The participation of phytochrome in the signal transduction pathway of salt stress responses in *Mesembryanthemum crystallinum* L.

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Abstract

Continuous irradiation of *Mesembryanthemum crystallinum* plants with light of equal amounts of photosynthetically active radiation, but widely different red:far red ratios was used to intervene in phytochrome-mediated signal transduction pathways in the presence and absence of salt stress. Light with a low ratio of red:far red (in contrast to light with a high ratio of red:far red), caused induction of PEP carboxylase activity, accumulation of the CAM isoform of PEP carboxylase, and the accumulation of malate anion. Taking these as indicators of CAM induction it is concluded that phytochrome can participate in the signal transduction pathway leading to CAM in *M. crystallinum*. A low ratio of red:far red light acted synergistically with salt stress in the induction of these CAM indicators. The simplest interpretation of this interaction is that the phytochrome-mediated effects and salt stress effects acted on the same signal transduction pathway.

The accumulation of pinitol was also increased by light with a low ratio of red:far red, consistent with the existence of a stress syndrome in *M. crystallinum* which utilizes a common transduction pathway.

A low ratio of red:far red light induced a strong shade avoidance response and, compared to light with a high red:far red ratio, modified chlorophyll content and betacyanin pigment complement.

Plants grown in light with a low ratio of red:far red flowered earlier than plants grown in light with a high red:far red ratio.

It is concluded that phytochrome can participate in the signal transduction pathway leading to the induction of both CAM and the processes which result in pinitol accumulation and pigmentation in *M. crystallinum*, as well as in the mediation of shade avoidance and flowering responses.

Key words: *Mesembryanthemum crystallinum*, CAM, phytochrome, signal transduction, drought stress.

Introduction

Following the application of water or salt stress, *M. crystallinum* displays a number of responses including the induction of CAM photosynthesis (with associated changes in carbohydrate metabolism, ion transport and stomatal mechanism); morphological changes such as reduced leaf area, increased succulence of stems and leaves; increase in the size of bladder cells; and the accumulation of pinitol and proline, presumed compatible solutes (Winter and Von Willert, 1972; Keiller *et al.*, 1987; Ratajczak *et al.*, 1994; Paul and Cockburn, 1989; Mawson and Zaugg, 1994; Thomas *et al.*, 1992).

The magnitude and controllability of CAM induction in *M. crystallinum* has made it a powerful experimental tool. It has been exploited in molecular studies of the regulation of genes encoding enzymes involved in this process (Bohnert *et al.*, 1988) (notably PEP carboxylase) and has also proved useful in other aspects of stress responses such as the synthesis of compatible solutes (Vernon and Bohnert, 1992; Yen *et al.*, 1995).

With the resulting accumulation of information on processes and genes involved in the stress responses of *M. crystallinum* it is now becoming practicable to undertake detailed analyses of the signal transduction pathways linking environmental signals to the responses of this

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plant. Studies of environmental effects on CAM induction in other plant species have revealed, however, that factors other than water/salt stress can significantly modulate the magnitude of the CAM induction response. In Kalanchoe blossfeldiana, for example, in addition to direct responses to water stress, there is a marked phytochrome-mediated effect of photoperiod on CAM induction (Queiroz and Brulffert, 1982). Optimization of the use of M. crystallinum as an experimental tool will require characterization of any such environmental effects.

Direct responses to water and salt stress, and their interaction with developmental and environmental factors, have been extensively studied in M. crystallinum and have proved to be complex. Vernon et al. (1993) showed, for example, in nuclear run-on experiments that a number of CAM-related genes are transcriptionally activated in a co-ordinated manner following salt stress. They also measured transcript levels of these genes following a range of treatments that affect water balance, such as drought, salinity and low temperature, and also treatment with the growth regulators 6-benzylaminopurine and abscisic acid (which cause CAM induction). These measurements indicated that despite the co-ordinated transcriptional activation of the genes during salt stress, their responses to other stimuli that affect water balance were not the same.

With respect to the effects of light on CAM induction in M. crystallinum, Cheng and Edwards (1991) have investigated the effects of duration of illumination. They showed that long days and continuous irradiation enhanced the induction of PEP carboxylase activity and caused malate anion accumulation. They interpreted their data on the basis of direct effects on photosynthetic production and did not explore the possibility of the involvement of phytochrome-mediated photoperiodic responses. McElwain et al. (1992) demonstrated that light quality also moderated the magnitude of the PEP carboxylase induction response to salt stress in M. crystallinum and, significantly in the context of the possibility of the involvement of phytochrome in the modulation of a co-ordinated manner following salt stress, their responses to other stimuli that affect water balance were not the same.

