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Modifications of photosynthetic activity and chloroplast morphology associated with *in vitro* trans-differentiation of *Zinnia* mesophyll cells into tracheary elements.

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ABSTRACT

Chloroplast morphology, chlorophyll dynamics and photosynthetic activity of *Zinnia* leaf mesophyll cells were studied during trans-differentiation into tracheary elements with the view of understanding the modification of these parameters in relation to TE differentiation. Size and the density (no. of chloroplasts/ cell) were drastically declined during the period from 48-72 hrs. of culture, which precisely overlaps with the initial deposition of wall bands, irrespective of light/ dark conditions. Distinct differences could be observed between nascent TEs and non-differentiating live cells in these events. Total photosynthetic activity was lost completely during the 1st 24 hrs. of culture, which coincided with de-differentiation of mesophyll cells, not necessarily be related to TE differentiation, but essentially to trans-differentiation. Both the PS I and PS II activity dropped rapidly between 48-72 hrs. of culture followed by complete loss of activity after 72 hrs (PS II) and 96 hrs (PS I), respectively. Total chlorophyll content was stable up to 48 hrs. and then declined sharply with a greater impact on chl-a content, as indicated by lowering chl-a:b ratio with time. There was no clear impact of light on these parameters except in the case of PS II activity where a faster rate of decline was observed in dark, right from the initiation of culture. As indicated by these findings, loss of PS activity and degradation of chloroplast structure of mesophyll cells are closely associated with the events of de-differentiation and secondary wall deposition, marking the arrest of metabolism in general, culminating cell death, accompanied with TE differentiation.

Keywords: *Zinnia elegans*, Mesophyll, tracheary element differentiation, Photosynthetic activity, chloroplast

INTRODUCTION

The classical pattern of plant development comprises the production of new cells, in specialized regions of cell proliferation, the subsequent co-ordinated expansion or elongation of these cells and finally, depending on their position with respect to their neighbors, a process of cell specialization or differentiation. Many differentiated plant cells are able to re-differentiate into new types of cell but in certain cases terminal type of differentiation could be seen. The classical example of terminal differentiation in plants is the tracheary element, a functional cell corpse that forms a single unit of water conducting vessels of the xylem. The xylem presents in vascular bundles includes xylem parenchyma, fibres, vessels and trachieds. Of these, the vessel elements and tracheids collectively known as tracheary elements, form a series of connected tubes for the xylem transport. The developmental programs producing such functional cell corpses involve the co-ordination of cell differentiation with programmed cell death (Groover and Jones 1999).

Xylem differentiation is a complex process, which is difficult to dissect in intact plants. Mesophyll cells that are formally adopted to perform photosynthesis can be induced to re-differentiate synchronously in a high percentage in cell suspension culture with auxin and cytokinin, offering the possibility to study biochemistry and molecular biology of this trans-differentiation process, free from the complexity of intact plant tissues (Fukuda 1996). Tracheary element formation is a terminal fate, culminating further development characterized by a programmed series of events including cytoskeletal re-arrangements, secondary wall formation, lignification and autolysis to form a hollow, water conducting cell corpse.

During the induction of differentiation, that corresponds to the de-differentiation process, isolated mesophyll cells lose their potential to function as photosynthetic cells and acquire the ability to grow and re-differentiate in a new

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environment to form tracheary elements (Fukuda 1996). The lose of photosynthetic activity may occur through the systematic degradation of photosynthetic machinery of mesophyll cells through the collapse of chloroplasts and enzymatic autolysis of the contents of it probably to be re-utilized elsewhere within the cell to contribute to the new developmental programs. At the same time, there are reports that, presence of light during the TE differentiation process seems a critical factor, even though it's role is not clear. Phillips and Dodds (1977) found higher cell and TE numbers in explants of *H. tuberosus*, excised under dim green light compared to those exposed to diffused day light. Burgess and Linstead (1984) reported an inhibition of xylogenesis in Zinnia system upon exposure to light. Several other studies have examined the role of light on PAL (phenyl ammonia lyase) activity, a sensitive marker for xylogenesis. In this study, we concentrated on morphological manifestations of chloroplasts and changes of photosynthetic activity during trans-differentiation of mesophyll cells in to tracheary elements and it's relationship to the process. Particularly changes in chloroplast morphology and structure, total chlorophyll content and chlorophyll a:b ratio, total photosynthetic activity as well as the activity of photosystem I and II were examined and their inter-relationship was discussed.

