SIGNALS MEDIATING ABA ACTION IN BARLEY GRAIN

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voor Timo

Contents

Chapter 1

General Introduction

Plants are unable to escape from unfavorable changes in their environment, they are tied to the place where they struck root. In order to survive, a plant species has to come through these periods of hardship, such as winter or drought. By multiplication via seeds (or spores), plants can ensure their progeny. Plant seeds consist of an embryo and food supply to support the initial growth of the embryo. Most plant seeds are able to survive for a long time under a variety of circumstances. The control of either quiescence or germination of a seed is essential for survival of the plant species. Usually, a seed will start germinating when conditions seem favourable for plant growth. In some circumstances, a grain remains quiescent although the environment seems suitable for germination. This phenomenon is known as dormancy. Dormancy guarantees the spread of germination over a period of many years. The mechanisms that control different types of dormancy are not yet clarified but seem to be determined by a combination of genotype and environmental conditions.

Fundamental research concerning the backgrounds of regulation of seed development and germination not only satisfies human curiosity, but it is of industrial interest as well. Increased understanding of germination may yield new ways to manipulate this process and can help agriculture in the propagation of crops. Moreover, control of germination of the barley grain is of great interest for the brewing industry. To produce a good malt, the main raw material for the production of beer, fast and even germination of barley grain are essential.

Introduction to the barley grain

The mature barley grain consists mainly of storage tissue: the starchy endosperm (Fig 1). This tissue provides nutrients required for the initial growth of the embryo during germination. The starchy endosperm is surrounded by three to four layers of thick-walled, brick-shaped cells, i.e. the aleurone layer, and it is separated from the embryo by the scutellum (Fig 1). The coating of the grain is formed by testa and pericarp, surrounded by the husk which is adhered to the underlying pericarp (Fig 1).

If a mature barley grain finds itself in favourable conditions, it will normally initiate germination. After sufficient rehydration, the embryo will

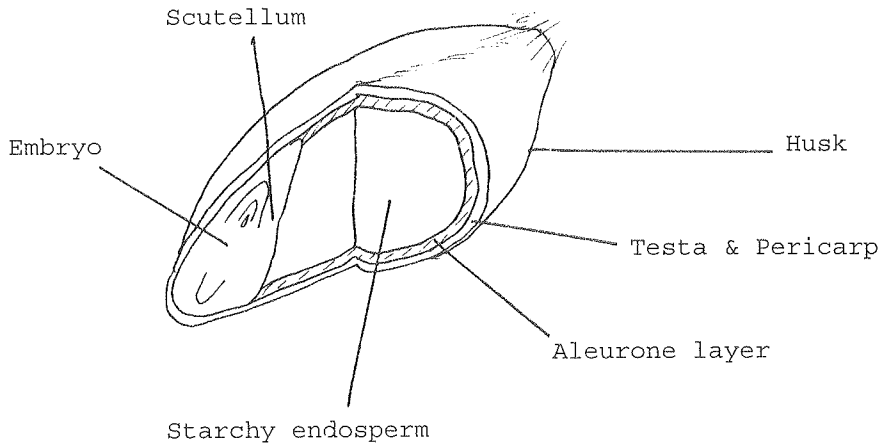

Figure 1. Schematic view of a barley grain.

start developing. Growth of the embryo requires nutrients, initially drawn from the embryo itself and later from the endosperm. Nutrients are stored in the endosperm mainly as starch and storage proteins. These reserves are mobilized during germination by hydrolytic enzymes and proteases which are released into the endosperm, first by the scutellum and shortly after by the aleurone layer. This mixture of enzymes is able to degrade cell walls and storage products yielding soluble molecules such as sugars, peptides and amino acids. These diffuse through the endosperm and are taken up by the scutellum. The nutrient sources in the grain enable the embryo to develop into a self-sustaining plant. The secretion of degrading enzymes seems to be the major function of the aleurone layer. Production and secretion of these enzymes is induced by gibberellins (GA) and can be inhibited by abscisic acid (ABA), both produced by the embryo. It is generally acknowledged that plant hormones, especially ABA and GA, play a pivotal role in the control of seed germination. Most processes during germination are stimulated by GA and inhibited by ABA.

The physiological significance of abscisic acid in seed development and germination

Abscisic acid, like all phytohormones, is involved in a variety of physiological responses. In roots and leaves its main regulatory role seems to be in the adaptation to stress conditions. It is believed that ABA plays a major role in acquiring resistance to salt-, osmotic-, cold (freezing)- and drought stress. These phenomena can be summarized as dehydration stress (e.g. Hartung and Davies, 1992, Hetherington and Quatrano, 1991, Leonardi *et al., in press*). The best-known action of ABA in coping with dehydration stress is its role in the regulation of stomatal opening. If a plant senses dehydration stress, either in leaves or in roots, it reacts with an increased ABA concentration. The elevated level of ABA mediates the closure of stomata, thus preventing water loss. Resistance or pre-adaptation to dehydration stresses can be acquired by pretreatment with ABA. It has been shown that ABA brings about the accumulation of proteins possibly responsible for such adaptation. A fine example is the induction of an aldose reductase in the resurrection plant *Craterostigma* (Bartels *et al.*, 1992). This protein is involved in the production of sorbitol, an osmo protectant. In addition, ABA is supposed to play a role in wound-responses (e.g Sanchez-Serrano *et al.*, 1992), gravitropism (e.g. Feldman and Sun, 1986), and plant-pathogen interactions (e.g. Fraser, 1992).

Mutant plants with altered ABA-metabolism or sensitivity have been very important in the study of the role of ABA in seed development and germination. The *Arabidopsis aba/abi3* mutant, which does not produce ABA and is insensitive for this hormone, was shown to lack certain storage proteins (Koorneef, 1986). Seeds of this, and other ABA-insensitive mutants exhibit no dormancy. Seeds of the maize ABA-insensitive "viviparous" mutants even tend to germinate before seed development is completed (McCarthy *et al.*, 1989). During "normal" seed development ABA levels peak just before the start of dehydration. Dehydration is necessary for the seed to be able to remain quiescent and obtain longevity. This makes survival during periods of drought or low temperature possible. The increased ABA concentration just before dehydration prevents precocious germination and induces the desiccation tolerance of the embryo. Seeds of the ABA-insensitive mutants of *Arabidopsis* have poorly developed desiccation tolerance and longevity (Ooms *et al.*, 1993). After the onset of dehydration, ABA levels decrease.

Germination of the mature seed starts with further development of the

embryo and the mobilization of reserve nutrients by the release of hydrolytic enzymes into the endosperm. Both processes are induced by GA and inhibited by ABA. The balance between these two hormones determines whether a seed remains quiescent or starts germination. This balance is partly determined by the concentrations of ABA and GA in the seed. Wang *et al.* (*in press*) found that embryos from dormant barley grains contain more ABA than nondormant embryos. In addition, dormant embryos synthesise ABA upon imbibition. However, the amount of ABA present in dormant embryos was not able to inhibit germination of nondormant embryos due to a difference in sensitivity to ABA (Van Beckum *et al.*, 1993). Moreover, Walker-Simmons (1989) found that embryos of dormant and nondormant wheat had similar levels of ABA but exhibited different sensitivity for this hormone. Apparently, sensitivity to ABA plays a more important role in the control of germination than ABA levels. Similar observations were made concerning GA-sensitivity of dormant and nondormant seeds (Hooley, 1992, Schuurink *et al.*, 1992).

To understand what determines the hormonal sensitivity of a seed, it is essential to understand the mechanisms by which a seed converts the perception of a hormone into a response. These mechanisms are tissue specific and are generally referred to as hormone signal transduction.

Signal transduction research in plants

Signal transduction starts with the perception of the "primary signal". In most cases, hormones are not perceived directly by the target enzyme(s) but by a protein that acts as a specific receptor. Upon binding of the hormone to a receptor protein, a cascade of so-called second messengers is triggered. This process offers the possibility to rapidly transfer the signal through the cell and, if necessary, over the plasma membrane. In addition, the initial signal can be amplified or attenuated.

Signal transduction is a relatively new field of research in plant sciences. This is reflected in the modest amount of knowledge on the subject as compared to that of other organisms. Most of the plant signal transduction research focuses on the search for similar mechanisms to those known from animal research. The result is a relatively large input in investigations involving hormone-binding proteins (for review: Palme, 1993) and Ca^{2+} (for review e.g. Allan and Hepler, 1989), inositol-1,4,5-trisphosphate (IP_3, for review Drøbak, 1993), GTP-binding proteins (e.g. Ma *et al.*, 1990; Wang *et al.*, 1993 and therein), cyclic nucleotides and

phosphorylation (e.g. Verhey and Lomax, 1993). All of these are well known second messengers in animals and have now been detected in plants as well, indicating that at least part of the signal transducing mechanisms in plants are similar to those in other organisms. That plants also may have pathways unique to their species is demonstrated by the identification of "CDPK"s, calcium-dependent protein kinases which are not found in other species. These kinases are Ca^{2+}-activated but calmodulin-independent (Harmon *et al.*, 1987; Harper *et al.*, 1991).

Contents of this thesis

The experimental system

This thesis describes research that was carried out to elucidate some of the processes in ABA signal tranduction with respect to seed development and germination. By obtaining knowledge of ABA-signal transduction we hope to understand what determines the responsiveness to ABA of a certain seed. As was stated earlier, the responsiveness of a seed to ABA and GA greatly determines whether a seed will germinate or remain quiescent under certain conditions.

The object of this research is the barley grain. Previous studies on climate-induced dormancy in barley revealed that dormant grains of barley showed increased ABA-sensitivity and response (Van Beckum *et al.*, 1993) and a decrease in GA-sensitivity and response (Schuurink *et al.*, 1992) as compared to nondormant grains. These batches of barley grain (cv Triumph) have an identical genetic background and thus provide excellent reference material for our studies. However, for practical reasons most of the experiments were performed with the "naked" barley variety Himalaya. In this variety, the husk is completely separated from the rest of the grain during harvest. The Himalaya grain can easily be separated into embryo + scutellum, aleurone and "rest grain" and as such has experimental advantage over other varieties.

Both embryo (and scutellum) and aleurone layer of barley are sensitive to ABA and GA. To study ABA responses at the cellular level we chose the aleurone layer as a model system. The aleurone layer consists of only one cell type, which makes it possible to extrapolate experimental results obtained from single protoplasts to predict tissue behaviour. In this respect it is important that aleurone protoplasts seem to be structurally and functionally comparable to their walled counterparts (Himmler *et al.*, 1990), although one should consider that protoplasts are severly stressed cells. In

addition, some hormone-induced responses of the aleurone layer can be relatively easily measured and quantified. GA induces the production and secretion of α-amylase (Jacobsen *et al.*, 1985) and ABA induces the expression of several genes (for review Skriver and Mundy, 1990).

The ABA-induced genes in aleurone mainly belong to two families, the LEA-genes (Late Embryogenesis Abundant) and Rab-genes (Responsive to ABA). High similarity exists between these gene families (Skriver and Mundy, 1990). In the aleurone, at least three mRNAs are recognized by the Rab 16 cDNA clone from rice. The expression of the corresponding barley genes is ABA inducible in complete aleurone layers as well as aleurone protoplasts. The induction by ABA takes place within 15 minutes and does not need *de novo* protein synthesis (unpublished data R.M. van der Meulen), as is the case for the Rab-gene from rice (Mundy and Chua, 1988). The induction of these mRNAs, therefore, seems a good marker for the study of ABA signal transduction mechanisms. Two of the mRNAs which hybridize with the rice Rab 16 probe were identified. Both appeared to be homologous to dehydrin clones from barley (Close *et al.*, 1989). One clone is 98.8% homologous to dehydrin DHN8. The other clone is 70 % homologous to dehydrin DHN3. All barley mRNAs recognized by the Rab 16 cDNA-clone from rice are denoted as "Rab mRNA" in this thesis.
The above-described characteristics of the aleurone layer, together with the fact that it is unable to produce ABA (unpublished data M. Wang), makes the aleurone layer a very attractive system to study the signal transduction of this hormone.

Outline

This thesis consists of six papers describing research on signals mediating ABA action in barley grain. Four of these papers deal with the effect of ABA on intracellular pH (pH_i). ABA was found to induce an increase of the pH_i of barley aleurone cells. The role of this cytosolic alkalinization in the induction of gene expression by ABA is discussed in chapter 2. In addition, it is shown that the pH_i-increase can be inhibited by H^+-ATPase inhibitors. In the following chapter, the suggestion that ABA activates the H^+-ATPases is studied. Chapter 3 describes how membrane potential measurements revealed that ABA induces a plasma membrane hyperpolarization. This hyperpolarization is probably brought about by activation of plasma membrane H^+-ATPases by ABA. The consequence of this hyperpolarization for the functioning of K^+-inward-rectifying ion

channels is also discussed in this chapter. In the appendix, the use of microelectrodes to measure membrane potential is described. These measurements were used to validate the method used in chapter 3. Activation of H^+-ATPases can be responsible for the ABA-induced pH_i-increase but will bring about extracellular pH (pH_e)-decrease as well. Chapter 4 describes the effect of ABA on pH_e. Study of the mechanisms underlying this pH_e-decrease revealed an important role for malic acid. The effect of ABA on malic acid release is probably mediated by the effect of ABA on pH_i. In this chapter the antagonistic role of GA on both pH_i and pH_e is described. The role of ABA and GA-induced pH_i-shifts in the induction of gene expression is discussed in chapter 5. The effects of artificial pH_i-shifts on an ABA-inducible promoter and a GA-inducible promoter was studied using CAT-reporter constructs.

The remaining two papers, chapter 6 and 7, discuss two additional signalling mechanisms which may play a role in ABA signal transduction. With the aid of phosphatase inhibitors a relation was found between dephosphorylation of certain proteins and ABA-induced gene-expression. These experiments and a first step towards the characterisation of these proteins are described in chapters 6. Finally, chapter 7 describes the identification of a cDNA clone encoding a protein which cross-reacted with an antibody against putative ABA-binding proteins from maize. Evidence is presented which suggests a role for this protein in ABA action in barley grain.

In the general discussion (chapter 8) some aspects of this study which are not yet discussed in the preceding chapters, are highlighted. Especially the tissue-specificity of hormone responses and the mechanism of interaction of ABA and its antagonist GA on the level of signal transduction are briefly discussed.

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The cytosolic alkalinization mediated by abscisic acid is necessary, but not sufficient, for abscisic acid-induced gene expression in barley aleurone protoplasts*

*Abstract***

We investigated whether intracellular pH (pH_i) is a causal mediator in abscisic acid (ABA)-induced gene expression. We measured the change in pH_i by a "null-point" method during stimulation of barley (*Hordeum vulgare cv* Himalaya) aleurone protoplasts with ABA and found that ABA induces an increase in pH_i from 7.11 to 7.30 within 45 min after stimulation. This increase is inhibited by plasma membrane H^+-ATPase inhibitors, which induce a decrease in pH_i, both in the presence and absence of ABA. This ABA-induced pH_i increase precedes the expression of Rab mRNA, as was measured by northern analysis. ABA-induced pH_i changes can be bypassed or clamped by addition of either the weak acids 5,5-dimethyl-2,4-oxazolidinedione (DMO) and propionic acid, which decrease the pH_i, or the weak bases methylamine and ammonia, which increase the pH_i. Artificial pH_i increases or decreases induced by weak bases or weak acids, respectively, do not induce Rab mRNA expression. Clamping of the pH_i at a high value with methylamine or ammonia treatment affected the ABA-induced increase of Rab mRNA only slightly. However, inhibition of the ABA-induced pH_i increase with weak acid or proton pump inhibitors treatment strongly inhibited the ABA induced Rab mRNA expression. We conclude that, although the ABA-induced pH_i increase is correlated with and even precedes the induction of Rab mRNA expression and is an essential component of the signal transduction pathway leading from the hormone to gene expression, it is not sufficient to cause such expression.

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**Abbreviations: BASI, bifunctional α-amylase subtilisin inhibitor; DES, diethylstilbestrol; DMO, 5,5-dimethyl-2,4-oxazolidinedione; EGTA, ethylene glycol-bis(ß-aminoethylether)N,N,N',N'-tetraacetic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; pH_e, extracellular pH; pH_i, cytoplasmic pH; Rab, responsive to ABA.

Introduction

The phytohormone abscisic acid (ABA) influences plant physiology, plant growth and development in a variety of ways. ABA, for example, plays a central role in stress responses and enhances adaptation to various stresses, such as desiccation and salt stress (Salisbury and Ross, 1992). ABA is also involved in the regulation of embryogenesis and grain development (Dure *et al.*, 1981; Skriver and Mundy, 1990). Most hormone responses are directly or indirectly connected with alteration of gene expression in plant tissue (Salisbury and Ross, 1992). The expression of hormone-regulated genes is controlled by a variety of processes. So far, knowledge about hormone signal transduction in plants and the signal accessibility to the gene level is limited.

Genes that are under ABA control have been isolated from different plant species. These include Rab genes (Baker *et al.*, 1988; Mundy and Rogers, 1986) and those encoding BASI (Mundy and Chua, 1988) and a 10 KDa soluble protein in wheat embryos (Em) (Marcotte *et al.*, 1989). The promoters of Rab genes are currently being studied to analyze ABA-regulated gene expression. ABA-responsive DNA elements and trans-acting hormone-activated regulatory proteins have been identified (Quatrano *et al.*, 1992).

