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Sink to Source Transition of *Pisum sativum* Leaves in Relation to Leaf Plastochron Index

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Abstract: The transition from a sink to a source state within developing leaves is synonymous with the maturation processes in leaves. We report on our investigation of the maturation of the first pair of leaflets on leaves of *Pisum sativum* L vis-à-vis the sink- source transition state, correlated to the leaf plastochron index. The study was carried out using the phloem-mobile fluorophore, 5,6-carboxyfluorescein (5,6-CF). Young leaves remain strong sinks until LPI 0 has been reached. The sink-to-source transition process then occurs until the leaf has reached LPI 1.8 and the transition of the leaflets to completely source state is completed by LPI 2.0. The sink to source transition in the pea plants takes place between LPI 0 and LPI 2.0 which represents 2.0 plastochrons or 4.68 days. Present results show that 1-2 leaves are simultaneously under transition in an intact plant. Present data provide convincing evidence that the leaf plastochron index is quantifiable and may be used to correlate the sink/source transition state in developing seedlings.

Key words: Sink to source transition, leaf plastochron index (LPI), sink, transition, source, 5,6-CF

Introduction

It has been long established that immature sink leaves import assimilates from source (older) leaves until they are able to produce sufficient assimilate to sustain growth and in turn, as maturation occurs, they themselves will become source of assimilates for the next generation of developing leaves. The passage from an entirely heterotrophic, carbohydrate-importing organ to an entirely autotrophic, carbohydrate-exporting organ was termed the sink-to-source transition and occurs in all expanding leaves (Turgeon, 1989; Robinson-Beers *et al*., 1990; Gagnon and Beebe, 1996). Therefore, leaves in the transition state are commonly defined as 'maturing' leaves, which import and export assimilates at the same time (Turgeon, 1989). Transition leaves thus have a dual function of being both assimilate exporter and importer but these processes are confined to separate source and sink regions within the leaf. These regions have been shown to support bidirectional transport through the petiole and major veins (Larson *et al*., 1972). Bidirectional transport occurs as a result of the simultaneous import into immature basal regions and concomitant export from mature apical regions (Jones and Eagles, 1962; Larson *et al*., 1972). Before the leaf is fully expanded, it must undergo a transition from a sink (net carbon importer) to a source (net carbon exporter) state. As the leaf expands, unloading from the major veins becomes reduced and eventually ceases (Turgeon, 1989; Roberts *et al*., 1997; Imlau *et al*., 1999; Oparka *et al*., 1999; Wright *et al*., 2003). It has been suggested that the structure and topology of the major veins facilitate export, rather than redistribution of photosynthate produced

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within the mesophyll of the precociously mature lamina tip (Larson *et al*., 1972). As a direct result of this, those portions of a leaf which do not synthesize sufficient carbohydrate to meet local requirements for growth must import it from other leaves (Penny and Nelson, 1970). Once fully matured, a leaf becomes an irreversible exporter and phloem loading and the subsequent export from source leaves is largely irreversible (Turgeon, 1989). Matured leaves thus function as the source of minor vein-loaded photoassimilate, which is transported towards the base of the leaf and which enters the long-distance transport compartment of the phloem (Ruiz-Medrano *et al*., 2001).

In dicotyledonous plants, the transition from photoassimilate sink to source status begins shortly after the leaf has begun to unfold (Turgeon, 1989). At this stage in development, the major morphogenetic events that determine leaf shape have already been completed. Maturation of the phloem and xylem in the class I and higher-vein orders, which occurs in an acropetal (lamina base to tip) direction, has been reported to be largely complete before the sink-to-source transition begins (Turgeon, 1989). In contrast, structural and functional maturation of the smaller veins proceeds in a basipetal (tip to base) direction as the leaf unfolds. Therefore, a gradient in the degree of leaf maturation exists from the base to the tip of the lamina during the sink-to-source transition (Turgeon, 1989). Transport must, therefore, occur simultaneously in opposite directions, at different locations within a transition leaf at any given time (Geiger, 1979; Turgeon, 1989; Minchin *et al*., 1993).