MATERIALS AND METHODS

Isolation and culture of cells

Mesophyll cells of Zinnia elegans CV Envy were isolated from the first pair of leaves of 2 weeks old seedlings and cultured as described by Fukuda and Komamini (1980). Light was provided at a photon flux density of 14.4 $\mu E\ cm^{-2}\ s^{-1}$ photosynthetically active radiation (Fluorescent light, Phillips), while flasks were covered with aluminium foils to provide darkness.

Fixation and observation of cells

Samples were taken at 24 hour intervals from thye initiation upto 120 hours of culture for chloroplast counts and fixed as follows. The cell suspension was centrifuged (150 g, 3 min) to remove the medium and the pellet was re-suspended in a few mls of 3.5% glutaraldehyde in phosphate buffer (pH 6) for 30 min at room T° on an orbital shaker (Queue shaker/ incubator, Queue systems). Then the cell suspension was centrifuged again (150 g, 3 min), the pellet was washed once with phosphate buffer to remove excess glutaraldehyde and re-suspended in a few ml of fresh phosphate buffer. Then the cells were examined and photographed (Zetopan microscope attached with Reichert Kam ES camera) using Normasky differential interference contrast microscopy. Each cell or group of cells was micrographed at two focal planes (upper and lower) to count chloroplasts. Then, the film was projected on a screen and the number of chloroplasts was counted. The total number of chloroplasts/ upper and lower focal planes was taken as an estimate of the total number of chloroplsats/ cell. Parallel counts of TEs and un-differentiated live cells were also taken to estimate the number of live cells and the % of TE formation.

Measurement of photosynthetic activity and chlorophyll content

Samples were taken from the beginning upto 120 hours from cell cultures maintained under dark and light conditions for the measurement of photosynthetic activity. Total PS activity, as well as the activities of photo system I (PSI) and photo system II (PSII) and total chlorophyll content were recorded. Cell suspension was centrifuged (150g, 3 min) and the pellet was re-suspended in the reaction medium (sucrose + EDTA + $MgCl_2$ + HEPES buffer (pH 7.6) + $MnCl_2$). Cell density was increased to 2 x 10^6 cells/ ml in the reaction medium and it was necessary to let the cells adjust to the reaction medium for 10-15 mints before measuring the photosynthetic activity. Total PS activity, PS I activity and PS II activity were measured with O_2 electrode (Digital Oxygen Systems, Model 10, England). O_2 evolution was measured over three, one-minute periods and the average of 2nd and 3rd measurements were considered. Total PS activity was measured by using cell suspension without adding any reagents. For the measurement of PS II activity 50 µl PPBQ (phenyl para benzoquinone) was added to the reaction medium. Total PS activity and PS II activity were expressed as nmols O_2 released/ min/ µg of chl a+b. PS I activity was measured by adding 100 µl ascorbate, 200 µl DCPIP (Dichloro phenol-indo-phenol) and 100 µl methyl violegen to the reaction medium and expressed as nmols O_2 used/ min/µg chl a+b. The total chlorophyll content of the cell suspension was estimated as follows: a volume of 0.5 ml of concentrated cell suspension (1 x 10^6 cells) was extracted in 80% v/v acetone for 10 mins, filtered in dark and then the absorbance was measured at 647 and 664 nm wavelengths with spectro-photometer (Zeiss PMG II). Based on the absorbance data, Chl a and b contents were calculated (Coombs *et al.* 1986).