Most research concerning the working mechanism of ABA has been focused on the expression of ABA-regulated genes and promoters. However, ABA is able to trigger a series of cellular responses which occur long before ABA-induced gene expression can be observed. It is also known that in rice cell suspensions, both salt stress and ABA are able to induce a common factor which triggers Em expression (Marcotte *et al.*, 1989). Experimental evidence indicates that second messengers like Ca^{2+} ions mediate ABA actions in response to extracellular signals (e.g. McAinsh *et al.*, 1990; Schroeder and Hedrich, 1989; Wang *et al.*, 1991a; Wang *et al.*, 1991b). Although it is difficult to conclude that cytosolic pH (pH_i) is also a second messenger in plant hormone signal transduction, pH_i changes are known to be induced by extracellular stimuli such as light (Felle, 1989) and hormones (Felle, 1986; Gehring *et al.*, 1990).

The eukaryotic pH_i is strictly regulated. Cells clamp their pH_i at 7.0-7.4 by ion transport mechanisms and a high buffering capacity of the cytosol (Haussinger, 1988). Proton transport across plant plasma membranes has been intensively studied. For example, plasma membrane-located H^+-transporters and H^+ co-transporters (Felle, 1988; Felle, 1989) have been well

studied and their immediate effects on pH_i have been reported. It has been reported that plant hormones are able to influence H^+-transport as well as pH_i (Felle, 1989). For example, auxin is involved in the activation of a plasma membrane H^+-ATPase (Cross *et al.*, 1978; Felle, 1989). In addition, some data show that auxin is able to induce a pH_i decrease by up to 0.2 pH unit (Felle *et al.*, 1986; Gehring *et al.*, 1990; Sanders *et al.*, 1981). It has also been demonstrated that ABA is able to induce an increase of pH_i in *Zea mays* coleoptile and hypocotyl cells (Gehring *et al.*, 1990). However, with regard to the importance of intracellular cytosolic events, we are still far from understanding the role of pH_i. For example, how do plant cells distinguish between pH shifts induced or triggered by a membrane-bound receptor, and pH shifts from metabolism or, pH shifts induced by extracellular pH changes? In conclusion, it is likely that additional intracellular information is necessary for a cell to react to a pH_i shift.

We have assessed the possible role of pH_i in ABA signal transduction leading to specific gene expression by measuring cytosolic pH and pH_i changes in nontreated and ABA-treated barley aleurone protoplasts. We also investigated the effect of manipulating the pH_i with weak acids, weak bases or proton pump inhibitors on specific gene expression ABA-controlled genes.

Materials and methods

Materials

[α-^{32}P]dCTP 3000 Ci/m mol was from Amersham (Buckinghamshire, UK). (R,S)-abscisic acid (chemical purity 99%), 5,5-dimethyl-2,4-oxazolidinedione (DMO), potassium propionate, methylamine, diethylstilbestrol (DES), zearalenone and digitonin were from Sigma (St. Louis, USA). Gene Screen plus was from DuPont (Boston, USA). Cellulase Onozuka R-10 was obtained from Yakult Honsha (Tokyo, Japan) and Gamborg B5 from Flow Laboratories (Irvine, UK). PVP K25 was from Fluka Chemie (Buchs, Switzerland) and PIPES from Janssens Chemical (Tilburg, the Netherlands). All other chemicals were from Merck (Darmstadt, Germany).

Isolation of protoplasts

Barley (*Hordeum vulgare L. cv.* Himalaya, harvest 1985; Department of Agronomy, Washington State University, Pullman, USA) aleurone protoplasts (containing only small vacuoles) were prepared essentially as

described by Wang *et al.* (1991a). The buffer we used for both washing and incubation of protoplasts was 10 mM Na/K phosphate buffer (10 mM KH_2PO_4/Na_2HPO_4, 0.5 M mannitol, 10 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$.

pH_i measurement by means of "null-point" method

Disruption of the plasma membrane of cells in a weakly pH buffered solution will, in principle, lead to a change of the extracellular pH (pH_e) unless pH_e is equal to the cytoplasmic pH (pH_i). The "null-point" method for determination of pH_i is based on this principle (Rink *et al.*, 1982). The pH_i can be determined by incubating protoplasts in weakly pH buffered solutions with different pH and subsequently disruption of the plasma membrane (i.e. pH_i is equal to the pH_e where no pH change could be observed after disruption). The null-point method was used to determine the pH_i in barley aleurone protoplasts. After different treatments, the protoplasts were washed and resuspended in a continuously and gently stirred weakly buffered phosphate buffer (the same phosphate buffer as described above but with 2mM KH_2PO_4/Na_2HPO_4) with different pH values at a density of 2×10^6 protoplasts/mL. The external pH was adjusted to the required value with HCl or NaOH and subsequently digitonin (0.005 % w/v) or Triton X-100 (0.02 % w/v) was added to permeabilize the plasma membranes of the protoplasts. The resulting pH changes were recorded with a combined pH electrode (Beckman, USA) coupled to a pH monitor (Pharmacia, Uppsala, Sweden) and a pen recorder. The values of the external pH at which permeabilization induced no apparent shift in external pH were taken as an estimate of pH_i. In all determinations, correction for the background acidification rate (mainly due to stirring CO_2 into the suspension) was made.

Experiments were performed at room temperature (about 22°C). Mean values ± SD are given (unless specified otherwise), with n=number of independent experiments.

RNA isolation and northern analysis

Barley aleurone protoplasts (4×10^5/mL) were incubated in 10 mM phosphate buffer with or without ABA and/or weak acids (DMO, at pH_e=6.0; propionic acid at pH_e=6.6) and bases (methylamine, ammonia at pH_e=7.4), in the dark at 25°C for 2 h or otherwise as stated in the text.

Total cellular RNA from 2×10^6 protoplasts was isolated and purified as described by Wang *et al.* (1991b). Agarose gel electrophoresis of

glyoxylated RNA and transfer to Gene Screen Plus was performed according to Sambrook *et al.* (1989) or to the instructions of the manufacturer. Hybridization was performed in 1% SDS, 1M NaCl, 10% dextran sulfate and 0.1 mg/mL sonificated salmon sperm DNA at 65 °C, with randomly primed, labeled cDNA hybridization probes (a Rab-16 cDNA probe from rice; a BASI cDNA probe from barley and a GAPDH cDNA probe from barley (Cojecki, 1986). The amount of ^{32}P-labelled probe hybridizing to the Rab mRNA was semiquantitatively determined by measuring the absorbency on autoradiographs with an Ultroscan KL densitometer (LKB).

Results

Measurement of pH_i

The null-point method, first developed to measure pH_i in animal systems (Rink *et al.*, 1982), was adapted for plant cells. To make the aleurone protoplast plasma membrane selectively permeable, the effects of both digitonin and Triton X-100 were investigated. Neither Triton X-100 nor digitonin affect the pH_e by themselves (data not shown). The amount of digitonin or Triton X-100 necessary to disrupt the plasma membrane was titrated; as examples, both Triton X-100 and digitonin titration are presented in figure 1A. As the concentration of either Triton X-100 or digitonin increases, we observed its effect on the disruption of protoplast plasma membrane by changes of the extracellular solution pH (Fig. 1A). After the first change of solution pH by either Triton X-100 or digitonin, any further addition of either Triton X-100 or digitonin induced a further change of solution pH (Fig. 1A). When the pH of extracellular solution is lower than the pH_i, disruption of protoplast plasma membranes leads to an increase of solution pH (Fig. 1B). When the pH of the extracellular solution is higher than the pH_i, permeabilization of protoplast plasma membrane causes a decrease of the solution pH (Fig. 1C). However, when the pH of the extracellular solution is equal to pH_i, selective disruption of the plasma membrane will not give any change of pH_e (Fig. 1D).

The basal barley aleurone protoplast pH_i established is 7.11 ± 0.01 (n=16) in the presence of digitonin and 6.87 ± 0.03 (n=12) in the presence of Triton X-100. The difference between the values obtained with digitonin and Triton X-100 may indicate that the action of Triton X-100, especially at higher concentrations, is not limited to the plasma membrane only (see "Discussion"); therefore, we mainly used digitonin in the experiments

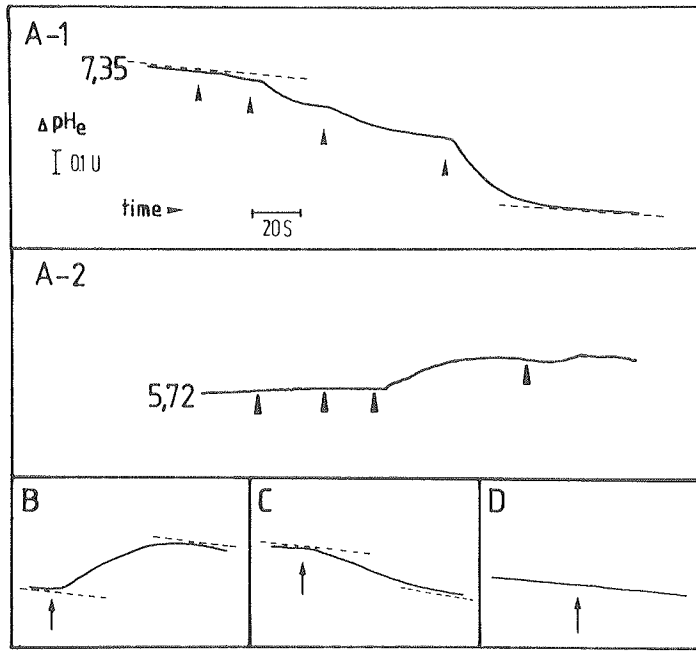

Figure 1. Determination of pH_i by the null-point method. Barley aleurone protoplasts were collected, washed and suspended in 2 mM phosphate buffer at various pH_e values. Changes in extracellular solution pH (pH_e) were induced by addition of Triton X-100 or digitonin to aleurone protoplasts. The arrows indicate the addition of Triton X-100 or digitonin. Time and ΔpH scale are indicated by bars and are identical for all panels. A-1: pH recording of titration curve for Triton X-100. The arrows indicate different amounts of Triton X-100 added to the protoplasts suspension (2 mM phosphate buffer, pH_e = 7.35) which are, from left to right, 0.005%; 0.01%; 0.02%; 0.05% (final concentration, w/v). A-2: pH recording of titration curve of digitonin. The arrows indicate different amounts of digitonin added to the protoplasts suspension (2 mM phosphate buffer, pH_e = 5.72) which are, from left to right, 0.0013%; 0.0025%; 0.005%; 0.01% (final concentration, w/v). B: pH recording of the change in pH_e induced by digitonin (0.005%, w/v) to protoplasts suspended in 2mM phosphate buffer at pH_e = 5.70 ($pH_e < pH_i$). C: pH recording of the pH_e change induced by digitonin (0.005%, w/v) in protoplasts suspended in 2mM phosphate buffer at pH_e = 7.32 ($pH_e > pH_i$). D: pH recording of the pH_e change, induced by digitonin (0.005%, w/v) in protoplasts as shown in panel C, but pH_e = 7.13 ($pH_e = pH_i$).

described below. The basal barley aleurone protoplasts pH_i values, obtained with the null-point method, are in rather good agreement with values reported by direct pH_i measurement with pH-sensitive micro-electrodes

(Felle, 1989; Kurkdjian and Guern, 1989).

The possibility of measuring pH_i changes with the null-point method had to be tested. In general, inhibition of the plasma membrane proton pump will cause an acidification of the cytoplasm. Therefore, we used the plasma membrane H^+-ATPase inhibitors DES and zearalenone. These inhibitors have been shown to induce a pH_i decrease in other systems (Inouye, 1988). Our measurements show that both DES- and zearalenone-induced pH_i changes can be detected with the null-point method (Table I). We conclude that the null-point method applied to barley aleurone protoplasts provides a realistic measurement of pH_i and is able to detect pH_i changes as well.

Are pH_i changes correlated with the induction of Rab mRNA expression?

It has been reported that ABA is able to induce both a pH_i increase (Gehring *et al.*, 1990), and specific gene (Rab, BASI) expression (Mundy and Chua, 1988; Mundy and Rogers, 1986). We have measured whether the induction of Rab mRNA expression by ABA is accompanied by sustained changes in pH_i. Barley aleurone protoplasts were incubated in 10 mM phosphate buffer (pH 6.8) with 5 μM ABA. At different incubation times a sample was taken and the pH_i was measured by means of the digitonin null-point method. Figure 2 shows that ABA indeed induces a significant increase of pH_i, which reached its maximal level after 45 to 60 min. In the

Table I. *Effect of proton pump inhibitors on ABA-induced pH_i and gene expression.* Barley aleurone protoplasts (4×10^5/mL) were incubated in 10 mM phosphate buffer (pH 6.8), to which 100 μM DES or Zearalenone and /or 10 μM ABA were added. After 1 h of incubation, samples were taken for pH_i measurement with the digitonin null-point method. The data represent the means ± SD of at least 12 indenpendent determinations. After 2 h of incubation, samples were taken for northern analysis. The level of mRNA was semiquantitatively determined. The gene expression data are presented as ranges. The Rab mRNA expression obtained with 10 μM ABA treatment was set to 100% and the GAPDH mRNA expression obtained with buffer treatment was set to 100%.

	pH_i	(n)	Rab(%)	(n)	GAPDH(%)	(n)
Buffer	7.08±0.02	(15)	9-12	(3)	100	(3)
10 μM ABA	7.21±0.01	(15)	100	(3)	95-105	(3)
100 μM DES	6.88±0.03	(12)	10-15	(2)	100-114	(2)
100 μM Zeara.	6.85±0.04	(12)	9-12	(2)	95-110	(2)
ABA + DES	6.91±0.03	(12)	18-29	(2)	90-113	(2)
ABA + Zeara.	6.89±0.05	(12)	23-39	(2)	97-110	(2)

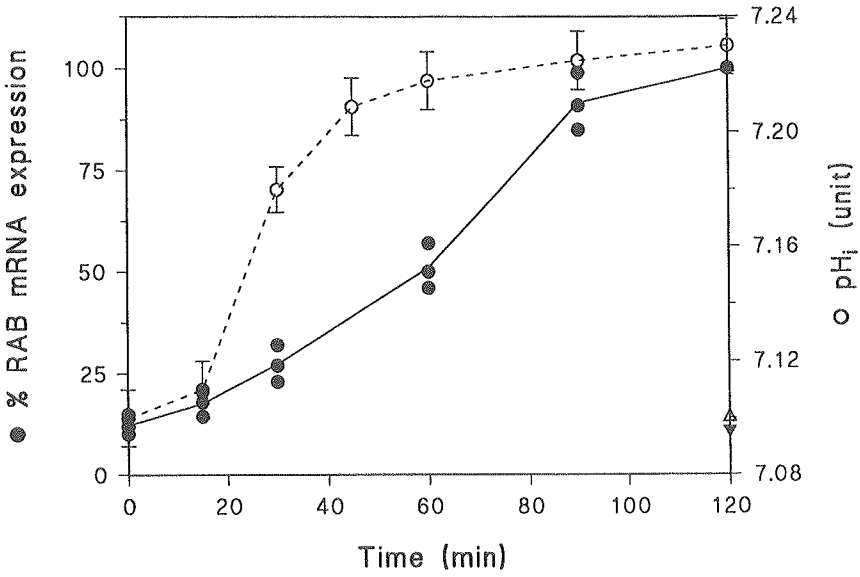

Figure 2. Time course of ABA-induced pH_i and Rab mRNA expression. Barley aleurone protoplasts (4 x 10^5/mL) were incubated in 10 mM phosphate buffer (pH 6.8) with or without 5 μM ABA. At different times, the samples were collected for both pH_i measurements and northern analysis. For pH_i measurements (open circles), the protoplasts were washed twice in 2mM phosphate buffer (pH 7.0) and the pH_i was measured with the digitonin null-point method (see "Materials and Methods"). The means ± SD of 6 independent experiments are presented. For northern analysis, the protoplasts were collected and the total RNA was isolated. The level of Rab mRNA expression (closed circles) was semiquantitatively determined and the mRNA expression obtained after 2 h with ABA was set to 100%. The means of three independent experiments are presented. The triangles (open for pH_i and closed for gene expression) represent control samples incubated without ABA for 2 h.

same experiment, the Rab mRNA expression was studied by northern analysis. The results of these experiments (Fig. 2) show that ABA induces a time-dependent increase in Rab mRNA levels, which reached its maximal level after 90 to 120 min. If we compare ABA-induced pH_i changes with Rab mRNA expression (Fig. 2), it is clear that the half maximal level of ABA-induced pH_i increase was reached at about 30 min., while the half maximal level of ABA-induced Rab mRNA synthesis was reached at about 60 min. These differences indicate that the ABA-induced cytoplasmic alkalinization could, at least in theory, be a prerequisite for Rab mRNA synthesis. In addition, the ABA-induced pH_i increase encompasses about

0.14 pH units, which is in good agreement with the results obtained by Gehring *et al.* (1990). In the absence of ABA no significant changes in either pH_i or Rab mRNA expression were observed (Fig. 2).

The concentration dependency of the ABA-induced pH_i increase and gene expression were studied as well. Protoplasts were incubated in phosphate buffer (pH 6.8) during 1 h with different concentrations of ABA, and subsequently pH_i was measured by the digitonin method. Figure 3 shows that under these conditions ABA-induced a half-maximal pH_i increase at about 1 μM, whereas at about 2 to 5 μM ABA was able to induce half-maximal Rab mRNA expression (Fig. 3). These results demonstrate that there is no significant difference in the capability of ABA to induce both pH_i changes and gene expression.

