Assimilate export from leaves of chilling-treated seedlings of maize. The path to vein

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Abstract – The structure of the vascular bundles of maize leaves was examined in order to compare it with the changes in [14C]-assimilate export at low temperature. Physiological measurements (photosynthesis, [14C]-assimilate export parameters) were made on two model inbred lines: chilling-tolerant KW 1074 (flint) and chilling-sensitive CM 109 (dent). When the temperature was decreased from 24 to 14 °C, the movement of [14C]-assimilates from the source cell to the phloem loading zone was inhibited more strongly in the dent-type CM 109 line than in the flint-type KW 1074 inbred line. CM 109 seedlings also showed a reduction in radioactivity exported from the 14CO2 feeding area, while KW 1074 seedlings did not. We determined the plasmodesmata frequency on four flint and four dent lines (including KW 1074 and CM 109 lines). This structural analysis indicated that the companion cell/thin-walled sieve tube complex was isolated symplastically from adjoining cells in flint-type inbred lines leaves, but it was not isolated in dent-type inbred lines. We hypothesise that the contribution of the symplastic route of phloem loading, being particularly susceptible to low temperature, might be one of the reasons for the higher chilling-sensitivity of dent genotypes, as compare to flint genotypes. © 2001 Éditions scientifiques et médicales Elsevier SAS.

long-distance transport / low temperature / maize taxonomy / phloem loading / plasmodesmata frequency

BSC, bundle sheath cells / MC, mesophyll cells / PC, parenchyma cells / PCMBS, p-chloromercuribenzenesulphonic acid / TEM, transmission electron microscope

1. INTRODUCTION

Maize (Zea mays L.) is a thermophilic plant which like many plants originated in the tropics. Despite this, strong intraspecies differences are observed in its tolerance to chilling. This diversity is correlated, to some extent, to the taxonomy of Zea mays L. Among four subspecies of maize, only flint (Zea mays ssp. indurata) and dent (Zea mays ssp. indentata) genotypes play an important economic role in temperate climates, particularly in North Europe. Flint genotypes generally demonstrate higher early vigour during cool springs than dent genotypes, although adult dent-type inbred line plants are always much bigger and produce higher yield than flint genotypes.

In spite of the progress in maize cultivation in cool climates, the mechanisms underlying the chilling-sensitivity of maize are still under discussion. Many authors have concentrated on the particular susceptibility of the photosynthetic processes to cold and the lack of efficient mechanisms of defence against photoinhibition [10, 16, 18]. In addition to photosynthesis, roots have been shown to play an important role in the performance of maize seedlings in cool climates [23]. Since both the root growth and root metabolism depend on the supply of carbohydrates from the shoot, attention has also been paid to the effect of chilling on assimilate transport from leaves to roots [25, 28, 30].

Studies on [14C]-assimilate transport kinetics in maize seedlings of several inbred lines of both flint and dent-type, treated with moderate low temperatures (10–15 °C) have not shown distinct differences in the
speed of assimilate transport to the roots. On the other hand, strong intraspecies differences were found in both the time of radiolabel movement into the phloem loading zone and the amount of [%14 C]-assimilates exported from leaves to roots. On the basis of a more detailed study, it was hypothesised that chilling-tolerant and chilling-sensitive maize inbred lines differ in the movement of assimilates from the place of synthesis to the phloem loading zone and the phloem loading rate at moderate low temperatures. Because no differences were found among the tested inbred lines both in photosynthetic rates and sucrose phosphate synthase activity, we focused on the structure of vascular bundles as one of the possible factors determining the short-distance transport and phloem loading. Small and intermediate bundle sheaths were studied because they are believed to be involved in assimilate loading, as opposed to the large bundles that serve in long-distance transport.

