leaf returned to atmospheric pressure (0.1013 MPa), while the pressure inside the leaf remained at $(0.1013 - P_1)$ MPa, resulting in the pressure difference by P_1. In contrast, when the syringe was pressurized by P_2, the pressure inside and outside the leaf was 0.1013 and $(0.1013 + P_2)$ MPa, respectively, hence the pressure difference P_2 was created. Water can infiltrate into stomatal pores when P_1 or P_2 exceeds ΔP. Note, however, that P_1, the pressure difference created by depressurization, cannot go beyond 0.1013 MPa by definition.

In plant species with a large stomatal opening, ΔP in Equation 1 or 2 could be less than the atmospheric pressure, in which case water can infiltrate with depressurization only. For rice, however, ΔP is estimated to be 0.124 MPa using Equation 2, with R of 0.59 (=1.18×0.5) µm and σ of 73 $mN\,m^{-1}$ of the surface tension of water at 18°C. This

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pressure difference cannot be achieved by depressurization, and, hence, pressurization is indispensable to infiltrate water into the stomata of rice leaves. For wheat, in comparison, ΔP is estimated to be 0.0209 MPa, which allows sufficient water infiltration by depressurization only.

Although the infiltration time for spinach, snap bean and wheat was 2–3 min with a couple of repetitions of manual depressurization and pressurization under laboratory room light, it took as long as 35 min for rice under the same conditions. We achieved a shortened infiltration time for rice (within 8 min) by the following three improvements: (i) infiltration under outdoor shade to prevent the stomatal closure; (ii) soaking of leaf segments with a surfactant solution prior to the infiltration to prevent the formation of thin air layers on the hydrophobic leaf surfaces; and (iii) facilitating continuous pressurization with a sealant injector. The shortening of the infiltration time has the advantage of decreasing submersion stress to the leaf tissue and improving the efficiency of the measurement.

The very small area of stomatal openings in rice slows the water infiltration through the stomata and, hence, the stomatal closure becomes a greater hindrance against the infiltration particularly in room light, as we observed in stomatal conductance. Infiltration in a brighter environment under outdoor shade was effective in shortening the infiltration time by preventing stomatal closure (**Table 1**).

The hydrophobicity of rice leaves also obstructed the infiltration by forming thin air layers on the leaf surfaces when the leaves were submerged in water. The air layers were hardly found on the leaves by 35 d after transplanting, and it seems that rice leaves acquire hydrophobicity along with plant growth. The gradual accumulation of silica in rice leaves (Yoshida et al. 1962) might be the cause of such hydrophobicity.

Cytoplasmic contamination due to centrifugation

A greater centrifugal force was needed with rice to collect the IWF in our previous study (3,000×*g* for 30 min) (Hayashi et al. 2008) and in the present study (6,000×*g* for 15 min) than with the other species. It is fortunate that a higher centrifugation up to 14,000×*g* for 15 min resulted in negligible cytoplasmic contamination, which was observed at a much lower centrifugal force (2,000–3,000×*g*) with wheat. It appears that the plasmalemma of rice leaves is very robust against such a large centrifugal force compared with many other species (Luwe and Heber 1995, Burkey 1999, Cheng et al. 2007).

Determination of apoplastic water volume

Cosgrove and Cleland (1983) and Husted and Schjoerring (1995) have shown that a solution of dye, such as Indigo carmine, can be used to measure the apoplastic water volume without having to use labeled radiocarbon sorbitol or mannitol (Cosgrove and Cleland 1983, Speer and Kaiser 1991, Husted and Schjoerring 1995). In our previous study (Hayashi et al. 2008) with rice, however, the use of Indigo carmine resulted in mean apoplastic water volumes of 0.25 $cm^3 g^{-1}$ LFW, which is greater than values reported for other species and is associated with a large standard deviation (0.22 $cm^3 g^{-1}$). Assuming that Indigo carmine is adsorbed or decomposed within the leaf apoplast during the infiltration–centrifugation processes, we used Blue dextran 2000 in the present study. Blue dextran 2000 is a glucosic polymer with a molecular weight of 2 million. With the large molecular weight, we presumed that the plasmalemma is impermeable to Blue dextran 2000, which is yet to be confirmed. The estimated water volumes in apoplast ranged from 0.109 to 0.170 $cm^3 g^{-1}$ LFW (**Table 2**) for spinach, snap bean, wheat and the two rice cultivars. The range of estimated water volumes (**Table 2**) is comparable with that (8–13%) reported in early studies (Speer and Kaiser 1991, Husted and Schjoerring 1995, Luwe and Heber 1995). The standard deviation for rice is also comparable with those for other species (**Table 2**). We therefore concluded that Blue dextran 2000 is suited to the measurement of the apoplastic water volume.