In addition, Table I shows that the ABA-induced pH_i increase is absent in protoplasts treated with the plasma membrane H^+-ATPase inhibitors DES and zearalenone. This suggests that the ABA-induced pH_i increase is correlated with an increase in plasma membrane H^+-ATPase activity.

Are pH_i changes the cause of specific gene expression?

To test for a possible causal relationship between the ABA-induced gene expression and the ABA-induced increase of pH_i, pH_i was manipulated by adding either weak acids (DMO or potassium propionate), or weak bases (methylamine or ammonia) to the protoplasts. The pH_i was decreased by incubating protoplasts in 10 mM phosphate buffer at pH 6.6 in the presence of DMO or at pH 6.0 in the presence of potassium propionate, and pH_i was increased by adding methylamine or NH_4Cl to protoplasts bathed in 10 mM phosphate buffer, pH 7.4 (Van Duijn and Inouye, 1991). Figure 4 shows that both weak acids and bases have significant effects on the pH_i. DMO and potassium propionate lower the pH_i to 6.91 and 6.89, respectively and prevent the ABA induced alkalinization. Methylamine and NH_4Cl raise the pH_i to 7.20 and 7.23, thereby mimicking the effect of ABA on pH_i. Simultaneous addition of methylamine or NH_4Cl and ABA has no significant influence on pH_i and demonstrates that both methylamine and NH_4Cl can effectively clamp the pH_i at a high value.

We used weak acids and bases to manipulate the pH_i. Because only the non-charged form of weak acids or bases are able to pass the protoplasts membrane, it is essential to apply the weak acids or bases at different pH_e according to their pK_a values. Therefore, we used the weak acids potassium propionate and DMO at pH_e 6.0 and 6.6, and the weak bases methylamine

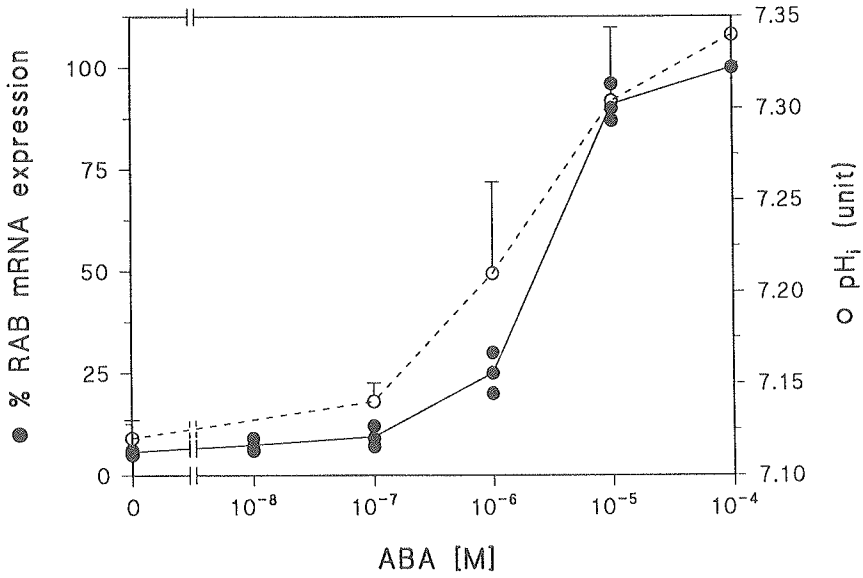

Figure 3. Dose responses of ABA-induced pH_i increase and Rab mRNA expression. Barley aleurone protoplasts were incubated in 10 mM phosphate buffer (pH 6.8) with different concentrations of ABA. After 1 h of incubation, the protoplasts were washed twice in 2 mM phosphate buffer (pH 7.0) and the pH_i (open circles) was measured with the digitonin null-point method (see "Materials and Methods"). The means ± SD of 6 independent experiments are presented. After 2 h of incubation, the protoplasts were collected for northern analysis (closed circles). The Rab mRNA expression at 10^{-4} M ABA was set as 100%. The means of three independent experiments are presented.

Table II. *Effects of extracellular pH on ABA-induced gene expression.*
Barley aleurone protoplasts (4 x 10^5/mL) were incubated in 10 mM phosphate buffer at different pH_e in the presence of 10 μM ABA. After 2 h of incubation, samples were taken for northern analysis. The level of Rab mRNA expression was semiquantatively determined. The gene expression data were presented as ranges. The mRNA expression obtained with pH_e 5.0 was set to 100%.

Buffer pH	Rab(%)	(n)
5.0	100	(2)
6.0	95-113	(2)
6.6	92-114	(2)
7.0	89-105	(2)
7.4	85-93	(2)
8.0	65-81	(2)

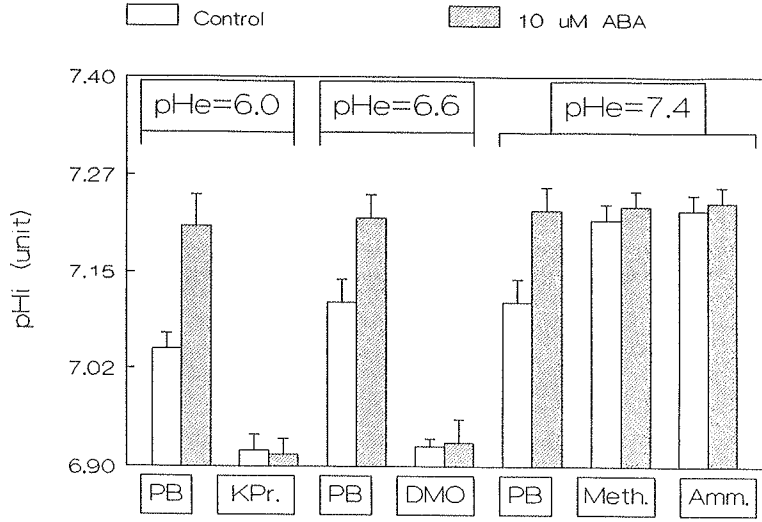

Figure 4. Effects of weak acids and bases on ABA-induced pH_i changes. Barley aleurone protoplasts were incubated in phosphate buffer (PB) with either weak acids (5 mM potassium propionate [KPr.] or 7.5 mM DMO) or weak bases (7.5 mM methylamine [Meth.] or 7.5 mM NH_4Cl [Amm.]) in the presence or absence of ABA (10 μM). During incubation, different pH_e required for loading weak acids or bases were used (see top of figure). After about 45 min of incubation, protoplasts were collected and washed twice in 2 mM phosphate buffer (pH 7.0) and the pH_i was subsequently determined as described in "Materials and Methods" with the digitonin null-point method. The data represent the means ± SD of 6 independent determinations.

and NH_4Cl at pH_e 7.4. Figure 4 shows that the necessary changes of the pH_e have only a small effect on the pH_i. The effects of weak acids or bases on pH_i are much more pronounced (Fig.4). This indicates that the strong effects of weak acids or bases on pH_i are not due to changing pH_e. We investigated whether changes of pH_e would influence ABA-induced gene expression as well. Table II shows that in the pH_e range from 6 to 7.4, ABA-induced Rab mRNA expression was not dramatically different.

To investigate the effects of manipulations of pH_i on gene expression, we examined whether the addition of weak acids or bases influences the ABA-induced Rab mRNA expression. Aleurone protoplasts were incubated in the presence and absence of ABA with different concentrations of the weak acid DMO. DMO is able to inhibit ABA-induced Rab mRNA

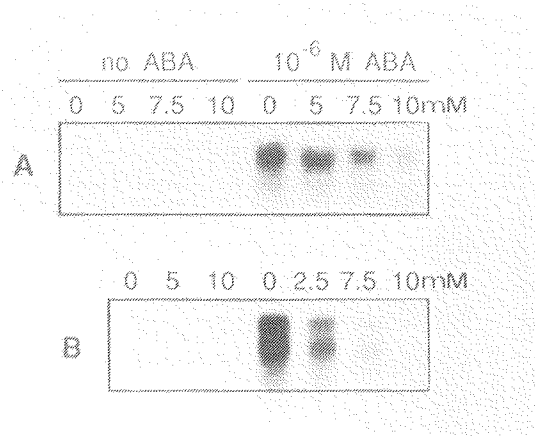

Figure 5. Effect of weak acids on Rab gene expression. **A** Barley aleurone protoplasts were incubated in 10 mM phosphate buffer (pH 6.6) in the presence of 0, 5, 7.5, or 10 mM DMO with or without ABA (5 μM). After 2 h of incubation the protoplasts were collected, and total RNA was isolated. The northern blot was probed with ^{32}P labelled rice Rab-16 cDNA. **B** As in A, but potassium propionate (0, 2.5, 7.5, 10 mM) was used as the weak acid. The incubation buffer is 10 mM phosphate buffer (pH 6.0). At least four independent experiments were done, and all gave similar results. One typical example is presented (the two bands that can be observed by hybridization with rice Rab-16 cDNA probe may represent barley mRNAs from the same Rab family, which are currently being investigated).

expression and this inhibition is DMO concentration dependent (Fig. 5A). Addition of DMO alone in the absence of ABA did not induce any Rab mRNA expression (Fig 5A). We observed similar results with potassium propionate (Fig. 5B). The inhibition of ABA-induced gene expression by weak acids is likely not due to the effect of pH_e, because the pH_e has only a very small effect on ABA-induced gene expression (Table II). In addition, both DES and zearalenone are able to prevent the ABA-induced pH_i increase as well as the ABA-induced gene expression (Table I).

Addition of methylamine to aleurone protoplasts, which clamps the pH_i at a high value in the presence or absence of ABA, resulted in no substantial effect on ABA-induced Rab gene expression (Fig. 6A). NH_4Cl, the second weak base used (Fig 6B), supports the methylamine data. However, neither methylamine nor NH_4Cl was able to affect the Rab mRNA level in the absence of ABA (Fig. 6), indicating that an artificially induced intracellular pH increase is not able to trigger Rab mRNA

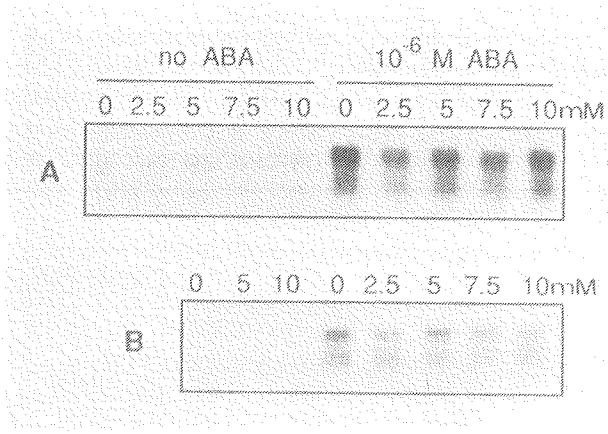

Figure 6. Effect of weak bases on Rab gene expression. **A** Barley aleurone protoplasts were incubated in 10 mM phosphate buffer (pH 7.4) in the presence of 0, 2.5, 5, 7.5 or 10 mM methylamine with or without ABA (5 μM). After 2 h of incubation, the protoplasts were collected, and total RNA was isolated. The northern blot was probed with ^{32}P-labelled rice Rab-16 cDNA.
B As in A, but NH_4Cl (0, 2.5, 5, 7.5, or 10 mM) was used as the weak base. At least four independent experiments were done, and all gave similar results. One typical example is presented (the two bands that can be observed by hybridization with rice Rab-16 cDNA probe may represent barley mRNA's from the same Rab family, which are currently being investigated).

expression.

We investigated whether another ABA-regulated gene might show the same type of effect as the Rab gene, when pH_i is altered by a weak acid or weak base. For this purpose, the expression of BASI mRNA was studied (Table III). An artificially induced decrease in pH_i by weak acids produced an inhibition of ABA-induced BASI mRNA expression, whereas an artificial increase of pH_i by weak bases produced no effect on ABA-induced BASI mRNA expression (Table III). These results demonstrate that the effects of pH_i changes on BASI gene expression are about the same as for Rab gene expression.

To ensure that the inhibition of ABA-induced gene expression by the above mentioned compounds (DMO, potassium propionate and H^+-ATPase inhibitors) is a specific inhibition instead of overall inhibition, the expression of a non-ABA-regulated gene was studied. We rehybridized our northern blots with a barley GAPDH cDNA probe (cDNA clone from

Table III. *Effect of weak acid and weak base on ABA-regulated and nonregulated gene expression.*
Aleurone protoplasts were incubated in 10 mM phosphate buffer, pH 6.6, to which 7.5 mM DMO and/or 5 μM ABA were added, or pH 7.4, to which 10 mM methylamine and/or 5 μM ABA were added. After 2 h of incubation, the protoplasts were collected for northern analysis. The levels of BASI mRNA were semiquantitatively determined. The mRNA expression obtained with ABA treatment was set to 100% and the GAPDH mRNA expression obtained with buffer treatment was set to 100%. The data represent the means ± SD of four independent determinations.

	BASI (%)	(n)	GAPDH (%)	(n)
pH_e 6.6				
Buffer	9.7±3.1	(4)	100.0	(4)
7.5 mM DMO	5.6±2.8	(4)	109.1±11.7	(4)
5 μM ABA	100.0	(4)	106.5±10.6	(4)
ABA+DMO	16.6±5.6	(4)	108.7±7.3	(4)
pH_e 7.4				
Buffer	3.7±0.5	(4)	100.0	(4)
10 mM Meth.	3.4±0.4	(4)	104.8±8.0	(4)
5 μM ABA	100.0	(4)	102.0±12.0	(4)
ABA+Meth.	96.0±7.0	(4)	108.9±9.8	(4)

Cojecki, 1986). The semiquantitative data of the northern blots are presented in Table III. These results indicate that an artificially induced decrease in pH_i by weak acids causes a reduction only in specific ABA-induced mRNA expression and that the overall transcription levels are not affected by the used decrease of pH_i. In unpublised experiments we have observed that GAPDH mRNA can be reduced (within 2 h) after treatment of protoplasts with calcium antagonsists or heavy metals (Cd^{2+}, La^{3+}). In addition, we rehybridized our northern blots with radioactive polyadenylated oligonucleotides (Sambrook *et al.*, 1989) and showed that there was no overall reduction in mRNA levels in pH_i-manipulated and H^+-ATPase inhibitor-treated protoplasts (data not shown).

Discussion

We used the "null-point" method to measure the pH_i of barley aleurone protoplasts. Our testing experiments demonstrated that (a) the measured basal pH_i (approximately 7.0) measured with the null-point method is in good agreement with values reported for plant cells when different methods were used (Felle, 1989); (b) weak acids or bases are able to influence the pH_i measured by null-point method; (c) proton pump inhibitors in long term (more then 45 min) are able to induce a decrease of pH_i that can be

detected by the null-point method; (d) changes of pH_e in the range of 6.0 to 7.4 lead (within 45 min) to small changes in pH_i which can be observed by the null-point method. This evidence shows that the null-point method is a realistic method for measuring plant protoplast pH_i. However, this method requires a large amount of protoplasts and is, therefore, especially for barley aleurone protoplasts, time consuming. In addition, this method does not allow a continuous pH_i measurement in time and has a rather poor time resolution. Nevertheless, this method seems to have no source of serious errors and will give relatively reliable measurements of the pH_i (Rink *et al.*, 1982; Inouye, 1988). It has been applied successfully in animal and cellular slime mould cells (Rink *et al.*, 1982; Aerts *et al.*, 1985).

Different fluorescent pH indicators developed for pH_i measurements are available. Although these fluorescent pH-indicators have some clear advantages over other methods (Bright *et al.*, 1987), there are several potential sources of error as well (e.g. Inouye, 1988). These errors include leakage of indicator from the cytoplasm to the extracellular medium and compartmentalization of the indicator within the cell. In addition, the calibration method make it difficult to obtain the absolute pH_i, and the preparation of cell samples and loading of the indicator can be difficult. Furthermore, environmental conditions may disturb the measurements and influence the required excitation and emission wavelengths. Compared with the fluorescent pH indicator methods, the null-point method is less complicated and has fewer sources of potential errors. We choose the null-point method for measurement of pH_i in barley aleurone protoplasts, because it could be applied easily without the problem of introduction of a measuring probe into the cytoplasm.

The null-point method requires a permeabilization of the plasma membrane. For this purpose we tested both Triton X-100 and digitonin. The difference in basal pH_i values obtained with digitonin and Triton X-100 might be explained by a possible action of Triton X-100, especially at higher concentrations, at internal membranes. This explanation is indirectly supported by the observation that at an extracellular pH which is higher than the pH_i, different steps in the decrease of solution pH were present at increasing Triton X-100 concentrations (Fig. 1A). Such steps in pH change were not observed when digitonin was used (Fig. 1A). The apparent absence of disrupting effects on internal membranes by digitonin is supported by the fact that digitonin is a steroid glycoside that forms insoluble complexes with cholesterol and other ß-hydroxysterols in the

plasma membrane (Akiyama *et al.*, 1980). In addition, in plant cells such as in carrot and maize, digitonin has been successfully used to introduce fluorescent dyes into the cytosol without interfering with intracellular vacuoles, suggesting that digitonin is able to permeabilize the plant cell plasma membrane effectively without affecting intracellular organelle membranes (Fiskum, 1985; Timmers *et al.*, 1991). Therefore, mainly digitonin was used for measurements of pH_i values in ABA-treated and pH_i-manipulated protoplasts. In addition, for this reasons no further attempts were made to establish directly possible effects of Triton X-100 on internal membranes.