RESULTS

Changes in morphology and density of chloroplasts

At the beginning, the cell suspension was homogenous with single mesophyll cells, but as differentiation started, two distinct groups of cells; differentiating and non-differentiating could be identified, besides the dead cells. A sample from a fresh cell suspension was fixed soon after the preparation and then, daily samples were taken for light and dark treatments. chloroplast number was counted taking into account the differentiated and non-differentiated cells separately. Changes in chloroplast density (mean number of chloroplasts/ cell) and morphological features are presented in Figure 1 and corresponding micrographs are shown in plates 1, 2 and 3. In freshly isolated cells, the mean number of chloroplasts/ cell was 53.9±6.8 and it did not change significantly until 48 hours of culture. But, with the onset of TE differentiation (48-72 hours of culture), chloroplasts density started to drop significantly in both light and dark treatments, compared to non-differentiating cells (Fig.1). It was continued to drop, as the differentiation proceeded, rapidly up to 96 hours and then gradually, up to 120 hours of culture, without showing a significant effect of light. The general picture that emerges from these results is that, the number of chloroplasts/ cell does not change very much in non-differentiating cells, while it is subjected to a drastic reduction with the onset of differentiation, from 48 hours of culture period onwards, irrespective to the light/ dark conditions.

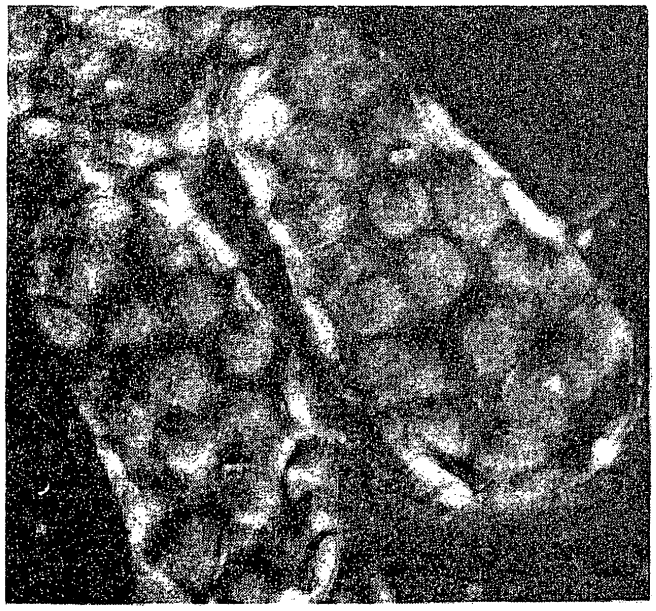


Plate1: Freshly isolated mesophyll cells. Cells appear fully packed with chloroplasts, which are arranged over the cytoplasmic layer that faces outer periclinal wall of the cell and anchored by a network of acting filaments

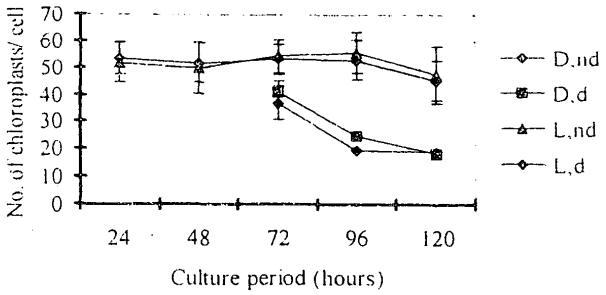


Fig.1. Changes in the number of chloroplasts/ cell in differentiating and non-differentiating cells under light and dark conditions.
D,nd Cultured in dark, non-differentiating cells
D,d Cultured in dark, differentiating cells (counting started after 72 hrs.)
L,nd Cultured in light, non-differentiating cells
L,d Cultured in dark, differentiating cells

A clear difference in morphological features of chloroplasts of nascent TEs and non-differentiating live cells could also be observed. The appearance of fresh mesophyll cells after fixing in glutareldehyde is shown in Plate1. Fixation in glutaraldehyde makes cells more flattened, facilitating the easy counting of chloroplasts. Cells appeared fully packed with chloroplasts completely encompassing the cell surface. The light micrograph of a differentiating cell after 72 hours of culture (Plate2) showed initial bandings of the secondary wall deposition. The distinct reduction in size as well as number of chloroplasts in differentiating cells was striking. As differentiation proceeds, chloroplasts rapidly disappeared and the deposition of wall bands

