Regulation of Photosynthesis and Water Use Efficiency in Relation to Stomatal Frequency and Intervereval Distance in C3- and C4-Grass Species

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The gas exchange rates of C3- and C4-grass leaves at low and high vapor pressure difference (VPD) conditions were measured and demonstrated the effects of anatomical and morphological features of the leaves, i.e. stomatal frequency and size and interveinal distance (IVD), on gas exchange characteristics. When the photosynthesis type was ignored, there was a positive correlation between stomatal frequency and stomatal conductance. However, there was less significant correlation between stomatal frequency and transpiration rate when plotted species within the same photosynthetic pathway. There was a negative correlation between stomatal frequency and net photosynthetic rate, suggesting that CO2 diffusion process from ambient to intercellular space was not much affected by the stomatal morphology, instead that a consequence of biochemical differences in photosynthetic characteristics of the mesophyll was more important. An increase in IVD significantly decreased net photosynthetic rate and water use efficiency (WUE). In addition to the regulation of CO2 concentrating mechanism in Kranz cells of the C4 subtypes, a rapid translocation of photosynthate from bundle sheath cells to the phloem in grasses with C4-MS type (NADP-ME) may contribute to maintain their high performances in photosynthesis and WUE through shorter IVD.

Keywords : guard cell, Kranz anatomy, net photosynthesis, stomatal conductance, transpiration, vapor pressure difference

INTRODUCTION

Previously, we found an inverse relationship between stomatal frequency and guard cell length in grass species (Kawamitsu et al., 1996), and that the distribution of subfamilies depicted in the figure agreed well with the dendrogram of the evolution in Gramineae (Tateoka, 1959). Even though it is anticipated that stomatal frequency and size of guard cells play an important role in photosynthesis and transpiration, few reports discussed them in terms of gas diffusions. The optimum number of stomata to maintain high photosynthetic rate with less transpiration was uncertain in respect to the water use efficiency (WUE). Yoshida (1976, 1977) reported that there was significant positive correlations between stomatal number and net photosynthetic rate, and transpiration rate in barley cultivars. On the other hand, Miskin and Rasmusson (1970) and Miskin et al. (1972) showed that stomatal frequency of barley had a positive correlation with transpiration rate, but not for photosynthetic rate, and suggested...
that a possibility of selection for a variety with a high WUE. When variations in stomatal frequency and size are extended more by increasing the number of species, this relationship may be modified.

The differences in interveinal distance (IVD) is one of the anatomical features in C₃- and C₄-grass species (Takeda and Fukuyama, 1971; Hattersley and Watson, 1976). In grass species with parallel venation, the anatomy of bundle sheath was classified into C₄-MS (mestome sheath), C₄-PS (parenchyma sheath), and C₄-NK (non-Kranz) types in relation to the mestome sheath cells located between metaxylem and bundle sheath cells (Brown, 1977), and the IVD was also different in these 3 types (Kawamitsu et al., 1985). Although both C₄-MS and C₄-PS types have Kranz structure in leaves, the IVDs of C₄-MS type are shorter than those of C₄-PS type (Kawamitsu et al., 1985) and their roles on physiological characteristics are uncertain. In C₄ species, the photosynthate finally produced in the bundle sheath cell is translocated to the sink organs by way of the phloem in vascular bundle. If the vein density per unit leaf area is increased, the translocation of photosynthate may be smooth and the feedback inhibition of photosynthate during the light period may be minimized. Therefore, it is speculated that photosynthetic rate in C₄-MS type, i.e. *Pennisetum purpureum*, *Saccharum officinarum*, *Sorghum bicolor* and *Zea mays* may be maintained at high level (Kawamitsu et al., 1985).

In the present work, we examined gas exchange rates, stomatal frequency and size, and IVDs of C₃- and C₄-grass species to relate their gas exchange rates and morphological characteristics.

**MATERIALS AND METHODS**

**Plant materials.** Plant materials used in the present work are listed in Table 1. Seeds of the materials except rice (*Oryza sativa*) were sown directly to 1/2 000 a pots which contained a mixture of sand and soil (1 : 1, v/v), and they were thinned to one per pot after germination. Compound fertilizer (N : P : K = 16 : 16 : 16, %) was given as basal fertilization (10 g). Rice plants at the 5th-leaf stage were transplanted and cultivated in flooding conditions with the same volume of pots containing paddy soil.