2. RESULTS

2.1. Photosynthesis and [%14 C]-assimilate export from leaves

The photosynthetic ($P_{a}$, table I) and [%14 C] incorporation rate ($I_{a}$, table I) decreased by about 50% in both lines at low temperature. The time necessary for radiolabel movement into the phloem loading region increased more in CM 109 than in KW 1074 (table I). The radioactivity exported from leaves ($R_{l}$) changed differently in both lines treated with low temperature. After chilling, $R_{l}$ increased slightly above the control level in KW 1074, while it decreased below in CM 109 (table I).

2.2. Anatomy of the leaf blade’s small and intermediate bundles

The structure of bundles in leaves of tested genotypes, typical for maize, was studied because they are believed to be involved in assimilate loading, as opposed to the large bundles that serve in long-distance transport.

<table>
<thead>
<tr>
<th></th>
<th>$P_{a}$ (µmol CO$_2$·m$^{-2}$·s$^{-1}$)</th>
<th>$I_{a}$ (kBq·min$^{-1}$)</th>
<th>AT (min)</th>
<th>$R_{l}$ (kBq)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KW 1074</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24°C</td>
<td>7.5 ± 0.5</td>
<td>1.13 ± 0.19</td>
<td>18.8 ± 3.0</td>
<td>3.12 ± 0.19</td>
</tr>
<tr>
<td>14°C</td>
<td>4.4 ± 0.8</td>
<td>0.63 ± 0.08</td>
<td>25.7 ± 2.5</td>
<td>3.71 ± 0.61</td>
</tr>
<tr>
<td>CM 109</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24°C</td>
<td>7.4 ± 0.9</td>
<td>1.11 ± 0.46</td>
<td>13.4 ± 0.9</td>
<td>3.16 ± 1.15</td>
</tr>
<tr>
<td>14°C</td>
<td>4.4 ± 0.6</td>
<td>0.56 ± 0.08</td>
<td>26.1 ± 4.0</td>
<td>1.83 ± 0.54</td>
</tr>
</tbody>
</table>

Table I. Photosynthesis and some [%14 C]-assimilate export parameters in the third fully developed leaves (middle part) of KW 1074 or CM 109 seedlings at control (24°C) and chilling (14°C) temperatures. $P_{a}$, Photosynthetic rate (µmol CO$_2$·m$^{-2}$·s$^{-1}$); $I_{a}$, [%14 C] incorporation rate (kBq·min$^{-1}$); AT, time interval between [%14 C] incorporation and appearance in phloem loading region (min); $R_{l}$, leaf radioactivity near the feeding region (kBq). Data represent means for nine plants ± SE and five plant ± SE for photosynthesis and export parameters, respectively.

The BSC exhibited a continuous suberin lamella crossed by numerous plasmodesmata, linking BSC with surrounding mesophyll cells (MC). Xylem, which was directly situated next to BSC, was composed only of one to three conducting elements. Phloem of the vascular bundles consisted of at least one thick-walled sieve tube, one thin-walled sieve tube and a companion cell. In some vascular bundles, there was more than one sieve element of both type. Sieve tubes were arranged in a row occupying the middle part of the bundle and they were separated from the bundle sheath by vascular parenchyma. A vascular parenchyma occupied most of the interfaces between sieve tubes and BSC. Only the last sieve tube from the row in some bundles had a cell wall interface with BSC; in others, the row was ended by a companion cell and only this cell had connections with BSC.

With the exception of S 195 having bigger BSC, sizes of bundle sheaths and vascular bundles were similar in tested genotypes (data not shown). No differences were recorded for cell-to-cell interfaces.
2.3. Plasmodesmata frequency

The frequency of plasmodesmata between MC and BSC as well as between BSC and vascular parenchyma was similar in tested genotypes, with the exception of the S 195 inbred line, which demonstrated more frequent connections between cells of that type (table III, columns b and c). There were also no differences in the plasmodesmata number joining both neighbouring BSC or neighbouring parenchyma cells (PC – data not shown).