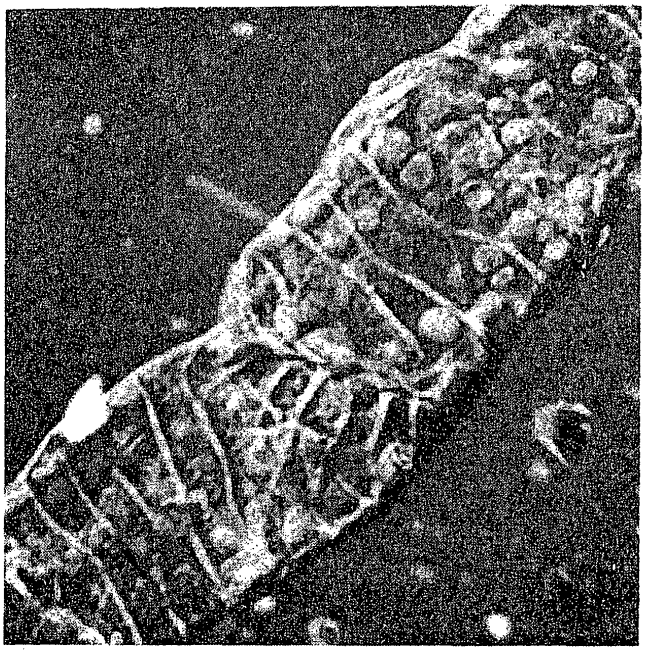


Plate 2: Appearance of a cell with the initiation of secondary wall deposition (72 hrs). Cell has elongated and deposition of parallel wall bands can be viewed very clearly (arrows). Chloroplast number has dropped considerably with a marked depreciation of it's contents and thus appear shrunken. Probably due to disruption of the network of actin filaments during de-differentiation, chloroplasats are dispersed or submerged in cytoplasm.

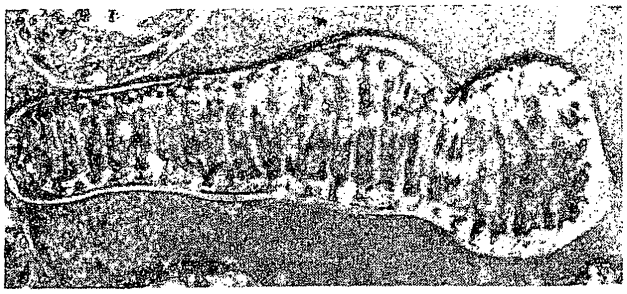
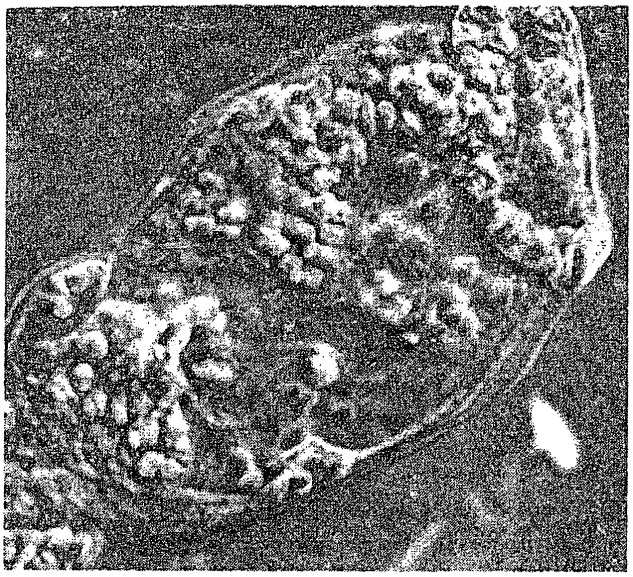


Plate 3: Contrasting morphological and structural differences between a tracheary element and non-differentiating live cell, at the same culture age (96 hrs).
Lower cell: Deposition of secondary wall bands has completely encompassed the cell surface in a reticulate pattern. Only a few chloroplasts, further shrunken in size can be seen.



