4. RESULTS

The monocot leaf offers an ideal model to examine interrelationship between cell maturity and various developmental and growth responses. In comparison to dicot leaves, studying these processes in monocot leaves has an advantage; for example, the dicot leaf initiates as a leaf primordia, thereafter its expansion is restrained, and its further expansion and development is dependent on availability of light. By contrast, the process of leaf expansion in monocot leaf is not obligatorily dependent on light, and leaf expansion continues, even in darkness without acquisition of photosynthesis. In addition, the monocot leaf develops from a meristem located at the leaf base. By virtue of this, the developing monocot leaves possess cells of different maturity along the length of the leaf. The existence of cell maturity gradient in the monocot leaf has been exploited to investigate the effect of light on gene expression in these leaves, particularly those related to development of photosynthesis. On exposing dark-grown monocot leaves to light, the initiation and magnitude of light-induced responses can be examined along the length of the leaf cells having different maturity.

Light-regulated responses in monocot leaves most prominently include the acquisition of photosynthesis. Therefore, in the present study an extensive characterization of this process was done by using key markers of this process, such as increase in the level of photosynthetic pigments and associated proteins. Since the influence of light on leaf development is not necessarily confined to chloroplast alone, we also examined the level of few cytosolic enzymes such as β-amylase, peroxidase and catalase.

In view of paucity of studies on pearl millet as a physiological system, in the first phase of the present study we made an extensive characterization of photosynthetic pigment formation in leaves to correlate with the observation made in other plant systems. The representative markers for chloroplast development are chlorophyll, carotenoid and protochlorophyllides. Of these carotenoid and protochlorophyllide is synthesized in darkness and are present in etioplast. The exposure of light accelerates the biosynthesis of these pigments. Exposure of dark-grown leaves to light also converts protochlorophyllide to chlorophyllide, and this
conversion in turn acts as a signal to stimulate chlorophyll biosynthesis. The light also stimulates carotenoid biosynthesis, which acts as a photosynthetic accessory pigments. Since the carotenoid and chlorophyll associate together to form an active photosynthetic antenna complex, these pigments are required to be synthesized in predetermined proportions. Therefore their biosynthesis is highly coordinated to maintain a strict stoichiometry between chlorophyll and carotenoid amounts. We also examined, the effect of light on levels of plastidic protein protochlorophyllide oxidoreductase (POR), which accumulates to high level in dark-grown seedlings and on exposure to light, the level of POR protein rapidly declines, marking the onset of chlorophyll biosynthesis and acquisition of photosynthesis. The effect of light on cytosolic compartment was investigated using \( \beta \)-amylase and peroxidase as a marker enzyme. The influence of light on other subcellular compartments such as peroxisome was analyzed using catalase as a marker enzyme.

4.1. Morphological features of the pearl millet seedlings

Pearl millet seeds are ovoid and about 2 mm in length. On imbibition radicle and shoot tip protrude from seed coat in about 24 h after sowing. During initial phase of seedling development, mesocotyl along with shoot tip expands and the leaf remained enclosed in coleoptile for 3-4 d from sowing. After that, the expanding leaf ruptures the coleoptile and continues its expansion. The light-and dark-grown seedlings manifest significant morphological differences in their developmental patterns, characteristic of photomorphogenic and scotomorphogenic development pathways. The light-grown seedlings have green expanded leaves and a short mesocotyl, because exposure to light suppresses elongation of mesocotyl. The mature pearl millet leaves after few days of expansion can be distinguished as consisting of two distinct portions, upper portion as leaf blade, and a lower portion as leaf sheath. In leaf blade portion, the leaf is wider, whereas leaf sheath at the base is narrow and tubular. Similarly the leaf blade portion is dark-green while the basal leaf sheath is pale green. In comparison to light-grown seedlings the dark-grown seedlings possess a long mesocotyl, but the first leaf is smaller in size than the light-grown leaves. The dark-grown leaf is yellow in color as it lacks chlorophyll and therefore accumulation of yellow colored cartenoid is visualized.
There was no apparent influence of light on germination as the germination percentage was similar in both light-and dark-grown seedlings. In this study, pearl millet seedlings were raised on only distilled water, therefore after 12-d in light and after 9-d in dark the leaf begin to senescence. Hence, experiments on first leaf of pearl millet seedlings were carried out only upto 9-d from sowing. The primary leaf was about 11 cm long in 10-d old light-grown seedlings, whereas in dark-grown seedlings, the leaf expansion ceased completely after 8-d, and the leaf size remained about 7 cm. In the present study effect of light on leaf development was studied by comparing continuous red light and white light-grown seedlings with seedlings grown in darkness.

4.2. Chlorophyll and Carotenoid

The marker of acquisition of photosynthesis in a developing leaf is initiation of chlorophyll accumulation. In order to characterize it, the profile of chlorophyll accumulation was analyzed in pearl millet leaf, from the day the first leaf begins expanding i.e. 3-d from sowing till 9th d. Since the leaves of dark-grown seedlings possess no chloroplasts, the accumulation of chlorophyll was monitored in the first leaf harvested from WL-and RL-grown seedlings. In initial period of leaf growth, leaves are shielded by coleoptiles up to 4-d from sowing, and then first leaf emerges out of the coleoptile and is directly exposed to light. Pearl millet leaf consists of two parts, the upper region as leaf blade and the lower region as leaf sheath, however for estimation of the pigment accumulation, chlorophyll and carotenoid content of the entire leaf was measured.

Fig. 4.1 shows the profile of chlorophyll accumulation in the first leaf excised from WL-and RL-grown seedlings from 3-9-d. It is evident that in both WL and RL light-grown leaves the amount of chlorophyll continually increases with increase in the age. However, the amount of chlorophyll was more in WL-grown leaves compared to RL-grown leaves. It is evident that among the two lights, exposure of seedlings to WL induced greater rate of chlorophyll accumulation, compared to seedlings exposed to the RL. The higher level of chlorophyll in leaf by ninth day signifies the fact that the chlorophyll level in the leaf increases along with the increase in the length of the leaf.
Figure 4.1. Time course of chlorophyll accumulation in the first leaf of pearl millet (WGC-75). Seedlings were grown under WL (○-) and RL (●-) from sowing. At the time point (days) indicated, the first leaf was excised at the mesocotyl junction, and the chlorophyll amount was estimated.
Since in higher plants chlorophyll consists of two species viz. chlorophyll a and chlorophyll b. We examined the profile of accumulation of chlorophyll a and chlorophyll b during the period of leaf expansion. Fig. 4.2 shows that both RL-and WL-grown leaves accumulate more chlorophyll a than chlorophyll b. It is evident that under WL higher level of chlorophyll b is accumulated than under RL. Basically the accumulation profile of both chlorophyll a and chlorophyll b follow a pattern similar to that observed for total chlorophyll.

We also examined whether the formation of chlorophyll a and chlorophyll b in pearl millet leaf maintains a co-ordinated balance in their accumulation, by examining Chl a/b ratio. Fig. 4.3 shows that Chl a/b ratio in RL-grown leaf varies between 1.63-3.77, with a highest value on 5th day from sowing. Thereafter the Chl a/b ratio declined and is stabilized at 2.45. Similarly Chl a/b ratio in WL-grown leaf also increased from a low level of 1.95 to highest level of 3.22 on 6th day and its value by 10th day was similar to that observed from RL-grown leaf.