Establishment of the involvement of phytochromes in the stress response of K. blossfeldiana benefited greatly from existing information derived from extensive studies of its commercially-important photoperiodic flowering responses. The photoperiodic characteristics of this plant were very well understood and this provided a valuable background for the CAM induction studies. Unfortunately, there is no equivalent information on phytochrome-mediated responses of M. crystallinum to support a study on the many and varied possibilities for the participation of phytochromes in the regulation of the stress syndrome.

Variations in the ratios of red:far red light are well known to lead to marked changes in phytochrome-mediated growth and development in a wide range of plant species. This has been exploited to study the possible involvement of phytochrome in the modulation of a number of indicators of CAM-induction and on other stress responses in M. crystallinum. Irradiation with light of high or low red:far red ratios and equal amounts of photosynthetically active radiation allowed direct experimental intervention in the signal transduction pathway at the level of phytochrome. Continuous irradiation avoided potential complications of interpretation arising from photoperiodic effects and the use of equal amounts of photosynthetically active radiation ensured that the observed effects did not result from different amounts of photosynthesis being powered by the different light treatments.

Thus the primary objective of the present work was to provide an initial broad exploration, not specific to any of the several phytochrome species or to any particular phytochrome-mediated process, of the possible involvement of phytochrome in the salt stress response signal transduction pathways of M. crystallinum.

In the course of these experiments observations were made on photomorphogenic responses such as induction of flowering, pigmentation and a shade avoidance syndrome. These responses are also discussed.

Materials and methods
Mesembryanthemum crystallinum plants were grown from seed for 6 weeks in the greenhouse with supplementary mercury vapour lighting, when necessary, to extend the photoperiod to 16 h.

Light treatments
Six-week-old plants were transferred to controlled environment cabinets which used combinations of fluorescent and tungsten lamps to provide equal amounts of photosynthetically active radiation (PAR) between 400 and 700 nm, but widely differing photon flux rate ratios of red light (R) to far-red (FR) light as described by Keiller and Smith (1989). Red and far red light, measured by spectroradiometer, comprised 10 nm bandwidths centred on 660 and 730 nm, respectively. The high and low red:far red ratios used in this work were 6.8 and 0.07. In all experiments PAR was 100 μmol m⁻² s⁻¹ except those investigating flowering in which PAR was 50 μmol m⁻² s⁻¹. Irradiation was continuous in all experiments and temperature was maintained at 25 °C.

Imposition of stress
Control plants were grown in compost kept moist with nutrient medium (Edwards and Walker, 1983). Salt stress was imposed by watering plants with nutrient medium containing 400 mM NaCl.

Light treatments
Malate anion, acid and pinitol analysis

Analyses were made on pooled extracts from at least 3 fully-expanded non-senescent leaves from separate plants. Acid content was measured by titration of a boiling water extract to the phenolphthalein end-point and total malate content was estimated enzymically using malic dehydrogenase and glutamate oxaloacetate amino transferase (Möller, 1974). Pinitol was identified and quantified by gas chromatography of the trimethylsilyl derivative and, since authentic pinitol standards were not available, expressed as myo-inositol equivalents (Paul and Cockburn, 1989).

Chlorophyll analysis

Leaf material was ground to a fine powder in liquid nitrogen, extracted with 80% acetone and clarified by centrifugation. Amounts of chlorophylls a and b were estimated by the method of Arnon (1949).

Measurement of PEP carboxylase activity

Fully expanded non-senescent leaf material was finely powdered in liquid nitrogen and 1.0 g of the powder was transferred to an ice-cold mortar and pestle and ground in 5.0 ml of cold 50 mM TRIS (pH 7.2) containing EDTA (2.0 mM), sodium isocitrate (30 mM) and 2% polyvinylpyrrolidone (average molecular weight 40 000). The extract was then centrifuged for 2 min at 12 000 rpm in a bench centrifuge, the supernatant decanted and used as the enzyme source. PEP carboxylase activity was measured spectrophotometrically by coupling to excess malic dehydrogenase activity and measuring NAD oxidation.

