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Abscisic-acid-induced drought tolerance in *Funaria hygrometrica* Hedw.

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Abstract. Three-week-old protonemata of *Funaria hygrometrica* Hedw. cultivated in Petri dishes tolerate slow drying (24 h to complete dryness) but not rapid drying (1 h to complete dryness). Slowly dried mosses show, on a dry-weight basis, a sixfold increase in abscisic-acid (ABA) contents during the drying process. Rehydrated, slowly dried protonemata have the ability to tolerate subsequent rapid drying. When ABA is added to three-week-old protonemata at a concentration of 10 μM for 16 h, tolerance to rapid drying is induced. These data indicate that the induction of drought tolerance in *Funaria hygrometrica* is mediated by ABA. Mosses treated with ABA loose their water as fast as controls do; therefore, ABA does not act via reduced water loss. However, induction of synthesis of new proteins by ABA may form an important part of the drought tolerance because 10 μM cycloheximide inhibits the ABA-mediated tolerance to rapid drying.

Key words: Abscisic acid (desiccation tolerance) – Desiccation tolerance – *Funaria*

Introduction

It is generally accepted that all higher plants contain abscisic acid (ABA) and that ABA plays an important role in the stress reactions of these plants (for a review, see Zeevaart and Creelman 1988; Creelman 1989). There are a few reports that the same is true for lower plants as well, e.g. for Hepaticae and Anthocerotae (Hartung et al. 1987) and Algae (Hirsch et al. 1989). However, our knowledge of the role of ABA in mosses is very limited (Bopp 1990). The ABA-mediated reactions of higher plants are much better understood than those of lower plants; the reactions of higher plants include stomata closure (reviewed in Zeevaart and Creelman 1988) and protein synthesis (Gomez et al. 1988; Mundy and Chua 1988; Harada et al. 1989; Bartels et al. 1990). With regard to water stress, poikilohydric or resurrection plants exhibit unique features (Walter 1955; Gaff 1977). They can tolerate complete drying and exist in a nearly water-free state for a long time. After rehydration, they recover completely within a few hours. This phenomenon is found in only a few vascular plants, but is a normal feature in mosses (Bewley 1979). For the resurrection plant *Craterostigma plantagineum* (Scrophulariaceae), it was reported that ABA induces the formation of a set of new proteins that may be responsible for the extreme desiccation tolerance (Bartels et al. 1990; Piatkowski et al. 1990). The possibility that the stomataless gametophytes of mosses react in a similar way prompted us to investigate whether ABA is involved in the induction of desiccation tolerance in *Funaria hygrometrica*.

Material and methods

Plant material and culture conditions. Spores of *Funaria hygrometrica* from the collection of the Botanical Institute Heidelberg, harvested in October 1987, were sown under aseptic conditions on Knop-agar plates (2% agar, w/v) covered with cellophane sheets and maintained at 20 ± 1° C at 1.5 $W \cdot m^{-2}$ and 20 h light per day. After 7 d the young protonemata were transplanted to new plates. All experiments were repeated at least three times.

Application of ABA and-or cycloheximide CHI. The cellophane sheets carrying the protonemata were transferred to Knop agar plates containing the various amounts of ABA and-or CHI given in the *Results*. 2-*cis*-(R,S)-abscisic acid and CHI (both from Sigma, München, Germany) were added as sterile solutions after cooling the autoclaved agar down to approx. 50° C. An ABA content of 10^{-5} M changes the pH of the Knop-containing agar only very slightly, not affecting the growth of the mosses.

Drying conditions. Slow drying consisted of transferring the cellophane sheets to empty Petri dishes. The Petri dishes were main-

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Abbreviations: ABA = abscisic acid; CHI = cycloheximide; DW = dry weight; FW = fresh weight; RWL = relative water loss

tained closed – but not sealed – in a growth chamber under the conditions given above. Mosses to be dried rapidly were put with their cellophane sheets in a laminar air flow and incubated for different times under a maximum stream of air.

Determination of water loss. The relative water loss (RWL, %) was calculated as 100–[FWx–DW]*100/(FWo–DW)] where FWo and FWx indicate the fresh weight at the beginning and end of the experimental time, respectively. Dry weight (DW) was determined after drying the mosses at 100° C for 24 h or after freeze drying, when the mosses were used for ABA estimation.

Rehydration of dried mosses. The cellophane sheets were dipped into sterile water until the protonemata were apparently soaked. The cellophane was then transferred to a new Knop-agar plate and the mosses maintained under standard conditions.