The accumulation of carotenoid in pearl millet leaf followed a profile similar to the accumulation of chlorophyll in developing leaf (Fig. 4.4). In dark-grown leaves the amount of carotenoid was quite low, however, its level increased slowly with the age of the seedlings. The exposure of seedlings to WL or RL enhanced the accumulation of carotenoid in the first leaf. Among the two light treatments used, WL stimulated carotenoid accumulation more significantly than RL. However, carotenoid accumulation follows a similar profile under both RL and WL.

Since carotenoid also acts as photosynthetic accessory pigments, a strict stoichiometry is required to be maintained between the levels of carotenoid and chlorophylls. Moreover in absence of carotenoids, chlorophyll molecules are photooxidized by light, so the absence of carotenoids leads to photooxidation of chlorophylls and loss of the functional chloroplasts. The profile of Chl/Car ratio is stabilized at nearing 5.0 in both continuous WL-and RL-grown seedlings showed in Fig. 4.5.

The influence of light on the level of chlorophyll was also examined by transferring dark-grown seedlings to light, to elicit the possible duration of lag between exposure to light and initiation of chlorophyll biosynthesis. The results presented in Fig. 4.6 shows that on exposure to WL or RL, there is a rapid formation
Figure 4.2. Time course of chlorophyll a and chlorophyll b accumulation in first leaf of pearl millet (WGC-75). Seedlings were grown under continuous WL (○, ⬤) or RL (•, ⬤) from sowing. At the time points indicated (days), the first leaf was excised at the mesocotyl junction, and the amount of chlorophyll a (○, ⬤) and chlorophyll b (○, •) was estimated.
Figure 4.3. Chl a/b ratio in the first leaf of pearl millet (WGC-75), during the period of leaf expansion. Seedlings were grown under WL (○) and RL (●) from sowing. At the time point (days) indicated, the first leaf was excised at the base and the Chl a/b ratio was estimated.
Figure 4.4 Time course of carotenoid accumulation in the first leaf of pearl millet (WGC-75). Seedlings were grown under WL (○), RL (●) and D (♦) from sowing. At the time point (days) indicated the first leaf was excised at the mesocotyl junction, and the carotenoid amount was estimated.
Figure 4.5. Chlorophyll/carotenoid ratio in first leaf of pearl millet (WGC-75), during the period of leaf expansion. Seedlings were grown under continuous WL (○) and RL (•) from sowing. At the time point indicated, (days) the first leaf was excised at the base and the Chl/Car ratio was estimated.
Figure 4.6. Time course of chlorophyll accumulation in the first leaf of pearl millet (WGC-75). Seedlings were grown in continuous darkness up to 6-d from sowing and then transferred to either continuous WL (○) or (●) RL. At the time points indicated (h), the first leaf was excised at the mesocotyl junction, and the chlorophyll amount was estimated.
of chlorophyll. Both in WL and RL the chlorophyll accumulation showed nearly equivalent levels at 2 h after transfer to light. Thereafter the chlorophyll accumulation was more rapid in WL-than in RL-grown seedlings. On comparing levels of chlorophyll after 24 h exposure of RL-and WL-grown seedlings (Fig. 4.6), with that of seedlings grown under continuous WL and RL (Fig. 4.1) it is evident that even after 24 h of light exposure the level of chlorophyll in seedlings transferred to light is only about half of that grown under continuous light.

The influence of light on synthesis of individual Chl species was examined by monitoring levels of chlorophyll a and chlorophyll b in first leaf after transfer of dark-grown seedlings to light. Fig. 4.7 shows that after 2 h of transfer from darkness to light the chlorophyll a and chlorophyll b amounts were nearly equal. However, on prolonged light exposure, chlorophyll a was predominantly synthesized than chlorophyll b. The profile of chlorophyll a and chlorophyll b accumulation were similar both in WL-and RL-grown seedlings.

The analysis of Chl a/b ratio in seedlings transferred to light shows that at 2 h (Fig. 4.8) after transfer to light, the Chl a/b ratio is close to 1.4. Thereafter, the leaves form more chlorophyll a than chlorophyll b. By 12 h of transfer a balance between Chl a and Chl b formation is achieved. (Fig. 4.8). Thereafter in WL Chl a/b ratio value stabilized at 2.0 whereas in RL stabilized at 2.50.

The transfer of etiolated seedlings to light also stimulated the carotenoid formation (Fig. 4.9). The dark-grown leaves possessed a basal amount of carotenoids, which however remains at a low level (Fig. 4.4) throughout the period of leaf expansion. The exposure to light stimulated the formation of carotenoid both in WL- and RL-grown seedlings, with WL exposure being more effective for stimulating carotenoid formation than the RL (Fig. 4.9).

The stoichiometry between carotenoid and chlorophyll formation was also analyzed (Fig. 4.10). In dark-grown seedlings at the time of transfer to light, during the initial phase of transfer Chl/Car ratio was low. Thereafter it increased rapidly due to accumulation of chlorophyll in the leaf upto 12 h. Thereafter Chl/Car ratio reached about 3.78, 3.58 for both WL and RL respectively.

While we examined the chlorophyll and carotenoid level in whole first leaf, a visual inspection of leaf clearly shows that leaf has more chlorophyll in the upper leaf
Figure 4.7. Time course of chlorophyll a and chlorophyll b accumulation in the first leaf of pearl millet (WGC-75). Seedlings were grown in continuous darkness up to 6-d from sowing and then transferred to either continuous WL (○, ○) and or RL (■, ●). At the time points (h) indicated the first leaf was excised at the mesocotyl junction, and the Chl a (○, ●) and Chl b (■, ○) was estimated.
Figure 4. 8.  Chl a/b ratio in first leaf of pearl millet (WGC-75) after transfer to light. Seedlings were grown in continuous darkness upto 6-d from sowing and then transferred to either continuous WL (○) or RL (•). At the time points indicated (h), the first leaf of pearl millet was excised at the mesocotyl junction, and the Chl a/b ratio was estimated.
Figure 4.9. Time course of carotenoid accumulation in first leaf of pearl millet (WGC-75) leaves. Seedlings were grown in continuous darkness (D) (•) upto 6-d from sowing, and then transferred to either continuous WL (•) or RL (o) for different period of time. At the time points (h) indicated the first leaf was excised at the mesocotyl junction, and the carotenoid amount was estimated.
Figure 4.10. Chl/Car ratio in first leaf of pearl millet (WGC-75) during the period of light exposure. Seedlings were grown in continuous darkness up to 6-d from sowing and then transferred to either continuous WL (○-) or RL (●-). At the time points (h) indicated, the first leaf was excised at the mesocotyl junction, and the Chl/Car ratio was estimated.
blade region than the lower sheath region. Since pearl millet leaf is continually expanding due to the activity of basal meristem. We examined the influence of cell position on accumulation of both chlorophyll and carotenoid in the leaf. To achieve this after harvesting the first leaf was segmented into 1 cm long segments numbered from base to leaf tip and pigment amount was estimated.

Fig. 4.11 shows that there is a distinct gradient of chlorophyll accumulation in first leaf harvested from seedlings grown under continuous WL and RL. The maximal amount of chlorophyll was observed near the leaf tip and a minimal amount was observed at the leaf base. The profile of chlorophyll accumulation was similar both in RL-and WL-grown leaves, but the amount of chlorophyll was lower in RL-grown leaves.