The most important difference between the tested genotypes was a higher frequency of plasmodesmata connections between companion cells and adjoining donor cells, such as BSC (table III, column d) and vascular PC (table III, column e), in dent-type inbred lines than in flint-type ones. The frequency of plas-

codesmata connections between BSC and companion cells (figure 1A) in the dent inbred lines was a few times higher than in flint ones (table III column d). Moreover, with the exception of S 335, dent inbred lines showed 4 to 5 plasmodesmata·µm–1 vein between companion cells and parenchyma (figure 1B); the connection was very rare or absent in flint inbred lines (table III column e). Symplastic connections between companion cells and their sieve tubes (figure 1C) were also more frequent in dent-type as compared to flint ones (table III column f). With respect to all these contacts, S 335 showed lower values than other dent-
type inbred lines, but higher than flint ones. There was no difference in the number of connections between PC and thick-walled sieve elements (figure 1D, table III column g).

Table II. Length of interfaces (in micrometers) between cells of different types in leaf sheath and vascular bundles of four flint- and four dent-type inbred line of maize. Results represent means ± SE. Number of vein transverse sections taken into account is indicated in column (a). MC, Mesophyll cells; BSC, bundle sheath cells; PC, vascular parenchyma cells; CC, companion cells; tnSE, thin-walled sieve elements; tkSE, thick-walled sieve elements.

<table>
<thead>
<tr>
<th>No. of veins</th>
<th>MC-BSC</th>
<th>BSC-PC</th>
<th>BSC-CC</th>
<th>PC-CC</th>
<th>CC-tnSE</th>
<th>PC-tkSE</th>
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<tbody>
<tr>
<td>Flint</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KW 1074</td>
<td>24</td>
<td>96.1 ± 26.6</td>
<td>24.6 ± 6.3</td>
<td>5.6 ± 2.1</td>
<td>2.7 ± 1.1</td>
<td>2.7 ± 1.1</td>
</tr>
<tr>
<td>S 311</td>
<td>25</td>
<td>96.7 ± 15.4</td>
<td>26.7 ± 6.6</td>
<td>7.5 ± 3.2</td>
<td>5.0 ± 2.8</td>
<td>5.0 ± 2.8</td>
</tr>
<tr>
<td>S 195</td>
<td>25</td>
<td>121.2 ± 17.4</td>
<td>39.3 ± 7.4</td>
<td>7.0 ± 2.7</td>
<td>5.9 ± 3.0</td>
<td>5.9 ± 3.0</td>
</tr>
<tr>
<td>Co 255</td>
<td>25</td>
<td>96.1 ± 13.8</td>
<td>24.1 ± 7.3</td>
<td>7.6 ± 2.8</td>
<td>4.4 ± 1.9</td>
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</tr>
<tr>
<td>Dent</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM 109</td>
<td>23</td>
<td>96.1 ± 32.0</td>
<td>22.5 ± 8.5</td>
<td>6.2 ± 2.4</td>
<td>3.3 ± 3.0</td>
<td>3.4 ± 1.8</td>
</tr>
<tr>
<td>S 160</td>
<td>25</td>
<td>85.2 ± 19.0</td>
<td>28.9 ± 7.1</td>
<td>6.3 ± 3.2</td>
<td>3.2 ± 1.5</td>
<td>3.2 ± 1.5</td>
</tr>
<tr>
<td>S 208</td>
<td>25</td>
<td>102.2 ± 14.9</td>
<td>26.7 ± 7.5</td>
<td>7.1 ± 2.7</td>
<td>4.7 ± 3.1</td>
<td>4.7 ± 3.1</td>
</tr>
<tr>
<td>S 335</td>
<td>25</td>
<td>95.1 ± 14.3</td>
<td>28.1 ± 4.9</td>
<td>8.4 ± 2.0</td>
<td>4.4 ± 2.3</td>
<td>4.4 ± 2.3</td>
</tr>
</tbody>
</table>

Table III. Plasmodesmata frequency (number of plasmodesmata per micrometer of vein) between cells of different type in bundle sheath and vascular bundles in leaves of four flint- and four dent inbred lines. Number of vein transverse sections taken into account is indicated in column (a). MC, Mesophyll cells; BSC, bundle sheath cells; PC, vascular parenchyma cells; CC, companion cells; tnSE, thin-walled sieve elements; tkSE, thick-walled sieve elements.