Extraction of ABA and purification. Mosses were frozen in liquid nitrogen and then freeze dried. The weight before and after freeze drying was determined to calculate the RWL. [^{3}H] Abscisic acid was added to methanolic extracts (70%, v/v) of the freeze-dried mosses as internal standard. The extracts were passed through C_{18} columns (Baker, Groß-Gerau, Germany) to remove pigments and other interfering substances. The methanol was removed by evaporation and 5 ml water added. The solvent was acidified to approx. pH 1.5 by the addition of HCl and then partitioned three times against ethyl acetate. The combined organic fractions were reduced to dryness. Finally, the sample was redissolved in Tris-buffered saline (50 µM Tris-HCl; pH 7,8; 100 mM NaCl) containing 5% methanol (v/v). The recovery was in the range of 80% as calculated from the internal standard.

Determination of ABA. Abscisic acid was analyzed by enzyme-linked immuno-sorbent-assay (ELISA) using monoclonal antibodies specific for 2-*cis*-(S)-ABA provided by E. Weiler (Lehrstuhl für Pflanzenphysiologie, Bochum, Germany). The protocol given by Weiler (1986) was followed. For *Funaria* extracts, high-performance liquid chromatography (HPLC) purification prior to ABA-ELISA could be omitted since the values obtained by ABA analysis of the semipurified extracts as described above were in the same range as HPLC-purified extracts (data not shown).

Results

Slowly dried, three-week-old protonemata of *Funaria* lost 50% of their initial water content within the first 4 h of the drying process. After 24 h, they had lost almost all of their water (Fig. 1). Rapidly dried mosses lost more than 90% of their water within 0.5 h and more than 95% within 1 h (Fig. 1). The mosses recovered completely after slow drying when they were rehydrated. Rapid drying within 0.5 h did not have irreversible effects. After 1 h of rapid drying the cells of the outer filaments of the protonemata that grow as circular areas died, while the central (older) parts were not severely affected. Rapid drying within 2 h killed the whole protonema (Table 1). The difference between living and dead cells can be seen very easily. All killed cells appear completely white and collapsed after a few hours whereas the living cells are green and turgescent.

The ABA content of slowly dried mosses increased from 1.7 nmol · g^{-1} DW in the control to 10.5 nmol · g^{-1} DW after 20 h of water loss (Fig. 2) which is approxi-

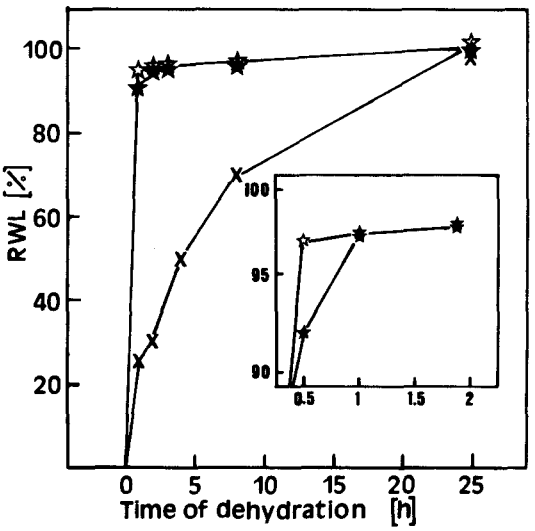

Fig. 1. Relative water losses of slowly dried protonemata ×—×, rapidly dried control protonemata (★—★) and rapidly dried protonemata that had been incubated for one week on 10^{-5} M (R,S)-ABA plates (☆—☆). Three-week-old *Funaria* protonemata were taken. The *insert* shows in more detail the situation of the rapidly dried protonemata during the first 2 h

Table 1. Survival of three-week-old *Funaria* protonemata after rapid drying. Moss plants were grown for two weeks on (R,S)-ABA of the given concentrations. 2, all cells (100%) recovered after rehydration; 1, only central parts of protonemata survived, all peripheral cells dead; 0, all protonema cells dead; –, not tested

Treatment	Drying time (h)						
	0.5	1	2	3	4	8	24
Control	2	1	0	0	0	0	0
10^{-7} M ABA	–	–	–	0	–	–	–
10^{-6} M ABA	–	–	–	1	–	–	–
10^{-5} M ABA	2	2	2	2	2	2	2