Potassium ion concentration in apoplast and leaf tissue

It is well known that K^+ is one of the major solute ions in leaf tissue and that its concentration is kept at 100–200 mM with variability among plant species (e.g. Speer and Kaiser 1991). While K^+ is present at high concentrations in the vacuole and cytosol (Speer and Kaiser 1991), its concentration in the apoplast ranges from below the detection limit to 8 mM only (Felle and Hanstein 2007). Our measured K^+ concentrations in apoplast and leaf tissue of rice are within the range reported by previous studies (Speer and Kaiser 1991, Felle and Hanstein 2007), which shows that our proposed IWF collection method is able to be used for IWF collection from rice leaves.

Merits and limits of this technique

This technique would facilitate studies with rice on the dynamic responses of mineral nutrients and various substances in the leaf apoplast to atmospheric and edaphic changes with IWF collection. For instance, measurements of ascorbic acid and other antioxidants in leaf apoplast will facilitate a better understanding of the defense mechanisms in rice against pathogens and air pollutants. This has been shown for ozone sensitivity with other species (e.g. Burkey 1999, van Hove et al. 2001, Cheng et al. 2007), but not with rice.

This technique may not be applicable, however, to the studies of stomatal behavior in relation to changes in leaf apoplast, since it needs open stomata, and the IWF collection would not be finished without altering the stomatal opening.

The fast infiltration through the cut edge of the rice leaves indicates the possibility of accelerating the infiltration by cutting the leaf sample into shorter segments. We have not taken this approach because the cutting operation may increase contamination of IWF with cytoplasmic contents.

Conclusion

Several unique aspects of rice leaves were identified in relation to the collection of IWF by the infiltration–centrifugation method. (i) Using a conventional method, the infiltration

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took a long time, approximately 35 min, for rice leaves, while it was finished within 2–3 min for leaves of other plant species under the same conditions. (ii) Depressurization was ineffective in rice leaves to complete the infiltration due to the very small stomatal pore, because of the large surface tension resistance. (iii) A thin layer of air was formed on the hydrophobic surfaces of rice leaves when the leaves were submerged in water. The air layer obstructed the infiltration of water into leaves. (iv) The IWF seeping from the leaf interior stuck to the leaf surfaces during centrifugation. It required a long time and tedious effort to collect the IWF. (v) A strong centrifugation at 6,000×*g* for 15 min was required to collect the IWF from rice leaves, whereas, for other species, relatively mild centrifugation at 400–1,000×*g* for 5–10 min was sufficient. With the set of improvements to overcome the above-mentioned difficulties, we have succeeded in collecting IWF from rice leaves without cytoplasmic contamination much more efficiently than before.

Materials and Methods

Sampling of plant leaves

Plant leaves of rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), snap bean (*Phaseolus vulgaris* L.) and spinach (*Spinacia oleracea* L.) were randomly sampled from experimental fields in the National Institute for Agro-Environmental Sciences, central Japan (36°01′N, 140°07′E; elevation, 25 m). These crops were cultivated using common agronomic practices in this region following the crop calendar shown in **Table 4**. For rice plants, two cultivars, Koshihikari, a Japonica type, and IR 72, an Indica type, were used in the present study.

Plant leaves were sampled between 09:00 and 10:00 h. The sampled leaves were placed in plastic bags for soybean and spinach and in 500 ml beakers with 50 ml of water for rice and wheat. The leaf samples were transported to the laboratory within 10 min after sampling, and were immediately cut into segments. Leaves of snap bean and spinach were cut into segments of 3 cm × 4 cm using a razor blade, avoiding the mid-vein. Leaves of rice and wheat were cut into segments with a length of 5 and 2 cm, respectively, using scissors. The upper half of the leaf blade having a thin mid-vein except for the apical region was used as the sample for rice and wheat. The fresh weight of each test sample was 1.0–1.5 g.