<table>
<thead>
<tr>
<th>No. of veins</th>
<th>MC-BSC</th>
<th>BSC-PC</th>
<th>BSC-CC</th>
<th>PC-CC</th>
<th>CC-tnSE</th>
<th>PC-tkSE</th>
</tr>
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<tr>
<td>Flint</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KW 1074</td>
<td>33</td>
<td>287</td>
<td>80</td>
<td>0.9</td>
<td>0</td>
<td>0.9</td>
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<tr>
<td>S 311</td>
<td>25</td>
<td>370</td>
<td>131</td>
<td>1.1</td>
<td>0</td>
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<tr>
<td>S 195</td>
<td>25</td>
<td>791</td>
<td>174</td>
<td>0.6</td>
<td>0.6</td>
<td>4.6</td>
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<tr>
<td>Co 255</td>
<td>25</td>
<td>327</td>
<td>70</td>
<td>1.7</td>
<td>1.1</td>
<td>3.4</td>
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<tr>
<td>Dent</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM 109</td>
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<td>426</td>
<td>99</td>
<td>8.3</td>
<td>5.1</td>
<td>7.9</td>
</tr>
<tr>
<td>S 160</td>
<td>25</td>
<td>301</td>
<td>94</td>
<td>5.7</td>
<td>4</td>
<td>5.1</td>
</tr>
<tr>
<td>S 208</td>
<td>25</td>
<td>439</td>
<td>83</td>
<td>9.1</td>
<td>4.6</td>
<td>6.9</td>
</tr>
<tr>
<td>S 335</td>
<td>25</td>
<td>347</td>
<td>107</td>
<td>5.1</td>
<td>1.1</td>
<td>2.3</td>
</tr>
</tbody>
</table>
3. DISCUSSION

We have examined the structure of vascular bundles of maize leaves in order to compare it with the chilling-induced changes in [14C]-assimilate export. In two model inbred lines, chilling-tolerant KW 1074 and chilling-sensitive CM 109 (Table I), we found no differences in either the photosynthetic rate or the rate of 14CO2 incorporation. On the other hand, we observed a clear cessation of short-distance transport as well as a decrease in [14C]-assimilates exported from the feeding area in the chilling-sensitive genotype CM 109 (Table II), suggesting an inhibition of phloem loading as proposed previously [26]. Similar differences in assimilate export from leaves of plants adapted to either cold or warm environments have been shown in an other C4 grass, Echinochloa crus-galli [21, 22].

Two pathways of phloem loading are discussed in the literature [17, 37, 39]. In symplastic loaders, sugars move through plasmodesmata from MC into the companion cell/sieve tube complex. In apoplastic loaders, sucrose leaks into the apoplast before its active uptake through the membrane of companion cells or sieve element or both. In dicotyledonous plants, different groups of plant species were distinguished on the basis of the number of plasmodesmata per µm2 at the companion cell/mesophyll interface [12]: type 1 (more than ten plasmodesmata); type 1-2a (1–10); type 2a (0.1–1); and type 2b (less than 0.1). Symplastic phloem loading seems to exist in type 1 species (using oligosaccharides as a transport form and possessing intermediary cells) while apoplastic phloem loading operates in type 2b species (using sucrose as a transport form and possessing transfer cells) [24]. In type 1-2a or 2a, sugars seem to be symplastically transported into the phloem, possibly along the sucrose concentration gradient [38]. An additional type, type 2c, has been described, consisting of many C4 and grass-sulcadian acid plants [39]. These plants are similar to the type 2a with respect to symplastic connections between companion cell/sieve tube complex and adjoining cells but have numerous plasmodesmata between BSC and MC.