The profile of chlorophyll a and chlorophyll b accumulation in leaf segment was followed along the length of leaf (Fig. 4.12). Similar to total chlorophyll the amount of both Chl a and Chl b increased from leaf base to leaf tip with more Chl being present in WL-grown leaves. In case of RL-grown leaves, Chl a level follows a profile similar to that of Chl a under WL, but the profile of Chl b distribution was less in RL-grown seedlings.

The fact that leaves of 7-d old RL-grown seedlings make less Chl b than WL-grown seedlings is evident by the comparison of Chl a/b ratio in different segments along the length of the leaf. In case of WL-grown seedlings showed a Chl a/b ratio between 2.13-2.5, while in RL-grown leaves showed a low Chl a/b ratio in basal segment (1.90), its level increased along the length of leaf and was stabilized at 3.17. It was evident that among the two light treatments, red light-grown leaf shows less formation of Chl b thereby leading to high Chl a/b ratio in RL-grown seedlings (Fig. 4.13).

The analysis of acetone extract of individual segments by absorption spectroscopy also correlated with the earlier results. The peak at both red and blue absorption regions, signifies the absorption of chlorophyll and carotenoid respectively, it is evident that the amount of both chlorophyll and carotenoid increases towards the tip of the leaf (Fig. 4.14).

Analogous to chlorophyll distribution, the distribution of carotenoid also showed increase in level towards the tip of the leaf. However comparison between
Figure 4.11. Distribution of chlorophyll along the length of first leaf of pearl millet (WGC-75) were grown under continuous WL (○) or RL (●) from sowing. The first leaves of 7-d-old seedlings were excised at the mesocotyl junction. The leaves were then excised to 1 cm long segments from leaf base to tip, and used for estimation of chlorophyll. Segments are numbered from base to the leaf tip.
Figure 4.12. Distribution of chlorophyll a and chlorophyll b along the length of first leaves of pearl millet (WGC-75). Seedlings were grown under continuous WL (o, ◇) or RL (*, •) from sowing. The first leaves of 7-d old seedlings were excised at the mesocotyl junction. The leaves were then cut into 1 cm long segments from the leaf base to tip, and used for estimation of chlorophyll a (o, •) and chlorophyll b (◇, •). The segments were numbered from base to the leaf tip.
**Figure 4.13.** Profile of chl a/b ratio in first leaf of pearl millet (WGC-75). Seedlings were grown under continuous WL (○) or RL (●). The first leaf of 7-d-old seedlings were excised at the mesocotyl junction. The leaves were then excised to 1 cm long segments from leaf base to tip, and used for estimation of Chl a/b ratio. Segments were numbered from base to the leaf tip.
Figure 4.14. Absorption spectra of 80% acetone extracts from first leaf of 7-d-old pearl millet (WGC-75). Seedlings were grown under continuous WL from sowing. The first leaf was harvested and excised to 1 cm segments from leaf base to tip, and used for pigment extraction for spectral analysis. Segments are numbered from base to the leaf tip. The number in spectra indicates the segment number.
dark-and light-grown leaves clearly show that transfer to light significantly effect the level of carotenoid accumulation in the leaf. Exposure to light stimulates carotenoid accumulation in a distribution profile, which is parallel to cell maturity gradient. It is evident that the older leaf segment shows a high accumulation of carotenoid level (Fig. 4.15).

Apparently the profile of carotenoid (Fig. 4.16) accumulation is imprinted and regulated by leaf development. The comparison of distribution of carotenoid in leaf of different age from 3-d to 9-d old shows a similar profile. In all cases the amount of carotenoid level at leaf tip is more than the leaf base. Fig. 4.16 clearly shows that carotenoid accumulation gradient are correlated to cell maturity in pearl millet leaf. The absorption spectra of acetone extracts of etiolated leaf clearly shows that leaf tip has higher amount of carotenoid than the leaf base (Fig. 4.17).

The Chl/Car ratio in pearl millet leaf showed that in WL-grown leaf Chl/Car amount was in the range of 1.17-0.94, in RL-grown leaves the Chl/Car ratio was 1.03 at leaf base and declines to 0.68 at the tip. It is evident that red light is not as effective as WL in stimulating Chl biosynthesis in basal segments of the leaf (Fig. 4.18).

4.3. Chlorophyll and carotenoids in pearl millet mutant

In yellow stripe mutant leaves, yellow and green tissues were dissected carefully, and its absorption was examined. Yellow portion contains very little amount of chlorophyll but show a considerable level of carotenoid (Data not shown) showing absorption spectra similar to wild type. In case of white stripe mutant, white portion of leaf contains insignificant amount of carotenoids and chlorophyll. The similar analyses were also done for zebra mutant that has transverse yellow zebra bands. The yellow stripe, which appears on low temperature treatment, has only carotenoids, and chlorophyll level could not be detected. The above studies highlighted that in zebra mutant on lowering temperature below 10°C shuts off chlorophyll biosynthesis but allowed normal carotenoid synthesis to proceed. Fig. 4.19 shows the quantitative analysis of chlorophyll and carotenoid pigments in green and yellow segments of stripe and zebra mutant. The result shows that light promotes the synthesis of chlorophyll and carotenoid levels in green tissues whereas in yellow tissues the chlorophyll development is arrested at the early stages of plastid
Figure 4.15. Distribution of carotenoid along the length of first leaf of pearl millet (WGC-75). Seedlings were grown under continuous WL (▼) or RL (△) or D (○) from sowing. The first leaves of 7-d-old seedlings were excised at the mesocotyl junction. The leaves were then excised to 1 cm long segments from leaf base to tip, and used for estimation of carotenoids. Segments were numbered from base to the leaf tip.
Segment distance from leaf base (cm)

Figure 4.16. Time course of carotenoid accumulation in first leaf of pearl millet (WGC-75) seedlings. Seedlings were grown under continuous darkness from sowing. At the time point indicated (days), the first leaves of seedlings were excised at the mesocotyl junction. The leaves were then excised to 1 cm long segments from leaf base to tip, and used for estimation of carotenoids. Segments were numbered from base to the leaf tip.
Figure 4.17. Absorption spectra of 80% acetone extracts from first leaf of 7-d-old pearl millet (WGC-75). Seedlings were grown under continuous WL from sowing. The first leaf was harvested and excised to 1 cm segments from leaf base to tip, and used for pigment extraction for spectral analysis. Segments are numbered from base to the leaf tip. The number in spectra indicates the segment number.
Figure 4.18. Profile of Chl/Car ratio in first leaf of pearl millet (WGC-75). Seedlings were grown under continuous WL (○) and RL (●) from sowing. The first leaves of 7-d-old seedlings were excised at the mesocotyl junction. The leaves were then excised to 1 cm long segments from leaf base to tip, and used for estimation of Chl/Car ratio. Segments were numbered from base to the leaf tip.
Figure 4.19. Distribution of chlorophyll and carotenoid in yellow (Y) and green (G) sector of yellow stripe (IP 8284) and zebra (IP 8283) mutant seedlings were grown under a photoperiod consisting of continuous white light for 16 h and complete darkness for 8 h at 25°C. In zebra four days under same conditions, thereafter seedlings were transferred to 8 h darkness at 10°C and 16 h continuous WL at 25°C. The green and yellow band leaf tissues were separated 1 cm below the tip of the leaves and estimated for chlorophyll and carotenoids.
development when insignificant amount of chlorophyll is present.