Measurement of stomatal diffusive conductance

Stomatal diffusive conductance to water vapor was individually measured for both abaxial and adaxial surfaces of five flag leaves in rice cultivar Koshihikari using a portable steady-state porometer (LI-1600, Li-Cor Inc.) with an attached 1600-01 narrow aperture. The measurements were carried out with pot-grown rice plants from 10:00 to 14:00 h on a clear day in October.

Collection of IWF

An infiltration–centrifugation method (Klement 1965, Luwe et al. 1993, Burkey 1999, Turcsányi et al. 2000, Hayashi et al. 2008) was applied to collect the IWF from spinach, snap bean and wheat. With rice, however, we encountered difficulties in collecting IWF from the leaves, and, hence, modifications were explored as noted later.

Infiltration for spinach, snap bean and wheat

Leaf segments were rinsed with deionized water and blotted dry with paper towels, and the initial fresh weight was determined. The leaf segments were suspended in 50 ml of 100 mM KCl using a 60 ml polyethylene syringe with a three-way stopcock and a stopper to maintain depressurization (DIK-8392-12, Daiki Rika Kogyo), as described previously (Hayashi et al. 2008); infiltration to replace the leaf air spaces with a 100 mM KCl solution was carried out manually under repeated applications of alternating vacuum and pressure.

The pressures for depressurization and pressurization by compressing 10 ml of air in the syringe corresponded approximately to 0.033 and 0.481 MPa, respectively. One cycle of vacuum and pressure took about 40 s. We determined the infiltration to be completed when the leaf color turned darker as the apoplastic air was replaced with the solution. The infiltration was completed within 2–3 min for the leaves of spinach, snap bean and wheat. Immediately after the infiltration, the leaf segments were blotted with paper towels and reweighed.

Infiltration in rice leaves

With the leaves of rice, the infiltration took much longer than for the other species. The infiltration time was particularly long as the rice plants became older: 9–12 min on days 37–46, 15–23 min on days 47–80 and 30–35 min on day 81 or later after transplanting, whereas, in the early growth stage of 31 d

Table 4 Species, cultivars and crop calendar of the plants used in this study

Species	Cultivar	Date			
		Sowing	Transplanting	Heading/flowering	Harvest
Spinach	Active	May 21, 2009	–	–	July 3, 2008
Snap bean	Aron	August 19, 2008	–	September 15, 2008	October 15, 2008
Wheat	Ayahikari	November 27, 2007	–	April 28, 2008	June 15, 2008
Rice	Koshihikari	April 15, 2008 April 15, 2009	May 12, 2008 May 12, 2009	August 1, 2008 August 2, 2009	September 16, 2008 September 18, 2009
Rice	IR72	May 12, 2008	June 4, 2008	August 25, 2008	October 14, 2008

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after transplanting, the infiltration time was comparable with that for the other species. To shorten the infiltration time for rice, we tried improvements in the following three aspects: (i) Light environment during the infiltration: infiltration time was compared between outdoor shade and laboratory room light. The outdoor shade enables infiltration under higher light intensity than the room light, while avoiding an extreme rise in the infiltration medium temperature under direct sunlight. (ii) Leaf surface wettability: in both rice cultivars of Koshihikari and IR 72, the leaves were strongly hydrophobic as evidenced by the silvery air layers on their surfaces when they are soaked in water. This phenomenon was more pronounced in hardened leaves of aged plants. To prevent formation of an air layer on leaf surfaces, we made leaf segments easily wettable by soaking either in deionized water for 20 min or in a surfactant solution for 2 min prior to the infiltration. We tested if the infiltration can be accelerated by increasing the wettability. (iii) Retention of high pressure: we used a sealant injector with a syringe holder of 280 mm in length and 53 mm in diameter to facilitate the retention of the pressure without the need for manual labor. See **Fig. 3** for a syringe set in a sealant injector.

Details of these methods are described in the Results section along with their efficacy in shortening the infiltration time.