Given the wealth of information that exists in the literature, the question which has to be faced is simply how to define the state of maturity relative to a quantified age parameter in a developing leaf. The plastochron index has been used to analyze all aspects of plant development including sink-to-source transition. Gagnon and Beebe (1996) used the plastochron index to study sink-to-source transition in leaves of *Moricandia arvensis* L., though only leaves 6-8 could be used, since the criteria governing PI were only fulfilled within that leaf range. Gagnon and Beebe's (1996) findings confirmed other previous studies that transition occurs in a basipetal fashion and that the transition had an approximate duration of 2.5 plastochrons, or approximately 5.6 days.

In order to be able to assess phloem transport capacity, the phloem mobile fluorophore 5,6-carboxyfluorescein (5,6-CF) was used to investigate the maturation of the first pair of leaflets on the compound leaves of *P. sativum* and the transition from sink to source state. Results were analyzed in relation to the leaf plastochron index and the data reported here, is strong evidence that changes in plant structure-function especially of the phloem is directly related to the plastochron index of the leaflets concerned.

Materials and Methods

Pisum sativum var. Green feast was grown from seed, under controlled environment in growth chambers (Conviron Model S10H, Controlled Environments Ltd., Winnipeg, Canada) at 25/18°C day/night at 16 h photoperiod with CO_2 maintained at 360 μmol mol^{-1} with fluctuations within ±15 μmol mol^{-1}. CO_2 was monitored using the integrated computer-controlled Horiba APBA-250 indoor CO_2 monitor (Horiba Ltd., Japan). Plants were illuminated using a combination of fluorescent tubes (F48T12.CW/VHO1500, Sylvania, USA) and frosted incandescent 60 W bulbs (Philips, Eindhoven, The Netherlands). Photosynthetic active radiation (PAR at 400-700 nm) was measured at about 250 μmol m^{-2} s^{-1}, 20 cm above soil level (Olivier and Annandale, 1998), with a Li-85A Quantum sensor (Li-Cor Inc, Nebraska, USA). Pot positions were changed every day in a matrix pattern, to avoid any chamber position effect. Plants were allowed to grow for at least two weeks before experiments were carried out. Unless otherwise stated, experiments were repeated six times and the micrograph are thus representatives of the treatment regimen described.

Plastochron Analysis

Plastochron index of plants (PI) per day was calculated for each plant based on the Erickson and Michelini (1957)'s formula, as revised by Ade-Ademilua and Botha (2005) where the reference length is 20 mm:

$$PI = n + \frac{\log L_n - \log 20}{\log L_n - \log L_{n+1}} \quad (1)$$

Where,
n is the serial number (counting from the shoot base) of that leaf which just exceeds 20 mm;
log L_n is the natural logarithm of the length of the leaf n and
log L_{n+1} is the natural logarithm of the next or subsequent leaf with a length that is less than 20mm.

Consequently, the Leaf Plastochron Index (LPI) is calculated as

$$LPI_n = PI - n \quad (2)$$

this is derived by simply subtracting the leaf's serial number from the plant's plastochron age (Erickson and Michelini, 1957).