### 4.4. Protochlorophyll(ide) Pchl(ide)

In angiosperms, biosynthesis of chlorophyll is obligatorily dependent on the light. When seedlings are grown in continuous darkness, the proplastids of the developing leaves differentiate into etioplast instead of chloroplasts. These etioplasts accumulate the precursor of chlorophyll biosynthesis Pchlide. The reduction of Pchlide to Chlide is a light dependent step in angiosperms. Chlide act as the intermediate form between the Pchlide and chlorophylls. When dark-grown seedlings are exposed to light the accumulation of chlorophyll is initiated. The phototransformation of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) also involves structural changes in the pigment protein complexes of etioplasts, which occurs during the conversion of Pchlide to Chlide and spectroscopically detected as Shibata shift (Shibata, 1957).

The accumulation of Pchlide in pearl millet leaves was examined by, dissecting 1 cm long segments of first leaves from base to tip, harvested from dark-grown seedlings. The accumulation of Pchlide in pearl millet leaves was found to be strongly influenced by cellular position in leaf. In dark-grown pearl millet leaves the maximal amount of Pchlide was observed in the leaf region near to the leaf tip (Table 4.1), while the same leaf possessed least amount of Pchlide at the base. In essence, the gradient of Pchlide accumulation in dark-grown leaves was similar to Chl accumulation gradient in light-grown leaves (Table. 4.1).

Similar examination of fluorescence spectra of leaves grown in continuous WL-and RL showed that the level of Pchlide was below the level of detectability, and the chlorophylls was predominantly present in Chlide form (Table. 4.2). Moreover these results also indicate that maximal amount of Chlide was present in leaf tip and least amount is present in the leaf base. To study the complete conversion of Pchlide to Chlide, 7-d-old dark-grown pearl millet control seedlings were transferred to continuous RL for 30 minutes. 30 min of RL is sufficient to complete the conversion of Pchlide to Chlide. It is evident that though maximal amount of chlorophyll in leaves belong to Chlide, a little amount of Pchl is also presents (Table. 4.3).
Table 4.1. Distribution of Pchlide and Chlide along the length of dark-grown pearl millet (WGC-75) leaves. Seedlings were grown under continuous darkness up to 7-day from the time of sowing. Thereafter first leaves of 7-day old seedlings were excised at the mesocotyl junction. The leaves were excised into 1 cm long segments from leaf base to tip, and used for estimation of Pchlide and Chlide. The segments were numbered from base to the leaf tip.

<table>
<thead>
<tr>
<th>Segment distance from leaf base (cm)</th>
<th>Chlide amount (µg/segment)</th>
<th>Pchlide amount (µg/segment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.0013</td>
<td>0.573</td>
</tr>
<tr>
<td>2.</td>
<td>0.0017</td>
<td>0.717</td>
</tr>
<tr>
<td>3.</td>
<td>0.0013</td>
<td>0.544</td>
</tr>
<tr>
<td>4.</td>
<td>0.0023</td>
<td>0.946</td>
</tr>
<tr>
<td>5.</td>
<td>0.0021</td>
<td>0.889</td>
</tr>
<tr>
<td>6.</td>
<td>0.0025</td>
<td>1.061</td>
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</tbody>
</table>
Table 4.2. Distribution of Pchlide and Chlide along the length of WL or RL-grown pearl millet (WGC-75) leaves. Seedlings were grown under continuous WL or RL upto 7-day from the time of sowing. Thereafter first leaves of 7-day old seedlings were excised into 1 cm long segments from leaf base to tip, and used for estimation of Pchlide and Chlide. The segments were numbered from base to leaf tip.

<table>
<thead>
<tr>
<th>Segment</th>
<th>White light</th>
<th></th>
<th></th>
<th>Red light</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chlide (peak nm)</td>
<td>Chlide (jug/segment)</td>
<td>Chlide (peak nm)</td>
<td>Chlide (jug/segment)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>669.0</td>
<td>0.043</td>
<td>668.0</td>
<td>0.070</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>670.0</td>
<td>0.139</td>
<td>669.0</td>
<td>0.206</td>
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<td></td>
</tr>
<tr>
<td>3</td>
<td>671.0</td>
<td>0.438</td>
<td>674.0</td>
<td>0.620</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>672.0</td>
<td>1.11</td>
<td>670.0</td>
<td>1.70</td>
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<td>2.53</td>
<td>670.0</td>
<td>2.54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>676.2</td>
<td>3.93</td>
<td>673.4</td>
<td>4.32</td>
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<td></td>
</tr>
<tr>
<td>7</td>
<td>676.4</td>
<td>4.73</td>
<td>673.3</td>
<td>3.45</td>
<td></td>
<td></td>
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</table>
Table 4.3. Distribution of Pchlide and Chlide along the length of dark-grown pearl millet (WGC-75) leaves. Seedlings were grown under continuous darkness upto 6-day from the time of sowing. Thereafter seedlings were transferred to continuous RL for 30 min and the first leaf was excised at the mesocotyl junction. The leaves were then cut into 2 cm long segments from leaf base to tip, and used for estimation of Pchlide and Chlide. The segments were numbered from base to the leaf tip.

<table>
<thead>
<tr>
<th>Segment</th>
<th>Chlide (µg/segment)</th>
<th>Pchlide (µg/segment)</th>
<th>Chlide peak position (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base</td>
<td>0.020</td>
<td>0.001</td>
<td>674.0</td>
</tr>
<tr>
<td>Middle</td>
<td>0.043</td>
<td>0.005</td>
<td>673.2</td>
</tr>
<tr>
<td>Tip</td>
<td>0.051</td>
<td>0.007</td>
<td>673.0</td>
</tr>
</tbody>
</table>
4.5. Protochlorophyllide in pearl millet mutant

Since accumulation of Pchlide in tissue signifies a block in chloroplast biosynthesis, we analyzed the zebra and yellow stripe mutants to examine this possibility. These yellow mutants which have yellow stripe parallel to green stripe were grown under WL and green and yellow leaf tissues were harvested and analyzed for Chlide amount (Tables. 4.4 and 4.5). This indicates that white segments of the leaf have no functional chloroplast, and Chl. It is therefore evident that defect in Chl synthesis in white segment of the leaf lies down stream to Pchlide formation step, therefore, Pchlide cannot be converted to Chlide due to the absence of the functional chloroplast.

4.6. Protochlorophyllide oxidoreductase (POR)

In etiolated leaves of monocot seedlings, proplastid develops into etioplast, possessing a central prolamellar body, consisting of several proteins. One of the important proteins in etioplast is POR, which accumulates at high level. POR also acts as one of the marker enzyme for initiation of light mediated transition of etioplast to chloroplast. The POR is primarily responsible for catalyzing reduction of Pchlide to Chlide on exposure to light. In etioplast, POR forms a ternary complex with NADH and Pchlide, which on illumination instantaneously reduces Pchlide to Chlide. Recent researches have shown that angiosperms have two POR proteins; namely POR A, and POR B. Of these POR A is present in etioplasts and exposure of leaves to light initiates a rapid decline in its level and it participates in chlorophyll biosynthesis only for a limited duration. POR B is present in chloroplasts and is responsible for the formation of chlorophyll in chloroplasts. In present study, we used POR as a marker enzyme to study the transition of etioplast to chloroplast. In addition we also compared the amount of POR in wild type chloroplasts versus mutant chloroplasts. The polyclonal antibodies used in this study were raised against a mixture of POR A and POR B of barley, which also cross-reacted with pearl millet POR.

