Comparative Analysis of Leaf Vein Density in Sri Lankan Traditional Rice Varieties

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ABSTRACT: *The C3 photosynthetic pathway of rice is less efficient than the C4 pathway. The possibility of conversion from C3 to C4 pathways in rice is currently under experiment. In plants undergoing such a transition, higher leaf-vein densities (LVD), and development of Kranz anatomy is expected to be present at an initial stage. High LVD could exist among traditional rice and those could be used as a starting point towards developing C4 rice. In the present study, LVD (estimated by average vein length between, two large longitudinal veins (LLV), two small longitudinal veins (SLV), and the vein length per unit area (TLV)) of 23 Sri Lankan rice accessions were analysed by performing a one-way ANOVA, with mean separation using Duncan's multiple range test. Among the tested varieties LLV, SLV, and TLV were found to be significantly (p<0.05) different among accessions. Based on the mean separation, the highest LLV was shown by Thanthiribalan, and the lowest by Dewareddhiri, Mahasuduwee, Suwadel and five other accessions. The highest SLV was reported in three accessions and the lowest was in Mahasuduwee, Suwadel and Mahakuruwee. The highest TLV was reported in Mahasuduwee, Suwadel and Mahakuruwee and the lowest in IR 64. Unlike TLV estimation, SLV can be measured directly. Given that TLV and SLV are negatively correlated (r=-0.9183) a low SLV will be a suitable parameter to select for high TLV. The accessions, Mahasuduwee, Suwadel and Mahakuruwee carried a higher LVD (lower SLV and LLV leading to higher TLV) and can be recommended as candidates for studying C3 to C4 photosynthetic pathway conversion in rice.*

Keywords: C3/C4 rice; Kranz anatomy; Leaf anatomy; Leaf vein density

INTRODUCTION

Rice (*Oryza sativa* L.), is a cereal crop that fulfils the main caloric requirement of over half of the world's population (Bhat *et al*., 2018). As we step into the year 2050, the world population is predicted to be 9.7 billion, a 34 % increase compared to today's population. With population growth, the demand for rice is expected to be doubled by 2050 (FAO, 2009). To meet this demand, rice yield needs to be increased significantly. Even though in the recent past the rice yield increased due to precise breeding techniques leading to the development of high yielding varieties and hybrids, currently, the rice yield has come to a plateau (Godfray *et al*., 2010). The unpredictable climate is one of the challenges faced by the farmers and plant breeders in

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today's agriculture. Climate change drastically affects the rice yield and hence, farmers struggle to obtain a good harvest under the constantly changing climatic conditions. Development of rice varieties that can produce better yields under adverse and erratic climatic conditions is therefore a current necessity.

The evolutionary trends of photosynthetic pathways have revealed that C_4 plants evolved from C_3 plants as an adaptation for high temperature and low CO_2 in the atmosphere (Christin *et al*., 2008). In doing so, the C_4 plants have adapted a unique tissue arrangement with bundle sheath cells and mesophyll cells, referred to as "Kranz anatomy"; an arrangement of two concentric rings of cells, inner bundle sheath cells containing most of the chloroplasts, encapsulated in a layer of mesophyll cells (Nelson, 2011; Lundgren *et al*., 2014). For the efficient function of the C_4 pathway, close contact and interaction between mesophyll and bundle sheath cells are important. When the two cell types are in close proximity, where each and every mesophyll cell is directly adjacent to at least one bundle sheath cell, interconnected *via* plasmodesmata, CO_2 get more efficiently accumulated in the bundle sheath cells (Dengler and Nelson, 1998). The Kranz anatomy and C_4 photosynthetic pathway lead to minimizing the photorespiration through compartmentalization and improves the efficiency of photosynthesis by minimizing the energy wastage. Of the cereals cultivated in the world, rice, wheat, and barley follow the C_3 photosynthetic pathway, and maize, sorghum, millet, and sugarcane, the C_4 photosynthetic pathway. As reviewed by Sage *et al.* (2011), higher plant lineages of C_3–C_4 intermediate photosynthesis is reported in several grass families, however, not in any of the crop species.