Rice leaf segments with a length of 5 cm were soaked in a 0.05% (v/v) Triton X-100 solution, a surfactant, for 2 min to increase leaf wettability, since the surfaces of rice leaves were hydrophobic. The segments were rinsed several times with deionized water and blotted dry with paper towels, and the initial fresh weight was then determined. The infiltration was carried out under relatively strong light in the outdoor shade to prevent stomatal closure without raising the solution temperature. Similarly to other plant species, rice leaf segments were manually infiltrated for 3 min using a 60 ml syringe with a three-way stopcock as described above. Ambient air with a volume of 10 ml was drawn into the syringe. The syringe containing infiltration solution and leaf segments was then set in a sealant injector, i.e. a hand-held apparatus for filling the gaps with silicone or acrylic sealant, to retain the pressurization for a long time. The inner pressure of the syringe was kept at maximum pressurization until the infiltration was completed (in 3–5 min). Immediately after the infiltration, the leaf segments were rinsed with deionized water, blotted with paper towels and reweighed.

Collection of IWF in spinach and snap bean leaves

Each leaf segment of spinach and snap bean was quickly rolled and inserted into a centrifuge tube with an airtight stopper [inside diameter, 1.4 cm; length, 6.5 cm (Ultrafree-CL, Millipore)] which had been weighed beforehand. The leaf segment became unrolled and stuck to the centrifuge tube wall. Centrifugation was conducted at $400\times g$ for 10 min for spinach (Speer and Kaiser 1991) and at $556\times g$ for 5 min for snap bean (Burkey 1999). The centrifuge tubes were reweighed following centrifugation to determine the volume of the collected IWF.

Collection of IWF in wheat

Several leaf segments of wheat were inserted into a centrifugal filter unit (inside diameter, 1.2 cm; length, 3.5 cm) attached to a centrifuge tube (Ultrafree-CL, Millipore). The microporous membrane of the filter unit was removed in advance to avoid the absorption loss of IWF. The IWF was collected at $1{,}000\times g$ for 10 min at 4°C.

Collection of IWF in rice leaves

In the case of rice leaves, placing more than one leaf segment in each centrifuge tube resulted in low recovery rates of the IWF because the IWF seeping from the leaves stuck to the interspaces of the leaves. The IWF that stuck to the leaf surfaces could be collected by using a pipet, which was very time-consuming and, therefore, inadequate for unstable constituents, such as ascorbic acid, a target substance. This problem was solved by inserting each leaf segment of a length of about 5 cm into a pipet tip of 1 ml, which was then placed into a centrifuge tube (Ultrafree-CL). Centrifugation was conducted at $6{,}000\times g$ for 15 min at 4°C. The IWF flowed out from the tip of the pipet tip without sticking to the leaf surfaces and accumulated at the bottom of the centrifuge tube. The operational sequence of the infiltration–centrifugation method for rice is shown in **Fig. 1**.

Determination of leaf apoplastic air and water volumes

The apoplastic air and water volumes were determined according to Husted and Schjoerring (1995), but a solution of Blue dextran 2000 (GE Healthcare) was used to determine the apoplastic water volume in place of Indigo carmine solution.

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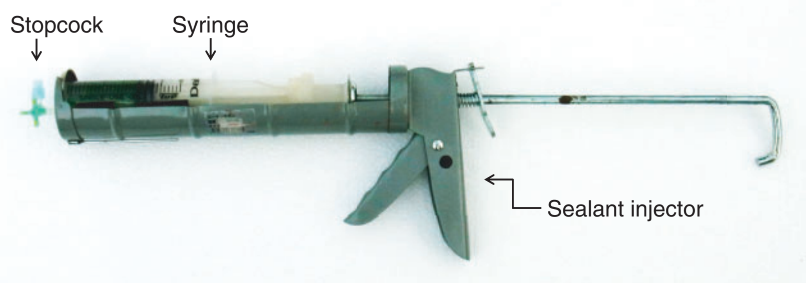

Fig. 3 A syringe installed in a sealant injector.

Apoplastic air volume

Five leaf segments with an area of 12 cm^2 (3 cm×4 cm) for spinach and snap bean and 10 leaf segments with a length of 5 cm for rice and wheat were weighed. The leaf segments were infiltrated with polydimethylsiloxane (silicone oil) with a kinetic viscosity of 5 $mm^2 s^{-1}$ (25°C; KF-96L-5cs, Shin-Etsu Chemical) using a 60 ml syringe with the same specifications as described above.