The POR protein levels were compared in WL-and dark-grown leaves of pearl millet by western blotting. Fig. 4.20 shows that in dark-grown leaves (WGC-75) POR A protein predominates, whereas in light-grown leaves POR B is a dominant protein. Since polyclonal antibodies were used in this study to recognize both POR A and
Table 4.4. Distribution of Chlide in green and white stripes of leaves of 7-d old pearl millet (SW-1) mutant. Seedlings were grown under a photoperiod consisting of 16 h light and 8 h dark at 25°C from the time of sowing. The green and white tissues of the leaves were excised and used for estimation of Chlide.

<table>
<thead>
<tr>
<th>Segment</th>
<th>Chlide (μg/segment)</th>
<th>Chlide peak position (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green</td>
<td>3.070</td>
<td>674.0</td>
</tr>
<tr>
<td>White</td>
<td>1.029</td>
<td>673.2</td>
</tr>
</tbody>
</table>
Table 4.5. Distribution of Chlide in green and yellow stripes of leaves of pearl millet (IP 8292) mutant leaves. Seedlings were grown under a photoperiod consisting of 16 h light and 8 h dark at 25°C for 4 days. Thereafter the seedlings were transferred to 16 h light at 25°C and 8°C for 8 h upto 7 day from the time of sowing. The yellow and green tissues of the leaves were excised and used for estimation of Chlide.

<table>
<thead>
<tr>
<th>Segment</th>
<th>Chlide peak position (nm)</th>
<th>Chlide (μg/segment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yellow</td>
<td>678.0</td>
<td>0.178</td>
</tr>
<tr>
<td>Green</td>
<td>675.0</td>
<td>1.35</td>
</tr>
<tr>
<td>Green</td>
<td>676.0</td>
<td>1.04</td>
</tr>
</tbody>
</table>
Figure 4.20. Quantification of POR protein in pearl millet (WGC-75) leaves. Seedlings were either grown in continuous white light (WL) or darkness (D) upto 7-d from the time of sowing. Thereafter first leaf was harvested, the POR level was determined by western blotting (Inset). The relative amount of POR was calculated using the intensity of POR band present in dark-grown leaf as 100%. Note the difference in mobility of POR in WL- grown leaf, which is light stable POR B.
POR B, the distinction between POR A and POR B was found to be primarily based on the respective MW. It is known that POR B of light-grown leaves has a MW which is about 2 kD less than that of POR A present in etioplast, whose molecular weight is about 36 kD. It is evident from results presented in Fig. 4.20 that POR of dark-grown leaves has higher MW than that of light-grown leaves indicating that these are POR A and POR B protein respectively.

The profile of distribution of POR protein along the length of dark-grown seedlings was analyzed by western blotting. The leaf was segmented in 1 cm long pieces numbered from base to the leaf tip, and the amount of POR was analyzed in each segment. Fig. 4.21 show that in dark-grown leaves the level of POR A protein increases from base to the leaf tip. In contrast the light-grown leaves, which has only POR B protein shows a massive decline in level of POR B protein (Fig. 4.22). Particularly in the segments near the leaf base the level of POR B protein is very low. However, inspite of decline in the level of POR B protein, its average pattern of distribution was not altered. Most interestingly the tip of the light-grown leaf showed a high content of POR B protein.

The analysis of POR protein in yellow stripe mutant showed that in dark-grown leaves POR A predominantly present. In contrast, in the green segment excised from leaf only POR B is present. Similarly in yellow segments excised from leaf only POR B appears to be present. On a comparative scale, though green and yellow sector of leaf are adjacent to each other, the yellow segment appears to have higher level of POR B protein than in green portion (Fig. 4.23). On a similar analysis of a white stripe mutant, the white portion of leaf showed no POR A but faint band of little POR B protein (Fig. 4.24). Evidently the white segment of leaves was not only devoid of chlorophylls, but also devoid of carotenoid and POR protein and apparently did not posses either etioplasts or chloroplasts.

4.7. LHCP

The LHCP is a marker protein for the development of thylakoid membranes in the chloroplasts. Its presence in the chloroplast marks the operation of an active photosynthetic system. The etiolated seedlings are devoid of LHCP and exposure of light to etiolated seedlings initiates LHCP formation. We used the LHCP in leaves as
Figure 4. Distribution of POR A protein in pearl millet leaves (WGC-75). Seedlings were grown under continuous darkness (D) for 7-d from the time of sowing. Thereafter, the first leaf was harvested and the leaf was dissected into 1 cm long segments and numbered from base to the tip. POR A level was determined by western blotting (Inset). The relative amount of POR A was calculated using the intensity of POR A band present in leaf tip as 100%.
Red Light (RL)

Figure 4.22. Distribution of POR B protein in pearl millet (WGC-75) leaves. Seedlings were grown under continuous RL for 7-d from the time of sowing. Thereafter the first leaf was harvested and the leaf was dissected in to 1 cm long segments and numbered from base to the tip. POR B level was determined by western blotting (Inset). The relative amount of POR B was calculated using the intensity of POR B band present in leaf tip as 100%.
Figure 4.23. Quantification of POR protein in yellow stripe (IP 8292) leaves. Seedlings were either grown in continuous white light or darkness (D) upto 7-d from the time of sowing. There after the first leaf was excised, yellow (Y) and green (G) band of the leaves were separated. The POR level was determined by western blotting (Inset). The relative amount of POR was calculated using the intensity of POR band present in dark-grown leaf as 100%.
Figure 4.24. Quantification of POR protein level in white stripe (SW-1). Seedlings were grown in continuous white light (W) or darkness (D) up to 7-d from sowing. Thereafter the first leaf was harvested, white (W) and green (G) bands of the leaves were separated. The POR level was determined by western blotting (Inset). The relative amount of POR was calculated using the intensity of POR band present in dark-grown leaf as 100%.
a marker to decipher the photosynthetic efficiency of green and yellow segments of mutant leaves. The influence of light on LHCP level in pearl millet leaves was examined by immunoblotting of extracts obtained from leaf of WCG-75 variety which was used as a control (Figs. 4.25 and 4.26). It is evident from that the formation of LHCP in pearl millet leaf is obligatorily dependent on exposure to light (Fig. 4.25). In dark-grown leaves only an insignificant amount of LHCP protein was detected. By contrast the light-grown leaves had a high level of LHCP protein indicating that LHCP protein is formed after exposure to light. The fact that light is essential for LHCP formation is evident since it was observed that the transfer of etiolated seedlings to light induces the formation of LHCP protein in leaves within few hours of exposure.