Upper cell: A non-differentiating live cell, chloroplast number seems not affected but the content seems somewhat depreciated, chloroplasts appear dispersed.

became more dense and prominent, gradually encompassing the cell, completely. Only a few chloroplasts could still be observed along the periphery of plasmalemma (Plate3). In non-differentiating cells, the chloroplast density remained almost unchanged, even though the size of the chloroplasts was very much reduced, contrary to the chloroplasts in differentiating cells. While the chloroplast envelop seemed intact, its content showed signs of degradation. Yet, no signs of complete degradation comparable to the chloroplasts of differentiating cells. The trend observed in Fig.1 agreed well with morphological observations of Plate 1 suggesting that, chloroplasts of differentiating and non-differentiating cells undergo two different pathways of degradation.

Changes in photosynthetic activity and chlorophyll content

PS activity and chlorophyll content were measured from the beginning up to 120 hours. The total PS

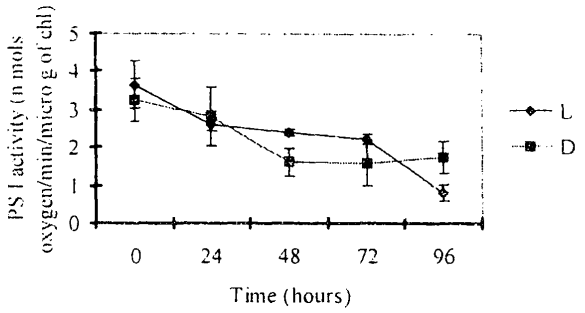


Fig.2. Changes in Photosystem-I activity of leaf mesophyll cells during tracheary element differentiation under light and dark conditions.
L- Light, cultures are maintained under 14.4 µE M^{-2} S^{-1} of photosynthetically active radiation (Fluorescent light, Phillips).
D- Dark, culture flasks were completely covered with aluminium foils at the beginning to ensure darkness and sampling was done under green light (445 nm).
PS-I activity is expressed as: n mols of O_2 used/ minute/µ g of Chl a+b.

activity could be measured only on the 1st day of culture (freshly isolated cells) and it had been lost completely during 24 hours under both the light/ dark conditions.

PS I activity also had dropped drastically, in slightly changing patterns under light and dark conditions, but could be detected up to 96 hours of culture (Fig.2). When cultured under light (14.4 µE M^{-2} s^{-1}), activity had dropped steadily during the first and last 24 hour culture periods with a gradual decline in between. But in darkness, there was a slight drop during 1st 24 hours with a sharp decline during the next 24 hours and remained somewhat stable up to 96 hours of culture. The overall loss of activity of PS I by the end of 96 hours period was about 53% and 39% under dark and light conditions, respectively.

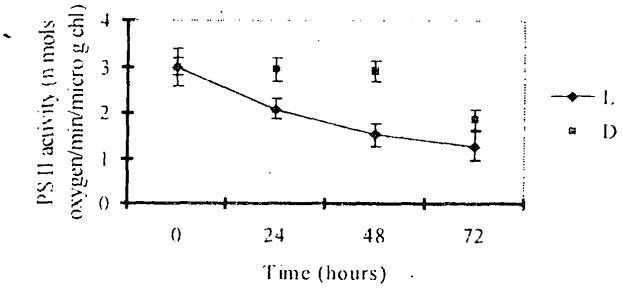


Fig.3. Changes in photosystem II activity of leaf mesophyll cells during tracheary element differentiation under light and dark conditions.
Legend: As in Figure2
PS II activity is expressed as: n mols of O_2 released/ minute/µ g of Chl a+b.

PS II activity could only be detected up to 72 hours of culture and responded quite differently to light and dark conditions (Fig. 3). In light, changes in activity were negligible up to 48 hours and then dropped sharply, while it started to fall significantly from the initiation of culture, in darkness. During the

last 48-72 hour period, an overall loss of activity by 40% and 50% was noticed under light and dark conditions respectively, which coincides with the onset of secondary wall deposition.