The C_4 pathway is characterized by high vein density (Gowik and Westhoff, 2011). Differentiation of bundle sheath cells and their localization is regulated through hormones (such as auxin, and cytokinin), brassinosteroids, small RNAs, peptides, transcription factors, and metabolites elicited by the leaf vascular centres (Nelson, 2011). Formation of veins, number or pattern of veins depends on the polar auxin transportation inside the newly developing leaf tissues (Sachs, 1991). Further, the vein development depends on the paths link to the auxin transport for the sinks and sources (Berleth *et al*., 2000). Auxin and its transportation pathways induce differentiation of mesophyll and bundle sheath cells to avoid the lateral spreading of auxin (Sachs, 1991; Berleth and Mattsson, 2000). Auxin transport pathways are regulated by small RNAs and a collection of transcription factors produce from the vascular centre (Lyer-Pascuzzi and Benfey, 2000).

Therefore, the high leaf vein density in plants is considered as a developmental indicator of an efficient photosynthetic pathway. In order to capture the desirable attributes of the C_4 plants that make them climate-ready, the International Rice Research Institute in Philippines is hosting a consortium to develop a "C_4 rice variety" through C_4 engineering (http://photosynthome.irri.org/c4j3/#). Even though C_4 engineering has met its fair share of challenges, the approach could lead to the next revolution in crop production (Von *et al*., 2012) The vein network of the leaf comprises of small longitudinal veins (SLV) and large longitudinal veins (LLV), interconnected *via* transverse veins. These collectively translocate the photosynthetic products assimilated from surrounding cells out of the leaf in to the phloem (McKown and Dengler, 2009). Most of the environmental factors (e.g. CO_2 concentration in atmosphere) did not change the leaf vein density of plants, therefore it is identified as a heritable trait (Uhl and Mosbrugger, 1998). Given that high leaf vein density (LVD) is identified as an evolutionary important trait in C_3 to C_4 conversion, mutation breeding attempts were made to develop lines with higher LVD (Feldman *et al*., 2014). However, there could be naturally occurring accessions with higher LVD. In the current study, the LVD of several Sri Lankan rice accessions was assessed to select germplasm with potentially higher LVD to perform further analysis on their suitability as a model for studying C_3 to C_4 conversion.

METHODOLOGY

Rice Materials

In the current study, *IR 64* and 23 Sri Lankan rice accessions sourced from the Plant Genetic Resource Centre, Gannoruwa, Sri Lanka were used for assessing the LVD (*Dewareddhiri* (*4610*), *Kallundai samba* (3381), *Kotathavalu* (3659), *Madayal vee* (3269), *Moroberekan* (6897), *Norunkan* (3241), *Pachchaperumal* (3136), *Pokkali* (3251), *Rathu heeneti* (4992), *Sivappu kuruvikkar* (4868), *Sudu heeneti* (2088), *Thavalu* (3634), IR-64 (9483), *Wannidahanala* (2053), *Batapolael* (2105), *Podihatatha* (3174), *Yakada wee* (3445), *Halsuduwee* (3877), *Kottiyaran* (4630), *Suwadel* (4802), *Herath banda* (2063), *Thanthiribalan* (3072), *Mahakuruwee* (3190), and *Mahasuduwee* (3662)). The study was conducted at the Department of Agricultural Biology, Faculty of Agriculture, University of Peradeniya during the period 2016 to 2017.

Planting and Experimental Design

The seeds were germinated in Petri-dishes and two-weeks old seedlings were transplanted in pots in 6 replicates according to a completely randomized design. The second week after germination, nutrients were applied through half strength Yoshida solution (Cock *et al*., 1976) and was switched to full strength Yoshida solution at two-weeks after transplanting. Urea was applied according to the recommendation of the Department of Agriculture in the fourth week and sixth week after transplanting.

Data Collection

For the LVD calculation, a one-centimetre leaf sample was collected from the widest middle portion of the first leaf (the leaf directly below the flag leaf) of the main culm of each rice plant from each species separately (Ueno *et al*., 2006). Permanent slides were prepared by mounting the leaf sections on 70 % glycerol. The slides were observed under a $20 \times$ magnification and imaged using a microscope eye-piece camera (AM7013MT Dino-light and Dino eye, AnMo Electronic Corporation, Torrance, California, USA). The average vein length between two LLV and two SLV, and the vein length per unit area (TLV) were calculated based on an image analysis conducted using software Dino-Capture 2.0 v1.5.18.A (European distributers, Europe).