**Gas exchange measurements.** The leaf gas exchange rates were measured by means of a chamber method for the fully expanded, youngest leaves according to Agata et al. (1986) and Kawamitsu et al. (1993). Gas exchange rates were measured at 2.8 and 1.4 kPa of vapor pressure differences (VPDs) in which stomatal conductance were low and high, respectively (Kawamitsu et al., 1987). The measurements started at 1.4 kPa of VPD and then changed to 2.8 kPa, thereafter the VPD returned to 1.4 kPa in order to check the stomatal response. The

<table>
<thead>
<tr>
<th>C₃-MS type (NADP-ME)</th>
<th>C₄-PS type (NAD-ME, PEP-CK)</th>
<th>C₄-NK type</th>
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<tbody>
<tr>
<td><em>Arundinella hirta</em></td>
<td><em>Brachiaria brizantha</em></td>
<td><em>Arundo donax</em></td>
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<tr>
<td><em>Cenchrus ciliaris</em></td>
<td><em>Chloris gayana</em></td>
<td><em>Saccharum indica</em></td>
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<tr>
<td><em>Hyparrhenia rufa</em></td>
<td><em>Eleusine coracana</em></td>
<td><em>Oryza sativa</em> cv. Nipponbare</td>
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<tr>
<td><em>Panicum antidotale</em></td>
<td><em>Eragrostis ferruginea</em></td>
<td><em>O. sativa</em> cv. Norin No.21</td>
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<tr>
<td><em>Paspalum dilatatum</em></td>
<td><em>Leptochloa chinensis</em></td>
<td><em>O. sativa</em> cv. Nagakurabozu</td>
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<td><em>Pennisetum dandestinum</em></td>
<td><em>Panicum dichotomiflorum</em></td>
<td><em>Phalaris arundinacea</em></td>
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<td><em>Miscanthus sinensis</em></td>
<td><em>P. maximum</em></td>
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<td><em>M. sinensis</em> var. condensatus</td>
<td><em>P. coloratum</em> var. makarikariense</td>
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<td><em>Setaria italica</em></td>
<td><em>P. coloratum</em> var. kabulabula</td>
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<td><em>Sorghum bicolor</em></td>
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<td><em>S. sudanense</em></td>
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recovery was observed in all species and it was confirmed that stomatal response to VPD was reversible. If the gas exchange rate did not recover in that process, the plant was replaced and measured again. All gas exchange rates were measured at 2000 μmol m⁻² s⁻¹ PPFD, 30±0.2°C leaf temperature, and 350±10 ppm CO₂. Stomatal conductance was obtained based on the leaf temperature, air temperature, humidity, and transpiration rate (Moss and Rawlins, 1963; von Caemmerer and Farquhar, 1981).

Measurements of stomatal frequency and IVD. After the gas exchange measurement, part of the measured leaf was cut and fixed in FAA solution, and total stomatal frequency (TSF) on abaxial and adaxial surfaces and guard cell length (long axis; GCL) were examined under optical microscope when chlorophyll was disappeared. The IVD of the leaf was measured after Kawamitsu et al. (1985).

RESULTS

The relationships between stomatal conductance and stomatal frequency in C₃- and C₄-grass species under two VPDs are shown in Fig. 1. There was a positive correlation between both parameters at 1.4 kPa, while it became less significant at 2.8 kPa (Fig. 1, Table 2). This indicated that the number of stomata did not affect the stomatal conductance when stomatal aperture was small. In addition, there was a high, positive correlation between TSF×GCL and stomatal conductance. It was speculated that, in C₃-NK type, increased

![Fig. 1 Relationship between stomatal frequency and stomatal conductance in grass species.](image1)