From the effect of the proton pump inhibitor, PCMBS, it appears that maize uses an apoplastic phloem loading mechanism [26, 34]. The recently identified ZmSUT1 protein may act as the sucrose/H+ symporter involved in this apoplastic pathway [11]. It was suggested that in grasses [3–5], including maize [6], plasmodesmata between companion cell/sieve tube and bundle sheath are rare or lacking. The apparent symplastic isolation of companion cell/thin-walled sieve tubes in grasses was assumed to be related to dryland habitat, where the apoplastic pathway would be an adaptive advantage for unfavourable conditions [5]. However, in some other C4 grasses the companion cell/thin-walled sieve element complex was shown not to be isolated symplastically from the rest of the leaf [2–4] which led to the conclusion that symplastic transport from MC to either type of sieve tubes could not be excluded [2].

In light of our results, it seems that intraspecies differences exist in the extent of symplastic isolation of companion cell/sieve tube complex in maize. In the case of KW 1074 and CM 109 inbred lines, plasmodesmata connections between donor cells and companion cells were about fifteen times more frequent in CM 109 (13.4 plasmodesmata per µm vein, Table II, columns d, e) than in KW 1074 (0.9 plasmodesmata per µm vein, Table II, columns d, e). The plasmodesmatal connections between companion cells and thin-walled sieve tubes were also much more frequent in CM 109 than in KW 1074 (Table II, column f). Therefore, the plasmodesmata network inside the phloem, as well as the contact between donor cells and phloem cells, is much more frequent in CM 109 than in KW 1074.

On the basis of our results, we hypothesise that in CM 109 inbred line, the symplastic pathway may participate in phloem loading in addition to the apoplastic pathway. If we combine the results shown in columns d and e of Tables II and III, the frequency of plasmodesmata between companion cells and donor cells (bundle sheath and PC) in CM 109 is 1.4 per µm2, which is similar to that in type 1-2a loaders. Sucrose transport into the companion cell/sieve tube complex in some woody plants in this group might be symplastic along the sucrose gradient [38]. Turgor-mediated symplastic flow of sucrose to the companion cell/sieve tube complex, contributing to phloem loading, was also demonstrated [19–21] in Ricinus communis, classified as type 1-2a plant. It seems that under conditions favouring photosynthesis, assimilate export from maize leaves might be exported from leaves along sucrose gradient [26–36]. It should be noted that the symplastic transport of solutes from the mesophyll to the sieve tubes has also been suggested in barley [19]; however, other authors [23] have postulated the symplastic isolation of the companion cell/sieve tube complex in that plant.

Our electron microscope observations are in accordance with the analysis of the export parameters [Table I] and earlier results concerning the kinetics of [14C]-assimilate transport [25, 28, 30]. The distinct cessation
of a short-distance transport as well as decrease of [14C]-assimilate exported from the feeding area observed in the chilling-sensitive genotype CM 109 may be related to the chilling-induced inhibition of the symplastic pathway for assimilate transport from source cells to the companion cell/sieve tube complex. Symplastic phloem loading seems to be strongly inhibited by a low temperature [40]. The mechanism of low temperature action on plasmodesmata is a matter of discussion. It was suggested that the low temperature induces a transformation of plasmodesmata to a non-functional state [14]. Furthermore, in symplastic loaders, companion cells collapse, as shown by cold-girdling experiments [13]. Cold temperatures were postulated to exert the selection pressure for the evolution from symplastic to apoplastic phloem loading mechanisms in dicotyledonous plants [39]; however, this hypothesis has been recently questioned [24].