We are able to measure the ABA-induced pH_i increase. This increase is both time and ABA concentration dependent. At 10 μM ABA, a 0.15 unit pH_i increase was observed within 45 min (Fig. 2). This response is equal in magnitude but much slower than that reported for an ABA-induced pH_i increase in maize coleoptiles and hypocotyls (Gehring *et al.*, 1990). However, the ABA concentration (100 μM) used by Gehring *et al.* (1990) was much higher than the concentration we used. Stimulation of barley aleurone protoplasts with 100 μM ABA induced a 0.1 unit pH_i increase within 15 min (Data not shown), which is comparable with the results reported for maize (Gehring *et al.*, 1990).

We manipulated the pH_i by adding weak acids, weak bases and proton pump inhibitors. According to our measurements, the effects of weak acids or bases on the pH_i are in agreement with results obtained from an other system (Aerts *et al.*, 1985; Van Duijn and Inouye, 1991). In addition, we showed that introduction of weak acids and bases into the cytoplasm increases the cytoplasmic buffer capacity, because the ABA-induced alkalinization could be inhibited by weak acids (Fig. 4). We have investigated the effects of pH_i modulation by weak acids and bases as well as the effects of proton pump inhibitors on gene expression and found that the weak acids (DMO and potassium propionate) inhibit ABA-induced expression of Rab and BASI genes. Inhibitions of Rab gene expression were half maximal at 5 mM DMO and at 2.5 mM potassium propionate. This difference in the half-maximal inhibition concentration of DMO and potassium propionate is probably due to the pK_a difference (for DMO the pK_a is 6.13; for potassium propionate the pK_a is 4.87). In addition, it has been reported that in cellular slime mould 5 mM DMO is able to induce a same pH_i decrease as 2 mM potassium propionate (Van Duijn and Inouye, 1991). At the concentrations we used, neither DMO nor potassium

propionate affect GAPDH expression or total mRNA level, suggesting that the inhibitory effects of DMO and potassium propionate on Rab mRNA expression are not due to a general inhibition of transcription. We cannot yet say whether the inhibitory effects of weak acids is at transcription level or at the mRNA stability level. Although the weak bases methylamine and ammonia are able to bypass ABA to induce an increase of pH_i, these weak bases neither induce Rab mRNA expression nor enhance ABA-induced Rab mRNA expression. These results demonstrate that an increase in pH_i alone is not sufficient to trigger gene expression.

We have shown that changes in pH_i are necessary but not sufficient to trigger specific gene expression. Therefore, it is likely that more complex regulatory mechanisms are involved in ABA-induced gene expression. Our future research line will be focused on the interrelationship of hormone-induced secondary messengers and their function on regulation of gene expression.

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Abscisic acid-induced membrane potential changes in barley aleurone protoplasts: a possible relevance for the regulation of Rab gene expression*

*Abstract***

The effect of ABA on the membrane potential of barley (*Hordeum vulgare cv.* Himalaya) aleurone protoplasts was studied by measuring the distribution of the lipophilic cation tetraphenylphosphonium (TPP^+). The resting membrane potential (E_m) according to our measurements with TPP^+ is about -53 mV and is in agreement with membrane potential values as measured with intracellular microelectrodes (about -55 mV). The TPP^+ measurements could demonstrate a clear dependence of the resting E_m on the external pH (pH_e).

Stimulation of the protoplasts with ABA induced a transient hyperpolarization of the membrane to -62 mV as measured with TPP^+. The hyperpolarization was ABA-concentration dependent.

Inhibition of the H^+-ATPases with the proton pump inhibitors diethylstilbestrol (DES) or Micanozole effectively prevented hyperpolarization. This indicates that the hyperpolarization is consistent with the activation of plasma membrane H^+-ATPases. The K^+-inward rectifier inhibitor $BaCl_2$ was able to prolong the hyperpolarization. This result suggests that the hyperpolarization causes the opening of K^+ channels.

The ABA-induced proton pump activation may be involved in ABA-induced gene expression, as DES was able to inhibit this gene expression. Ba^{2+} did only show a slight inhibitory effect on ABA-induced gene expression.

Introduction

Abscisic acid (ABA) plays a major regulatory role in important processes such as seed development and germination. The effects of ABA

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**Abbreviations: TPP^+, tetraphenylphoshonium; E_m, membrane potential; pH_e, external pH; DES, diethylstilbestrol; FDA, fluorescein diacetate; R_s, shunt resistance; R_m, membrane resistance.

on these processes are widely studied. The last decade, research in this direction has focused on studies on ABA regulated genes such as Rab (responsive to ABA, from rice, Mundy and Chua, 1988), Em (an ABA induced gene from wheat (Marcotte *et al.*, 1989), and BASI (bifunctional α-amylase subtilisin inhibitor from barley, Mundy and Rogers, 1986) and analysis of ABA-regulated promoters. So far, studies on the signal transduction pathway(s) of ABA are scanty.

Obvious candidates for second messengers in signal transduction are hormone-induced ion fluxes. Several ion fluxes are reported to be influenced by ABA. Recently, reports have shown an effect of ABA on pH_i; both intracellular alkalinization (in corn and parsley hypocotyls, Gehring *et al.*, 1990 and in barley aleurone protoplasts, Van der Veen *et al.*, 1992) and acidification (in barley leaf segments, Beffanga, 1992) by ABA have been reported. The contradictory results are reflected in the ambiguous results from studies on the role of H^+-ATPases. Studies in different cell types show that these can either be activated (e.g. Hartung *et al.*, 1980; MacRobbie, 1981) or inhibited (e.g. Schroeder,1990; Serrano, 1989) by ABA. Raschke stated that in stomata guard cells ABA reduces H^+-ATPase activity, and (thereby) induces K^+ efflux. Ca^{2+} fluxes are believed to play a major part in this process (for review: Hetherington and Quatrano, 1991). ABA is known to affect intracellular Ca^{2+} as well (McAinsh *et al.*, 1990; Wang *et al.*, 1991).

In a previous study we have suggested a role for H^+ fluxes in the signal transduction of ABA leading to gene expression (Van der Veen *et al.*,1992). In the present paper we report on ABA-induced changes in the plasma membrane potential, suggesting a stimulation of H^+-ATPases, and its relation to specific gene expression. We have used the distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) as an indicator for membrane potential and compared this method with intracellular microelectrode measurements. The membrane potential measurements indicate that ABA causes a rapid transient hyperpolarization, probably by activation of plasma membrane H^+-ATPases, followed by K^+ influx. We discuss the possibility that these ion fluxes are involved in ABA signal transduction leading to Rab gene expression.

Materials and methods

Materials

Tritiated Tetraphenylphosphonium bromide and tritiated water were

obtained from Amersham Int. (England). Silicon oil AR 20 and AR 200 were from Wacker Chemie (Munich, FRG). DES, MES, ABA and fluorescein diacetate (FDA) were obtained from Sigma Chemical Co (St. Louis, U.S.A.) and PIPES from Janssen Chimica (Tilburg, the Netherlands). Cellulase Onozaka R-10 was purchased from Yakult Honsha (Japan). Gamborg B5 was obtained from Flow Laboratories (Irvine, Scotland). All other chemicals were from Merck (Darmstadt, FRG).

Isolation of protoplasts

Barley (*Hordeum vulgare cv.* Himalaya) aleurone protoplasts were prepared essentially as described by Wang *et al.* (1991). The obtained protoplasts were washed three times with washing buffer (0.5 M mannitol, 10 mM KCl, 1 mM $MgCl_2$, 1.1 mM $CaCl_2$, 0.1 mM EGTA, 0.5 mM K_2HPO_4, 10 mM PIPES-HCl (pH 6.8), the osmolarity being approximately 800 mOsm).

Measurements of membrane potential

TPP^+-distribution measurements.

Membrane potentials were measured essentially as described by Van Duijn and Wang (1990). In short, 80 µl barley aleurone protoplast suspension (1.6 x 10^6 protoplasts/mL) in washing buffer (unless stated otherwise) was incubated with 360 µM ^{3}[H]-TPP^+ for 5 min. At different times after TPP^+ addition ABA was added to the protoplasts. After 5 min the suspension was layered on top of 400 µl silicon oil (AR20:AR200=1:9) and 10 µl 10% sucrose (at the bottom of the tube). The tubes were then immediately centrifuged for 15 s at 10.000x g in a swing-out rotor to separate the cells from the buffer. The tubes were frozen in liquid nitrogen and the tip containing the protoplast pellet (in sucrose) was cut off. The radioactivity in both protoplast pellet (cpm TPP^+_{in}) and supernatant (cpm TPP^+_{out}) was measured with a liquid scintillation counter (LKB, type 1209 rackbeta) after addition of 2 mL scintillation liquid (Lumasafe, Lumac LSC).

In order to determine the volume ratio of protoplasts and supernatant, parallel experiments were performed in which ^{3}[H]-H_2O was used instead of ^{3}[H]-TPP^+, resulting in cpm H_2O_{in} and cpm H_2O_{out}.

The membrane potential was calculated by substitution of R_{TPP} and R_V into the Nernst equation:

$$E_m = \frac{RT}{F} \ln \frac{R_{TPP}}{R_V}$$

in which R_{TPP}= cpm TPP^+_{out}/cpm TPP^+_{in}, R_v= cpm H_2O_{in}/cpm H_2O_{out}, R=gas-constant, T=absolute temperature; F=Faraday's constant. The whole procedure was performed at room temperature (ca. 22 °C).

Microelectrode measurements

For intracellular microelectrode measurements protoplasts were transferred to a glass bottom teflon culture dish (Ince *et al.*, 1985) filled with washing buffer. Membrane potential recordings were made with fine tipped glass microelectrodes with wide-angle tapers, filled with 3 M KCl (mean resistance 41 MΩ ± 13 MΩ, n=17) and a microelectrode amplifier with capacitance compensation (WPI Series 700 Micro Probe Model 750, WP Instruments, New Haven, CT). Microelectrode capacitance was compensated maximally to obtain rise times lower than 0.05 ms (=time to reach 66% of the potential response upon a current pulse). A piezo-stepper device (Piezo-stepper P-2000, Physik Instrumente (PI) Gmbh Co., Waldbronn-Karlsruhe, F.R.G.) was used to ensure rapid (4μm/0.1ms), radial (at an angle of 60° from the horizontal) impalements of protoplasts with minimal vibration as opposed to impalement by hand. Measurements were carried out at room temperature.

Upon impalement of the microelectrode a shunt resistance (R_s) occurred. The shunt resistance is caused by the hydration mantle surrounding the glass microelectrode and can be much lower then the protoplast-membrane resistance (R_m) itself. The decrease of the input resistance by introducing R_s causes the measured initial membrane potential to depolarize rapidly to a new steady state level, via a discharge of the membrane capacitance via the input resistance (see e.g. Ince *et al.*, 1985; Van Duijn *et al.*, 1988). The initial potential upon microelectrode impalement can be measured if sufficiently fast responding microelectrodes are used. It appears as a peak-shaped potential transient with peak value (E_p) rather than a immediate (step wise) depolarization. As this E_p represents the membrane potential before depolarization due to the shunt resistance, we used this value as the best estimate of the intact cell E_m in our system (Ince *et al.*, 1985; Van Duijn *et al.*, 1988), unless, of course, resealing appears resulting in a $R_s >> R_m$.

Protoplast viability staining

FDA solution (5 mg/mL in acetone) was diluted 1:20 in washing buffer. A quarter volume of cell suspension was added and the cells were incubated at room temperature for some seconds. Viable cells colour bright green upon UV illumination while dead cells show no staining.

RNA analysis

Total cellular RNA was isolated and purified as described by Van der Meulen *et al.* (1993). Northern blots were made by separating 10 µg RNA samples in a glyoxal/DMSO 1 % agarose gel (Sambrook *et al.*, 1989) and subsequent blotting onto a nylon membrane as suggested by the manufacturer (Hybond). Hybridization of the blots was carried out at 60°C in 6x SSC (1x SSC: 0.15 M NaCL, 0.015 M sodium citrate pH 7.0), 0.5% SDS, 5x Denhardt's and 0.1 mg/mL sonificated salmon sperm DNA. The blots were washed at 60°C, twice in 5x SSC, twice in 2x SSC, and finally in 0.2x SSC. The intensities of the autoradiographs of these northern blots were semi quantitatively analyzed with an Ultrascan Kl densitometer.

Statistics

Mean values ± standard error (SE) are given, with n= number of independent experiments, unless stated otherwise. Significance of differences between mean values was tested with Student's t-test.

Results

ABA-induced membrane potential hyperpolarization

The membrane potential (E_m) of barley aleurone protoplasts was calculated from the distribution of the lipophilic cation TPP^+. The resting membrane potential was measured to be -53.1 ± 6.0 (pH_e 6.8, n=26) and varied from batch to batch between ca. -40 and -60 mV. Upon addition of ABA, a rapid transient hyperpolarization of the E_m was observed (Fig. 1). The peak value of this hyperpolarization was reached at about 20 seconds after addition of the hormone and had an average value of -62.0 ± 7.2 mV (pH_e 6.8, n=16). This ABA-induced hyperpolarization was consistently observed throughout the different protoplast batches, and is significant ($p<0.01$), as the peak value varied proportional with the resting E_m.

The transient hyperpolarization was dependent on the ABA concentration (Fig. 2). The dose-response curve suggests Michaelis Menten kinetics. Assuming such kinetics, the half-maximum induction was reached

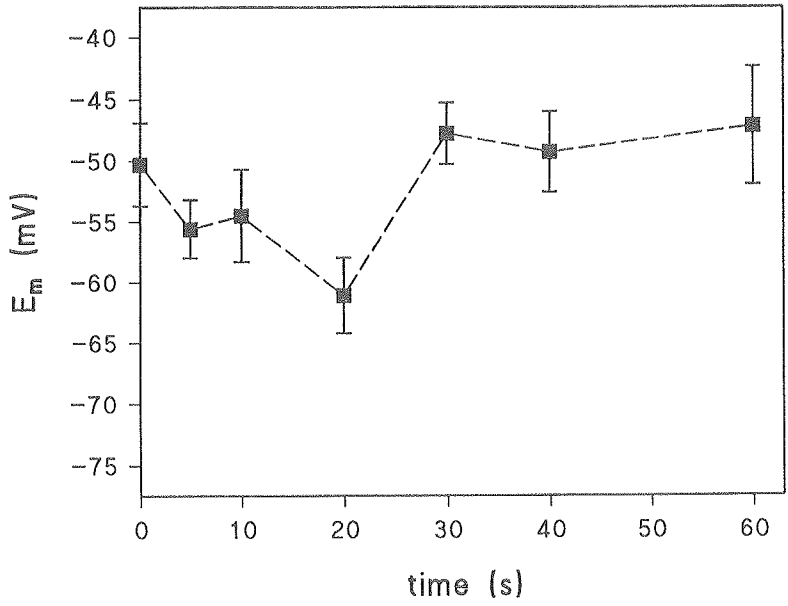

Figure 1. E_m response to ABA as measured with TPP^+. $5x10^{-5}$ M ABA was added at t=0. The mean of four experiments ± S.E. is given (pH_e=6.8).

at an ABA concentration of $1.4x10^{-9}$ M. The specificity of the ABA-induced hyperpolarization was tested using the ABA analogues (±)cis/trans α-ionyledene acidic acid and (±)cis/trans ABA glucose ester. Both analogues have structure similarities with (±)cis/trans ABA, but were reported to be hardly effective with respect to the induction of Rab-gene expression in barley aleurone protoplasts (Van der Meulen *et al.*, 1993). Since neither α-ionyledene acidic acid nor ABA-glucose ester were able to induce a significant

Table I. *Effect of ABA and ABA analogues (5x 10^{-5}M) on membrane potential 20 sec after addition.*
The mean ΔE_m (E_m at 20 sec- E_m at 0 sec in mV) of at least 4 experiments ± S.E. are given.

	ΔE_m (mV)
± cis trans abscisic acid (ABA)	-10.8 ± 3.1
α-iononyledeneacidic acid	-2.4 ± 1.7
ABA-glucose ester	-1.6 ± 3.4

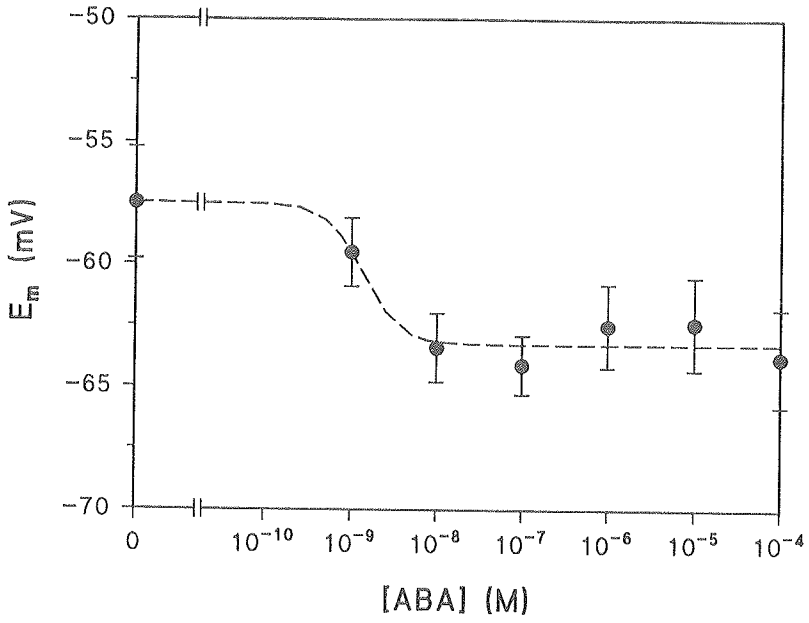

Figure 2. Concentration dependency of ABA-induced hyperpolarization at pH_e=6.8. The mean of four independent experiments ± S.E. is given. The line is a fitted curve, $E_m = (E_{min} + \{E_{max}-E_{min}\}) / (1 + \{[ABA]/[ABA]_{50}\}^2)$. The ABA concentration giving half maximal induction $[ABA]_{50}$ was $1.4 .10^{-9}$ M. The fit was within a confidence bound of 0.95% (r=0.97).

hyperpolarization ($p<0.10$) (Table I), we conclude that the ABA-induced hyperpolarization was rather specific.