The total chlorophyll content (chl-a+b, µgs/ 10^6 cells) and chlorophyll a:b ratio (µgs of chl-a/ 1 µg of chl-b) decreased with the initiation of culture, in a

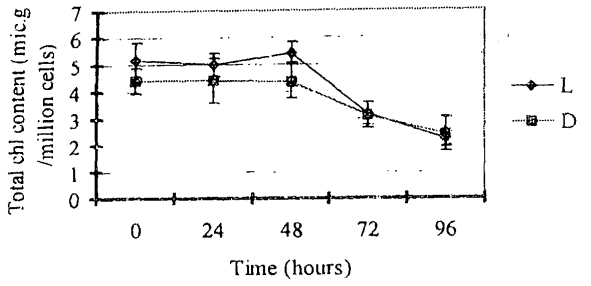


Fig. 4. Changes in total chlorophyll content (chl a+b) of leaf mesophyll cells during tracheary element differentiation under light and dark conditions.
Legend: As in Figure3
Total chlorophyll content is expressed in µ gs of chl a+b/ 10^6cells.

differentiation specific manner. Total chlorophyll content started to decrease slowly at first and then steeply (Fig. 4). A slight increase was observed in total chl content at day 2 in the light treatment which was not significant and then dropped with culture period. Moreover, the photosynthetic activity was more sensitive than the other parameters and the

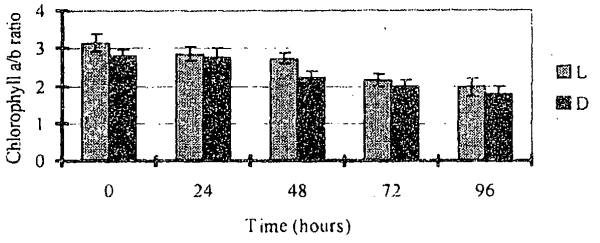


Fig.5. Changes in chlorophyll a/b ratio of leaf mesophyll cells during tracheary element differentiation under light and dark conditions.
Legend: As in Figure 4.

reduction of it precedes the gradual decline of total chl. content and the chl a:b ratio. Both the chl a and b contents decreases with culture age, but the rate of decline in chl a was rapid compared to chl b, causing a gradual decline in chl a:b ratio (Fig. 5). These changes indicated a gradual breakdown of photosynthetic machinery that coincided with the initiation of differentiation. Surprisingly, a considerable amount of chlorophyll could be found even in day 4 of culture (after the TE formation is completed). This must have been due to the non-differentiating cells in which the changes in chloroplast density was rather marginal. Data presented here are based on the analysis of mixed (differentiating and non-differentiating) cell populations. The decline in photosynthetic parameters would have been more drastic and clear, had it been possible to separate differentiating and non-differentiating cell populations. Despite several attempts using percoll and sucrose density gradients, such a separation could not be achieved.

Facts presented in Figure1 and light micrographic observations confirmed that changes in the number of choroplasts/ cell during TE formation vary in differentiating and non-differentiating cells. This behavior could be clearly observed when the two cells viewed in Plate3 are compared; the lower cell is differentiated to a TE while the upper cell remained almost unchanged. In the upper cell, chloroplasts appear shrunken but the chloroplast density seems not changed considerably while in the lower cell, both the size and density of chloroplasts were considerably reduced even though, cells were at the same culture age.

DISCUSSION

Chloroplasts consisted of reticulate arrays of actin filaments around it, which may anchor them to plasma membrane. During the events of de-differentiation, which corresponded to the induction of xylogenesis those actin filaments turn in to a three dimensional network over the entire cell, which caused chloroplasts to leave the vicinity of plasma membrane and mesophyll cells to lose their photosynthetic capacity (Kobayashi *et al.* 1987). De-differentiation process completed within first 24 hour period (Fukuda 1996) and our observation of the complete loss of total photosynthetic activity (flow of electron from water to NADP) of mesophyll cells after 24 hours of culture, was confirmatory in nature with above explanation. Distinct reduction in the size and density of chloroplasts observed later, as the differentiation proceeds in coincidence with secondary wall formation seems well correlated with the disruption of the network of actin filaments leaving the chloroplasts in a disoriented and dispersed state. Appearance of chloroplasts in Plates 1 & 3 is in agreement with this view and provided evidence for this morphological disruption accompanied by the onset of TE differentiation. Fukuda (1994) reported that, the disruption of chloroplasts and the loss of photosynthetic activity was not TE differentiation specific, but related to trans-differentiation i.e. change of the fate of cells from one functional role to another. The trend observed in Fig.1 agreed well with morphological observations of Plate 1, suggesting that, chloroplasts of differentiating and non-differentiating cells