PAGE analysis of PEP carboxylase isoforms

Leaf material was finely powdered in liquid nitrogen and 1.0 g samples were extracted by vortexing in 200 µl of 100 mM TRIS (pH 8.3) containing 100 mM NaCl, 10 mM dithiothreitol, 5 mM EDTA and 100 µg leupeptin. After centrifugation for 2 min at 12 000 rpm in a bench centrifuge an aliquot of the supernatant solution was subjected to Western blot analysis as described in Slocombe et al. (1993). Aliquots of the extract were separated on 8% polyacrylamide gels and the separated proteins electroblotted on to nitrocellulose membrane and visualized by ponceau red staining. After destaining, PEP carboxylase isoforms were immunolocated using rabbit anti-Kalanchoe daigremontianum PEP carboxylase polyclonal antibody followed by commercial anti-rabbit antibody conjugated to alkaline phosphatase then BCIP/NBT chromogenic substrate.

Results and discussion

Effects of high and low red: far red ratio and salt stress on PEP carboxylase activity and monomer isoform complement

The activity of PEP carboxylase, a key enzyme in CAM, has been shown to increase dramatically during the induction of CAM in M. crystallinum (Holtum and Winter, 1982) and is, therefore, used in the present work as an indicator of the CAM induction process. Table 1 shows that the application of salt stress, as anticipated, caused an increase in PEP carboxylase activity. The magnitude of this increase is, however, much influenced by the experimental light treatments. Leaves of plants subjected to salt stress with low red: far red ratio treatment contained more than three times the level of PEP carboxylase activity of salt-stressed plants which received the high red: far red treatment. Indeed low red:far red ratio treatment alone, in the absence of salt stress, resulted in a 9-fold increase in PEP carboxylase activity over unstressed plants which received high red: far red ratio treatment. Simultaneous treatment with low red:far red ratio and salt stress acted synergistically and resulted in the accumulation of substantially more PEP carboxylase activity than the sum of the activities measured in plants receiving either low red: far red ratio treatment or salt stress alone.

In M. crystallinum PEP carboxylase is believed to be tetrameric in its active form. Four holoenzyme isoforms and at least six monomeric PEP carboxylase subunits differing in molecular weight or charge have been identified in extracts of this plant (Slocombe et al., 1993). The molecular weights of the four molecular weight isoforms are 105, 108, 113, and 116 kDa. The 108 kDa isoform increases markedly in amount with CAM induction and the 113 kDa isoform decreases in amount. To date, only two genes encoding PEP carboxylase subunits have been identified in M. crystallinum, namely Ppc1, expression of which is markedly increased by CAM induction, and Ppc2 in which expression is slightly reduced by CAM induction. The molecular weight of the product of Ppc1 is given as 100–105 kDa and the molecular weight of the product of Ppc2 is 110 kDa (Cushman et al., 1989).

Table 1. Effects on phosphoenolpyruvate carboxylase activity, acid accumulation and malate anion accumulation in M. crystallinum plants following 3 weeks of treatment with high and low red:far red ratio light in the presence and absence of salt stress: standard errors were less than 12% and n = 3

<table>
<thead>
<tr>
<th>Ratio of red:far red light</th>
<th>Salt stress</th>
<th>PEP carboxylase activity (nmol OAA min⁻¹ g⁻¹ FW)</th>
<th>H⁺ (µmol g⁻¹ FW)</th>
<th>Malate (µmol g⁻¹ FW)</th>
</tr>
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<tbody>
<tr>
<td>Low</td>
<td>−</td>
<td>64</td>
<td>40</td>
<td>353</td>
</tr>
<tr>
<td>Low</td>
<td>+</td>
<td>252</td>
<td>53</td>
<td>375</td>
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<td>High</td>
<td>−</td>
<td>7</td>
<td>50</td>
<td>50</td>
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<tr>
<td>High</td>
<td>+</td>
<td>70</td>
<td>61</td>
<td>355</td>
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relationships between the products of Ppc1 and Ppc2 as reported by Cushman et al. (1989) and the monomer isoforms described by Slocombe et al. (1993) are unresolved, but may involve variations in RNA splicing, post-translational modifications, the expression of as yet uncharacterized PEP carboxylase genes or a combination of these processes. However, the present experiments simply utilize the 108 kDa and 113 kDa isoforms as indicators of the induction of CAM-related processes and detailed information on the relationships of the isoforms is not required for this purpose.