The leaf segments were floated on the water surface in a tray after infiltration to prevent leaf desiccation and rolling, which tends to take place more easily in leaf segments infiltrated with silicone oil than in those infiltrated with water. The oil and water on the leaf segments were wiped away with tissue paper, and the leaf segments were reweighed. The apoplastic air volume (V_{air}, $cm^3 g^{-1}$ LFW) was determined by dividing the increase in leaf weight by the density of silicone oil (0.915 g cm^{-3}; 25°C) and the leaf fresh weight. The amount of silicone oil adsorbed to the cuticle made an insignificant contribution (Husted and Schjoerring 1995).

Apoplastic water volume

Five leaf segments with an area of 12 cm^2 (3 cm×4 cm) for spinach and snap bean, 16 leaf segments with a length of 5 cm for rice, and 32 leaf segments with a length of 2 cm for wheat were weighed (W_{INI}, g LFW). The leaf segments were infiltrated with 0.20% (w/v) Blue dextran 2000 dissolved in a 100 mM phosphate buffer solution at pH 6.2 using a 60 ml syringe with the same specifications as described above.

After the infiltration, the leaf segments were washed with deionized water, blotted dry with paper towels and reweighed. Assuming a specific density of 1 g cm^{-3}, the infiltration volume (V_i, cm^3) was estimated from the difference in leaf weight before and after the infiltration. Next, the leaf segments were put into the centrifuge tube, and the tube was centrifuged at the same centrifugation force described above for each plant procedure. The absorbance of about 100 µl from the collected IWF at 610 nm was determined using a spectrophotometer (DU800, Beckman Coulter). The apoplastic water volume (V_{wat}, $cm^3 g^{-1}$ LFW) was calculated by the following equation (van Hove et al. 2001),

$$V_{wat} = \frac{V_i\left(\left(A_{610,b} - A_{610,a}\right)/A_{610,a}\right)}{W_{INI}}, \quad (3)$$

where $A_{610,b}$ and $A_{610,a}$ denote the absorbance of Blue dextran 2000 solution at 610 nm before and after infiltration, respectively.

Cytoplasmic contamination in IWF and leaf tissue extracts

The G6PDH activity was used to assess the degree of cytoplasmic contamination in IWF (Lyons et al. 1999). The activity was measured following the reduction of NADP at 340 nm using a solution with a final volume of 1 ml, which contained 66 mM KH_2PO_4/K_2HPO_4 (pH 7.6), 10 mM $MgCl_2$, 300 µM NADP, 2 mM glucose-6-phosphate (G6P) and 100 µl of IWF or 50 µl of leaf tissue extracts. The G6PDH activity in the IWF was calculated using a molar absorption coefficient of 6.22 $mM^{-1} cm^{-1}$ for NADPH at 340 nm. All assays were performed in triplicate. Since the IWF is a mixture of the infiltrated solution and the original apoplastic solution, the measured G6PDH activity was multiplied by the sum of the apoplastic water and air volumes ($V_{wat} + V_{air}$) to yield G6PDH activity per gram of leaf fresh weight.

In addition, leaf tissue extracts were obtained by homogenizing 0.25 g LFW of leaf tissue in 5.0 ml of 100 mM KCl using a mortar and pestle. The homogenate was transferred to a 10 ml centrifuge tube and centrifuged at 10,000×g for 10 min at 4°C. The G6PDH activity of the supernatant was also measured.

Potassium concentration in apoplast and leaf tissue

K^+ concentrations of apoplastic solutes were measured. K^+ concentrations in leaf tissue were also measured for comparison with those of the apoplast. For measurement of the K^+ concentration, 350 mM sorbitol solution was used as the infiltration solution instead of 100 mM KCl solution (Hayashi et al. 2008). Sixteen leaf segments of 5 cm in length were used for the IWF collection as described above. On the other hand, leaf tissue extracts were obtained by homogenizing 0.35 g LFW of leaf tissue in 5.0 ml of 350 mM sorbitol using a mortar and pestle. The K^+ concentrations were determined using capillary electrophoresis (G1600A, Agilent Technologies). The apoplastic K^+ concentration was calculated by multiplying the K^+ concentration of IWF by the dilution factor F_{dil} (Husted and Schjoerring 1995):

$$F_{dil} = \frac{(V_{air}+V_{wat})}{V_{wat}} \quad (4)$$

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