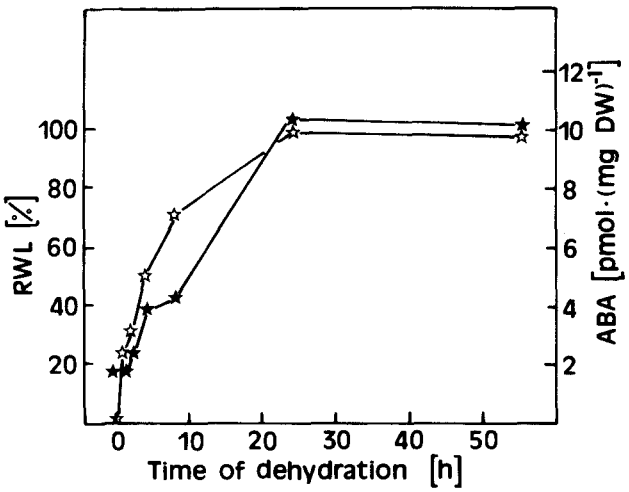

Fig. 2. Increase of 2-cis-(S)-ABA levels in slowly dried *Funaria* plants mg^{-1} DW) (★—★) in comparison to the relative water loss (☆—☆)

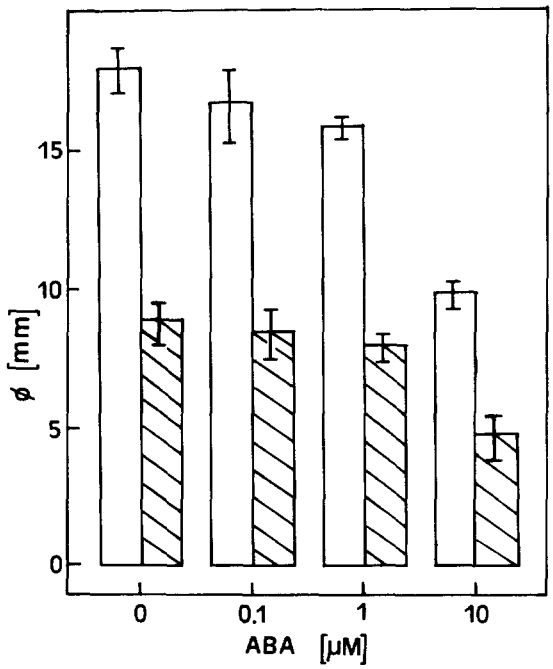

Fig. 3. Reduction of protonema size by the addition of ABA. After one week on Knop agar *Funaria* protonemata were isolated and transferred to plates containing the given amounts of (R,S)-ABA. Protonemata grow as a circular area. The diameters of these areas after one *(hatched bars)* and two *(open bars)* more weeks are given with the standard deviations (± SD)

mately a sixfold increase. After reaching this level, the amount of ABA remained constant.

The question arose whether ABA has any effect in enhancing desiccation tolerance in *Funaria*. To test this, we transferred 7-d-old protonemata to agar plates containing various amounts of ABA. Concentrations of ABA of 10^{-7} M and 10^{-6} M had almost no effect on the growth of the protonemata while a concentration of 10^{-5} M ABA drastically reduced the size of the protonemata, measured after one and two weeks of incubation (Fig. 3). This confirms the data of Lehnert and Bopp (1983). The mosses were then exposed to 3 h of rapid drying conditions. Controls and plants grown on 10^{-7} M ABA died. Plants on 10^{-6} M ABA survived but exhibited severe damage while plants on 10^{-5} M ABA recovered completely (Table 1). Treatment with ABA did not slow down the rate of water loss. On the contrary, we observed an even more rapid loss of water in ABA-treated plants (Fig. 1, insert).

Mosses treated with 10^{-5} M ABA could resist up to 24 h of rapid drying conditions and still recovered completely upon rehydration. To elucidate whether slowly dried mosses show an elevated ability to resist rapid drying because of the enhanced ABA content after slow drying, we rehydrated 3-week-old protonemata that had been exposed to slow drying conditions for 3 d, allowed them to recover for 1 d and dried them rapidly for various times. The results (Table 2) are somewhat heterogeneous. However, the important point is that none of the controls could resist 2 h or more of rapid drying while some survivors did exist among the predried plants. The heterogeneity in the predried mosses may be the result of damage caused by the slow drying process. We suppose that some of the protonemata had suffered more than others and were not able to repair this damage so that they were even more sensitive to rapid drying than the controls.

