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Ferredoxin NADP$^+$ oxidoreductase regulation in the developing wheat leaf.

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Introduction

Ferredoxin NADP$^+$ oxidoreductase (FNR) is an important enzyme in plant metabolism, involved in supplying chemical energy in the form of reducing power for a range of different metabolic processes throughout the plant (Arakaki *et al*., 1997). There are two forms of FNR, a photosynthetic FNR ($_p$FNR) and a heterotrophic FNR ($_h$FNR). Both forms are localised in organelles called plastids. $_p$FNR catalyses the reduction of NADP$^+$ in photosynthetic tissue eg mesophyll cells, whilst $_h$FNR catalyses the reduction of ferredoxin in heterotrophic tissues (Figure 1). In the latter case the reduced ferredoxin is then available for enzymes such as nitrite reductase (Bowsher *et al*., 1989) and glutamate synthase (Bowsher *et al*., 1992) of the nitrogen assimilatory pathway.
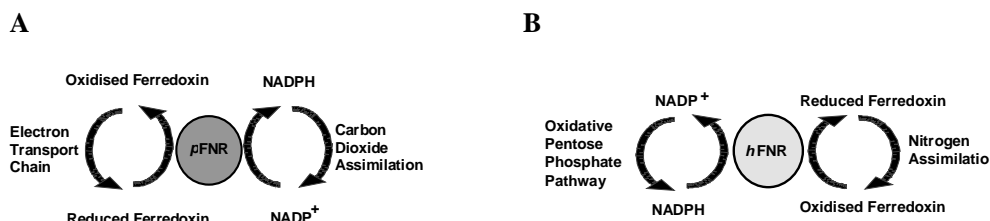

Figure 1. The electron flow from (A) the electron transport chain to carbon dioxide assimilation mediated by photosynthetic FNR and (B) the oxidative pentose phosphate pathway to nitrogen assimilation mediated by heterotrophic FNR. Arrows indicate the direction of electron flow.

Materials and methods

Preparation of nutrient media and growth conditions: The concentration of macronutrients and micronutrients needed for plant growth were based on those described by Paul and Stitt (1993). Where appropriate, and depending on the specific growth conditions of each experiment, 10mM KNO$_3$ was added to the nutrient solution. 0.2% (w/v) Phytagel media was added to the nutrient solution and this suspension was autoclaved. The media was left to cool to 40 °C and 50ml was added to each sterile magenta vessel (7.5cm x 7.5cm x 20cm). After the gel had solidified, five dry wheat seeds (*Triticum aestivum* L. cv. Maris Huntsman) were sown in each magenta vessel using aseptic techniques. Plants were grown at 20 °C, in a controlled environment cabinet in (a) 10mM KNO$_3$ with a 16 hour light / 8 hour dark regime; (b) 10mM KNO$_3$ in darkness; (c) no nitrogen source with a 16 hour light / 8hour dark regime; (d) no nitrogen source in darkness. The light levels in the controlled environment cabinet

(measured as photosynthetic photon flux density) were 240 μmol/m^2/sec in the light. Plants were harvested between 4 to 5 hours into the photoperiod, after 6 or 7 days, when leaf height reached approximately 12cm. Seedlings were removed from the Phytagel and the roots were separated from the shoots with a razor blade. The roots were then rinsed in deionised water and excess water was removed by blotting them on a paper towel pad. Primary wheat leaves were dissected out from the seedling and then cut into 2cm sections (Figure 2). All samples were either used immediately or frozen in liquid nitrogen and stored at – 80 ℃ until required.
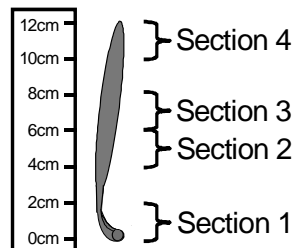

Figure 2. A representation of the sections dissected from the 12cm primary wheat leaf.
The brackets indicate the 2cm sections that were cut from the primary wheat leaf and used for subsequent analysis.