![Fig. 2 Relationship between stomatal frequency × guard cell length and stomatal conductance in grass species.](image2)
<table>
<thead>
<tr>
<th></th>
<th>E(1.4)</th>
<th>gs(1.4)</th>
<th>WUE(1.4)</th>
<th>P(2.8)</th>
<th>E(2.8)</th>
<th>gs(2.8)</th>
<th>WUE(2.8)</th>
<th>IVD</th>
<th>TSF</th>
<th>AVEGCL</th>
<th>TSF × GCL</th>
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<tbody>
<tr>
<td>P(1.4)</td>
<td>0.161</td>
<td>0.136</td>
<td>0.536**</td>
<td>0.956***</td>
<td>0.471**</td>
<td>0.453**</td>
<td>0.347</td>
<td>-0.607***</td>
<td>-0.259</td>
<td>0.179</td>
<td>-0.239</td>
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<tr>
<td>E(1.4)</td>
<td>0.987***</td>
<td>-0.630***</td>
<td>0.099</td>
<td>0.825***</td>
<td>0.834***</td>
<td>-0.581**</td>
<td>0.366*</td>
<td>0.586**</td>
<td>-0.164</td>
<td>0.663***</td>
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<tr>
<td>gs(1.4)</td>
<td>-0.602***</td>
<td>0.080</td>
<td>0.825***</td>
<td>0.844***</td>
<td>-0.576**</td>
<td>0.391*</td>
<td>0.607***</td>
<td>-0.173</td>
<td>0.690***</td>
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<tr>
<td>WUE(1.4)</td>
<td>0.553**</td>
<td>-0.235</td>
<td>-0.241</td>
<td>0.687***</td>
<td>-0.685***</td>
<td>-0.502**</td>
<td>0.165</td>
<td>-0.552*</td>
<td>0.187</td>
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<tr>
<td>P(2.8)</td>
<td>0.464*</td>
<td>0.444*</td>
<td>0.995***</td>
<td>-0.490**</td>
<td>0.095</td>
<td>0.365*</td>
<td>-0.066</td>
<td>0.446*</td>
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<tr>
<td>E(2.8)</td>
<td></td>
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<td>-0.487**</td>
<td>0.114</td>
<td>0.384*</td>
<td>-0.070</td>
<td>0.466*</td>
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<tr>
<td>gs(2.8)</td>
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<td>-0.710***</td>
<td>-0.417*</td>
<td>0.040</td>
<td>-0.505**</td>
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<td>IVD</td>
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<td>0.347</td>
<td>0.078</td>
<td>0.498**</td>
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<td>TSF</td>
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<td></td>
<td>-0.730***</td>
<td>0.897***</td>
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<tr>
<td>AVEGCL</td>
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<td>-0.421*</td>
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P, net photosynthetic rate; E, transpiration rate; gs, stomatal conductance; WUE, water use efficiency; IVD, interveinal distance; TSF, total stomatal frequency; AVEGCL, averaged guard cell length; TSF × GCL, stomatal frequency times guard cell length. (1.4) and (2.8) mean that the parameters measured at 1.4 and 2.8 kPa VPD.

*, **, ***: significant at the 0.05, 0.01, 0.001 porbability levels, respectively. n = 36.
stomatal number per unit leaf area directly correlated to the high stomatal conductance (Fig. 2, Table 2). The correlation decreased when VPD increased (Table 2). In C₃-NK types, there are species growing in winter or in summer. The relationship between stomatal frequency and guard cell length of the winter C₃-NK types was close to that of C₄ types in addition to the lower stomatal conductance. The correlation between stomatal frequency and photosynthetic rate were not recognized (figure not shown, Table 2).

The IVDs of C₄ species are shorter than those of C₃ species and ascribed to their anatomical characteristics of the species with C₄ photosynthesis (Takeda and Fukuyama, 1971; Hattersley and Watson, 1976). The IVD and net photosynthetic rate had a negative, significant correlation regardless of VPD conditions (Fig. 3, Table 2). Among plots, the C₄-MS type located in the left portion, C₃-NK type in the right portion, and C₄-PS type in the middle portion.

When measured simultaneously, there was a significantly positive correlation between stomatal conductance and net photosynthetic rate (Table 2). The relationship in C₄ plants was close to linear regression, however, that in C₃ plants was a saturated curve (Fig. 4). Among C₄ species, both MS and PS types were on the same regression line, suggesting that the difference in Kranz anatomy does not affect the relation. When VPD increased, the trend of saturation curve in C₃ species did not change although the initial slope became steeper. The differences in initial slopes between C₃ and C₄ species reflect characteristics of the CO₂ fixation enzyme in the mesophyll cell and agree well with differences in the affinity of ribulose
1,5-bisphosphate carboxylase/oxygenase (Rubisco) and phosphoenolpyruvate carboxylase (PEPCase) to $\text{CO}_2$.

Figure 5 showed that the effect of IVD on instantaneous WUE, the ratio of net photosynthetic rate to transpiration rate. Regardless of VPD, there was a significant correlation between both parameters (Table 2). In Fig. 6, the relationship between IVD and stomatal frequency was classified by the forms of vascular bundle. As a result, each type had own linear regression, indicating that there was a close relationship between Kranz anatomy and stomata. Table 2 shows the correlation matrix between parameters measured, and it is effective when the above-mentioned results are examined in detail. Especially, there was a significant correlation between IVD and WUE when VPD was 2.8 kPa ($r = -0.710^{***}$). The decreasing order in coefficients was clearly shown between IVD and WUE at 1.4 kPa VPD ($r = -0.685^{***}$), IVD and net photosynthetic rate at 2.8 kPa VPD ($r = -0.649^{***}$), and IVD and net photosynthetic rate at 1.4 kPa VPD ($r = -0.607^{***}$). In addition, stomatal conductance at 1.4 kPa VPD had high correlations between TSF ($r = 0.607^{***}$), and TSF $\times$ GCL ($r = 0.690^{***}$), respectively. There was a significant correlation between stomatal conductance and transpiration rate because all measurements were conducted under stable VPD in the assimilation chamber.
DISCUSSION