Fig. 4.26 shows that in light-grown leaves the level of LHCP increases from leaf base to leaf tip. In the segments near the leaf base level of LHCP is low, and it gradually increase towards the leaf tip. The content of LHCP was also examined in leaves of pearl millet mutant such as zebra, which possesses alternate yellow and green stripes of leaf. Immunoblotting of extracts of yellow and green sectors of leaf excised from zebra mutant showed that yellow sector of leaf contains little amount of LHCP as compared to green sector (Fig. 4.27). Similarly in yellow stripe mutant the LHCP level was low in yellow region and high in green region (Fig. 4.28). These results clearly indicate that yellow region of leaves of zebra and yellow stripe mutant is deficient in LHCP a marker for functional chloroplast and performs photosynthesis at a reduced rate.

4.8. Phosphoenol pyruvate (PEP) carboxylase

Since pearl millet is a $C_4$ plant, the primary fixation of $CO_2$ in leaves is carried out by the activity of enzyme PEP-carboxylase. In $C_4$ plants PEP-carboxylase activity is localized in M cells, and this enzyme can be considered as a marker for cytosolic enzyme in leaves. The relative level of PEP-carboxylase was examined in dark-and light-grown leaves of pearl millet, using antibody raised against Amaranthus species. The observations indicated that Amaranthus antibody was able to detect pearl millet PEP-carboxylase. It is well known that in $C_4$ plants like Amaranthus and maize, the formation of PEP-carboxylase is induced by light. In dark-grown leaves PEP-
Figure 4.25. Quantification of LHCP level in pearl millet (WGC-75) leaves. Seedlings were either grown in continuous WL or darkness upto 7-days from the time of sowing. The first leaves were harvested and the level of LHCP was determined by western blotting (Inset). The relative amount of LHCP was calculated using the intensity of LHCP band present in the WL-grown leaf tissue as 100%.
Figure 4.26. Distribution of LHCP protein in pearl millet (WGC-75) leaves. Seedlings were grown in continuous WL for 9-days from the time of sowing. The first leaves were harvested and the leaf was segmented into two cm long segments from base to the tip. The segments were numbered from base to the leaf tip. The LHCP level in segments was determined by western blotting (Inset). The relative amount of LHCP level was calculated using the intensity of LHCP band present in the leaf tip (segment 4) as 100%.
Figure 4.27. Quantification of LHCP level in zebra (IP 8283) mutant leaves. Seedlings were grown under a photoperiod consisting of 16 h light and 8 h dark at 25 °C for 4 days thereafter seedlings were transferred to photoperiod consisting of 16 h light at 25 °C and 8 h dark at 10 °C. The yellow (Y) and green portion (G) of the leaves were dissected carefully and the LHCP level was determined by Western blotting (Inset). The relative amount of LHCP was calculated using the intensity of LHCP band is present in green leaf tissue as 100%.
Figure 4.28. Quantification of LHCP level in yellow stripe (IP 8292) mutant leaves. Seedlings were grown under a photoperiod consisting of 16 h light and 8 h dark at 25 °C for 4 days thereafter seedlings were transferred to a photoperiod consisting of 16 h light at 25 °C and 8 h dark at 10°C. The first leaf was harvested, yellow (Y) and green portion (G) of the leaves were dissected carefully. The LHCP level was determined by western blotting of protein (Inset). The relative amount of LHCP was calculated by using the intensity of LHCP band in the green leaf tissue as 100%.
carboxylase level was below the level of detectability. In comparison, light-grown pearl millet seedlings showed a distinct band of PEP-carboxylase in leaf (Fig. 4.29).

In light-grown pearl millet leaves, distribution of PEP-carboxylase was similar to that observed for LHCP. As a representative example, Fig. 4.30, shows PEP-carboxylase distribution in glossy mutant leaves where its level increases from leaf base to leaf tip.

4.9. Oxygen evolution

In order to determine the photosynthetic capacity of the pearl millet leaf, light mediated O₂ evolution was examined from leaf harvested from etiolated-and WL-grown seedlings. The leaf segments from etiolated seedlings displayed no light-dependent oxygen evolution (data not shown). However light-grown leaf segments on exposure to light showed a light-dependent O₂ evolution. Using photosynthetic O₂ evolution as a marker for operation of functional chloroplasts, we compared the photosynthetic O₂ evolution of leaf strips excised from green and yellow leaf region of zebra mutant. Fig. 4.31 shows that the yellow segments, has low ratio of photosynthetic O₂ evolution compared to green segments. Interestingly the yellow region of the leaf also showed sluggish respiration rate, which was nearly half of the green segment (data not shown).

4.10. α-Amylase

In pearl millet leaves α-amylase is present both in extra-plastidic fractions and in chloroplasts (Vally and Sharma, 1995). The distribution profile of total α-amylase activity in pearl millet leaves show increasing activity towards the leaf tip. Pearl millet leaf a-amylase is immunologically similar to maize seed a-amylase, which is evident by the fact that the antibodies raised against maize seed a-amylase also recognizes pearl millet leaf α-amylases (Vally and Sharma, 1995). Our earlier studies showed that in pearl millet leaves light stimulates the α-amylase synthesis, inducing the appearance of a new isoform of a-amylase, which is exclusively localized in chloroplast. In this study the correlation of light with existence of functional chloroplast and of α-amylase protein was examined by using western blotting.

In dark-grown seedlings the level of α-amylase protein in pearl millet leaf is
Figure 4.29. Quantification of PEP carboxylase level in pearl millet (WGC-75) leaves. Seedlings were grown either in continuous (W) or darkness (D) for 7-days. The first leaves were harvested and the PEP-carboxylase level was determined by western blotting (Inset). The relative amount of PEP-carboxylase was calculated using the intensity of PEP-carboxylase band present in light grown leaf as 100%.
Figure 4.30. Quantification of PEP-carboxylase level in glossy mutant (IP 8282) leaves. Seedlings were grown in continuous white light (WL) for 9-days from the time of sowing. The first leaves were harvested and the segments were dissected into two cm long segments and were numbered from base to the leaf tip. The PEP-carboxylase level was determined by western blotting (Inset). The relative amount of PEP-carboxylase was calculated using the intensity of PEP-carboxylase band present in leaf (segment 5) tip as 100%. 

Segment distance from leaf base (cm)
Figure 4. Light dependent oxygen evolution in leaves of zebra (IP 8284) mutant. Seedlings were grown under a photoperiod consisting of 16 h light and 8 h dark at 25 °C for 4 days after it transferred to 16 h light at 25 °C and 8 h dark at less than 10°C. The yellow (Y) and green portion (G) of the leaves were excised into 1-3 mm long stripes and O₂ evolution was measured using a clark type O₂ electrode.
quiet low, but in light-grown seedlings the \textit{\textalpha{}-amylase} protein level is about two folds difference (Fig. 4.32). This pattern is also observed in mutant seedlings of pearl millet when the \textit{\textalpha{}-amylase} protein level is compared between light-and dark-grown seedlings. The examination of a-amylase in \textit{early flowering}, mutant showed that it was similar to wild type a low level of a-amylase protein in dark-grown seedlings (Fig. 4.33).

The possibility that the presence of chloroplast is necessary for a-amylase induction was ascertained by estimating the a-amylase protein in yellow and green sector of the \textit{yellow stripe} mutant of pearl millet. The yellow and green sectors were carefully dissected and the a-amylase protein was determined by western blotting. Fig. 4.34 show that in the same leaf, the green portion has high level of a-amylase protein compared to yellow portion of the leaf. Quantitatively the level of a-amylase protein in green portion was about twice than the yellow portion.