Surface Loading of Phloem Transport Tracer

All experiments were carried out using intact plants. The leaf loading protocol used to load 5,6-CF was adapted from that of Grignon *et al*. (1989) and Roberts *et al*. (1997). Experiments were carried out by applying 5,6-carboxyfluorescein diacetate (5,6-CFDA) to source leaflets and monitoring the transport of the fluorophore in leaflets above the fed leaflet. Experiments involving the application of 5,6-CFDA were undertaken 7 h after the beginning of the photoperiod. In all cases, the adaxial surface of an attached leaflet was gently abraded with fine sandpaper and rinsed with distilled water, Lamina zones lacking large veins were chosen as abrasion sites. After abrasion, 100 µL of working strength 5,6-carboxyfluorescein diacetate (5,6-CFDA) was applied directly to the abraded area to the leaflet surface and covered with transparent polythene film (Housebrand, Brackenfell, South Africa) to prevent evaporation. At the end of the leaf loading experiments, the leaflets and petioles of interest, were detached 30 min to 3 h after application of 5,6-CFDA and observed for 5,6-CF distribution, using an Olympus BX-61 (Tokyo, Japan) epifluorescence microscope fitted with a U-YFP filter set (10C/Topaz 41028, Chroma Technologies, Battlebro USA). Several montages of all regions of each leaflet were taken using an Olympus PlanApo 2x objective (Tokyo, Japan) and the whole leaflet images were reconstructed using the multiple image alignment (MIA) routine in analSiS 3.2 (Soft Imaging System GmbH, Münster, Germany).

Preparation of Working Strength 5,6-CFDA

5,6-CFDA (C-195) was purchased from Molecular Probes, Eugen, Oregon USA in 100 mg units, to which 1 mL of 0.2% dimethylsulphuroxide (DMSO) was added. This stock solution was foil-wrapped and stored at -4 ºC until needed. Working strength 5,6-CFDA was prepared by taking a 1 µL aliquot of the stock solution and adding to 1 mL of distilled water. Working strength 5,6-CFDA was foil-wrapped and stored at -4 °C until needed. This resultant mixture was applied directly to abraded leaflet surfaces. Once loaded the acetate moiety is cleaved and the resultant 5,6-CF is transported within the phloem.