Data Analysis

In all 24 varieties LLV, SLV and TLV were statistically analysed using a one-way ANOVA with PROC GLM function, and mean separation was carried out using Duncan's multiple range test. The correlation between LLV, SLV and TLV were assessed using SAS v9.1.3 (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

The plants with a C_4 photosynthetic pathway are more efficient in assimilating carbon compared to those with a C_3 photosynthetic pathway. While in most plants the leaf anatomy is reflective of the photosynthetic pathway that it adopts (i.e. C_3 or C_4), there are plants with anatomy that is in the transition from that of C_3 to C_4. The evolution of C_3 to C_4 is said to be governed by the development of leaf anatomy with a high LVD, a characteristic of C_4 plants

(Feldman *et al*., 2014). The LVD impacts the photosynthetic efficiency by influencing the development of Kranz anatomy and by increasing the bundle sheath to mesophyll cell ratio through hormonal and transcriptional regulation. It also increases the translocation of photosynthates for phloem loading. So high LVD in a rice plant indicates prospects towards engineering a rice plant with better photosynthetic capacity.

Sri Lankan rice germplasm is a diverse genetic resource which may harbour accessions with higher LVD. Such accessions could be ideal candidates to initiate further research towards engineering a rice plant with high photosynthetic capacity. According to Ueno *et al*. (2006), rice plants with high LVD has shown a reduction of the length between LLV, and an increase in the length between SLV and the TLV. In the current study, 23 Sri Lankan rice accessions were analyzed considering, mean length of the LLV, SLV and TLV as indicator parameters to select accessions with high LVD. The variety *IR 64* was used as a reference accession, given that most of the earlier studies on vein density analysis in rice were conducted using mutant populations derived from a single *IR 64* plant produced at IRRI.

The LLV length is inversely related to the LVD. Among the rice accessions evaluated in the current study, the highest LLV was shown by *Thanthiribalan*, however, the accession is not significantly different ($p \geq 0.05$) to *Herath Banda*, *IR 64*, and *Moroberekan*. The lowest LLV was reported by *Dewareddhiri* (Figure 1), while it was not significantly different ($p \geq 0.05$) from LLVs of *Suwadel*, *Mahasuduwee*, *Yakadawee*, *Kottiyaran*, *Kotathawalu*, *Pokkali*, *Madayal wee*, *Batapolael*, *Kallundai samba*, and *Norunkan*. The highest and lowest LLV groups were significantly ($p \leq 0.05$) different from each other.
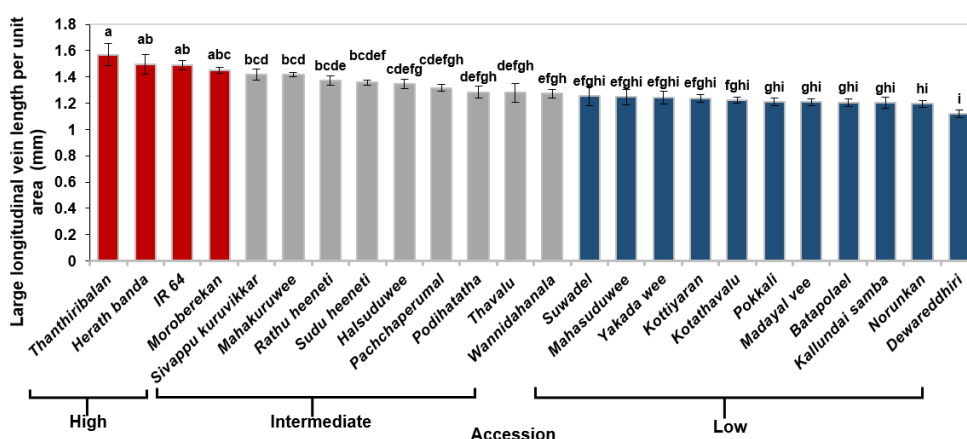
Figure 1. The variation of the mean length of the large longitudinal veins (LLV) of the 7th leaf of the selected 24 rice accessions.Values with the same letter (a-i) are not significantly different (*p*≥0.05). Error bars indicate the standard error.

