Observations of Cross Veins of the Second Foliage Leaf Blade in the Rice Plant by Use of a Revised Method for Clearing Leaves*

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Received November 9, 1981

The photosynthetic rate largely depends on the following two processes; the CO₂-fixation in mesophyll and the transport of photosynthetic products from mesophyll. The latter process seems to be closely related to the development and the arrangement of veinlets within a leaf, since the veinlets occupy terminal positions and are surrounded with mesophyll.

The terminal veinlets of the plant with netted venation are named vein-endings,³⁾ and those of the plant with parallel venation are named variously; commissural vascular bundle,¹⁰⁾ connecting strand,¹⁶⁾ cross vein,¹⁾ transverse strand¹²⁾ or transverse bundle.²⁾ Hereafter, we will call them cross veins.

These two kinds of veinlets have a similar mode of origin and structure,^{1,3,8,12)} excluding whether they have free-ends or not; veinlets of the plant with netted venation have their ends open to the mesophyll, while veinlets of the plant with parallel venation do not have the open ends because their ends join with neighbouring longitudinal bundles.

Results on experiments of vein-cutting,^{9,15)} and dye¹⁶⁾ and ion¹²⁾ movement along veinlets suggest that the veinlets provide the route for lateral conduction of water and nutrients within the leaf. The vein-endings of the sugar beet leaf were demonstrated to absorb and transport rapidly ¹⁴C-photosynthates from the mesophyll.⁵⁾ These results indicate that veinlets, including vein-endings, play important roles in the trans-

port of photosynthate as well as in the conduction of water and nutrients. As compared with such roles of vein-endings, however, those of cross veins have not yet been investigated thoroughly.

Most leaf-clearing techniques have been developed in connection with studies on the venation of dicotyledonous leaves.^{4,11)} However, these techniques do not always give good results for the graminaceous leaf because its texture is different from that of dicotyledonous leaf.

In the present study, the revised method of whole leaf clearing was tested to observe the venation pattern in the rice leaf. For this study, the venations of the second foliage leaf blades of seedlings originating from seeds of different nitrogen contents were comparatively observed. The results obtained are discussed in relation to the arrangement of cross veins within a leaf and the interconnecting patterns among the veins, as compared with those obtained by other investigators.^{1,2,10,12,16)}

Materials and Methods

Two kinds of seeds with different nitrogen contents, used in this experiment, were prepared as follows; rice plants (*Oryza sativa* L. cv. Chyukyoasahi) were grown under two nitrogen regimes under field conditions. One of these groups of plants was top-dressed with 6.8 kg of ammonium sulfate per 10a at the heading stage and others were left untreated. Seeds of the first, fourth, fifth and sixth insertions from the top of the uppermost primary branch of forty panicles in both plants were selected. The average nitrogen contents of the seeds of the top-dressed plants and the untreated

* The outline of this paper was presented at the 172th Meeting of the Crop Science Society of Japan (Oct. 7, 1981).

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ones were $353.3 \pm 2.0 \mu\text{g}$ and $285.8 \pm 3.1 \mu\text{g}$ per seed, respectively.

Selected seeds were soaked in tap water for 48 h in the dark at 30°C , then placed on a salan net floating in a vat with tap water in a growth cabinet set at 33°C for 16 h in the light (10 klx intensity) and 25°C for 8 h in the dark.

At the ninth day after planting, the second foliage leaf blades were sampled and stored in a mixture of glacial acetic acid-95% ethanol, 1:3. Stored laminae were used to test several clearing reagents which render a leaf transparent and allow the venation to be seen *in situ*.

The cleared lamina specimens were photographed with the aid of an optical microscope and macrophotographic equipment (OLYMPUS Model PMT-35). Drawings of the venation patterns of the second lamina were made from tracings of the enlarged positives. The lamina was divided into three equal parts, top, middle and basal parts, along the longitudinal axis of lamina, then distribution of cross veins was determined. By the use of curvimeter and scale, the measurement of total lengths of vascular bundles by categories for the second lamina was made from the drawings of the venation pattern. Epidermal cell size of the lamina was measured as follows; excluding rows containing trichomes and stomata, cells were selected randomly from several rows of epidermal cells between small longitudinal bundles on either side of the lamina midrib on the adaxial (upper) surface.