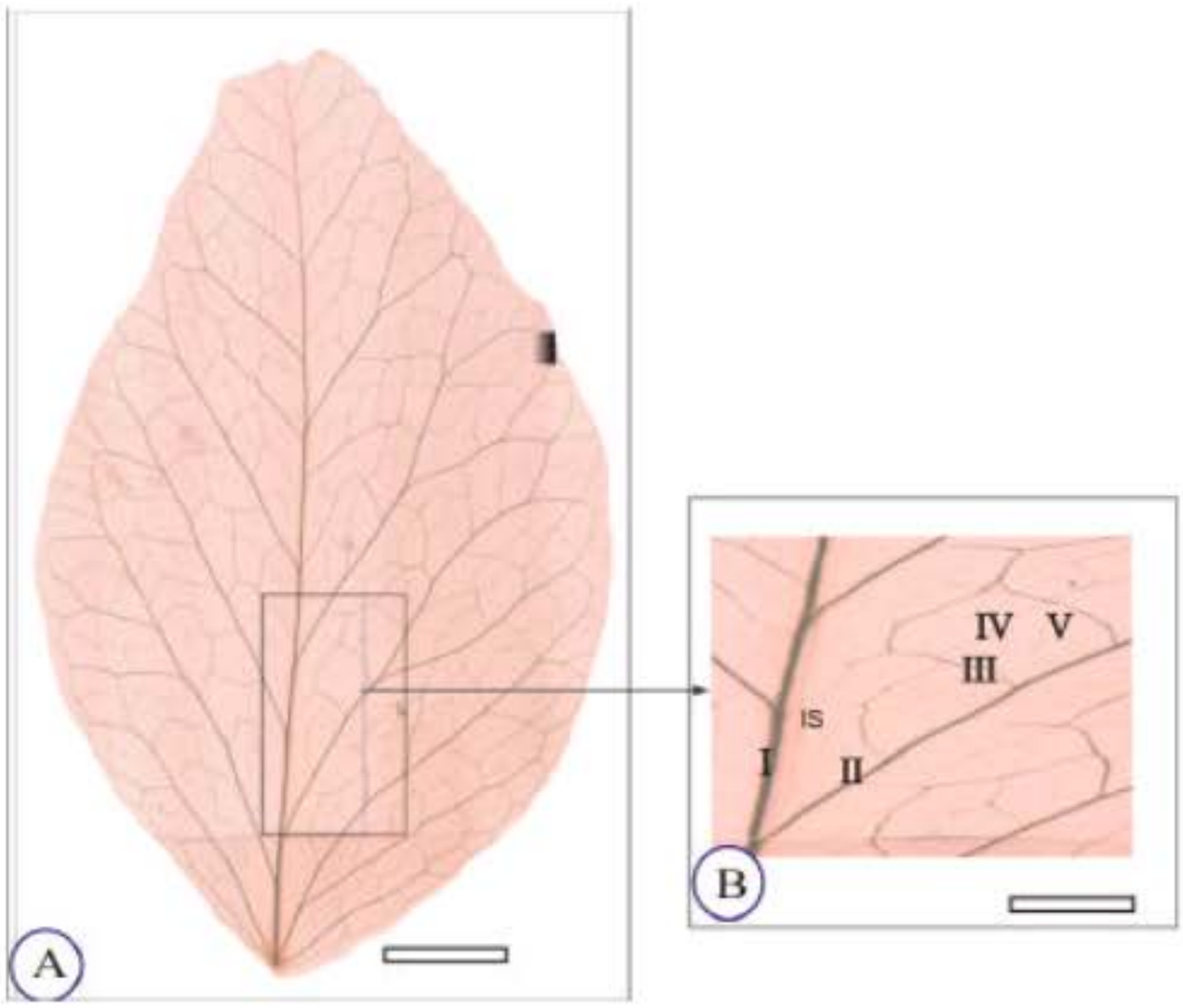

Fig. 1: Cleared leaf, stained in safranin to highlight the xylem, which shows the venation pattern in a mature leaflet of *Pisum sativum* L. (A). Detail (B) shows the class I vein (I) and associated connections to a class II, intersecondary (IS), class III, class IV and class V veins. Note that class V veins delimit the leaf areoles within the leaf. Bar in A = 4 mm; bar in B = 1 mm

Results

The individual leaflets making up the compound leaf of *P. sativum* contain a primary vein and four lateral vein orders (Fig. 1). The primary vein runs the length of the leaf and class II veins develop from it. These class II veins frequently overarch and interconnect at the leaf margins, providing a loop between the veins. Figure 1 also illustrates that the space between class II veins is often interconnected directly via intersecondary veins, which delimit the broad, large areolar regions within the leaf blade. These intersecondary veins interconnected with the class II veins at frequent intervals in the interstitial lamina zone between successive class II veins. Clearly, the intersecondary vein must have an important load balancing and assimilate distribution function. The class III veins derived from the class II veins subdivide at regular intervals to outline the form of the minor vein network, which comprises the class IV and the class V veins which in turn delimit the areolar regions responsible for unloading and uptake of assimilates, which for many species have been demonstrated to occur predominately from the minor vein network. As in all other species reported to date, maturation of the phloem and xylem in the primary vein and class II veins occurs in the acropetal (lamina base to tip) direction and is largely complete before the transition from sink to source begins. While the leaf unfolds, structural and functional maturation of the smaller veins proceed in the basipetal (tip to base) direction (Avery, 1933; Turgeon, 1989).

Pea leaflets began to unfold at about LPI -0.5 and were fully unfolded by LPI 0. Full expansion was achieved at LPI 2. At LPI 0, leaflets had attained about 66% of their final size. Figure 2A shows the distribution of the fluorescence associated with 5,6-CF in a leaflet at LPI 0. It was apparent that all vein classes visible in this yet immature and expanding leaflet contain 5,6-CF. Figure 2G shows a