Protein extraction: Leaf proteins were extracted by grinding leaf sections to a fine powder in liquid nitrogen using a mortar and pestle. 1ml of extraction buffer (100mM KH_2PO_4, 0.5mM Na_2EDTA pH 7.8) was then added and the tissue ground up and transferred to an eppendorf tube. The extracts were then centrifuged at 13,000g for 30 minutes at 4 ℃. The pellet was discarded and the supernatant was frozen in liquid nitrogen and stored at -20 ℃, until required for further analysis.

FNR assay and immunoprecipitation: FNR activity was assayed by measuring the ferredoxin dependent reduction of cytochrome C in the presence of NADPH (Suzuki *et al*., 1985). For immunoprecipitation studies $_h$FNR antiserum (Bowsher *et al*., 1993b) was diluted with 100mM tris-HCl, pH 7.6, to give a final percentage of antiserum in each extract of 11.5% and 0%. The extract and antiserum were then incubated on an orbital shaker for 90 minutes at 4°C. Following centrifugation at 13,000g for 5 minutes at 4°C to remove the antibody-antigen complex, the supernatant was carefully transferred to a clean eppendorf tube and assayed for FNR activity.

Results and discussion

Total FNR activity increased from the base of the leaf blade to the tip (Table 1). This pattern of activity is similar to the increase in activity of other photosynthetic enzymes during leaf development including ribulose-1-5 bisphosphate carboxylase (Dean and Leach, 1982; Viro and Kloppstech, 1980). It is also consistent with a rise in CO_2 dependent oxygen evolution (Tobin *et al*., 1988) and the Calvin cycle enzymes, fructose 1,6 bisphosphatase and glyceraldehyde 3 phosphate dehydrogenase (Sibley and Anderson, 1989).

Table 1. Ferredoxin NADP$^+$ oxidoreductase activity in proteins extracted from four sections along 12cm primary wheat leaves. 2cm leaf sections were taken 0-2cm (section 1), 4-6cm (section 2), 6-8cm (section 3) and 10-12cm (section 4) respectively. The FNR activity was determined by a cytochrome c (Cyt c) reductase assay and expressed as per g fw. The mean of 4 measurements is shown ± SEM.

Leaf section (cm from leaf base)	FNR activity (nmol Cyt C reduced/min /g fw)
1 (0-2 cm)	1710±56
2 (4-6 cm)	2480±61
3 (6-8 cm)	3430±64
4 (10-12 cm)	3890±140

Total FNR activity was determined in the primary leaves of wheat seedlings grown in different environments (Table 2). Plants grown in a 16 hour photoperiod or in the dark in the presence of 10mM KNO$_3$ had significantly higher FNR activity than when nitrate was denied. Furthermore, FNR activity was significantly higher when plants were grown in the light. The analysis of total FNR activities in wheat seedlings, grown in different environments suggests that there are differently regulated FNR activities within the primary wheat leaves.

Table 2. FNR activity in the primary leaves of 12cm seedlings grown in different environments. Plants were grown until they reached 12cm in the light and dark and in the presence and absence of 10mM KNO$_3$. Each value represents the mean of 4 measurements from independent treatments ± SEM. Light means that plants were grown in a 16 hour photoperiod and dark means that plants were grown and harvested in complete darkness.

Growth conditions	FNR activity (nmol Cyt C reduced /min /g fw)
Light, 10mM nitrate	3225±307
Light, 0 mM nitrate	2272±75
Dark, 10 mM nitrate	1298±89
Dark, 0 mM nitrate	712±117