Results and Discussion

1. Preparations of the whole-lamina clearing for the observation of venation patterns.

In this experiment the following six clearing reagents of the plant organs were compared; sodium hydroxide,^{4,16)} lactophenol,⁶⁾ Eau de Javelle (sodium hypochlorite),¹¹⁾ lactic acid,¹⁴⁾ methyl benzoate⁷⁾ and chloral hydrate.¹³⁾

The procedure involving the use of lactic acid and chloral hydrate gave the most distinct specimens to observe the venation patterns on the rice lamina, as compared with the other reagents. This procedure

consists of following three steps.

In the first step, the stored lamina was plunged into fresh fixation solution in a boiling water bath for 10 min. This period should be prolonged until 20 min, when a fresh lamina is used. Almost all chlorophyll was extracted, and the lamina became white and opaque in this step. After rinsing with distilled water, the lamina was transferred into a boiling 85% lactic acid in a water bath for 30 min. During this second step, the lamina was thoroughly decolorized and became translucent. Then, in the third step, the lamina was soaked in ethanol saturated with chloral hydrate for periods up to 1 h. The lamina was spread out flat on a deck glass and mounted in ethanol saturated with chloral hydrate. Then a coverslip was added. Staining was unnecessary.

Photographs of the specimens which were prepared by this procedure are shown in Figs. 1 and 3.

In contrast with the above procedures, when the rice lamina was treated with 5-10%⁴⁾ and 2%¹⁶⁾ aqueous solution of sodium hydroxide and with sodium hypochlorite, the softend lamina was too fragile and easily fractured. In these treatments, the progression of the decolorizing reaction within the lamina was sometimes uneven. In contrast, the lactic acid treatment was superior in completeness and in stability of the decolorizing process to the other reagents. Moreover, the lamina treated with lactic acid could be handled easily without breakage.

Although the testing of lactophenol and methyl benzoate gave good results with reference to transparency, the lamina treated with these reagents was apt to shrink and became too hard. Therefore, they were easily fractured in the mounting step. On the other hand, the lamina treated with ethanol saturated with chloral hydrate gave excellent results without shrinkage and folding-down of the leaf edges.

2. Shapes of cross veins and types of termination of small longitudinal vascular bundles.

There were no differences in morphological appearance of cross veins between the

Fig. 1 The second leaf blade (LN-lamina*) of rice cleared to show cross veins. a, whole lamina; b, c and d show partially magnified photographs of top, middle and basal of the lamina, respectively. Magnifications of a and b-d are $\times 11.5$ and $\times 40.5$, respectively. * see Table 1.

Fig. 2. Interconnection pattern of vascular bundles in the second leaf blade (HN-lamina*) of rice. The total lengths for the large longitudinal vascular bundles, the small longitudinal vascular bundles and the cross veins of the leaf shown in this figure were 71.5 mm, 155.5 mm and 52.0 mm, respectively. Numbers of the cross vein in top, middle and basal parts of the lamina were 70, 148 and 107, respectively. Densities** of the cross vein in top, middle and basal parts of the lamina were 11.1, 11.7 and 8.1, respectively.

* See Table 1. ** See Table 2.

Table 1. Quantitative leaf characters of the second foliage leaf blade (lamina) of rice.

Plot	Length in mm	Width in mm	Leaf area in mm ²
LN	13.7±0.5	1.9±0.0	23.5±2.0
HN	14.7±0.3	2.3±0.1	27.2±1.9

Note. Each value represented by Mean ± Standard error was calculated by the data from ten leaves. LN and HN mean the second leaf blades (laminae) of seedlings originating from seeds of low nitrogen content and those of high nitrogen content, respectively.

second lamina of the seedling originating from the seed of high nitrogen content (HN-lamina) and that from the seed of low nitrogen content (LN-lamina). The average length and thickness of cross veins for both laminae were more or less similar.

The cross veins were usually links between two longitudinal bundles. Their connecting forms were various; straight and at right angles to the longitudinal bundles, straight but oblique, variously curved or

sigmoid (Figs. 1, 2 and 3). Such shapes of cross veins in the lamina of rice and wheat are illustrated by KAUFMAN¹⁰⁾ and BLACKMAN,¹⁾ respectively. BLACKMAN suggested that these various shapes in the cross veins are produced as a result of minor inequalities during elongation of the mesophyll and the longitudinal vascular tissues of the leaf.