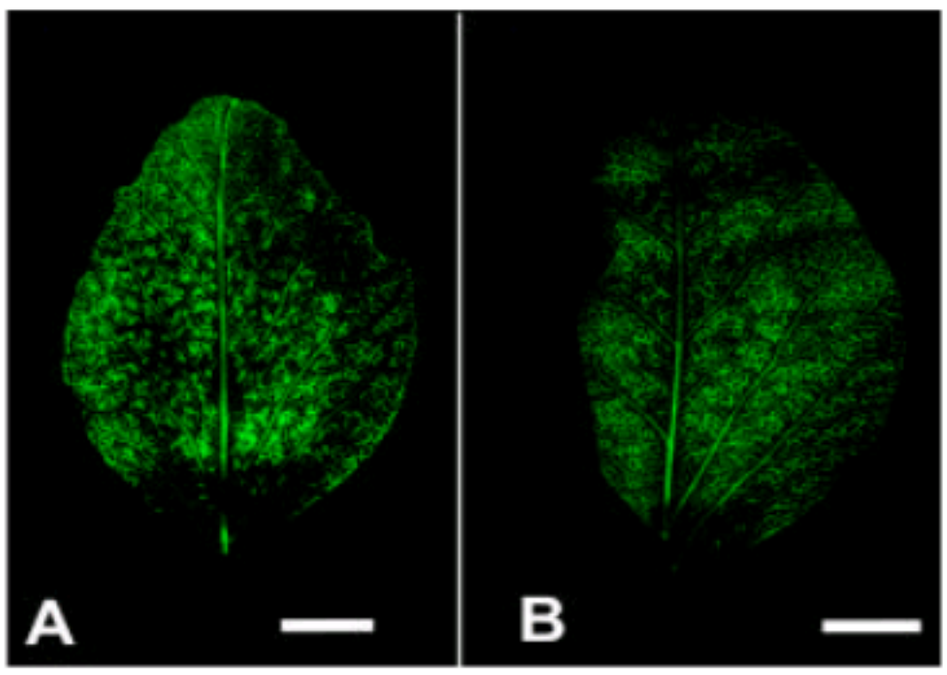

Fig. 2A-D:Epifluorescence micrographs which illustrate the distribution of the phloem-mobile probe, 5,6-CF in intact leaflets of P. sativum, 3 h after application of the fluorophore to distant (source) leaflets. Each leaflet is reconstructed from montages of overlapping regions of the leaf surface. Bar in A-F = 4 mm; in G = 40 mm; in H = 16 mm. Fig. 2(A) shows the distribution of 5,6-CF in leaflets at LPI 0. Note that the imported 5,6-CF was transported through all vein groups in all regions of the lamina and once inside the class III vein network, the veins appeared as if bleeding (Fig. 2G, arrows) indicating that 5,6-CF was being unloaded into the mesophyll. This pattern of distribution of the fluorophore is typical of a leaflet in the sink state and was observed in all sink state leaves. Fig. 2(B) shows the distribution of 5,6-CF in leaflets at LPI 0.5. The distribution of the imported fluorochrome in the leaflet is generally similar to that in leaflets in the sink state (Fig. 2. Whilst import is evident across the lamina, there was no evidence of the fluorophore towards the tip of the leaflets (Fig. 2H, arrows) and where present, it appears threadlike with no evidence of bleeding or thus unloading into the mesophyll; which means the tip is no longer importing. Transition from sink to source has commenced in the tip and is proceeding basipetally in this leaflet. Fig. 2(C) shows the distribution of 5,6-CF in leaflets from LPI 1-1.4. The fluorophore was not observed in the tip and mid region of the lamina except in a few higher vein orders, indicating the progressive transition of the upper half of the lamina from sink to source state. Leaflets from LPI 1-1.4 are thus considered to be at an advanced sink to source transition state. Fig. 2(D) shows the distribution of 5,6-CF in leaflets from LPI 1.6 -1.8. Distribution and the unloading of the fluorophore via the class III vein network is restricted and is limited portions of the lower half of the lamina. Leaflets between LPI 1.6 and 1.8 are thus at the late sink -source transition stage