Plate 1 illustrates that the PEP carboxylase isoform complement of salt-stressed and unstressed M. crystallinum is substantially influenced by red: far red ratio. Plants subjected to high red:far red ratio treatment alone contained approximately equal amounts of four bands running as two doublets with apparent molecular weights of 113 and 116 kDa (Plate 1, arrow a) and 105 and 108 kDa (Plate 1, arrow b). This is the complement which has consistently been found in plants exhibiting the C₃ mode of photosynthesis. Salt stress, low red:far red ratio treatment in the absence of salt stress, and the combination of low red:far red ratio treatment and salt stress, all result in an increase in the 108 kDa isoform. This is the isoform which increases in amount with the induction of CAM. Consistent with the activity measurements described above, the most substantial increase in the amount of the 108 kDa isoform was observed in plants subjected simultaneously to both low red:far red ratio treatment and salt stress. The increase in amount of the same monomer isoform following separate application of salt stress and low red:far red ratio light treatment demonstrates that these two treatments exert their effects on the level of accumulation of the same gene product. Furthermore, the synergism of the effects of simultaneous application of low red:far red ratio and salt stress on PEP carboxylase activity suggests that these two environmental factors are not acting independently, but are both contributing to the activity of a convergent signal transduction pathway.

Treatment with light of low red:far red ratio alone also caused the disappearance of the 113 kDa isoform, again mimicking changes which occur during induction of CAM by salt stress (Slocombe et al., 1993). This is also consistent with the view that the observed phytochrome-mediated effects on PEP carboxylase isoform complement involve components of the normal CAM induction pathway.

These data demonstrate that phytochrome can play a part in the signal transduction pathway leading to the accumulation of the CAM-associated form of PEP carboxylase in M. crystallinum.

Effects of salt stress and high and low red: far red ratio on malate anion and acid accumulation

The substantial diel fluctuations of malic acid characteristic of CAM could not be expected to occur in the continuous irradiation regimes of the present experiments. However, Cheng and Edwards (1991) showed that malic acid and malate anion accumulate in M. crystallinum in continuous light under CAM-inducing conditions. Accordingly, the accumulation of malic acid and malate anion are used here as an indicator of the status of the malate synthesizing and accumulating components of CAM. Table 1 shows that low red:far red ratio treatment, salt stress and the combination of these two treatments resulted in the accumulation of large and more or less

![Plate 1. Immunoblot of phosphoenolpyruvate carboxylase monomer isoforms of M. crystallinum following 1 week of treatment with low red:far red ratio light (Low R. Fr) or high red:far red ratio light (High R. Fr) in the presence and absence of salt stress (Salt). Arrow a indicates higher molecular weight doublet comprising 116 and 113 kDa bands. Arrow b indicates lower molecular weight doublet comprising 105 and 108 kDa bands.](https://academic.oup.com/jxb/article-abstract/47/5/647/514361/The-participation-of-phytochrome-in-the-signal)
equal amounts of malate anion, some 7-fold greater than in plants receiving high red: far red ratio treatment alone. Levels of free acid accumulation were smaller and similar in all of the experimental treatments.

Low red: far red ratio treatment alone caused a large increase in the level of accumulated malate anion. This is again consistent with the involvement of a phytochrome-mediated signal transduction pathway in *M. crystallinum* which interacts with the CAM-inducing effects of salt stress and can also cause salt stress-independent induction.

These data demonstrate the involvement of phytochrome in the induction of components of CAM related to the synthesis and storage of malate. Further work will be required to determine if any or all of the components of CAM involved in the consumption of malate are similarly influenced.

**Effects of high and low red: far red ratio and salt stress on pinitol accumulation**

Pinitol (1-D-3-o-methyl-chiro-inositol) accumulates in salt-stressed *M. crystallinum* and, as a putative compatible solute, is believed, like CAM, to be a component of a stress syndrome associated with adaptation to arid conditions. Like CAM, the synthesis of pinitol in response to salt stress involves enzyme induction, namely myoinositol methyl transferase (Vernon and Bohnert, 1992). This association of pinitol accumulation with CAM and the induction of these two phenomena by the salt-stress signal led us to make measurements of pinitol accumulation in the present experiments. Figure 1 shows that, as expected, substantial pinitol accumulation resulted from the application of 3 weeks of salt stress. However, plants receiving both salt stress and low red: far red ratio treatment contained some five times the amount of pinitol contained by plants which received both salt stress and high red: far red ratio treatment. Furthermore, although pinitol accumulation was not detected in plants receiving high red: far red ratio treatment in the absence of salt stress, plants receiving low red: far red ratio treatment in the absence of salt stress accumulated around the same amount of pinitol as plants receiving salt stress plus high red: far red ratio treatment. These findings indicate that accumulation of the putative compatible solute pinitol is modulated by phytochrome-mediated processes. Indeed the data may indicate that the processes regulating both CAM induction and pinitol accumulation operate through an environmental signal transduction pathway which shares at least some components. This adds to the complexity of the overlapping responses in *M. crystallinum* highlighted by Thomas and Bohnert (1993) and Vernon et al. (1993).