Figs. 2, 3-a, 3-b and 3-c show that most of the longitudinal bundles which do not extend to the leaf tip or leaf sheath, join into one or both of the neighbouring bundles by means of a veinlet or veinlets which are of the same order of size as the cross veins. When there are two, they manifest a Y- or T-shaped arrangement. Consequently, most of the tips of these longitudinal bundles end not freely.

Using the second lamina of the rice plant, cv. Sasanishiki, CHONAN *et al.*²⁾, Fig. 4, illustrate that more than half the number of the tips of large and small longitudinal bundles at the leaf tip end freely. This observation is apparently different from

Fig. 3. Terminals of small longitudinal vascular bundles at top (a and b) and at base (c) of the second leaf blade (HN-lamina*). A surface view of the epidermis in adaxial side of the second leaf blade (HN-lamina*) is shown in d.

Y- and T-shaped arrangements of veinlets are observed on the right side in b and on the left side in c, respectively. The arrow in b indicates that the tip of the small longitudinal vascular bundle slightly protrudes beyond the joining point with both interconnecting veinlets. Arrows in d indicate the two cross veins out of the plane of focus.

MR, Midrib; S, Stomata; T, Trichome. Magnifications of each photograph are × 140 in a and b, × 55 in c, and × 280 in d. * See Table 1.

Table 2. Longitudinal distribution of the cross veins in the second leaf blade of rice.

Part	LN*			HN*		
	No. of cross veins	Leaf area in mm ²	Density of cross veins	No. of cross veins	Leaf area in mm ²	Density of cross veins
Top	99.0±13.5	4.8±0.4	20.4±2.0	93.0±7.4	5.9±0.5	16.5±2.3
Middle	107.0±5.1	9.3±0.9	12.0±0.7	138.3±11.9	10.6±0.9	13.1±0.8
Base	91.0±6.7	9.3±0.2	9.8±0.7	104.7±6.7	10.7±0.7	9.8±0.8
Whole lamina	291.0±18.0	23.5±2.1	12.7±0.2	335.0±20.3	27.1±3.4	12.5±1.0

Note. Density is represented by number of cross vein per unit area (mm²). Each value is shown by Mean ± Standard error calculated by the data from five leaves. There was no significant difference found at the 5% level in number of the cross veins in top, middle and basal parts of the lamina and between corresponding parts of both laminae.

* See Table 1.

(Significance at 5% level (*) in the density of cross vein.)

Table 3. Comparison of the distances between successive cross veins and between longitudinal bundles at top, middle and basal parts of the second leaf blade of rice.

Plot	Top Distance between		Middle Distance between		Base Distance between	
	C.V.	L.V.	C.V.	L.V.	C.V.	L.V.
LN*	453.9±26.8	118.0±3.1	580.0±22.2	129.2±4.0	643.8±37.5	153.3±8.1
HN*	498.0±23.8	129.4±4.5	603.9±22.0	115.8±4.2	690.4±29.7	143.6±6.8

Note. Each value is represented by Mean ± Standard error (μm). They were calculated by the data from the second leaf blades of three plants in which the respective distances were measured for thirty points. C.V., Cross vein; L.V., Longitudinal vascular bundle. * See Table 1.

(Significance at 5% level. *) C.V. ° L.V.

ours. This disagreement may be due to the varietal difference and/or different growth conditions of seedlings.

In Fig. 3-b, the tip of the small longitudinal bundle, indicated by the arrow, slightly protrudes beyond the joining point with interconnecting veinlets. This type of termination of small longitudinal bundles was sometimes observed.

3. Distribution pattern of cross veins.

Table 1 indicates that the leaf length, width and area of the HN-laminae were larger than those of LN-laminae.

Table 2 shows that the total number of the cross veins was larger in HN-laminae than in LN-laminae. This is mainly due to the presence of a larger number of cross veins in the middle part of the HN-laminae. However, no significant differences between both laminae in each corresponding part were found in total number of the cross veins as well as in the density of cross veins. In both HN- and LN-laminae, the density of cross veins in the top part was distinctly higher than in the basal part, and the distribution pattern showed a decreasing

Table 4. Distribution in the number of the cross veins on either side of lamina midrib of the second leaf blade of rice.