undergo two different pathways of degradation.

Two hypotheses have been proposed to explain the changes in the number of chloroplasts/ cell during leaf senescence (Wardly *et al.* 1984). According to the first, the decline in chloroplasts was believed to be due to lysosome action, a view supported by the studies of Lamppa *et al.* (1980) and Wittenbach *et al.* (1982). According to the other, the chloroplast was viewed as an autonomous organelle with its contents of chlorophyll, RuBISCO and the other constituents being degraded in an orderly fashion. It has been explained as a process of dismantling rather than a decomposition. Thus, the chloroplast number would remain unchanged at least up to the point of the general autolysis. Martinoia *et al.*(1983) reported that there was no change in chloroplast number/ mesophyll cell in senescing barley leaves in spite of the loss of over 80% of the chlorophyll and RuBISCO. Ultra-structural studies of senescing chloroplasts showed that, internal structures were degraded well before the disruption of chloroplast envelope. Chloroplasts contained a broad spectrum of hydrolytic enzymes degrading the internal features (Wardly *et al.* 1984). It is probable that, during the trans-differentiation, chloroplasts were subjected to a gradual enzymatic dismantling until the entire content of cytoplasm collapsed and disappeared. This was the result of selective gene expression, which governed biochemical and morphological events leading to the complete trans-differentiation.

Demura and Fukuda (1993, 1994) have shown that, differentaition of specific genes was expressed at specific stages, preferentially in cells in which, differentiation has induced, 12-24 hours before the onset of secondary wall thickenings. It has been further reported that, the expression of these genes was restricted to cells that were involved in vascular differentiation even during the development of intact plants (Ye and Verner 1993). Thus, it was obvious that, the nascent TEs and non-differentiating live cells undergo two different developmental pathways. This differentiation specific gene expression of induced cells may lead the events towards TE formation (plate-3, lower cell) while other cells continue to remain undifferentiated but live getting adopted to a different pattern of growth.

Since the TE differentiation process was completed within 72-96 hours, rapid autolysis of chloroplasts and other cytoplasmic organelles could be expected in differentiating cells, by this time. The situation in non-differentiating, live cells could be similar to those in senescing leaf mesophyll cells where the chloroplasts remained autonomous even while the internal contents were being degraded. Most probably, the chloroplast density in differentiating and non-differentiating cells followed two trends. In differentiating cells, the chloroplast number decreaseed rapidly due to the breakdown of chloroplasts probably according to the first hypothesis. In non-differentiating cells, changes in chloroplast number agreeed better with the second hypothesis. The fluorescence micrographic evidence further supported the view of two distinctly different patterns of change in chloroplast density during TE formation. There seemed to be no marked effect of light or the intensity of light used for this series of experiments on the pattern of change in the number of chloroplasts/ cell.

The first morphological manifestation of differentiation occured approximately 72 hours after isolation, when nascent TEs synthesize an elaborate secondary cell wall between their primary cell wall and plasma membrane (Groover and Jones 1999). Our observations presented in Plate2 (after 72 hours of culture), illustrated this initial deposition of wall bands explicitely and Figure 1 showed the drastic nature of the reduction of chloroplast number which precisely coincided with this new developmental event (initiation of wall deposition). Groover *et al.* (1997) reported that, approximately 6 hours after the appearance of visible cell wall thickenings, the large central vacuole collapsed rapidly and cytoplasmic srteaming ceases simultaneously terminating the normal metabolism irreversibly, marking the critical event of TE differentiation. We have noticed a complete arrest of PS II activity and PS I activity by 72 and 96 hours of culture respectively (the total PS activity has already lost) with a substantial decrease in total chlorophyll content providing clear evidence for such kind of termination of metabolic activities, in a differentiation specific manner.