Model inbred lines KW 1074 and CM 109 are typical representatives of Z. mays ssp. indurata and Z. mays ssp. indentata, respectively. Our observations showing higher plasmodesmata frequency between companion cells and adjoining cells in the CM 109 inbred line relative to the KW 1074 inbred line are in agreement with the data on plasmodesmata frequency in three flint and three dent genotypes (Table II) columns d, e). Thus, it seems that observed differences in plasmodesmata frequencies might reflect more general differences between Z. mays ssp. indurata and Z. mays ssp. indentata. The dent materials originated from the warmer climate of the New World. Some of the flint lines that are the source of the currently used maize inbred lines have been adapted to mountainous regions and are generally more chilling-tolerant than dent genotypes. We believe that part of the chilling-tolerance in flint-type maize inbred lines is related to more efficient export of assimilates from the leaf.

4. METHODS

Seedlings (3rd leaf stage) of maize (Zea mays L.) were used in the experiments. Physiological measurements (photosynthesis, [14C]-assimilate export parameters) were performed on model inbred lines: chilling-tolerant KW 1074 (flint) and chilling-sensitive CM 109 (dent). Growth and photosynthesis were tested earlier under both laboratory [15–25] and field conditions [41]. Structural studies (plasmodesmata frequency) were carried out for eight inbred lines (four flint-type inbred lines: KW 1074, S 311, S 195, Co 255; and four dent-type inbred lines: CM 109, S 160, S 208, S 335). The lines S 311, S 195, Co 255, S 160, S 208, S 335 are currently used in a maize breeding programme in Poland. The performance of these inbred lines under field conditions has been described elsewhere [29, 31]. Four of the lines (three flint-type inbred lines: S 311, S 195, Co 255; and one dent-type inbred line: S 335) showed high (8–9, on a 1–9 degree scale) early vigour under cold spring conditions, while two others (dent: S 208, S 160) demonstrated weak early vigour (5–6, on 1–9 degree scale).

4.1. Plant growth

After 4 d of germination in darkness, seedlings were grown for 7–8 d in a growth chamber (photoperiod: 12 h day/12 h night, temperature: 24/22 °C; irradiance: 200 µE·m−2·s−1; relative humidity: 70 %) on Knop water nutrient solution supplemented with Hoagland’s micro-nutrients, until the appearance of the fourth leaf.

4.2. Photosynthesis

The photosynthetic rate of intact leaves was measured in a closed system using an infrared gas analyser (AirTECH 2500-P, GAZEX Co., Poland). During the measurements, seedlings were kept in the thermostated experimental chamber with roots kept in Knop water solution. Photosynthesis was measured in the middle of the third fully developed leaf. Irradiance was the same as during growth. Temperature during the measurements was 24 (control) or 14 °C. Before measurements, seedlings were acclimated to the chamber for 60 min. Experiments were repeated three times independently, with three plants per inbred per experiment.