Part	LN*		HN*	
	Left	Right	Left	Right
Top	48.3±6.1	50.7±7.6	45.3±3.1	47.7±4.5
Middle	53.3±2.3	53.7±3.4	70.7±4.8	67.3±7.2
Base	45.0±3.9	46.0±2.9	52.7±2.2	52.0±4.5
Total	146.6	150.4	168.7	167.0

Note. Each value is shown by Mean ± Standard error calculated by the data from five leaves. * See Table 1.

Table 5. Comparison of the total lengths of three kinds of vascular bundles in the second leaf blade of rice.

Plot	L.V.	S.V.	C.V.	Total
LN*	64.5±1.2	129.4±7.9	44.9±1.8	238.8±5.0
HN*	71.2±0.9	150.6±10.2	50.8±1.9	272.6±6.2

Note. Each value is shown by Mean ± Standard error (mm) calculated by the data from five leaves. L.V., Large longitudinal bundle; S.V., Small longitudinal bundle; C.V., Cross vein. * See Table 1.

trend basipetally.

YAMAZAKI¹⁶⁾ and CHONAN *et al.*²⁾ showed that the distribution pattern of cross veins was nearly uniform within a lamina of the rice plant. The results of our quantitative analysis does not agree with the results obtained by them.

The differentiation of cross veins, trichomes and stomata proceeds basipetally.¹⁷⁾ This fact suggests that the cell number and cell size in successive parts of the lamina along the longitudinal axis depends on the growth conditions, which may affect the cell division and cell enlargement. Therefore, in each part of the lamina along the longitudinal axis, cell number per unit leaf area (cell density) should have a close relationship with the cross vein density.

Table 3 shows the distances between successive cross veins and between the longitudinal bundles in both laminae. The distance between successive cross veins of HN-lamina is greater than that of LN-lamina. Though the distance between successive cross veins in both laminae increased basipetally, this distance was always greater than that between the longitudinal bundles.

Table 4 shows the number of the cross veins on both sides of the midrib of both

laminae. There was only minor variation in the distribution in number of the cross veins on the right and left sides of each lamina. Differences observed are mainly attributed to variation in the number and the extent of the small longitudinal bundles on either side of the midrib as observed in Fig. 2.

4. Total lengths of veins by categories for the second lamina.

Table 5 shows that the total length of all veins was larger in HN-laminae than in LN-laminae. This is mainly due to the larger increment in the length of small longitudinal bundles in HN-laminae. The total lengths of the large longitudinal bundles, the small longitudinal bundles, and the cross veins of both laminae made up approximately 26%, 55% and 19% of the total length of all veins, respectively. This ratio of the cross veins was larger than that of CHONAN *et al.*²⁾ who estimated the ratio at 14.9%.

PLYMALE and WYLIE¹⁵⁾ have determined the total length of veins of various categories in several dicotyledonous leaves and showed that the vein-endings constituted approximately 95% of the total length of all veins of the laminae. The ranking of cross veins

Table 6. Comparison of the cell size of long epidermal cells at top, middle and basal parts of the second leaf blade of rice.

Plot	Top		Middle		Base	
	Length	Width	Length	Width	Length	Width
LN*	56.6±2.1	12.1±0.4	72.9±2.6	15.0±0.4	72.2±2.4	16.2±0.3
HN*	64.1±2.0	11.9±0.3	66.8±2.7	13.1±0.2	74.4±2.4	18.9±0.2

Note. Each value is shown by Mean ± Standard error (μm) calculated by the data from three leaves. Thirty cells were measured in each of three parts of the leaf blade respectively. * See Table 1.

(Significance at 5% level.)
* length ° width

in total length of all veins in the rice lamina was markedly lower, as compared with that of vein-endings of dicotyledonous leaves.

5. Epidermal cell size patterns as an index of cross vein density in distribution.

In addition to the cross veins, the epidermal cells could also be observed very clearly in the cleared specimens, as shown in Fig. 3-d. By use of the same specimens in which the cross veins were counted, the positional differences in size of long epidermal cells along the longitudinal axis on the adaxial surface of the lamina were used as an index of cell growth.