CONCLUSION

Trans-differentiation of *Zinnia* leaf mesophyll cells in to tracheary elements was accompanied by systematic degradation in chloroplast morphology and loss of photosynthetic activity. Total photosynthetic activity was arrested within 24 hrs. of culture probably indicating that, the e⁻ transport connecting PS I and PS II was liable to biochemical re-orientation during de-differentiation, dismantling the photosynthetic machinery. Time-frame for the decline of PS I and PS II activity convingcingly overlapped with secondary wall deposition with a higher impact on chl-a content, contributing to the loss of photosynthetic capacity of mesophyll cells. These findings proved that, chloroplast morphology and photosynthetic capacity of mesophyll cells were

subjected to a drastic decline in coincidence with the initiation of secondary wall deposition, marking the termination of metabolic activity en-route differentiation in to tracheary elements.

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REFERENCES

Burgess J and Linstead P 1984 *In-vitro* tracheary element formation: Structural studies and the effect of triiodobenzoic acid. Planta. 160: 481-489.

Coombs J Wall DO Long SP and Kurlock JMO 1986 Techniques in bioproductivity and photosynthesis. 2nd edition, Pergmon press, pp 224.

Demura T and Fukuda H 1993 Molecular cloning and characterization of cDNA associated with tracheary element differentiation in cultured *Zinnia* cells. Plant Physiol. 103: 815-821.

Demura T and Fukuda H 1994 Novel vascular cell-specific genes whose expression is regulated temporally and spatially during vascular system development. Plant Cell. 6: 967-981.

Fukuda H 1994 Redifferentiation of single mesophyll cells into tracheary elements. Int. J. Plant Sci. 155:262-271.

Fukuda H 1996 Xylogenesis: Initiation, progression and cell death. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47: 299-325.

Fukuda H and Komamine A 1980 Establishment of an experimental system for the tracheary element differentiation from single cells isolated from the mesophyll of *Zinnia* elegans. Plant Physiol.. 65: 57-60.

Groover A DeWitt N, Heidel A and Jones A 1997 Programmed cell death of plant tracheary elements differentiating *in vitro*. Protoplasma 196:197-211.

Groover A and Jones AM 1999 Tracheary element differentiation uses a novel mechanism coordinating programmed cell death and secondary cell wall synthesis. Plant Physiol. 119:375-384.

Kobayashi H Fukuda H and Shibaoka H 1987 Reorganization of actin filaments associated with the differentiation of tracheary elements in *Zinnia* mesophyll cells. Protoplasma. 138:69-71.

Lampa GK Elliot LV and Benedich AJ 1980 Changes in chloroplast number during pea leaf development, an analysis of a protoplast population. Planta. 148:437-444.

Martinoia E Heck U Dalling MJ and Matile PH 1983 Changes in chloroplast number and chloroplast constituents in senescing barley leaves. Biochem. Physiol. Pflanzen 178: 147-155.

Phillips R and Dodds JH 1977 Rapid differentiation of tracheary elements in cultured explants of Jerusalem artichoke Planta. 153:210-212.

Wardley TM Bhallu PL and Dalling MJ 1984 Changes in the number of chloroplasts during senescence of mesophyll cells of attached and detached primary leaves of wheat (*Triticum aestivum L*). Plant Physiol. 175:421-424.

Wittenbach VA Lin W and Herbert RR 1982 Vacuolar localization of protease and degradation of chloroplasts in mesophyll protoplasts from senescing primary wheat leaves. Plant Physiol. 69:98-102.

Ye Z H and Verner J E 1993 Gene expression patterns associated with *in vitro* tracheary elemenmt formation in isolated single mesophyll cells of *Zinnia elegans*. Plant Physiol. 103: 805-813.