**Effects of high and low red: far red ratio and salt stress on morphological features**

In the course of the experiments aimed to investigate the possible involvement of phytochromes in the regulation of CAM and pinitol accumulation, changes in plant morphology and pigmentation were observed. These differences were manifested quickly and were very obvious after 3 weeks of treatment.

The effects of the light treatments on the gross morphology of *M. crystallinum* may be summarized as representing a strong shade avoidance syndrome. Plants which received low red: far red ratio treatment were 'straggly' and those which received high red: far red ratio treatment were bushy with the length of internodes being some 6-fold longer in the low red: far red treated plants.

These effects of low red: far red ratio are typical of the phytochrome-mediated vegetational shade avoidance response seen in many plants and indeed represent a strong example of the response. In its natural ecological niche *M. crystallinum* may not often suffer shading by other plants and the existence of such a strong shade avoidance response appears enigmatic. One speculative interpretation, however, is that a powerful response to self shading is involved in the generation of the closed canopy characteristic of stands of *M. crystallinum*. It is possible that this closed canopy reduces the evaporation of soil water. Thus the phytochrome-mediated canopy formation can also be considered as a component of the *M. crystallinum* drought stress response syndrome.

**Effects of high and low red: far red ratio and salt stress on chlorophyll and betacyanin content**

Chlorophyll content was 86 and 64 mg g$^{-1}$ dry weight in the high and low red: far red-treated plants, respectively. Salt stress in the low red: far red-treated plants caused a dramatic decrease in chlorophyll content to 16 mg g$^{-1}$ whereas salt-stressed plants receiving the high red: far red...
treatment contained a similar amount to the unstressed plants (61 mg g⁻¹). Chlorophyll a:b ratio was also influenced by these treatments. The ratios observed were 3.5 and 4.5 in plants receiving low and high red:far red treatments, respectively. In salt-stressed plants receiving low and high far red treatments, respectively, the ratios were 1.8 and 5.4.

It was also noted that the tips and abaxial surfaces of leaves of plants given low red:far red treatment contained orange pigment whereas the leaves of plants receiving the high red:far red treatment contained the red pigment characteristic of mature greenhouse-grown *M. crystallinum* plants. These differences were not affected by salt stress. Phytochrome-mediated effects on betacyanin accumulation have been commonly observed (Guidici de Nicola *et al.*, 1974; Woodhead and Swain, 1974) and afford a general explanation of these effects. This represents an additional phytochrome-mediated process in *M. crystallinum*. apparently distinct from the water/salt stress syndrome or shade avoidance responses described above.

**Effects of high and low red:far red ratio on flower induction**

The onset of flowering in *M. crystallinum* was also influenced by the experimental light treatments. Of four plants receiving low red:far red ratio treatment all had flowered within 31 d whereas of four plants receiving the high red:far red treatment none had flowered after that period. After 116 d when the experiment was terminated only one of the plants receiving high red:far red ratio had flowered. These data indicate a role for phytochrome in the regulation of the switch from vegetative to floral development in *M. crystallinum*.

Herppich *et al.* (1992) propose that CAM induction in *M. crystallinum* is developmentally modulated and occurs regardless of the presence or absence of water stress when the plant reaches a particular stage of development (indicated by the commencement of branching). Environmental factors such as water and salt stress drastically influenced the magnitude of acid fluctuation, but could not induce CAM before the critical stage of development was attained. In contrast, Schmitt and Piepenbrock (1992) and Piepenbrock *et al.* (1994) found no such correlation between the induction of PEP carboxylase mRNA or acid fluctuation with developmental stage and concluded that induction is environmentally rather than developmentally controlled.

In the present experiments, important components of CAM were induced by salt stress-independent light treatments which also accelerated the onset of floral development. If the switch to floral development is taken as a general indicator of stage of development then it can be argued that the induction of CAM in low red:far red ratio treatment has occurred because the onset of the appropriate stage of development has been accelerated by the light treatment. This is consistent with a link between developmental stage and CAM induction, but is equally consistent with phytochrome-mediated phenomena separately influencing both CAM induction and flowering.

*M. crystallinum* is a much used and powerful model for the study of salt/water stress responses. The present work demonstrates for the first time that phytochrome-mediated light effects can very significantly influence the induction of a number of important components of these responses. It will be necessary, therefore, to take account of the involvement of phytochrome in the salt/water stress signal transduction pathways in the design of future experimental work utilizing this plant.

**References**


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