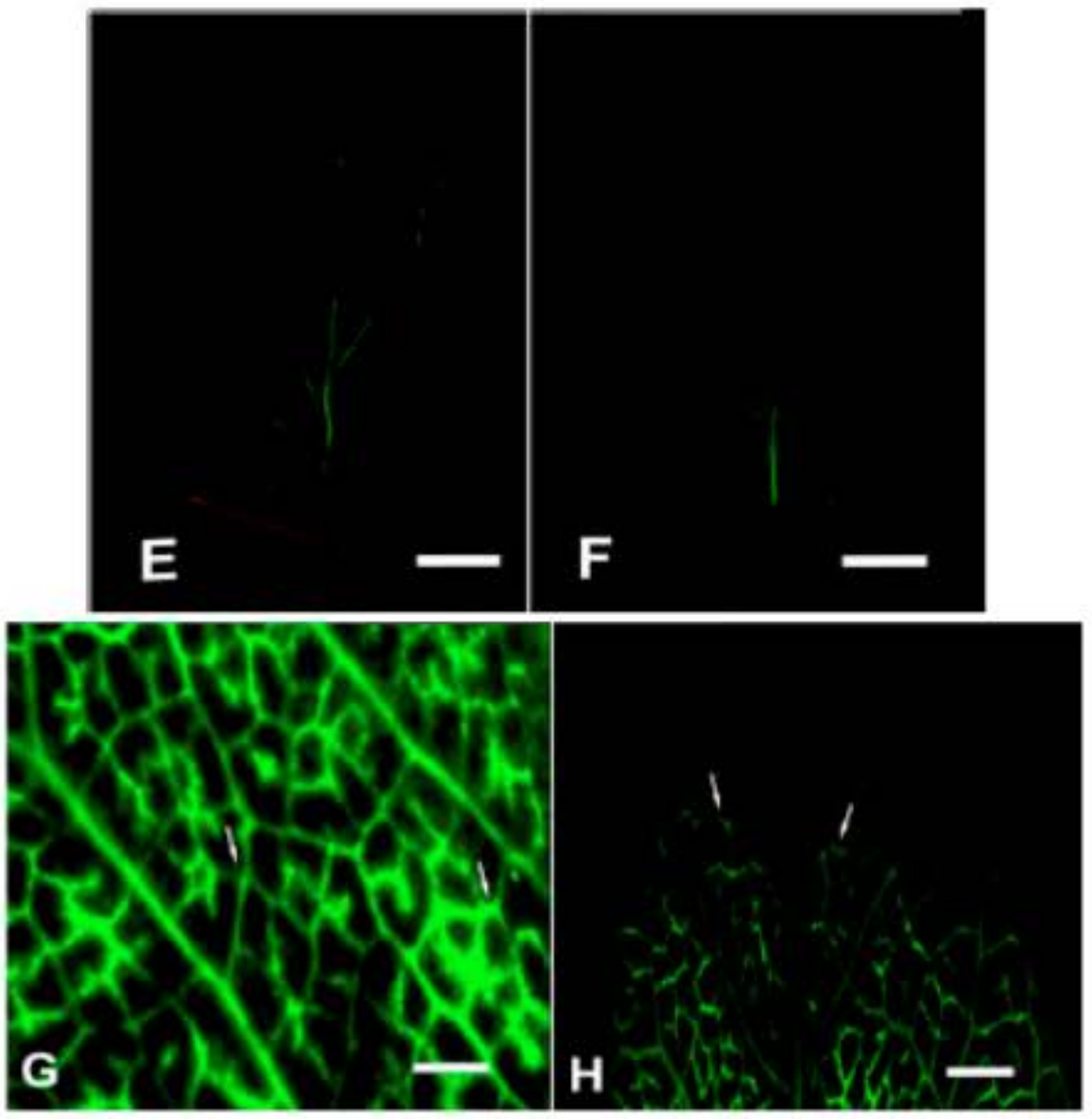

Fig. 2E-H: (E) and (F) show the inflow of 5,6-CF into leaflets at LPI 2 and 2.5, respectively. In leaflets at LPI 2, the fluorophore did not move beyond the primary vein and few class II veins (2E); movement of the fluorophore into these veins was even more restricted in the leaflet at LPI 2.5 (2F). Leaflets at this LPI range, are thus in an early source development state