The stomata surrounded by pairs of guard cells on the leaf surface are the only moving portions of immovable plants. In general, stomata control both photosynthesis and transpiration, simultaneously, and are always exposed to the dilemma, since impossible to give the priority to each of them (Meidner and Mansfield, 1968). However, CAM species have very high WUE because of their peculiar stomatal reactions (Ting, 1987). At night when transpiration requirement decreases, their stomata open and fix ambient CO₂ by PEPCase as malic acid. During the light period, the decarboxylated CO₂ from the stored malic acid is refluxed as starch and sucrose under conditions when stomata are closed to minimize the loss of water (Kluge and Ting, 1978; Winter and Smith, 1996). On the other hand, mesophyll cells in C₄ species efficiently fix CO₂ by PEPCase, which has a higher affinity to CO₂, to overcome the conditions of partly closed stomata to minimize the disadvantageous loss of water by transpiration. Then, the WUE in C₄ species is maintained at higher level than that in C₃ species (Long, 1999).

To begin with, we examined the factors, except for the biochemical ones, which affect WUE in C₃- and C₄-grass species having a parallel venation. It has been indicated that WUE in C₃ species was lower than those in C₄ species (Long, 1999). However, reports dealing with the relation between stomatal frequency, the gateway of CO₂ and water vapor, and IVD, or between WUE and vascular bundle including phloem and xylem are not known so far.

In C₃- and C₄-grass species, both of stomatal conductance and transpiration rate increased when the stomatal number on the leaf surface increased (Fig. 1). In contrast, the TSF had less effect on net photosynthetic rate, and it seemed to have closely or entirely connected with the factor(s) concerning water vapor diffusion (Table 2). The TSF×GCL of C₃-NK type, the proportion of stomatal area per unit leaf area, was higher in the summer-growing species. The correlation between TSF×GCL and stomatal conductance had a higher value when compared with that between TSF and stomatal conductance (Table 2). From these results, the TSF×GCL was the best parameter expressing the open area of stomata in comparison with stomatal frequency. In addition, it is anticipated that correlation coefficient increases when VPD is low, and decreases when VPD is high. One of the causes of lowering the correlation coefficient may be the non-uniform stomatal closure when VPD is high (Terashima et al., 1988). Significant correlation between TSF and net photosynthetic rate, or between TSF×GCL and net photosynthetic rate were not observed in the present experiment (Table 2). Therefore, one can ignore the stomatal frequency to maintain or elevate net photosynthetic rate in a breeding program.

In grass species, transverse vascular bundles with parallel venation system play an important role in photosynthesis (Altus and Canny, 1982). There were significant, negative correlations when the effects of IVD on net photosynthetic rate were examined among all grass species under two contrasting VPDs (Fig. 3, Table 2). Although we can not deny that the biochemical factor affects this relation, the effect of morphological factors seems to be very important because the effect of VPD is disregarded.

Vascular bundle tissue of the leaf is structurally important as a skeleton which supports the leaf. The long leaf must have sturdy mid-rib and high-density vascular bundles, to support the structure and simultaneously, to transfer the photosynthe and water smoothly. *Pennisetum purpureum*, *Saccharum officinarum*, *Sorghum bicolor* and *Zea mays* have large leaves and belong to C₄-MS type. On the other hand, *Chloris gayana*, *Panicum dichotomiflorum* and *Panicum maximum* have smaller leaves and belong to C₄-PS type. In C₄-MS type, the bundle sheath cell seemed to originate from mestome sheath cell of the C₃-NK type.
type and the cell wall is thicker than that of C₄-PS type. The Kranz anatomy of C₄-MS type is single sheathed cells and that of C₄-PS type is double sheathed with one from the mestome sheath cells (Hattersley and Watson, 1976; Brown, 1977). The fiber in the leaf of C₄-PS type is less than that of C₄-MS type and good for the digestion of domestic animals, according to the research on the livestock production (Hattersley and Watson, 1976).