To examine whether the relative contribution of $_h$FNR activity to total activity changed with respect to development, proteins were extracted from leaf sections of 12cm wheat seedlings grown under different environmental conditions and incubated with $_h$FNR antiserum (Figure 3). The level of FNR activity in extracts incubated with $_h$FNR antiserum was expressed as a percentage of the activity in extracts incubated in the preimmune serum (shown as 100% activity). In wheat seedlings grown in the presence of light and 10mM nitrate the $_h$FNR antiserum was able to remove FNR activity from sections 1, 2 and 3 compared with the control extracts incubated with preimmune serum. However, in the extract taken from tissue at the tip of the leaf (section 4), no FNR activity was removed by the addition of $_h$FNR antiserum. The $_h$FNR activity (the activity removed by the $_h$FNR antiserum) decreased from the base of the leaf to the tip. In wheat seedlings grown in identical light levels but denied nitrate, the $_h$FNR antiserum removed a maximum of 15% activity from the leaf sections. In leaf proteins extracted from wheat seedlings grown in the dark in the presence of nitrate, 0-45% of FNR activity was removed from the tip to the base by treatment with the $_h$FNR antiserum. When dark grown plants were deprived of nitrate, only 2%-10% of activity could be removed by preincubation with $_h$FNR antiserum in leaf extracts.
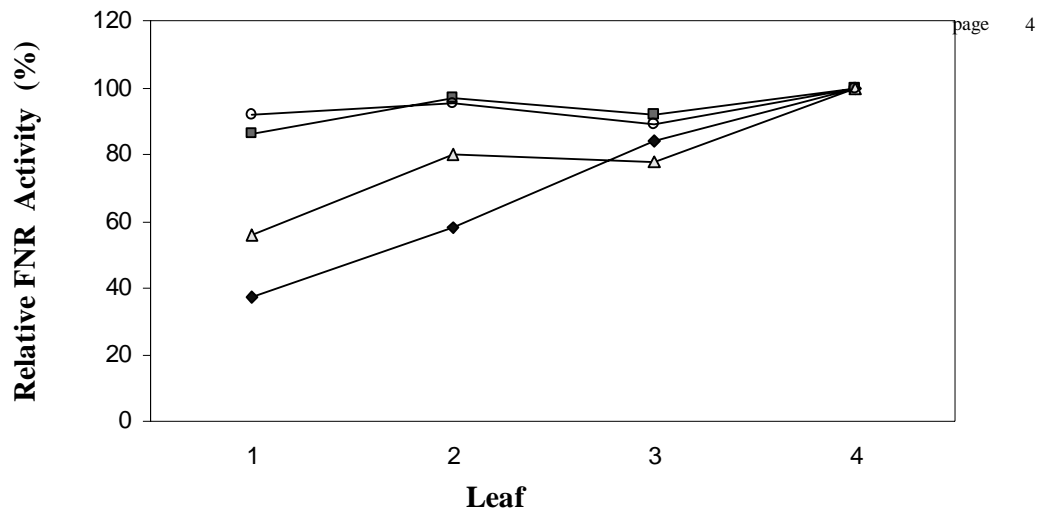
Figure 3. Immunoprecipitation of FNR activity from wheat root extract using the $_h$FNR antiserum.   The graph shows the level of FNR activity remaining after immunoprecipitation with the $_h$FNR antiserum (described as $_p$FNR activity). Seedlings grown in either a 16 hour photoperiod supplemented with 10mM KNO_3 (♦), a 16 hour photoperiod in the absence of KNO_3 (■), in the dark with 10mM KNO_3 (▲) and in the dark in the absence of KNO_3 (o).

From these immunoprecipitation experiments the total FNR activity can be attributed to $_p$FNR and $_h$FNR and was dependent upon growth conditions and development. $_p$FNR increased from the base of the leaf to the tip, whilst $_h$FNR decreased in the presence of light and nitrate. This is consistent with work on mung bean leaves where both $_h$FNR and $_p$FNR were present in young leaves, but $_h$FNR disappeared as development proceeded (Jin *et al*., 1994). Similarly immunoprecipitation studies with radish $_p$FNR antiserum showed $_p$FNR activity was induced by light (Wada *et al*., 1997). $_p$FNR in immature tissue at the base of the leaf (sections 1,2) was reduced in the presence of nitrate. $_h$FNR activity in all leaf sections was reduced in the absence of nitrate, suggesting that this enzyme is induced by nitrate. This study suggests the relative importance of $_p$FNR and $_h$FNR changes during development. Work is currently underway to investigate the implication of this change in proportions of isoforms on metabolic demands of the leaf.

Acknowledgements

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