higher magnification of a portion of the lamina. Some diffusion of 5,6-CF from the class III vein into the mesophyll was evident, indicating unloading. It was apparent that the fluorophore was being imported into all regions of the lamina of leaflets at LPI 0. Figure 2B shows the reduction of fluorescence in the tip of the leaflets at LPI 0.5. Note that the distribution of fluorescence decreased as the fluorochrome move from the base towards the tip of leaflets. A higher magnification of the tip of the leaflet is shown in Fig. 2H. The fluorophore was absent in most veins at the tip and in class III veins where present, they appeared threadlike with no unload of the fluorochrome into the mesophyll. A restriction in the flow of the fluorochrome towards the upper region of the leaflet points to the possibility that the region had commenced export as against import at LPI 0.5.

Figure 2C shows the presence of fluorescence only in very few higher vein orders in the upper and most part of the mid regions of leaflets at LPI 1-1.5. There was no visible unloading of the fluorochrome into class III veins within these regions. However, fluorescence appeared in the class III veins at the basal region of the leaflet. Note some diffusion of 5,6-CF into the mesophyll around the class III veins. The upper region had ceased to import the fluorochrome completely, while the middle region had commenced gradual transition from import to export. The latter was made evident by the gradient restriction in the flow of the fluorochrome in the upper end of the mid region. However, while the matured upper half of the lamina had ceased importing, the basal half of the lamina was still actively importing 5,6-CF.

Complete absence of fluorescence in the upper, mid and part of the lower regions of the lamina was evident in leaflets at LPI 1.6-1.8 as shown in Fig. 2D. Reduction in fluorochrome in the class III veins and subsequent diffusion into the mesophyll at the basal region of the leaflets was also apparent. The maturation of the lamina occurred even further basipetally, capturing the upper end of the lower region of the lamina at LPI 1-6-1.8; this signaled the commencement of transition of the lower region of the pea leaflets.

Figure 2E shows 5,6-CF in the primary vein as well as in some of the class II veins, up to the mid region of leaflets at LPI 2 while Fig. 2F shows that the primary vein in the basal region of the leaflet is fluorescent at LPI 2.3. Fluorescence was not visible within the leaflets by the time they attain LPI 2.5. Unload of the fluorochrome did not occur in any region of the lamina of leaflets, as the class III veins throughout the lamina, have stopped importing fluorophore completely. This indicated the attainment of maturity by the remaining basal portion of the lamina and thus the maturation of the leaflet.

Discussion

The results reported in this study demonstrate that sink-to-source transition in *P. sativum* can be related to Leaf Plastochron Index (LPI). The gradual cessation of import from the tip to the base of leaflets was studied using the phloem mobile fluorophore, 5,6-CF. In essence, the results confirm those of Gagnon and Beebe (1996) that sink-to-source transition is directly correlated to leaf plastochron index.

All regions of lamina of leaflets at LPI 0 were shown to import 5,6-CF from a distant source leaflet and to unload it into the mesophyll, via the class III vein network. The class IV and class V veins were clearly not involved in the import process and as expected, they are only functional during export in this case, as well. Unloading of the fluorochrome by the class III veins was evident through a phenomenon described previously by Roberts *et al*. (1997) as a bleeding appearance of the class III vein network in young Nicotiana benthamiana leaves. The import and diffusion of 5,6-CF into the mesophyll in leaflets at LPI 0 are thus a strong indication that the leaflets are still in the sink state.