Ions involved in the ABA-induced hyperpolarization

Intracellular concentrations of Ca^{2+}, K^+ and H^+ are reported to be affected by ABA (e.g. McAinsh *et al.*, 1990; Wang *et al.*, 1990; Blatt, 1990; Raschke, 1987). We systematically tested the working hypothesis that changes in the efflux of these ions were responsible for the ABA-induced hyperpolarization.

To investigate the role of Ca^{2+}, we added Cd^{2+}, which was shown to be a Ca^{2+} antagonist in aleurone protoplasts (Wang *et al.*, 1992). The rationale behind this experiment was that in case this Ca^{2+}-antagonist had no effect on the ABA-induced hyperpolarization, it would be very unlikely that increased efflux of Ca^{2+} was involved in the electrophysiological response

Table II. *The effect of K^+ and Cd^{2+} on the resting membrane potential (E_m) and the membrane potential 20 seconds after addition with ABA (stimulated E_m).*
The mean of at least 4 experiments ± S.E. is given.

	E_m	stimulated E_m
buffer	-53.6 ± 3.4	-62.3 ± 4.3
1 mM Cd^{2+}	-54.1 ± 4.0	-58.0 ± 2.3
100 mM K^+	-47.8 ± 3.0	-57.7 ± 2.3

of ABA. As shown in table II, Cd^{2+} had a slight effect on the ABA-induced hyperpolarization. This could be consistent with an effect of ABA on Ca^{2+} as described by Wang *et al.* (1991). Next, we applied high extracellular concentrations of K^+ (100 mM) to block the efflux of this cation. We found that the presence of such high extracellular K^+ had only a relatively small effect on the resting E_m and that ABA could still induce a significant membrane hyperpolarization ($p<0.05$) (Table II). Bush *et al.* (1988) reported that 100 mM $[K^+]_{out}$ did not alter the barley aleurone membrane conductance. The relatively small depolarization of E_m in response to the K^+ increase might therefore be explained by increased pump activity. Such relatively small depolarizing effects of K^+ on E_m were also encountered in other plant cells, especially in the presence of Ca^{2+} (for review: Highinbotham, 1981).

However, the specific plasma membrane proton pump inhibitors DES and Micanozole suppressed ABA-induced hyperpolarization in a concentration dependent manner (Fig. 3). As is shown in figure 3, 40 μM Micanozole or 100 μM DES significantly inhibited the ABA-induced hyperpolarization, without significantly inhibiting the resting membrane potential ($p<0.05$). This (indirect) evidence indicates that activation of the plasma membrane H^+-ATPase was the main mechanism behind the ABA-induced hyperpolarization.

The transient nature of the hyperpolarization indicates that the suggested activation of proton pumps is accompanied by changes in other ion fluxes, abolishing the initial hyperpolarization. Indeed, there are examples of other systems in which proton pump activation is followed by an increased K^+ influx (e.g. Rascke, 1987). In order to test wether our system responded likewise, we used $BaCl_2$ to block the K^+ influx. This bariumsalt is known as a specific inhibitor of hyperpolarization-activated K^+ inward rectifying conductances in different plant cells (Schroeder *et al.*, 1987; White and

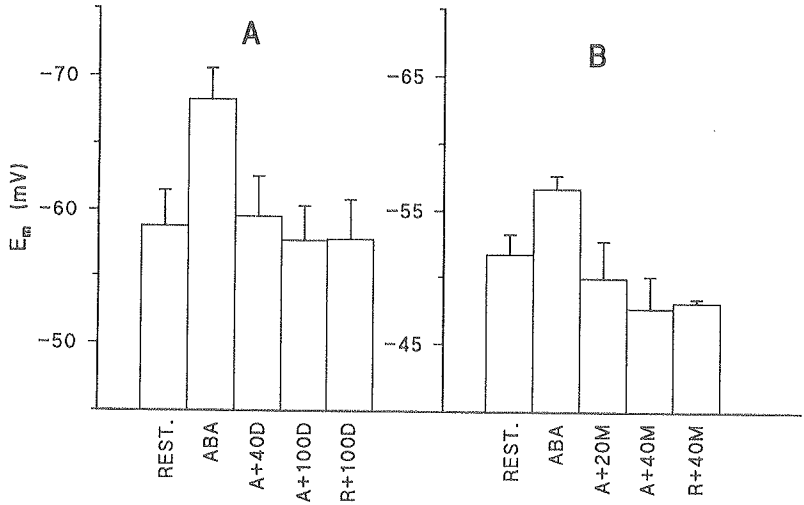

Figure 3. Effect of proton pump inhibitors on ABA induced hyperpolarization. Panel A: "Rest.": resting membrane potential (without ABA), "ABA": ABA-induced hyperpolarization, 20 sec. after addition of ABA, "A+40D": ABA induced E_m in the presence of 40 μM DES, "A+100D": idem, in the presence of 100 μM DES, "R.+100D" resting membrane potential in the presence of 100 μM DES. Panel B: "Rest.": resting membrane potential, "ABA": ABA-induced hyperpolarization, "A+20M": ABA-induced hyperpolarization in the presence of 20 μM Micanozole, "A+40M": idem, in the presence of 40 μM Micanozole, "R.+40M": resting membrane potential in the presence of 40 μM Micanozole. In both A and B the mean of at least 4 independent experiments ± S.E. are given.

Tester, 1992), as well as barley aleurone (Heimovaara-Dijkstra *et al.*, 1993). As shown in figure 4, 2 mM $BaCl_2$ substantially delayed the return to the resting potential. It seems likely that the ABA induced hyperpolarization and/or increased ΔpH over the plasma membrane activated a K^+ inward rectifying conductance.

Involvement of the transient hyperpolarization in the induction of Rab-gene expression

Incubation of barley aleurone protoplasts with ABA resulted in the induction of several mRNA's, at least three of which appear upon hybridization with the Rab 16 clone from rice (Fig. 5). We were interested

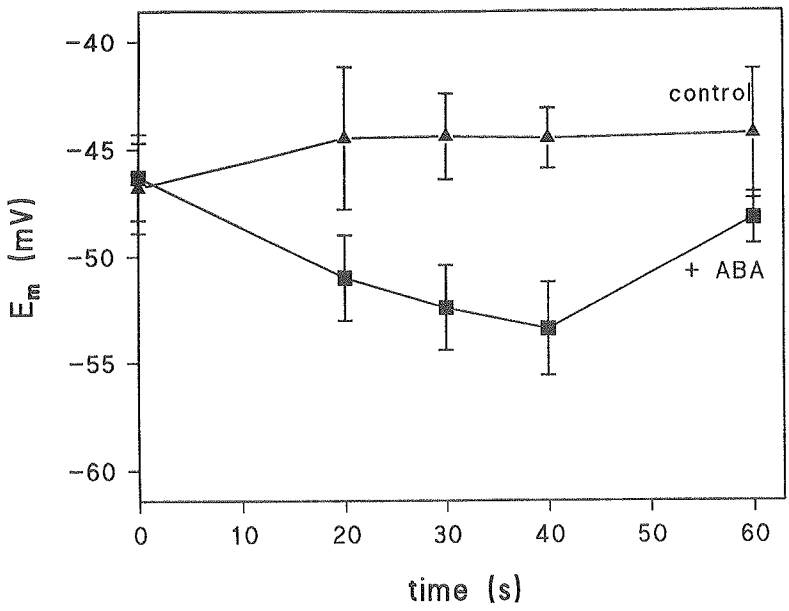

Figure 4. E_m response to ABA ($5x10^{-5}$ M) in the presence of 2 mM $BaCl_2$. Squares indicate the ABA-induced response. Triangles represent a control without ABA. The mean of at least five independent experiments ± S.E. is given, E_m values at 20, 30 and 40 seconds are statistically different ($p>0.05$).

to know whether the suggested H^+ or K^+ fluxes could act as a step in the signal transduction of ABA leading Rab-gene expression. We found that if proton pump activity was inhibited with DES, Rab gene expression was reduced (Fig. 5). The inhibition of Rab gene expression by DES was concentration dependent, rather like the hyperpolarization. Addition of $BaCl_2$ had a slight effect on the Rab gene expression level. $BaCl_2$ applied together with DES did cause a strong decrease in Rab mRNA (more effective than DES alone) but this treatment did also affect the expression level of another gene that is not regulated by ABA: GAPDH (Cojecki, 1986, Fig 5B). This indicated that the combination of DES and $BaCl_2$ might affect the general transcription level rather than specific ABA induced gene expression. Incubation with either DES or $BaCl_2$, did not interfere with GAPDH mRNA expression.

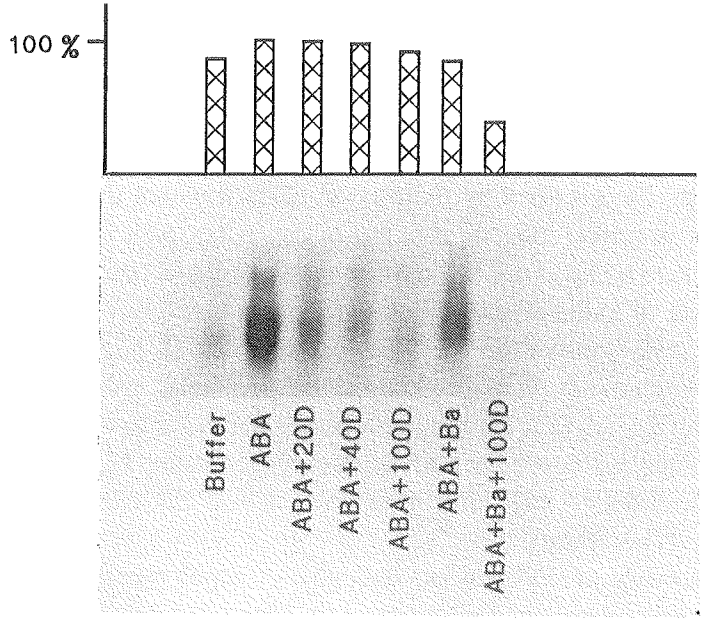

Figure 5. Effect of the proton pump inhibitor DES and the K^+-channel blocker $BaCl_2$ on ABA-induced gene expression. Protoplasts were incubated for 1.5 h in unsupplemented buffer (Buffer), or with ABA (5 x 10^{-6} M) (ABA), with ABA and DES (40 or 100μM; ABA+40 D and ABA+100 D, respectively), with ABA and 2 mM $BaCl_2$ (ABA+Ba), with ABA and 100 μM DES and 2 mM $BaCl_2$ (ABA+100D+Ba). RNA was isolated, separated, blotted and hybridised with a cDNA probe for Rab 16 (rice), lower panel, or GAPDH. GAPDH mRNA levels were semiquatitatively determined with a LKB densitometer and are presented as % expression, ABA=100%, upper panel. A ribosomal probe was used to confirm that RNA loading was equal (data not shown).

Reliability of TPP^+ as a probe for E_m

We used TPP^+ distribution as an indicator of the plasma membrane potential. The method has been successfully used in several plant species (Lin, 1985; Rubenstein, 1978), but was reported to be less successful in some other plant material, like algae (Gimmler and Greenway, 1985). Since this is the first report on this method being used in barley aleurone protoplasts we did some experiments to verify its reliability in this material.

We found that a 5 min incubation with TPP^+ did not inhibit O_2 uptake (data not shown). In addition, FDA viability staining showed that about 80% of the cells were enzymatically active. This percentage did not change

after a 30 min incubation with TPP^+. From these results it was concluded that the used amount of TPP^+ had no harmful effect on the metabolism or viability of the protoplasts within our measuring time.

In order to estimate the most probable value of E_m one should consider several factors which will influence the calculated value. The presence of 20% "dead" cells will cause an underestimation of both resting and stimulated E_m. On the other hand, an overestimation is caused by uptake in the organelles, as described by Saito *et al.* (1992). As we used TPP^+ as an indicator for hormone-induced changes in E_m, rather than to approach the true E_m value, we chose not to consider either of these corrections.

The effect of extracellular pH on the E_m was measured with TPP^+. An increase in pH_e resulted in a more negative E_m (Fig. 6). This is in agreement with reports on other plant cells (e.g. Beilby, 1984). Addition of the uncoupler FCCP to the protoplasts resulted in a decrease of the E_m of 17%. From this we conclude that the measured resting E_m was probably mainly determined by diffusion potentials, with a minor contribution of electrogenic H^+-ATPases.

The reliability of TPP^+ as an indicator of rapid membrane potential changes is dependent on the uptake and exchange speed of TPP^+. To determine these parameters, the protoplasts were incubated in $[^3H]$-TPP^+ until an equilibrium was reached (after 2 min) after which the $[^3H]$-TPP^+ was chased with an excess of unlabelled TPP^+. The resulting exchange curve is shown in figure 7. We calculated the initial exchange speed from this curve. It was found that an amount of TPP^+, equivalent to 4 mV, could be exchanged within one second. This indicates that short, transient potential changes can be measured with this method. Very fast transient E_m changes will probably be underestimated with this method, as there will be a delay in TPP^+ exchange.

Finally, we compared the barley aleurone protoplast E_m as calculated from the TPP^+ measurements with intracellular glass-microelectrode measurements. The procedure and interpretation of data are described in materials and methods. The resting membrane potential measured with microelectrodes was -54.6 ± 17.8 mV. ($n=31$, $pH_e=6.8$). This value is in agreement with that obtained with the TPP^+ method. Microelectrode measurements have several disadvantages as well; obviously the method is laborious and in addition the interpretation of the data is troubled by the induction of a shunt resistance upon microelectrode impalement (Ince *et al.*, 1986; Van Duijn *et al.*, 1988). The obtained shunt resistance (R_s) varied

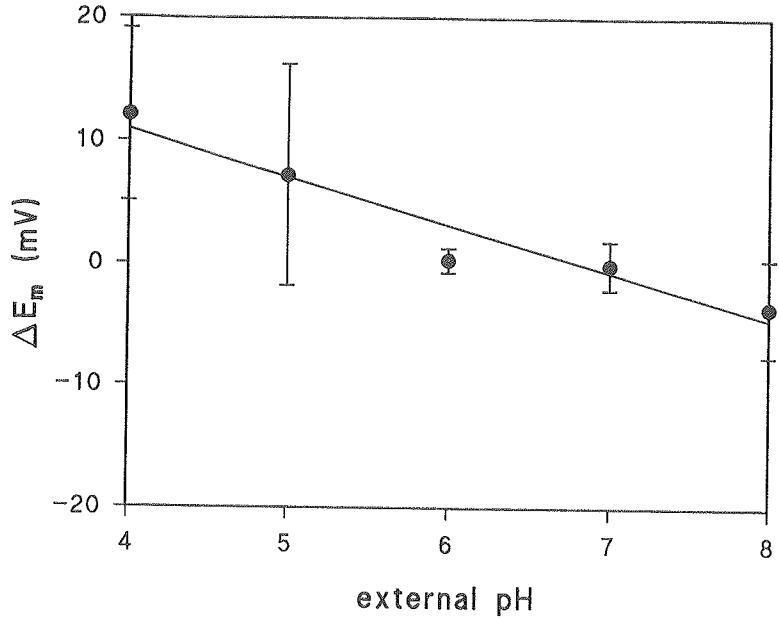

Figure 6. The pH_e effect on E_m as measured with TPP^+. The mean effect on the E_m in relation to the E_m at pH_e 6.8 of at least 4 experiments ± S.E. are given ($\Delta E_m = E_m$ at pH_e6.8 - E_m at pH_e). Lines were fitted with linear regressions, r>0.94.

per protoplast and varied during prolonged measurements. Therefore, it was difficult to perform continuous timecourse measurements. As microelectrode impalement is unsuitable for very rapid sampling of cells, we did not use this method to study the very rapid ABA-induced transient hyperpolarization.

From the above we conclude that TPP^+ distribution can be used to estimate the plasma membrane potential and that it is a reliable indicator of membrane potential changes in barley aleurone protoplasts.