Table 6 shows that the long epidermal cell length and width in both HN- and LN-laminae increased basipetally, especially in HN-lamina; i.e., the cell density in both laminae decreased basipetally. This result indicates that the density of cross veins has a close relationship to the epidermal cell density. However, in Table 6, no differences in the cell size were found among the corresponding parts of both laminae which grew under different amounts of nitrogen supplied from the endosperm. From these results, questions arise as to whether the cross veins of the second lamina had already been differentiated in the embryo, and whether the level of nitrogen supplied by seeds of low nitrogen content was sufficient for the differentiation cross veins of the second lamina. Further investigations are necessary on this point.

All of the observations of these experi-

ments were carried out cleared and unstained lamina specimens. This simple method for the clearing of whole lamina specimens in leaves of the rice plant will surely offer a sound structural basis for studying transport routes in the leaves.

Summary

The revised method of the whole leaf clearing was tested to observed the venation pattern in the rice leaf. For this study, the venation of the second foliage leaf blades (laminae) of the seedlings originating from the seeds of high nitrogen content(HN-laminae) and those from the seeds of low nitrogen content(LN-laminae) were comparatively observed.

The procedure involving the successive use of glacial acetic acid-95% ethanol, 1:3, 85% lactic acid, and ethanol saturated with chloral hydrate gave the most distinct specimens to observed the venation pattern in the rice lamina.

Most of longitudinal bundles which do not extend to the leaf tip or the leaf sheath join into one or both of the neighbouring bundles by means of a veinlet or veinlets. Therefore, most of the tips of these longitudinal bundles ended not freely(Figs. 1, 2 and 3).

Total number of the cross veins was larger in HN-laminae than in LN-laminae. In both laminae, the number of the cross veins in the middle part was higher than in the top and the basal parts. However, no

significant differences between both laminae in each corresponding part were found in total number of the cross veins as well as in the density of cross veins. In both laminae, the density of cross veins in the top part was distinctly higher than in the basal part, and the distribution pattern showed a decreasing trend basipetally (Table 1 and 2). The distance between successive cross veins in both laminae increased basipetally. This distance was always greater than that between the longitudinal bundles (Table 3). There was only minor variation in the distribution in number of the cross veins on the right and left sides of the midrib of both laminae (Table 4).

The total length of all veins was clearly longer in HN-laminae than in LN-laminae. This is mainly due to the larger increment of the length of small longitudinal bundles in HN-laminae. The total lengths for the large longitudinal bundles, the small longitudinal bundles and the cross veins of both HN- and LN-laminae made up approximately 26%, 55% and 19% of the total length of all veins, respectively (Table 5).

The long epidermal cell length and width in both laminae increased basipetally, *i.e.*, the cell density in both laminae decreased basipetally (Table 6). This result indicates that the density of cross vein has a close relationship to the epidermal cell density.

Acknowledgement

We are grateful to Dr. Peter B. KAUFMAN, University of Michigan, and Dr. E. MAEDA, Nagoya University, for their help in reviewing the manuscript. We are also grateful to Dr. Y. TAKEOKA, Nagoya University, for his advice.