\textbf{4.11. \textbeta{}-Amylase}

An analysis of intracellular distribution of p-amylase in pearl millet leaves indicated that p-amylase is predominantly localized in M cells and is absent in B cells. Additionally in pearl millet leaves p-amylase is localized outside the plastid, most likely in the vacuole. Our earlier studies showed that p-amylase level in dark-grown leaves increases from base to tip, whereas in light-grown leaves it is predominantly localized at the leaf base (Vally, 1995).

The quantitation of P-amylase protein in pearl millet leaves was carried out with polyclonal antibodies raised against maize seed \textit{\textbeta{}-amylases} and these antibodies have been shown to specifically cross react with pearl millet leaf P-amylase in the double immunodiffusion reaction and also by immunoblotting. It is evident that in NF-treated light-grown leaves, the decline in p-amylase activity is arrested in all segments (Fig. 4.35). The analysis of p-amylase distribution along the length of leaf by western blotting revealed that the level of p-amylase protein in dark-grown pearl millet seedlings increases from base to the tip of the leaf (Fig. 4.36). In light-grown leaves P-amylase protein was predominantly localized near the base of the leaf. The level of p-amylase protein was maximal in the first segment and declined in more mature segments (Fig. 4.37).
Figure 4.32. Quantification of α-amylase level in pearl millet (WGC-75) leaves. Seedlings were grown in continuous white light (WL) or darkness (D) upto 7-d from the time of sowing. Thereafter first leaf was harvested, the level of α-amylase was determined by Western blotting (Inset). The relative amount of α-amylase level was calculated using the intensity of α-amylase band present in WL- grown leaves as 100%.
Figure 4.33. Quantification of α-amylase level in *early flowering* (IP 4021) mutant leaves. The mutant seedlings were grown in 16 h white light (WL) and 8 h darkness (D) at 25°C or continuous darkness (D) up to 9 d from the time of sowing. Thereafter first leaf was harvested, the level of α-amylase was determined by Western blotting (Inset). The relative amount of α-amylase was calculated using the intensity of α-amylase band present in WL- grown leaves as 100%.
Figure 4.34. Quantification of α-amylase level in yellowstripe (IP 8292) mutant leaves. The mutant seedlings were grown under a photoperiod consisting of 16 h light and 8 h dark at 25°C upto 9-d from the time of sowing. Thereafter first leaf was harvested, yellow (Y) and green (G) leaf tissues were separated carefully, the level of α-amylase was determined by Western blotting (Inset). The relative amount of α-amylase level was calculated by using the intensity of α-amylase band present in green leaf as 100%.
Figure 4.35. Distribution of P-amylase in 0.4 mM NF treated pearl millet (WGC-75) leaves. Seedlings were grown under continuous red light (RL) for 7-d from the time of sowing. Thereafter the first leaf was excised and the leaf was dissected into 1 cm long segments from base to the tip. The level of P-amylase was determined by western blotting (Inset). The relative amount of p-amylase was calculated using the intensity of P-amylase band present in leaf tip (segment 6) as 100%.
Seedlings were grown in continuous darkness upto 7-d from the time of sowing. The first leaf was harvested and the leaf was dissected into 1 cm long segments which were numbered from leaf base to the leaf tip. Thereafter the level of P-amylase was determined by western blotting (Inset). The relative amount of P-amylase level was calculated using the intensity of p-amylase band present in leaf tip (segment 5) as 100%.
Figure 4.37. Distribution of β-amylase level in pearl millet (WGC-75) leaves. Seedlings were grown in continuous red light (RL) for 7-d from the time of sowing. The first leaf was harvested and the leaf was dissected in to 1 cm long segments and numbered from base to the tip. Thereafter the level of p-amylase was determined by western blotting (Inset). The relative amount of P-amylase was calculated using the intensity of P-amylase band present at leaf base (segment 1) as 100%.
The studies on regulation of P-amylase in pearl millet leaves revealed that in light-grown seedlings NF-treatment, which generate albino seedlings due to photooxidation of chloroplasts, stimulates P-amylase activity in pearl millet seedlings by 7 fold, when compared to light-grown seedlings grown in distilled water. Since NF-treatment significantly stimulates P-amylase level, the likely effect of loss of chloroplast on P-amylase distribution was examined by quantifying along the length of the leaf.

The stimulation of p-amylase level in NF based light-grown leaves indicated an inverse relationship between the presence of functional chloroplasts and level of P-amylase protein. The fact that NF-treatment stimulates P-amylase only in light-grown seedlings, which had no functional chloroplasts, reinforced this view. The possibility of p-amylase induction level by loss of chloroplasts was examined by using mutant of pearl millet, which have lost chloroplasts in certain sector either due to mutation or it has reduced chloroplast functions due to reduction of photosynthetic protein. The analysis of pigment protein i.e. LHCP and O2 evolution as presented in earlier sections indicated that yellow sector of zebra and yellow stripe leaf are deficient in photosynthetic functions. We examined the P-amylase level in yellow and green sector of mutant leaves by western blotting. The above analysis revealed that the yellow leaf sector had a significant higher level of p-amylase protein than green sector. The western blotting analysis revealed that, yellow sector has about 2 fold difference in p-amylase protein than green sector (Fig. 4.38).

The analysis of P-amylase level in zebra mutant, which makes alternate yellow and green sector, was also done. The yellow and green bands were excised and P-amylase level was examined. The profile of p-amylase protein for zebra mutant is similar to that observed with yellow and green sector isolated from yellow stripe mutant leaf. In this mutant too, yellow band has higher level of p-amylase protein than green band indicating that loss of functional chloroplasts stimulates p-amylase level in yellow band (Fig. 4.39).

4.12. Peroxidase activity

Peroxidase is an enzyme, which has multifarious function in plants, such as biosynthesis of lignin, polymerization of extensin, defense against attack by
Figure 4.38. Quantification of β-amylase level in zebra (IP 8284) leaves. The mutant seedlings were grown under a photoperiod consisting of 16 h white light (L) and 8 h darkness (D) at 25°C for 4 days. Thereafter the mutant seedlings were transferred to a photoperiod consisting of 16 h light at 25°C and 8 h darkness at 10°C up to 9-d. Thereafter the first leaf was excised and the yellow (Y) and green (G) tissues separated carefully. The level of P-amylase was determined by western blotting (Inset). The relative amount of P-amylase level was calculated using the intensity of p-amylase band present in yellow leaf tissue as 100%.
**Figure 4.39.** Quantification of p-amylase level in *yellow stripe* (IP 8292) mutant leaves. The mutant seedlings were grown under a photoperiod consisting of 16h light and 8 h darkness at 25°C upto 9-d from the time of sowing. Thereafter the first leaf was excised and the yellow (Y) and green (G) tissues were separated carefully. The level of β-amylase was determined by western blotting (Inset). The relative amount of P-amylase level was calculated using the intensity of P-amylase band present in yellow leaf tissue as 100%. 