We have previously proposed the possibility of the relation of IVD to the differences of translocation rate between C₄-MS and C₄-PS types (Kawamitsu et al., 1985). Sucrose and starch produced by photosynthesis must translocate rapidly out of the chloroplasts in order to maintain the source activity. Photosynthetic rate of C₃-NK species such as rice is maintained a higher level in the morning, and the feedback inhibition by photosynthate accumulation is considered as the mid-day depression of photosynthesis (Hirasawa et al., 1992). In the C₃-NK type, the IVD of the rice plants is very long and the distance between mesophyll cell and vascular bundle phloem is also 100–200 µm (Kawamitsu et al., 1996). In case of C₄ species, the distance between mesophyll cell and phloem tissue is at most 60 µm, and that of bundle sheath cell and phloem is 40 µm due to the Kranz structure. The differences of these IVDs, in conjunction with TSF (Fig. 6), seem to affect net photosynthetic rate in both types of species. If the IVD of rice plant could be shortened equal to or close to C₄-PS type, the translocation rate might be remarkably promoted and net photosynthetic rate could be elevated substantially. Therefore, the physiological, biochemical and morphological characteristics of the leaves concerning C₄-MS and C₄-PS types seem to give these effects significantly.

Parameters such as net photosynthetic rate, transpiration rate, stomatal conductance and intercellular CO₂ concentration were monitored simultaneously during our gas exchange measurements. The stomatal conductance and net photosynthetic rate had a linear regression in many cases, and the correlation coefficient was significant. When plotted them on figures, the saturated curves for C₃ species and linear regressions for C₄ species were obtained (Fig. 4). In addition, when the high VPD increased the gradient of regression, the lines shifted to left portions, with the difference between C₃ and C₄ species remained unchanged. Therefore, the difference in the substrate kinetics of CO₂ fixing enzyme in both species reflected on the gradient of initial slope. It was recognized that there was two times difference between net photosynthetic rates in C₃ and C₄ species under identical stomatal conductance. Net photosynthetic rate showed the rectilinear increase regardless of the form of vascular bundle, when stomatal conductance increased up to 200 mmol m⁻² s⁻¹ at 2.8 kPa VPD and 400 mmol m⁻² s⁻¹ at 1.4 kPa VPD. Thereafter, the gradient decreased and photosynthesis attained at saturation in C₃ species. Up to the turning point in which the relation between photosynthetic rate and stomatal conductance is straight line, where the stomatal control is significant, followed by the other factors seemed to lower net photosynthetic rate. The data of rice varieties in Fig. 4 were well fitted to the regression line of C₃-NK type plants. Therefore, it may be difficult to promote net photosynthetic rate in case of rice variety even if the stomatal numbers are increased, and even if the stomatal conductance are elevated as a result. In C₄-grass species, their stomatal conductance did not saturate.

The increase in IVD remarkably lowered the WUE (Fig. 5). In comparison with the relationship between TSF and WUE, the correlation coefficients of the relation between IVD and WUE are more significant ($r = -0.685^{***}$ at 1.4 kPa VPD, $r = -0.710^{***}$ at 2.8 kPa VPD; Table 2). There is a report that in wheat (C₃-NK type), the IVD of drought-resistant variety is shorter than that of drought-sensitive variety, and that grain yield of the former is also higher than the latter (Hattalli et al., 1993).

It is concluded that the IVD is one of the factors which elevates the WUE of grass species and a useful characteristic in the breeding programs.
We thank Prof. Katsu Imai, Meiji University, and anonymous reviewers for editing English and constructive comments on the manuscript.

REFERENCES


<和文抄録>

イネ科 C₃, C₄植物における光合成および水利用効率の制御と気孔密度 および維管束間距離の関係

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本研究は，葉の内外部形態が純光合成速度と水利用効率に及ぼす影響を明らかにするため，気孔開度の著しく異なる葉面表裏下で葉のガス交換速度を測定し，検討を加えたものである。光合成型を無視した場合，気孔密度と気孔コンダクタンスとの間に有意な相関が認められ，気孔数が増大すると蒸散速度が増大することが示された。同じ光合成型植物内では，気孔密度と気孔コンダクタンスおよび蒸散速度との間に有意な相関は認められなかった。気孔密度と純光合成速度との間には負の相関があり，外圏空気から気孔間隙までの CO₂の拡散には気孔形態の影響は小さいことがなく，むしろ葉肉細胞の光合成特性に関連する生化学の違いが重要と考えられた。維管束間距離が増大すると純光合成速度と水利用効率は低下した。これらより，C₃植物ではクランツ細胞内の CO₂濃縮機構に加え，とくに維管束間距離が短い C₄-MS 型は，維管束鞘細胞から師部への光合成物の迅速な移動が光合成速度と水利用効率を高く維持するものと結論された。