References

1. BLACKMAN, E. 1971. The morphology and development of cross veins in the leaves of bread wheat (*Triticum aestivum* L.). *Ann. Bot.* **35**: 653—665.
2. CHONAN, N., H. KAWAHARA and T. MATSUDA 1974. Morphology on vascular bundles of leaves in gramineous crops. I. Observation on vascular bundles of leaf blades, sheaths and internodes in rice plants. *Proc. Crop Sci. Soc. Japan* **43**: 425—432.*
3. ESAU, K. 1977. *Anatomy of seed plants* (2nd ed.). John Wiley and Sons, Inc., New York. 321—374.
4. FOSTER, A. S. 1949. *Practical plant anatomy* (2nd ed.). D. Van Nostrand Company, Inc., Princeton New Jersey. 216—217.
5. GEIGER, D. R., R. T. GIAQUINTA, S. A. SOVONICK and R. J. FELLOWS 1973. Solute distribution in sugar beet leaves in relation to phloem loading and translocation. *Plant Physiol.* **52**: 585—589.
6. GODDY, J. B. 1963. *Laboratory methods for work with plant and soil nematodes*. Her Majesty's Stationary Office, London. 26—28.
7. HACKETT, C. and H. E. STEWART 1969. A method for determining the position and size of lateral primordia in the axes of roots without sectioning. *Ann. Bot.* **33**: 679—682.
8. HARA, N. 1968. Procambium differentiation of veins in the leaf. *Bot. Mag. Tokyo* **81**: 158—169.*
9. HARBERLANDT, G. 1914. *physiological plant anatomy* (translated from the 4th German edition by M. DRUMMOND, 1965). Today and Tomorrow's Book Agency, New Delhi-5. 386—389.
10. KAUFMAN, P. B. 1959. Development of the shoot of *Oryza sativa* L. II. Leaf histogenesis. *Phytomorphology* **9**: 227—311.
11. KONOSHIMA, M. 1968. *Laboratory manual of botany*. Hirokawa Publishing Co., Tokyo. 66—78.**
12. KUO, J., T. P. O'BRIEN and S. Y. ZEE 1972. The transverse veins of the wheat leaf. *Aust. J. Biol. Sci.* **25**: 721—737.
13. LERSTEN, N. R. 1967. An annotated bibliography of botanical clearing methods. *Iowa State Jour. Sci.* **41**: 481—486.
14. PETERSON, C. and A. FLETCHER 1973. Lactic acid clearing and fluorescent staining for demonstration of sieve tubes. *Stain Techn.* **48**: 23—27.
15. PLYMALE, E. L. and R. B. WYLIE 1944. The major veins of mesomorphic leaves. *Amer. J. Bot.* **31**: 99—106.
16. YAMAZAKI, K. 1960. Studies on the connecting strand of the vascular system in rice leaves. *Proc. Crop Sci. Soc. Japan* **29**: 400—403.*
17. ——— 1963. Studies on the leaf formation in rice plants. I. Observation on the successive development of the leaf. *Proc. Crop Sci. Soc. Japan* **31**: 371—378.*

* In Japanese with English summary.

** In Japanese.

〔和 文 摘 要〕

葉身透明化法による水稻第2葉身の結合脈の観察

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水稻葉の脈系を観察するために、全葉身の透明化法を検討した。この方法を用いて高い窒素含量の種子からのめばえの第2葉身(HN)と、低い窒素含量の種子からのめばえの第2葉身(LN)の脈系を比較観察した。

全葉身の脈系を観察するのに、もっとも鮮明な標本は、葉身を氷酢酸・95%エタノール混合液(1:3)―85%乳酸―抱水クロラル飽和エタノールの順序で処理する方法によってえられた。

葉身の先端部または葉鞘中へ走行しない縦走維管束のほとんどは、1ないし2本の細脈によって隣接する1ないし2本の縦走維管束と結合した(第1, 2, 3図)。

結合脈の数はLNよりもHNで多かった。両葉身の中央部における結合脈の数は、葉身先端部や基部にくらべて多かった。しかし、両者の葉身のそれぞれ対応する部位間には結合脈の密度だけでなく、結合脈の数でも統計的有意差はなかつた。両者の葉身で結合脈の密度は、葉身基部より葉身先端部で明らかに高く、その分布パターンは向基的に低くなる傾向を示した(第1, 2表)。

HNとLNで結合脈間の距離は、向基的に大きくなつた。しかし、この距離はけっして縦走維管束間の距離より小さくなることはなかつた(第3表)。

HNとLNともに、葉身中肋をはさんだ左右両側の葉身部分の結合脈の数は、わずかに相違した(第4表)。

各維管束の長さの合計は、明らかにLNよりHNで大きかつた。これは主として縦走小維管束の全長の増加によつていた。両葉身とも縦走大維管束、縦走小維管束および結合脈のそれぞれの全長は、全維管束長の約26%, 55%および19%を占めていた(第5表)。

HNとLNともに、長い表皮細胞の長さや幅は、向基的に増加した。すなわち細胞密度は向基的に減じ(第6表)、結合脈の密度と密接に関係していた。