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**β-Amylase Relative amount (%)**

- **Yellow**
  - Bar graph showing 90% relative amount.
- **Green**
  - Bar graph showing 30% relative amount.
pathogens, in the healing response to wounding and the oxidative metabolism of auxin. In plants most peroxidase activity is localized in cytosol, therefore we used it as a marker enzyme to examine effect of cell maturity on the level of a typical cytosolic enzyme. Analysis of peroxidase activity in first leaf of pearl millet seedlings during the course of leaf elongation revealed that in young leaf the level of peroxidase is significantly higher in light-grown leaves compared to dark-grown leaves. With the increase in the leaf length, peroxidase activity declined in the light-grown leaves. In contrast the dark-grown leaves had low level of peroxidase activity and the peroxidase activity increased with the age. Interestingly 9-d old light-grown leaf had peroxidase activity equivalent to 3-d old dark-grown leaves (Fig. 4.40).

Since the young dark-grown leaves possessed a low level of peroxidase activity compared to light-grown leaves, we examined the effect of NF treatment on peroxidase activity of pearl millet seedlings. The NF mediated loss of carotenoids in pearl millet seedlings significantly stimulated peroxidase activity in dark-grown seedlings (Fig. 4.41), However it had no drastic effect on the level of peroxidase activity in RL-grown leaves. In both cases the peroxidase activity declined to a low level with increase in the age of the seedlings.

The 7-d-old light-grown leaves of pearl millet posses nearly similar activity of peroxidase in dark-grown leaves, therefore pearl millet leaves were dissected to examine the distribution of peroxidase activity in leaf. Fig. 4.42 shows that though the peroxidase activity in 7-d old RL-and dark-grown seedlings are nearly equal (Fig. 4.40), their distribution in leaf is opposite to each other. In dark-grown leaf peroxidase activity increases from leaf base to tip, and in contrast RL-grown leaf peroxidase activity declines from leaf base to tip.

The isozyme profile of peroxidase in different segment of leaf was analyzed using the native PAGE. The analysis of peroxidase activity by PAGE along the leaf axis showed that in dark-grown leaves the basal segment possess low activity, and it increases towards tip of the leaf (Fig. 4.43). On the contrary, in the light-grown leaves the peroxidase distribution was different from the dark-grown leaf. In light-grown leaf maximal peroxidase activity was observed in the base of the leaf and then it gradually declined towards the leaf tip (Fig. 4.44). The above isozyme profiles on PAGE also clearly correlated with peroxidase activity distribution along the length of pearl millet
Figure 4.40. Time course of peroxidase activity in first leaf of pearl millet (WGC-75). Seedlings were grown under continuous RL (•) or darkness (○) from the time of sowing. At the time point indicated (days) first leaf was excised at the base and the peroxidase activity was determined.
Figure 4.41. Time course of peroxidase activity in first leaf of pearl millet (WGC-75). Seedlings were grown under continuous RL (○) or darkness (●) with 0.4 mM NF solution from the time of sowing. At the time point indicated (days) first leaf was excised at the base and the peroxidase activity was determined.
Figure 4.42. Distribution of peroxidase activity in first leaf of pearl millet (WGC-75). Seedlings were grown under continuous RL (○) or continuous darkness (●) upto 7-d from the time of sowing. At the time point indicated the first leaf was excised at the base and segmented into 1 cm long segments from leaf base to tip. The segments were numbered from leaf base to tip and the peroxidase activity was determined in segments.
Figure 4.43. Distribution of peroxidase isozymes along the length of pearl millet (WGC-75) leaves. The leaf was excised from 7-d old dark-grown pearl millet seedlings. The leaf was excised at the base and cut into 1 cm long segments and numbered from base to the leaf tip. The segments were homogenized and centrifuged. The amount of proteins were calculated in supernatants and equal amounts of proteins were loaded onto each lane and after completion of native PAGE, stained for peroxidase activity.
Figure 4.44. Distribution of peroxidase isozymes along the length of pearl millet (*WGC-75*) leaves. The leaf was excised from 7-d old RL-grown pearl millet seedlings. The leaf was excised at the base and cut into 1 cm long segments and numbered from base to the leaf tip. The segments were homogenized and centrifuged. The amount of proteins were calculated in supernatants and equal amounts of proteins were loaded onto each lane and after completion of native PAGE, stained for peroxidase activity.
leaf (Fig. 4.42).

4.13. Catalase Activity

The catalase enzyme is predominantly present in peroxisomes in leaves and act as a scavenger of $\text{H}_2\text{O}_2$ generated in plant metabolism. It is also believed that it may act as a salicylic acid binding protein \textit{in vivo} and plays a role in defense mechanism. In the present study, we examined that the light induced expression of catalase activity in the pearl millet leaves. Fig. 4.45 shows the time course of catalase activity during the period of leaf elongation in pearl millet seedlings. The exposure of light had no effect on the level of catalase activity. In both dark-and light-grown leaves catalase activity increased with age. However at 8-d old RL-grown leaf had significantly higher catalase activity than dark-grown leaves.

The distribution of catalase activity as well as its \textit{isozymes} was studied along the length of the 7-d old leaves. The primary leaf was dissected into one cm long segments, numbered sequentially from base to tip, and the catalase isozyme level was measured by native PAGE. The pearl millet leaf showed two catalase isozyme both in dark-and light-grown leaves. The analysis of catalase isozymes by native PAGE along the length of light-grown leaf showed a distinct gradient of catalase level from leaf base to tip (Fig. 4.46). In contrast dark-grown leaf did not show a distinct gradient of catalase accumulation. It had nearly the same catalase activity on PAGE except in the leaf tip region (Fig. 4.47).

Since the effect of light on catalase activity was noticed only after 6-d, the effect of light on catalase activity was examined after transferring dark-grown seedlings to RL for 12 h. In seedlings exposed to RL the level of catalase activity increased along the length of the leaf compared to dark-grown leaves and showed a pattern similar to light-grown leaves. This indicated that the level of catalase activity was positively regulated by light (Fig. 4.48) during the later phase of leaf elongation.
Figure 4.45. Time course of catalase activity in first leaf of pearl millet (WGC-75). Seedlings were grown under continuous RL (○) or complete darkness (●) from the time of sowing. At the time point indicated (days), the first leaf was excised at the base and used for estimating the catalase activity.
Figure 4.46. Distribution of catalase isozymes along the length of pearl millet (WGC-75) leaf. The leaf was excised from 7-d old RL-grown pearl millet seedlings. The leaf was excised at the base and cut into 1 cm long segments and numbered from base to the leaf tip. The segments were homogenized and centrifuged. The amount of proteins were calculated in supernatants and equal amounts of proteins were loaded onto each lane and after completion of native PAGE, stained for catalase activity.
Figure 4.47, Distribution of catalase isozymes along the length of pearl millet (WGC-75) leaf. The leaf was excised from 7-d old dark-grown pearl millet seedlings. The leaf was excised at the base and cut into 1 cm long segments and numbered from base to the leaf tip. The segments were homogenized and centrifuged. The amount of proteins were calculated in supernatants and equal amounts of proteins were loaded onto each lane and after completion of native PAGE, stained for catalase activity.
Figure 4.48. Distribution of catalase activity in first leaf of pearl millet (WGC-75). Seedlings were grown under continuous darkness (•) upto 7-d from the time of sowing and then transferred to continuous RL (○) for 12 h. The first leaf was excised at the base and segmented into 1 cm long segments from leaf base to tip. The segments were numbered from leaf base to tip and the catalase activity was determined in segments.