4.3. In vivo study of [14C]-assimilate transport

Export of [14C]-assimilate from the third fully developed leaf was studied in vivo as described in detail elsewhere [26, 27]. The method is based on the fact that after a short incorporation of 14C (‘pulse’) by a maize leaf, labelled assimilates are exported within several minutes [35] and radioactivity is thus translocated throughout a plant as a ‘wave’ [26, 27]. The experimental conditions were the same as during the growth. The first run was the control conducted at 24 °C, while the effect of chilling (14 °C) was studied in the second one. Each run started with a short (max. 5 min) photosynthetic incorporation of 14CO2 into a small area in the middle of the third leaf. Radioactivity (Bremsstrahlung radiation originated from interaction of 14C β− particles with the leaf tissue) was
counted by three proportional detectors \[22\] attached tightly to the leaf. The first detector measured radioactivity of the feeding area and allowed an estimation of the \(^{14}\text{CO}_2\) incorporation rate as well as the starting time point of the \([^{14}\text{C}]-\text{assimilate export}. The second counter was installed 4.5 cm below the first one. It detected the radioactivity exported from the feeding area \((R_1)\). The second counter was also used to evaluate the time interval of assimilate movement from the place of synthesis (the first detector) to the loading zone. This parameter \(\text{(appearance time)}\) was corrected for the time necessary for the label to move in the leaf between the first and second detectors. To estimate that correction, the third detector was positioned 4.5 cm below the second one. In all three cases, time intervals were calculated between the moments when the radioactivities detected by separate counters reached the halves of their maximum values. As the radioactivity measured by the third detector dropped from its maximum to the minimum indicating that the radioactivity ‘wave’ had passed on, the run was stopped. After the first run \((24 \, ^\circ\text{C})\) had been completed, leaves and roots were cooled to \(14 \, ^\circ\text{C}\) in 30 min. Before the start of the second run, plants were acclimated to the new conditions for 60 min. The second run started with \(^{14}\text{CO}_2\) feeding into the leaves \(\text{when experiments were conducted at the same temperature conditions. Data are shown as difference between the first and second detectors. To estimate that correction, the third detector was positioned 4.5 cm below the second one. In all three cases, time intervals were calculated between the moments when the radioactivities detected by separate counters reached the halves of their maximum values. As the radioactivity measured by the third detector dropped from its maximum to the minimum indicating that the radioactivity ‘wave’ had passed on, the run was stopped. After the first run \((24 \, ^\circ\text{C})\) had been completed, leaves and roots were cooled to \(14 \, ^\circ\text{C}\) in 30 min. Before the start of the second run, plants were acclimated to the new conditions for 60 min. The second run started with \(^{14}\text{CO}_2\) feeding into the leaves \(\text{as described above}. The same level of radioactivity incorporation into the leaves was ensured as in the first run, as well as the same counting geometry. As it was reported in earlier studies \[26, 30\] no differences were found in the transport parameters between the first and the second run performed between 3 and 7 h after the light was switched on, when experiments were conducted at the same temperature conditions. Data are shown as difference between the first and the second run, expressed as a percentage of the first run. Experiments were repeated in five independent experiments, one plant per inbred per experiment.

4.4. Electron microscope observations

The material for electron microscopical examination was taken from leaf blades of the fully expanded third leaf. The samples were cut from the middle part of the blade \(\text{(half way from the tip to the base). The small veins between the third and the fourth large ones counting from the blade edge were selected. Samples of three leaves per harvest per inbred line were taken in three independent experiments. Specimens for electron microscopical examination were fixed in 2.5 \% glutaraldehyde in 0.1 M phosphate buffer \(\text{(pH 7.2)}\) for 4 h, at \(4 \, ^\circ\text{C}\) and postfixed in 1 \% osmium tetroxide for 2 h at \(4 \, ^\circ\text{C}\). The material was dehydrated in ethanol \((10-70 \%)\), acetone \((70-100 \%)\) and embedded in Spurr’s resin \[32\] The ultrathin \((70 \text{~nm)}\) sections on copper grids were stained with uranyl acetate and lead citrate. Sections were visualised with a transmission electron microscope JEM-1200 EX \(\text{(JEOL, Japan)}\) at 80.0 kV.

4.5. A length of cell interfaces

The lengths of cell-to-cell interfaces were measured from micrographs of low magnification \(1 \, 000 \times\) with the use of Analysis 2.1 \(\text{(Soft-imaging Software GmbH). Linear dimensions were analysed for 24, 23 and 25 vascular bundles of KW 1074, CM 109 and other lines, respectively.}

4.6. Plasmodesmata frequency

The plasmodesmata number was estimated directly under the electron microscope with the magnification of \(50 \, 000 \times\) \(\text{(5000 TEM and 10× optical magnification). Only plasmodesmata extending more than half-way across the cell interface were counted. Branched plasmodesmata were scored as one. Plasmodesmatal frequencies were determined in 33 bundles in the case of KW 1074 and CM 109 and 25 bundles in the case of other lines. Three bundles per single leaf section were counted for different plants selected from three independent experiments.}

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