Discussion

We obtained evidence that TPP^+ can be used as an indicator of relatively rapid changes of the plasma membrane potential of barley aleurone protoplasts. The absence of large vacuoles and a cell wall probably adds to the suitability of this method in these cells (see Gimmler and Greenway, 1985). The sensitivity of the aleurone protoplasts to ABA makes it an ideal system to study ABA action on the plasma membrane. Using

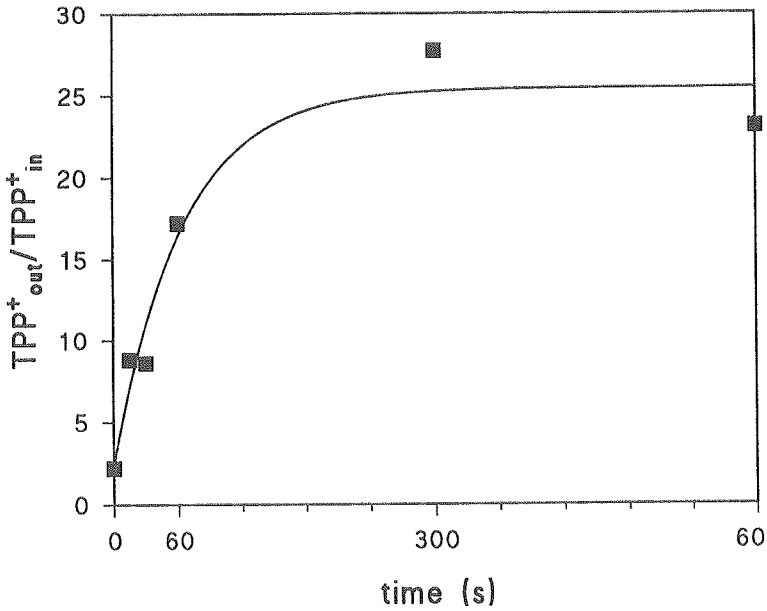

Figure 7. TPP^+ exchange curve. Protoplasts were incubated with [^{3}H]TPP^+ (360 μM) until an equilibrium distribution was reached (≤ 5 minutes). At t=0, 10 mM unlabelled TPP^+ was added and at t=15, 30, 60, 300 and 600 s protoplasts and buffer were separated and the ratio of TPP^+_{out}/TPP^+_{in} was determined. The values were corrected for the amount of TPP^+ which is expected to remain after total exchange due to the [^{3}H]TPP^+/TPP^+ ratio.

TPP^+, we found a resting membrane potential close to the expected nonelectrogenic membrane potential. Glass microelectrode measurements confirmed this value of the resting E_m. Patch clamp recordings on the same material revealed an E_m-value of -45 mV (Bush *et al.*, 1988) and several reports describe membrane potentials of the same order of magnitude in protoplasts and suspension cells (e.g.: Briskin and Leanard, 1979; Van Duijn *et al.*, 1993). The use of the uncoupler FCCP showed that the resting membrane potential was indeed only for ca 17% dependent on (H^+)-ATPase activity. The plasma membrane H^+-ATPase inhibitors DES and Micanozole suppressed ABA-induced hyperpolarization. These inhibitors had only a minor effect on the resting E_m, which is consistent with a small contribution of the plasma membrane ATPase activity to the E_m of unstimulated protoplasts. Our conclusion that ABA activates the plasma membrane proton pump in aleurone protoplasts is in agreement with our earlier observations

showing that ABA induces a cytosolic alkalinization in such protoplasts. This alkalinization could also be inhibited by DES (Van der Veen *et al.*, 1992). Gehring *et al.* (1990) demonstrated a cytosolic alkalinization in maize hypocotyls in response to ABA, using a pH-sensitive dye.

The timescale of the pH_i increase in barley aleurone protoplasts (maximal increase after approximately 1 h, Van der Veen *et al.*, 1992), suggests that the transient nature of the ABA-induced hyperpolarization is not due to a transient activation of the H^+-ATPases. Our experiments with $BaCl_2$ indicate that the subsequent depolarization is caused by an activation of an inward conductance of K^+ ions. Both membrane hyperpolarization and an increased pH gradient over the plasma membrane could be responsible for the opening of K^+ channels. Treatment with $BaCl_2$ caused a prolongation of the hyperpolarization, but was not able to completely prevent subsequent depolarization.

Activation of H^+-ATPases seems to be a rather common response of plant tissue to plant hormones. Apart from the effect of ABA on aleurone cells, both auxins and fusicoccin are reported to have such effect in several plant tissues (e.g. review by Zocchi, 1990). Abscisic acid, however, is also reported to inhibit H^+-ATPases in guard cell protoplasts. This inhibition is accompanied by a K^+ efflux (e.g. Rascke, 1987). This effect of ABA on H^+ and K^+ fluxes is contrary to that in aleurone cells, suggesting a tissue-specific response. It is interesting to see wether such a response can have a specific role in ABA signal transduction. The ABA-induced ion fluxes reported here are very rapid responses and as such precede ABA-induced gene expression.

We were able to show that the proton efflux is important for Rab gene expression and that both responses were DES-concentration dependent. This is consistent with our earlier observation (Chapter 2: Van der Veen *et al.*, 1992) that there was no causal relationship between pH_i increase and Rab gene expression. The K^+ inward rectifying conductance inhibitor Ba^{2+} had a small inhibitory effect on the ABA-induced transcription levels. The inhibition observed when adding both DES and $BaCl_2$ at the same time seems to be caused by inhibition of general transcription levels rather than ABA-induced gene expression.

The physiological role of these ABA-induced ion fluxes in barley aleurone protoplasts is still unclear. The role of the H^+-ATPase activation and K^+ influx in ABA induced gene expression might be the catalysation of a signal transduction step or in affecting mRNA stability. These

possibilities are currently being investigated.

Aknowledgements

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Counteractive effects of abscisic acid and gibberellic acid on extracelullar and intracellular pH and malate in barley aleurone*

*Abstract***

Barley aleurone layers are known to constitutively acidify their surroundings, primarily by L-malic acid release (Mikola and Virtanen, 1980). Here we demonstrate the antagonistic effects of the plant hormones gibberellic acid (GA_3) and abscisic acid (ABA) on the regulation of extracellular pH (pH_e) of barley aleurone layers. We observed a strong correlation between ABA-induced enhancement of extracellular acidification and an ABA-induced increase in L-malic acid release. In addition, ABA caused an increase in intracellular L-malate level. GA_3 caused a slight decrease in intracellular L-malate level and was able to inhibit the ABA-induced increase in L-malate intracellular concentration and release. In addition, this ABA-induced L-malate release could be completely inhibited by GA_3. The ABA-induced release of L-malic acid could not account for the total ABA-induced pH_e decrease, suggesting the existence of an additional mechanism involved in the regulation of pH_e. It has been reported that ABA-induces an intracellular pH (pH_i)-increase, possibly due to the activation of plasma membrane proton pumps (chapter 2: Van der Veen *et al.*, 1992). A pH_i increase, such as that caused by ABA, might be correlated with the intracellular L-malate increase as suggested by the pH-stat model of Davies (1986). We studied if the effects of GA_3 on L-malate concentration were correlated with changes in pH_i and found that GA_3 caused a pH_i-decrease and that GA_3 and ABA could interfere in the regulation of pH_i. In addition, we were able to mimic the effect of both hormones on L-malate release by bringing about artificial pH_i changes with the weak acid 5,5-dimethyl-2,4-oxazolidinedione and the weak base methylamine. The physiological meaning of the effects of GA_3 and ABA

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**Abbreviations: DES, diethylstilbestrol; DMO, 5,5-dimethyl-2,4-oxazolidinedione; pH_e, external pH; pH_i, intracellular pH; MDH, malate dehydrogenase.

on the regulation of both pH_e and pH_i during grain germination are discussed.

Introduction

Several processes during barley (*Hordeum vulgare* L.) grain germination are influenced by pH: α-amylase and several proteases have acidic pH optima and Ca^{2+} liberation and metabolite uptake by the scutellar epithelium are facilitated by low pH (Hamabata *et al*, 1988). In addition, the response of barley aleurone layers to GA, a phytohormone known to play an important role in stimulation of grain germination (Akazawa, 1972), is enhanced at low external pH (Sinjorgo *et al*, 1993). Therefore, changes in pH could be a mechanism by which processes during germination are controlled.

Barley aleurone layers are generally known to acidify their surroundings, mainly due to a constitutive release of L-malic acid (Mikola and Virtanen, 1980). Macnicol and Jacobsen (1992) reported that during grain maturation the pH of the endosperm decreases. This acidification seems to be brought about by the aleurone and involves malic acid secretion. ABA and GA_3 have been reported to increase the extracellular acidification of mature barley aleurone layers (Drozdowicz and Jones, 1993). These authors suggested that GA_3 stimulates phosphate and organic acid release by the aleurone layers. No stimulation of extracellular acidification was observed when aleurone layers of wheat were treated with GA_3 (Hamabata *et al.*, 1988).

Since we are interested in the mechanism of GA and ABA action in barley aleurone cells, we studied the effect of these hormones on the regulation of extracellular pH and L-malate release by barley aleurone. In addition, we investigated the possibility that some of the antagonistic actions of ABA and GA are achieved by counteractive effects on the regulation of pH_i.

Materials and Methods

Materials

L-malic acid was from Sigma (St. Louis, USA). Cellulase R-10 was from Yakult Honsha (Tokyo, Japan). Gamborg B5 was from Flow Laboratories (Irvine, UK). PVP K25 was from Fluka Chemie (Tilburg, the Netherlands) and PIPES was from Janssen Chemicals (Tilburg, the Netherlands). Malate dehydrogenase, citrate synthase, acetyl-CoA and NAD were from Boehringer Mannheim (Mannheim, Germany). All other

chemicals were from Merck (Darmstadt, Germany).

Isolation of aleurone layers and aleurone protoplasts

Barley (*Hordeum vulgare* L. cv Himalaya, harvest 1985; Department of Agronomy, Washington State University, Pullman, USA) grains were de-embryonated and cut into halves longitudinally. To sterilize the half-grains, they were briefly rinsed with 70% ethanol and incubated for 30 min in 0.1% hypochlorite while shaking. After washing (H_2O) they were rinsed for 10 min in 10 mM HCl, washed again and then incubated for 3 d in water at 25°C in the dark. After this, the aleurone layer and starchy endosperm could be easily separated. Aleurone protoplasts (containing only small vacuoles) were prepared as described by Wang *et al.* (1991). The buffer used for washing and incubation of protoplasts was a 10 mM Na/K phosphate buffer (0.5 M Mannitol, 10 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM KH_2PO_4/ $NaHPO_4$, pH 7.0, 830 mOsm).

Measurement of pH_e

Ten aleurone layers were incubated in 1 mL H_2O for 1 day at 25°C in the dark. The pH_e was measured with a standard Pharmacia glass electrode. For measurement of the effect of L-malic acid on the pH_e, a sample of 500 µl was taken from the medium after the incubation period, and supplemented with a certain amount of L-malic acid, after which the pH was again registered.

Measurement of pH_i, null-point method

The null-point-method is based on the principle that when the plasma membrane of cells in a weakly buffered solution is permeabilized, the pH_e will change unless it is equal to the pH_i of the cells. After subjecting the protoplasts to the different treatments, described in the text, they were washed and resuspended in weak phosphate buffer at a concentration of 2 x 10^6 protoplasts/mL. This was a similar buffer as described above but with only 2 mM Na/K phosphate. The pH_e was adjusted to the desired value with NaOH or HCl and subsequently digitonin (0.005%, w/v) was added to permeabilize the plasma membrane. The resulting pH changes were recorded with a combined pH monitor (Pharmacia, Upssala, Sweden) and a pen recorder. The values of the pH_e, at which no apparent shift of pH was recorded after permeabilization, were taken as a close estimate of pH_i. In all determinations correction for the background acidification rate (mainly

due to stirring CO_2 into the suspension) was made. Experiments were performed at room temperature (about 22°C). A more detailed study of this method was presented by Van der Veen *et al.* (1992, chapter 2).

Measurement of L-malate

The determination of malate and malate dehydrogenase (MDH, EC 1.1.1.37) activity were both based on the measurement of ΔA_{340}, which resulted from NAD reduction in the coupled reaction with citrate synthase (EC 4.1.3.7). For determination of the extracellular malate, the incubation medium (H_2O, see "measurement of pH_e") was assayed. For intracellular malate measurements, the aleurone layers were ground under liquid nitrogen and the powder was dissolved in 400 µl distilled water (0°C) and sonicated for 5 s. Intracellular MDH was inactivated by incubation for 5 min at 100°C and then the slurry was centrifuged (5 min, 4°C). The final reaction mixture consisted of 100 mM KH_2PO_4 (pH 7.4), 300 µM acetyl-CoA, 750 µM NAD, 0.2 units citrate synthase and 0.3 units MDH in a total volume of 200 µl. A_{340} was measured at t=0 and after an incubation of 45 min at 25°C. ΔA_{340} was related to a standard curve of L-Malate.

Statistics

Data are presented as means ± SE, with n=the number of measurements. Differences between values were tested with Student's t-test with a confidence boundary >0.95.

Results

The effects of GA_3 and ABA on pH_e

We measured the effect of GA_3 and ABA on pH_e of barley aleurone layers and found that ABA (10 µM) stimulated extracellular acidification, as was reported earlier by Drozdowicz and Jones (1992). The presence of GA_3 (10 µM) alone did not bring about any significant change in extracellular acidification, but it did partly inhibit the extracellular acidification caused by ABA (Fig. 1). Addition of $CaCl_2$ (20 mM) to the aleurone layers, as is often done to increase the effect of GA on α-amylase induction (e.g. Drozdowicz and Jones, 1992), did not influence these pH_e changes (data not shown).

A correlation between pH_e-decrease and L-malate release

A possible mechanism to achieve extracellular acidification could be the

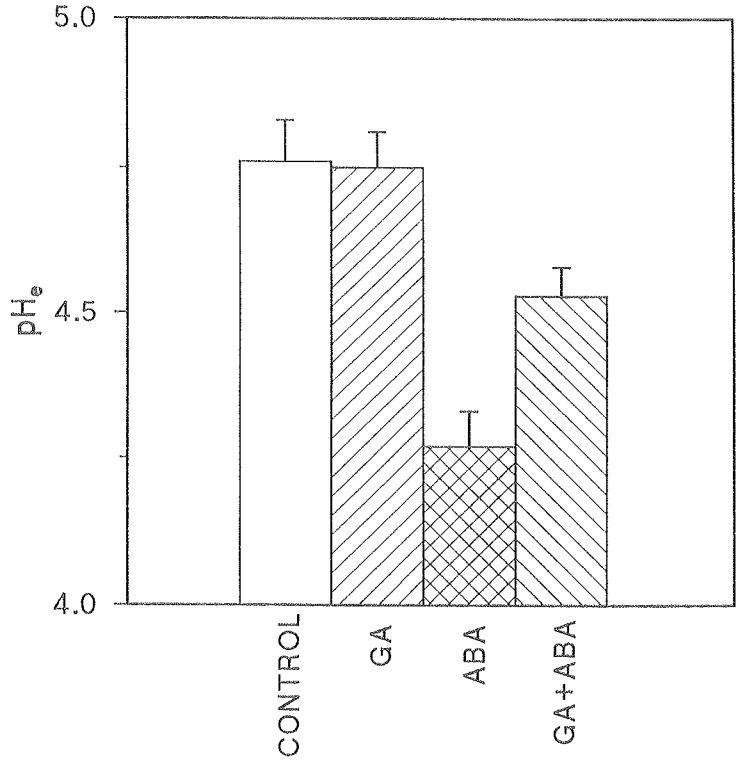

Figure 1. The effect of GA_3 and ABA on pH_e. Ten barley aleurone layers were incubated in 1 mL H_2O for 16 h in the absence or presence of 10 μM GA_3 or 10 μM ABA. The pH_e of the medium was measured using a glass pH electrode. Control, nontreated layers; GA, incubated with GA_3 ; ABA, incubated with ABA; GA+ABA, incubated with GA_3 and ABA. The mean values ± SE of eight independent experiments are presented.

stimulation of organic acid release. Since the organic acid L-malic acid is reported to be the main acidifying component secreted by aleurone layers (Mikola and Virtanen, 1980), we studied the effect of ABA and GA_3 on the level of extracellular L-malate. ABA (10 μM) increased the amount of released malate to 400% as compared to nontreated aleurone layers, whereas GA_3 (10 μM) inhibited L-malate release by the aleurone layer by about 50%. The stimulation of L-malate release, brought about by ABA, could be completely overridden by GA_3 (Fig. 2).

The time course of the pH_e decrease and extracellular L-malate increase shows that ABA causes a detectable (and significant) pH_e-decrease when

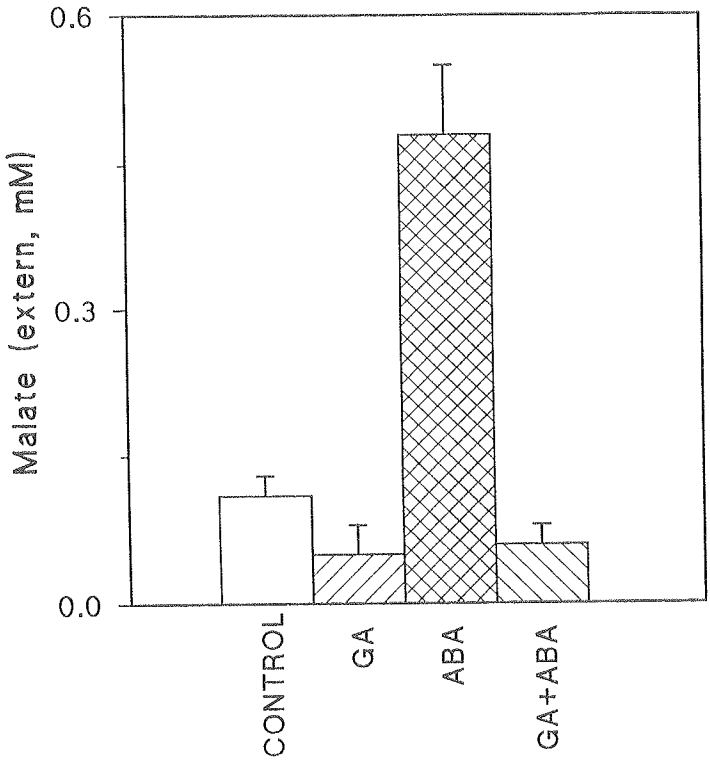

Figure 2. The effect of GA_3 and ABA on extracellular malate concentration. Ten barley aleurone layers were incubated in 1 mL H_2O for 16 h in the absence or presence of 10 μM GA_3 or 10 μM ABA. The L-malate content in the incubation medium (H_2O) was measured as described in "Materials and Methods". The mean values ± SE of eight independent experiments are presented.

incubated for 4 h with ABA. The L-malate increase shows a detectable (and significant) difference between ABA-treated and nontreated layers when incubated for more then 4 h (Fig. 3).