An acropetal reduction in fluorescence was observed when leaflets reached LPI 0.5, suggesting that a restriction in the flow of the fluorochrome towards the tip was taking place. In all cases, 5,6-CF was absent at the tip of leaflets at LPI 0.5 but it was still evident in the more distal ends of the class III vein network. It is important to stress that no apparent unloading of the fluorophore into the mesophyll around the veins occurred. Thus, whilst class III veins in the lamina tip region may continue to import 5,6-CF, there is no evidence of unloading associated with these veins. The lamina tips of leaflets at LPI 0.5 have thus transitioned to an export phase. It is therefore reasonable to argue that sink-to-source transition commence between LPI 0 and 0.5 in the pea variety used in these experiments.

5,6-CF was absent in the apical as well as in parts of the mid region of the lamina by LPI 1 to 1.5. However, very few higher vein orders showed evidence of 5,6-CF fluorescence which suggests that import into the upper half of the lamina was restricted by this stage. In contrast, fluorescence was present in the basal region and unloading of 5,6-CF via the major vein network, is indicative that the basal region of the lamina is still immature and as such is still importing 5,6-CF from the fed distant source leaflets and that the transition from sink to source state had advanced basipetally. Based on the data obtained in these experiments, the leaves which are at LPI 1-1.5 are classified as being at an advanced sink-to-source transition stage.

Between LPI 1.6 and 1.8, the transition from sink to source was near-completion in the basal regions. Some evidence of continued off loading was observed (Fig. 2D), but only at the extreme marginal base of the leaflets, signifying the near-completion of the leaflet's transition from sink to source.

However, there was still evidence of the movement of fluorophore into the higher vein orders, up to the mid region of the leaflet in leaflets at LPI 2 and the fluorochrome moved outwards from the primary veins into few class II veins in these leaflets. By LPI 2.3, little evidence of 5,6-CF uptake by the primary vein at the basal region of leaflets could be seen and by LPI 2.5, no fluorescence was evident in the primary vein. The presence of the fluorophore in the primary and class II veins as observed in leaflets which are at LPI 2-2.3 is not necessarily an indication of active import as pointed out by Turgeon (1989). According to Turgeon (1989), though there is usually a clear boundary between the importing and non-importing regions, label frequently extends into the non-importing zone in the larger veins. Therefore it is presumed that the larger veins of leaflets at LPI 2-2.3 are actually within exporting zones thus, the leaflets at this stage are possibly at an early stage of transition to the source state. This data finds support from the earlier work by Wimmers and Turgeon (1991) who used [^{14}C] sucrose to examine sink-to-source transition in *P. sativum* L. cv. Little Marvel. Wimmers and Turgeon (1991) reported that the transition process occurs between LPI 1.6 and 1.8. The authors however used leaf length in calculating plastochron index as against average leaflet length used in this study which might account for the discrepancy of approximately 0.4 to 0.5 plastochrons. As such, direct results comparison of Wimmers and Turgeon's (1991) experiments with those reported here cannot be made.

From the literatures it is evident that some variability exist in the number of consecutive leaves which are in sink-to-source transition at any one time. For example, Gagnon and Beebe (1996) reported that four successive leaves were found to be in transition simultaneously in *Moricandia arvensis* L. Four leaves were also in transition in sugar beet and tobacco (Turgeon and Webb, 1973). In contrast, the squash plant has only one leaf at a time in sink-to-source transition (Turgeon and Webb, 1973). The results of the experiments reported here, reveal that 1-2 leaves are under transition at the same time on the pea plant, at any given time. The duration of sink-to-source transition appears to be species dependent. For example, Gagnon and Beebe (1996) reported that sink-to-source transition took an approximate duration of 2.5 plastochrons in *M. arvensis* L., while results of the experiments reported in this paper show that sink-to-source transition in *P. sativum* takes about 2 plastochrons

The data that is presented in this research show that sink-to-source transition in pea leaves is directly related to leaf plastochron index. The sink/source state of leaflets at various LPIs, as well as the duration of the sink-to-source transition process and the number of leaves under transition at a given time may be determined by using the plastochron index.

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