Table 2. Survival of slowly predried *Funaria* protonemata (three weeks old) upon subsequent rapid drying for different periods of time. Moss plants were predried for 3 d, rehydrated and kept for 1 d under normal conditions and then exposed to rapid drying conditions for the given times. At each time 12 controls (not predried) and 12 predried protonemata were examined. The number of protonemata in the three possible states (see Table 1) is given

Treatment	State	Time (h)				
		0.5	1	2	4	8
		Number of protonemata				
Controls	2	12	0	0	0	0
	1	0	8	0	0	0
	0	0	4	12	12	12
Predried	2	8	7	2	1	0
	1	2	3	6	1	8
	0	2	2	4	10	4

The induction of desiccation tolerance by ABA is a slow process. Incubation for 8 h or less with 10^{-5} M ABA was not sufficient to induce tolerance to rapid drying since all cells of the protonemata died. After 16 h of incubation the protonemata survived 3 h of rapid drying but with damage, while 24 h of incubation allowed complete recovery under these conditions. This period could be sufficient time to produce protective proteins in larger amounts. The formation of new proteins is an important component in the induction of desiccation tolerance. This is supported by the fact that mosses growing for 2 d on plates containing 10 μM CHI and which were then transferred to plates containing 10 μM CHI plus 10 μM ABA for 24 h did not survive 1.5 h of rapid drying as did controls treated only for 24 h with ABA at the given concentration. Death of the CHI-treated plants was obviously not caused by the CHI itself, for control plants survived 16 d on CHI-agar in a viable state but at a drastically reduced growth rate; protonemata incubated first for 24 h with ABA and then with ABA plus CHI survived 2 h of rapid drying (data not shown).

Discussion

Three-week-old protonemata of *Funaria hygrometrica* cultivated in Petri dishes are able to tolerate complete desiccation when the drying process is slow. Rapidly dried mosses die under these conditions. It is known from many other plant systems that the rate of desiccation influences the conservation of stressed plants (Dhindsa and Bewley 1977; Oliver and Bewley 1984a; Eickmeier

1988). In our system, tolerance to rapid drying of more than 1 h duration can be induced by ABA at concentrations of 10^{-6} M or 10^{-5} M (Table 1) or by drying mosses slowly (Table 2). These results support the hypothesis that mosses produce substances when suffering drought stress. Although the ABA content increased parallel to the relative water loss, the protective substance cannot be ABA itself. The inhibition by CHI of tolerance induction demonstrates the participation of proteins in this process, perhaps as protective substances themselves. This is proposed for *Craterostigma* (Piatkowski et al. 1990) and for plants suffering cold stress (Guy 1990). Another possible role of newly formed proteins may be an involvement in the formation of proline or sucrose as protective substances. Dhindsa (1991) showed that the increase in activity of some enzymes of glutathione metabolism in slowly dried *Tortula ruralis* depends upon protein synthesis. However, nothing is known about a possible ABA dependence of this effect.

That the ABA response takes more than 8 h is in good agreement with the supposed key role of de-novo protein synthesis. However, these conclusions are not in agreement with the interpretation of desiccation experiments with the tolerant moss species *Tortula ruralis* (Oliver and Bewley 1984a, b). These authors have suggested that "rehydration proteins" rather than proteins synthesized during drying are the important factor in desiccation tolerance. The main argument was that drying in this system took not more than 15–60 min, which allows no new proteins to be synthezied in sufficient amounts. However, they used outdoor-collected mosses that probably had suffered drought stress and so had already produced protective proteins. It is a well-known fact from occasional observation that *Funaria* cultivated in the greenhouse is much more drought tolerant than plants grown in Petri dishes.

Even untreated *Funaria* protonemata exhibit a high desiccation tolerance when they are dried rapidly. They can survive water losses (with regard to their initial water content) of 91% without irreversible damage after 30 min of rapid drying, and of 97% after 1 h, if only the peripheral cells of the protonemata are killed (Fig. 1, Table 1). This might result from the presence of a certain level of constitutive protective substances, induced by a minimum concentration of endogenous ABA. Further research to test this will be needed.

In contrast to vascular plants, moss gametophytes are not able to control their water loss by stomata and our data indicate that the addition of ABA does not lower the rate of water loss in moss gametophytes. Whether the ABA-mediated closure of stomata in *Funaria* sporophytes (Garner and Paolillo 1973) has a significant influence on the rate of water loss in the sporophyte is not known.

The data available so far show that desiccation tolerance of *Funaria* resembles in many aspects the situation found in the poikilohydric vascular plant *Craterostigma,* where ABA induces desiccation tolerance in callus cultures, probably by inducing the synthesis of new proteins (Bartels et al. 1990; Piatkowski et al. 1990). Therefore, the situation in mosses and higher plants may be the same or similar at the cellular level. The further study of drought tolerance in mosses may thus allow a deeper understanding of the evolution of this process. Furthermore, the relative morphological simplicity of mosses, the easy handling and the availability of advanced methodologies for genetic analysis may offer certain advantages for further investigations (Ashton et al. 1990; Bopp 1990).

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