To investigate the quantitative role of malic acid in the ABA-induced extracellular acidification, we treated aleurone layers either with or without GA_3, ABA, or both and measured the pH_e and L-malic acid in the incubation medium (water). We then removed the aleurone layers and added malic acid to the medium to reach the same concentration of L-malate as was present in the medium of ABA-treated layers (0.48 mM). If L-malic acid was added to the medium of untreated aleurone layers, the pH_e dropped

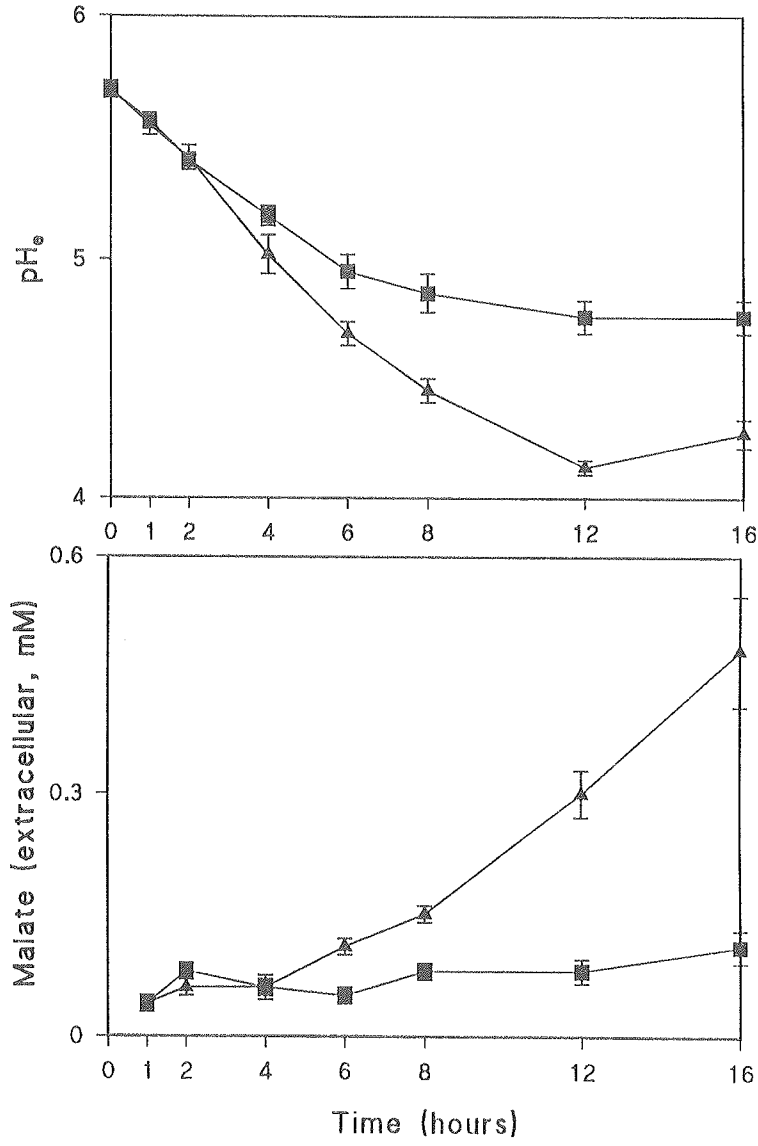

Figure 3. Time course of ABA-induced pH_e decrease and extracellular L-malate increase. Ten barley aleurone layers were incubated in 1 mL H_2O for 16 h in the absence (squares) or presence (triangles) of 10 μM ABA. The pH_e of the medium was measured using a glass pH electrode, the L-malate in the incubation medium was measured as described in "Materials and Methods". The mean values ± SE of three independent experiments are presented.

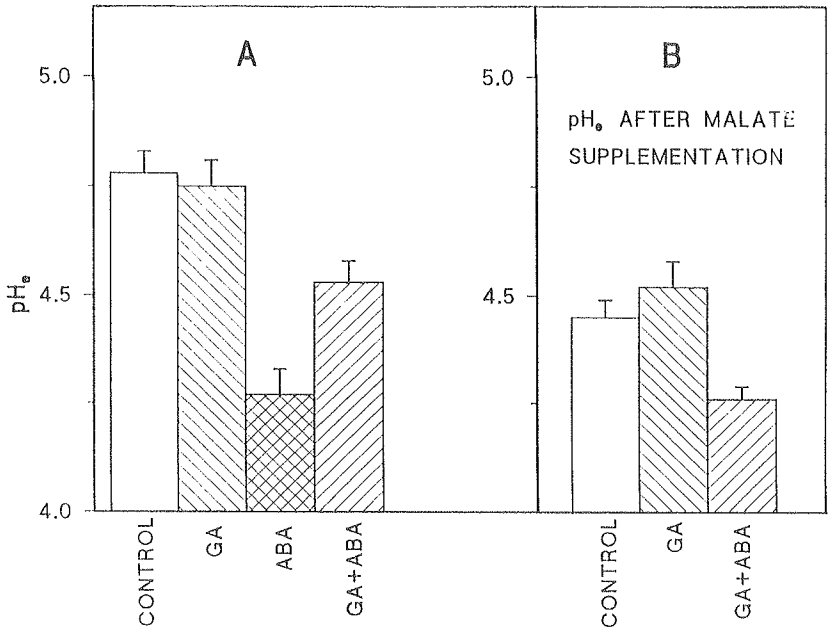

Figure 4. The contribution of the ABA-induced malate release to the ABA-induced pH_e decrease. Ten barley aleurone layers were incubated in 1 mL H_2O for 16 h in the absence or presence of 10 μM GA_3 or 10 μM ABA. The aleurone layers were then removed from the medium and L-malate concentration and pH_e were measured as described in "Materials and Methods". A shows the pH_e of the medium just after incubation; control, nontreated control; ABA, incubated with ABA; GA, incubated with GA_3; GA+ABA, incubated with GA_3 and ABA. B shows the pH_e of the same media, obtained after supplementation of these media with L-malic acid. An amount of L-malic acid was added to achieve a final concentration which was equivalent to that of the medium of ABA-treated layers (0.48 mM). The mean values ± SE of three independent experiments are presented.

approximately 0.3 unit. We found a similar drop of pH if L-malic acid was added to the medium of GA_3-treated layers (Fig. 4A). This indicates that an increase of L-malic acid in the medium, up to the concentration of ABA-treated layers, was not sufficient to completely mimic ABA's acidifying effect.

Although GA_3 itself had no effect on extracellular acidification (Fig. 4A), it partly counteracted the effect of ABA on pH_e (Fig. 4A) and it completely inhibited the L-malate release caused by ABA (Fig. 2). If we added L-malic acid (to a final concentration of 0.48 mM) to the medium of

ABA+GA_3-treated aleurone layers, the pH dropped to the same value as observed in the medium of ABA-treated layers (Figs. 4A and B). These observations strongly suggest that extracellular acidification induced by ABA was brought about by L-malic acid release, which could be inhibited by GA_3, and by an additional mechanism which was unaffected by GA_3.

The effect of GA_3 and ABA on pH_i and L-malate concentration

Intracellular L-malate measurements showed that ABA treatment increased the intracellular malate content, whereas GA_3 brought about a decrease (Fig. 5). Again, GA_3 was able to completely inhibit the ABA-induced increase. Hence, the ABA-induced release of L-malate as described in the preceding section, as well as the inhibition of the L-malate release by GA, are correlated with the effects of these hormones on intracellular L-malate concentrations. Davies (1986) suggested that modulation of cytosolic malic acid concentrations plays a crucial role in the biochemical pH-stat of the cell. pH_i increase would bring about malic acid increase, thus stabilizing pH_i. Since ABA is reported to increase pH_i (e.g. Gehring *et al.*, 1990 and chapter 2: Van der Veen *et al.*, 1992), the L-malate increase caused by ABA might be brought about via an ABA-induced pH_i increase. GA_3 has been reported to cause a short, transient acidification of the cytosol of maize hypocotyls (Irving *et al.*, 1992). If GA has an acidifying effect in barley aleurone cells, then the inhibitory effect of GA on the ABA-induced L-malate increase might be mediated by a counteractive effect on the ABA-induced pH_i increase. Therefore, we investigated the effect of GA_3 on the ABA-induced pH_i increase.

The effect of GA_3 on the intracellular pH in barley aleurone protoplasts was studied using the null-point method. The same method was used successfully to study the ABA-induced pH_i changes in barley aleurone protoplasts (chapter 2: Van der Veen *et al.*, 1992) and is discussed in the report of that study in more detail. The average pH_i of untreated protoplasts was 6.99 ± 0.01 (n=24). Different batches of protoplasts had a slightly different basal pH_i, resulting in small variations in the mean pH_i between the different experiments. Addition of GA_3 (10 μM) induced a decrease in pH_i of 0.18 pH unit (± 0.02, n=22) on average, reaching a new steady-state level at 45 min after addition of the hormone (Fig. 6). The acidification was GA_3 dose dependent (Fig. 7), with a half maximal induction at 4×10^{-9} M.

The effect of GA_3 on pH_i was opposite to that of ABA and was achieved in about the same time span (see also chapter 2: Van der Veen *et*

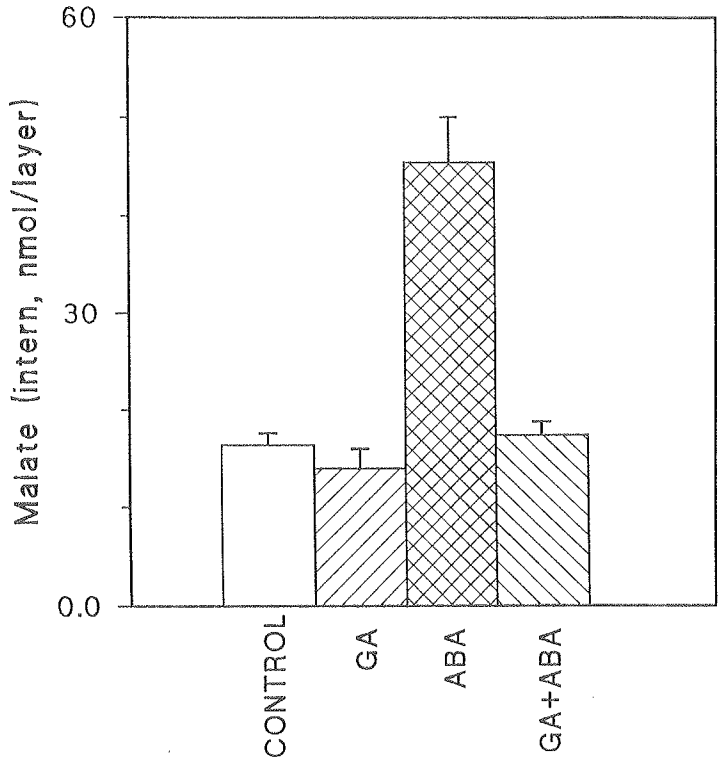

Figure 5. The effect of GA_3 and ABA on the intracellular malate concentration in barley aleurone layers. Ten barley aleurone layers were incubated in 1 mL H_2O for 16 h in the absence or presence of 10 μM GA_3 or ABA. The aleurone layers were extracted and L-malate content measured as described in "Materials and Methods". Control, nontreated layers; ABA, incubated with ABA; GA, incubated with GA_3; GA+ABA, incubated with GA and ABA. The values ± SE of six independent experiments are presented.

Table I. *The long-term effects of GA_3 and ABA on pH_i of barley aleurone protoplasts.* Aleurone protoplasts (8 x10^5/mL) were incubated in 10 mM phosphate buffer (pH 7) at 25°C, with or without 1 μM ABA or GA_3, and harvested after 1 or 6 h. pH_i was measured as described in "Materials and Methods". The mean values ± SE of three independent experiments are presented.

	1 h	6 h
Control	6.97 ± 0.01	7.00 ± 0.01
ABA (1μM)	7.10 ± 0.01	6.99 ± 0.03
GA_3 (1μM)	6.76 ± 0.02	7.06 ± 0.01

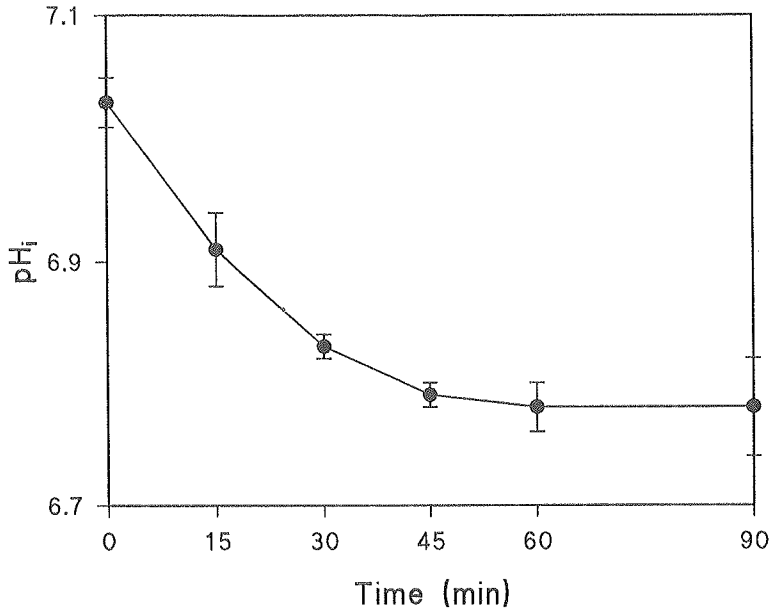

Figure 6. Time course of GA_3-induced pH_i decrease. Barley aleurone protoplasts (8 x10^5/mL) were incubated in 10 mM phosphate buffer (pH 7.0) with or without 10 µM GA_3. At different times samples were collected for pH_i-measurements. The samples were washed twice with 2 mM phosphate buffer (pH 7.0) and pH_i was determined with the null-point method (see "Materials and Methods"). The mean values ± SE of three independent experiments are presented.

al., 1992). The counteractive effects of GA_3 and ABA were studied by adding these two hormones simultaneously to the protoplasts. The results, presented in figure 8, show that the combination of both hormones brought about intermediate shifts in pH_i. Looking at long-term effects of ABA and GA_3 on pH_i we found that approximately 6 h after the addition of the hormones the pH_i of the protoplasts had returned to the level of untreated protoplasts (Table I).