The SLV also has an inverse relationship with leaf vein density (Figure 4). The highest SLV was shown by *Pachchaperumal, Sivappu kuruvikkar* and *Moroberekan*. However, the accessions *Wannidahanala*, *Suduheenati* and *IR64* were not significantly ($p \geq 0.05$) different from the highest SLV group. The lowest SLV was reported in *Mahasuduwee*, however, it was not significantly different from SLV of

Suwadel and *Mahakuruwee* (Figure 2). The highest and lowest SLV groups were significantly ($p \leq 0.05$) different from each other.
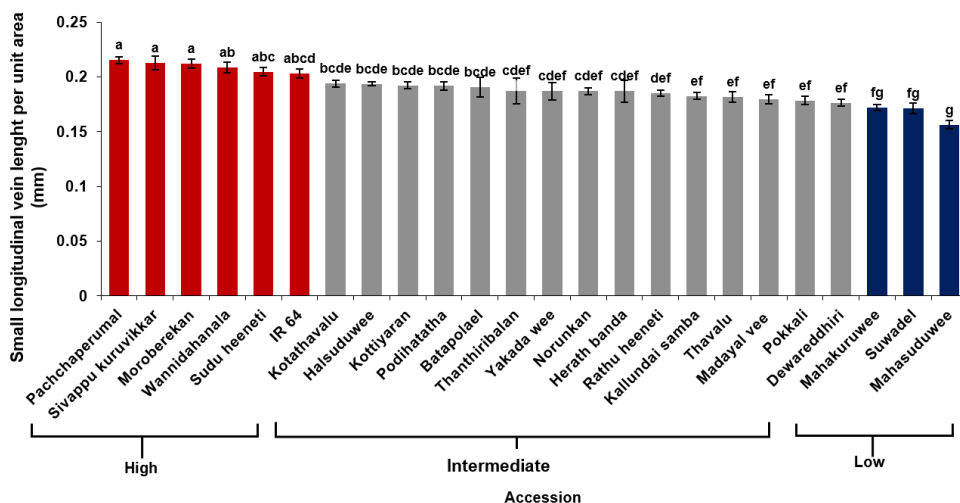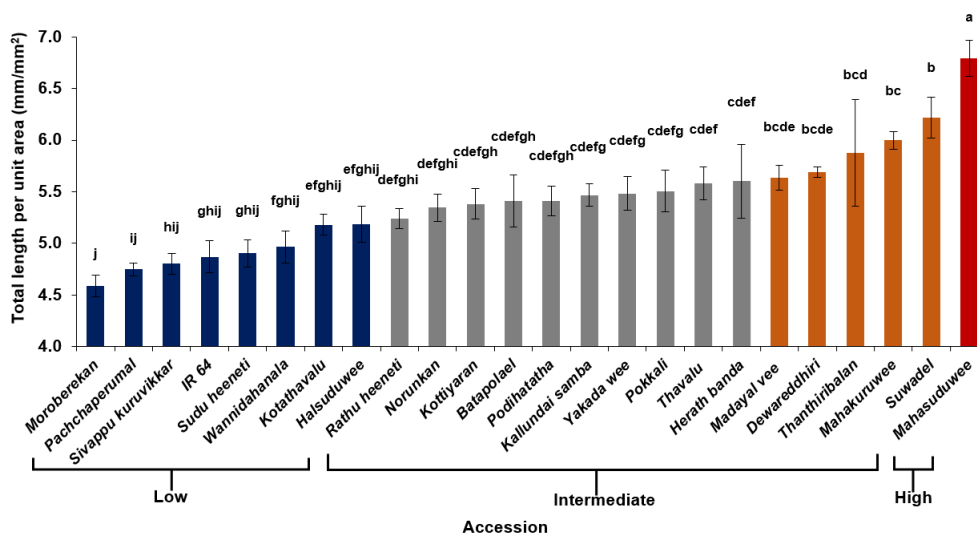

Figure 2. The variations of the mean length of the small longitudinal veins (SLV) of the 7th leaf of the selected 24 rice accessions. Values with the same letter (a-g) are not significantly different ($p<0.05$). Values are given as ± standard error.

The lowest mean LLV and SLV lengths are important for the increasing number of LLV and SLV in a unit area of the rice leaf, hence, indicates a higher LVD *(*Ueno *et al.*, 2006). According to the result, *Mahasuduwee*, *Suwadel* and *Mahakuruwee* are identified as rice accession with lowest SLV. *Dewareddhiri* showed the lowest SLV in the intermediate group (Figure 2). Further, *Mahasuduwee* and *Suwadel* were identified as having lowest LLV along with *Dewareddhiri* and five other accessions (Figure 1). Therefore, these three traditional accessions could be considered as rice accessions carrying higher LVD based on LLV and SLV.

The TLV variation of the 24 accessions ranging from 4.5 to 7.0 mm/mm^2 is shown in Figure 3. The highest TLV was in the accessions *Mahasuduwee* and it is 6.7 mm/mm^2. The accessions *Suwadel*, *Mahakuruwee*, *Thanthiribalan*, *Dewareddhiri* and *Madayal vee* were significantly ($p \leq 0.05$) different from *Mahasuduwee*, however, was included in the second highest TLV group (Figure 3). The higher TLV indicates high LVD. So, accessions *Mahasuduwee*, *Suwadel* and the *Mahakuruwee* can be considered as potential high LVD candidates.

The correlation between SLV, LLV and TLV analysis is important to know the relationship between above-mentioned leaf parameters. According to the Pearson's correlation analysis, the correlation between SLV and LLV is positive, and the correlation between TLV and LLV is negative. Further, the correlation between TLV and SLV is strongly negative (r=-0.9; Figure 4). Therefore, low SLV can be used as

an indicator of high TLV, and hence can be used as an indirect measurement instead of calculating the TLV.

Figure 3. The variations of the total length per unit area (TLV) of the 7th leaf of the selected 24 rice accessions. Values with the same letter (a-j) are not significantly different (*p<0.05*). Values are given as ± standard error.

Based on the SLV, LLV and TLV analysis, the lowest SLV and LLV, as well as highest TLV, were shown in accessions *Mahasuduwee*, *Suwadel* and *Mahakuruwee.*

Figure 4. Correlation analysis heat map for large longitudinal vein (LLV) length, small longitudinal vein (SLV) length, and the vein length per unit area (TLV). Each value represents the Pearson correlation coefficient (*r*) at *p<0.05* between each measurement. Positive values represent positive correlation and the negative values represent the negative correlation.

As reviewed by Karki *et al*. (2013) by introducing C_4 photosynthetic pathway in to C_3 photosynthetic plants the nitrogen use efficiency can be enhanced and the water losses can be reduced, leading to a yield increase. The initial step towards genetically engineering a rice plant with high photosynthetic capacity begins with increasing the leaf vein density. Therefore, the identification of rice accessions carrying high leaf vein densities in the current study will benefit the C_4 rice consortium efforts as well as studies that focus on understanding the anatomy and physiology of vein development in rice.

CONCLUSIONS

The parameters SLV and TLV showed a significant negative correlation, and therefore, low SLV can be used as an estimate of a high TLV. Among the tested 24 accessions the highest LLV was shown by *Thanthiribalan*, and the lowest was shown by *Dewareddhiri*, *Mahasuduwee*, *Suwadel* and eight another accessions such as *Yakadawee, Kottiyaran, Kotathavalu, Pokkali, Madayal vee, Batapolael, Kallundai samba, Norunkan*. The highest SLV was reported in the accessions *Pachchaperumal*, *Sivappu kuruvikkar*, and *Moraberakan* along with another three accessions such as *Wannidahanala, Suduheeneti, IR64* and the lowest was represented in three accessions *Mahasuduwee*, *Suwadel* and *Mahakuruwee*. The highest TLV was reported in *Mahasuduwee*, *Suwadel* and *Mahakuruwee* and the lowest in *IR 64*. Therefore, *Mahasuduwee*, *Suwadel* and *Mahakuruwee* carried the highest LVD, and they can be recommended as potential accessions for studying the C_3 to C_4 photosynthetic pathway conversion in rice.

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