We have shown that L-malate release induced by ABA was detectable 6 h after addition of the hormone. The increase of intracellular L-malate induced by ABA was not yet detectable 4 h after addition of the hormone (data not shown). Thus, it appears that modulation of the pH_i induced by either GA_3 or ABA occurs before we can detect changes in intracellular (and extracellular) L-malate concentration. This points at an underlying mechanism as described by Davies' pH-stat model (Davies, 1986), in which

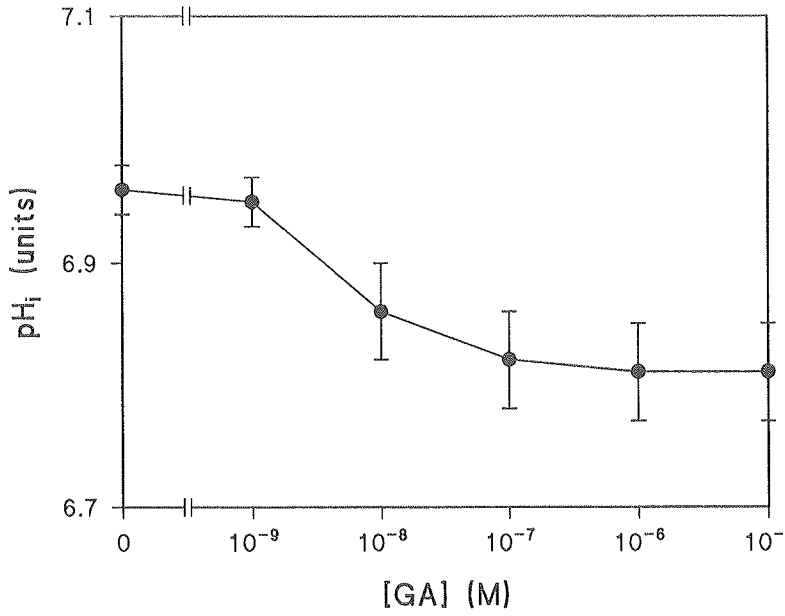

Figure 7. Dose-response curve of the GA_3-induced pH_i decrease. Barley aleurone protoplasts (8x 10^5/mL) were incubated in 10 mM phosphate buffer (pH 7.0) with indicated concentrations of GA_3. After 1 h, the protoplasts were washed twice in 2 mM phosphate buffer (pH 7.0) and pH_i was measured with the null-point method (see "Materials and Methods"). The mean values ± SE of four independent experiments are presented.

modulation of L-malate concentrations is triggered by changes in pH_i. To determine if changes in pH_i, as those brought about by ABA and GA, can be responsible for the respective increase or decrease in L-malate concentrations, we artificially modified pH_i using the weak base MA and the weak acid DMO. The effect of MA and DMO (7.5 mM) on pH_i in barley aleurone protoplasts has been described by Van der Veen *et al* (1992, chapter 2). We studied the effect on intact aleurone layers. Although we were not able to measure the effect on pH_i in walled cells, we expect the effect to be comparable to that in protoplasts, since these chemicals should be able to pass the cell wall. MA is able to cause an increase in pH_i comparable to that of ABA (chapter 2: Van der Veen *et al*, 1992). When applied to aleurone layers, 5 mM MA caused an increase in the amount of L-malate released (Table II). This increase was seen consistently throughout separate experiments, although the extend of the increase varied. When

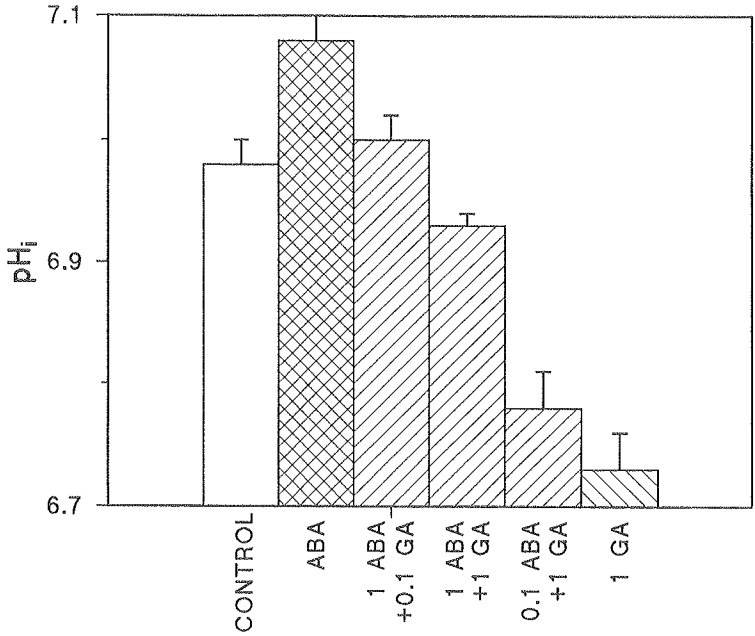

Figure 8. Opposing effects of GA_3 and ABA on pH_i. Barley aleurone protoplasts (8x 10^5/mL) were incubated in 10 mM phosphate buffer (pH 7.0) with different concentrations of GA_3 and ABA. After 1 h, the protoplasts were washed twice with 2 mM phosphate buffer (pH 7.0) and pH_i was measured with the null-point method (see "Materials and Methods"). Control, non-treated layers; 1 ABA, incubated with 1 μM ABA; 1 ABA + 0.1 GA, incubated with 1 μM ABA and 0.1 μM GA_3; 1 ABA + 1 GA, incubated with 1μM ABA and 1μM GA_3; 0.1 ABA + 1 GA, incubated with 0.1 μM ABA and 1 μM GA_3; 1 GA, incubated with 1 μM GA_3. The mean values ± SE of three independent experiments are presented.

applied together with ABA it did not significantly affect L-malate release. This might be explained by the finding of Van der Veen *et al.* (1992, chapter 2) that 7.5 mM MA does not further increase the ABA-induced alkalinization of pH_i. In addition, we found that 5 mM DMO, which is able to decrease pH_i by ca. 0.2 pH unit (similar to GA's effect) and is also able to do so in the presence of ABA (chapter 2: Van der Veen *et al*, 1992), could inhibit L-malate release both in tne absence and in the presence of ABA (Table II). These data support Davies' hypothesis that increase in pH_i inhibits MDH, thus causing an increase in L-malate. Acidification of the pH_i, on the other hand, would be favourable for MDH activity, thereby

Table II. *The effect of artificially-induced pH_e-changes on the amount of L-malate release by barley aleurone layers.*
Ten aleurone layers were incubated in 1 mL 10 mM phosphate buffer (pH 6.6 or 7.4) at 25°C, with or without 10 μM ABA, 5 mM DMO or 5 mM MA. After 16 h samples were taken to determine L-malate concentration as described in "Materials and Methods". Results ± SE of four independent experiments are presented.

		L-malate (mM)
control	(pH_e 6.6)	0.19 ± 0.04
DMO	(")	0.09 ± 0.01
ABA	(")	0.54 ± 0.17
ABA+DMO	(")	0.13 ± 0.05
control	(pH_e 7.4)	0.27 ± 0.05
MA	(")	0.41 ± 0.08
ABA	(")	0.61 ± 0.15
ABA+MA	(")	0.55 ± 0.11

causing a decrease in L-malate concentration.

Discussion

GA and ABA are widely studied antagonists (e.g. Jacobsen and Beach, 1985 and Skriver and Mundy, 1990). The interaction between GA_3 and ABA is complex, as shown by their effect on gene-expression; ABA is able to completely inhibit GA-induced gene expression (α-amylase), whereas GA does not have any effect on ABA-induced gene expression (Rab) (e.g. Skriver and Mundy, 1990; Van Beckum *et al.*, 1993).

The opposite effects of ABA and GA on pH_e and L-malate concentration described here illustrate the complex interaction between both hormones; GA_3 appears to completely inhibit ABA-induced L-malate-accumulation (Figs. 2 and 5). To our knowledge this is the first report of an action of ABA which can be "overruled" by GA_3. GA_3 itself did not induce a significant decrease of pH_e. Drozdowics and Jones (1992) claimed that GA_3 caused an acidification of the pH_e, but whether this effect was significant was not reported. Although GA_3 is able to inhibit the ABA-induced L-malate release completely, it inhibited the ABA-induced extracellular acidification only partly. This suggests that L-malic acid release can account for part of the ABA-induced pH_e drop and that ABA induces an additional acidifying mechanism. Other organic acids have been reported to take part in the acidification of the endosperm by the barley

aleurone layer (Macnicol and Jacobsen, 1992) but seemed to play a minor role compared to malic acid. Mikola and Virtanen (1980) reported that phosphate and amino acids contribute to the constitutive acidification caused by mature aleurone layers, albeit to a minor extent. It was later suggested that the release of these components was inhibited by ABA (Drozdowics and Jones, 1992). Van der Veen *et al.* (1992, chapter 2) demonstrated that ABA-induced an increase of pH_i in barley aleurone. This intracellular alkalinization could be inhibited by DES and Zearalenone, two plasma membrane H^+-ATPase inhibitors. Therefore, it is tempting to propose that ABA stimulates the plasma membrane proton pump, possibly causing the pH_i increase. The ABA-induced extracellular acidification could then consist of a dual mechanism: induction of L-malic acid release, which can be completely inhibited by GA_3, and stimulation of plasma membrane H^+-ATPases, which seems unaffected by GA_3.

The mechanism by which malic acid is released is not clear. L-malate could be released as an organic acid or could be transported as a divalent anion, balancing (separate) H^+ release. The latter option seems to be more likely as malate is mainly present as the divalent anion at a pH_i around 7. One transport mechanism by which malate can be transported out of the cell is the Guard Cell Anion Channel 1, which is permeable for malate (Hedrich and Marten, 1993). In either case the mechanism would apparently be completely inhibited by GA.

The pH_i increase caused by ABA could be the trigger for L-malate increase, as suggested by Davies' pH-stat model (Davies, 1986). We have shown that GA_3 induces a decrease of pH_i and that it counteracts the effect of ABA on pH_i (Fig. 8). If the (transient) pH_i increase caused by ABA is the trigger for L-malate increase, then the opposing effect of GA_3 on pH_i might well explain its inhibitory effect on L-malate production and release. This hypothesis is supported by the data obtained with the metabolically inert weak acid DMO and the weak base MA. By artificially mimicking GA's effect on pH_i, we observed an effect on the L-malate release similar to that of GA: the L-malate release was inhibited in the absence of ABA and did completely inhibit the ABA induction of L-malate release. Moreover, we could mimic the ABA-induced release of L-malate by increasing pH_i with the aid of MA (Table II). Van der Veen *et al.* (1992, chapter 2) reported that 7.5 mM MA was not able to increase pH_i to a level higher than that achieved by ABA alone. In our system it did indeed not significantly affect the ABA-induced L-malate release. To obtain favourable

conditions for uptake of the weak acid or base, the extracellular pH was adjusted to values more close to their respective pK_a's. In this way the amount of acid or base that can enter the protoplast is increased, since these compounds are able to pass the membrane in their uncharged form. The increase of extracellular pH, used to facilitate MA-uptake, also seemed to cause a slight increase in L-malate release itself. This might be caused by a small effect of this pH_e (which is higher than pH_i) on the pH_i. The pH stat model of Davies (1986) based on the effect of pH_i on malate dehydrogenase can explain the effects on the L-malate concentration but does not account for the increased release of L-malate, unless this can be considered as an "overshoot" of the mechanism.

Another question that arises from this study is the mechanism behind the GA-induced intracellular acidification. If activation of plasma membrane proton pumps would indeed be responsible for the part of the ABA-induced extracellular acidification that is not affected by GA_3, then the GA_3-induced pH_i decrease cannot be explained by inhibition of the same H^+-ATPases. Possibly there are more or less independent, opposing mechanisms by which ABA and GA_3 modulate pH_i. The precise mechanism by which GA_3 and ABA modulate pH_i is open for further investigation.

The effect of plant hormones on pH_e is suggested to play a role in the regulation of germination (e.g. Hamabata *et al.*, 1988). In principle, acid surroundings are favourable for several processes during germination, such as hydrolase activity, Ca^{2+} release and metabolite uptake by the scutellar epithelium (Hamabata *et al*, 1988). On the other hand, without addition of hormones to aleurone layers, extracellular acidification is achieved to approximately pH 4.8, quite sufficient to create such favourable surroundings. There may be something significant about the ABA-induced L-malate increase apart from its suggested function in a biochemical pH-stat. Hedrich and Marten (1993) showed that extracellular malate increased the anion permittivity of the anion Guard Cell Anion Channel 1 and induced closure of the stomata, thereby mimicking ABA's effect. The fact that the change in L-malate concentration described here is not restricted to the intracellular concentration, suggests that L-malate has other functions as well. It would be interesting to determine if malate has a role as signal molecule in barley aleurone.

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The effect of intracellular pH on the regulation of the Rab 16A and the α-amylase 1/6-4 promoter by ABA and GA*

Abstract

The intracellular pH (pH_i) of barley aleurone cells is known to be affected by hormones and plant growth conditions. The role of pH_i shifts in gene expression, induced by abscisic acid (ABA) and gibberellins (GA), was investigated. Weak acids and weak bases were used to artificially mimic the pH_i changes brought about by ABA and GA. We found that Chloramphenicol acetyltransferase (CAT) expression controlled by the Rab promoter was affected by pH_i shifts while the α-amylase promoter seemed insensitive. CAT fused to the 35S promoter was used as a control which is not inducible by ABA or GA_3. The expression of this construct was not significantly affected by artificial pH_i changes.

Results

Abscisic acid (ABA) and gibberellin (GA) are two key hormones in the regulation of cereal grain germination. Both hormones exhibit part of their action by the regulation of gene expression. Many genes are now documented that are induced by ABA, among which are the members of the Rab gene family (Responsive to ABA; for review; Skriver and Mundy, 1990). GA is known to induce α-amylase genes (e.g. Jacobsen and Beach, 1985). Several possible signal transduction mechanisms in the cascade leading from perception of these hormones to gene induction have now been reported. Both intracellular Ca^{2+} concentration and pH_i are affected by ABA and GA in several plant species (e.g. McAinsh *et al.*, 1990, Gehring *et al.*, 1990, Wang *et al.*, 1991, Gilroy and Jones, 1992 and chapter 4: S. Heimovaara-Dijkstra *et al.*, 1994). It was shown that ABA increases the pH_i of barley aleurone cells from the basal level of 7.0 to 7.2, whereas GA_3 causes a decrease to 6.8 (chapter 2: Van der Veen *et al.*, 1992 and chapter 4: S. Heimovaara-Dijkstra *et al.*, 1994). Some reports suggest a role for the pH in the regulation of grain germination (e.g. Hamabata *et al.*, 1988). The

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fact that the two antagonistic hormones bring about counteractive effects on pH_i and pH_e of barley aleurone layers (chapter 4: S. Heimovaara-Dijkstra *et al.*, 1994) makes it tempting to suggest a pivotal role for pH_i in hormonal regulation of some processes during germination. Furthermore, it was found by Van Beckum *et al* (1993) that aleurone protoplasts, isolated from dormant barley grains, had a higher pH_i than aleurone protoplasts from nondormant barley grains of the same variety. These dormant and nondormant grains exhibit a difference in ABA and GA responsiveness (Schuurink *et al.*, 1993; Van Beckum *et al.*, 1993). It is still not clear what role basal pH_i and hormone-induced pH_i changes play in the signal transduction cascade.

In an earlier attempt to define the effect of pH_i changes on the induction of gene expression by ABA, the pH_i of barley aleurone protoplasts was artificially manipulated and the effect on Rab mRNA levels was studied (chapter 2: Van der Veen *et al.*, 1992). It was shown that clamping the pH_i at a relatively high level (a similar level as brought about by ABA, approximately pH 7.2), could not mimic ABA in inducing Rab gene expression. This relatively high intracellular pH_i did not affect the level of Rab mRNA which could be induced by ABA. On the other hand, clamping pH_i at a relatively low level (approximately. 6.9), did cause a decrease of the level of ABA-induced Rab mRNA. However, this approach could not discriminate between an effect of pH_i on signal transduction leading to gene expression, or effects of pH_i on mRNA stability. To get a better understanding of the role of the basal pH_i and/or hormone-induced pH_i changes in ABA and GA regulation of gene expression, we used a model system in which protoplasts were transfected with constructs of ABA- or GA-inducible promoters fused to a CAT reporter gene. These constructs show hormone-dependent induction of CAT expression after 16 h and the results can easily be quantified. As a control we used a CaMV 35S-CAT construct.

Manipulation of the intracellular pH with DMO and Methylamine

The effect of intracellular pH shifts on hormone induction of the Rab and α-amylase promoter was studied by artificially changing the pH_i with the metabolically inert weak acid 5,5-dimethyl-2,4-oxalzolidinedione (DMO) or the weak base Methylamine (MA). As these chemicals are only able to pass the plasma membrane in their uncharged form, the extracellular pH was adjusted according to their pK_a values. The effect of the addition of DMO and MA (and other weak acids or bases) on the pH_i of barley aleu-

rone protoplasts was described earlier (chapter 2: Van der Veen *et al.*, 1992). Since the period of incubation was substantially longer in our present study, we first tested the long-term effect of DMO and MA on pH_i.

We measured pH_i with the Null-point method (chapter 2: Van der Veen *et al.*, 1992) with one adjustment: the amount of digitonin used to permeabilise the plasma membrane had to be increased to 0.01%, probably due to the recovery of the cell-wall structure by the protoplasts after 16 h of incubation. We found that the resting pH_i of the unmanipulated batch was virtually unchanged after 16 h of incubation (7.02 at t=16 h vs 7.01 at t=0). In the presence of 5 mM DMO it had dropped to 6.87 and in the presence of 5 mM MA it had increased to 7.21 (both at t=16 h). Those pH_i shifts are similar to those reported to be brought about by DMO (7.5 mM) and MA (7.5 mM) in barley aleurone after 1 h of incubation (chapter 2: Van der Veen *et al.*, 1992). These artificial pH_i shifts were used to study the significance of physiological differences in pH_i on promoter induction.

35S CaMV-controlled CAT expression is not affected by pH_i changes

The cauliflower mosaic virus 35S promoter (-941 to +9), fused to the chloramphenicol acetyltransferase (CAT) coding region followed by a pea *rbc*S-E9 polyadenylation site, was used as a control. This promoter construct was made and described by Skriver *et al.* (1991). They reported that it was affected neither by GA, nor by ABA. We transfected aleurone protoplasts with the 35S-CAT construct using essentially the same materials and methods as described by Skriver *et al.* (1991). The transfected protoplasts were treated with either 5 mM DMO or 5 mM MA. The effect of the weak acid and weak base treatment on the expression of the CAT marker enzyme was studied. It appeared that neither acidification of the cytoplasm with DMO nor alkalinization with MA did significantly affect the induction of the 35S promoter (Fig 1). There was an effect of extracellular pH on the expression level however; i.e. CAT expression was significantly higher at pH_e 7.4 than at pH_e 6.6. Van der Veen *et al.* (1992, chapter 2) reported that the pH_i was hardly affected by such changes in pH_e. Thus, the observed increase does not seem to be caused by a direct effect of pH_i on the level of CAT activity.

Induction of the Rab 16A promoter by ABA is inhibited at low pH_i and stimulated at high pH_i

Both the proton pump inhibitor Diethylstilbestrol (DES) and the weak

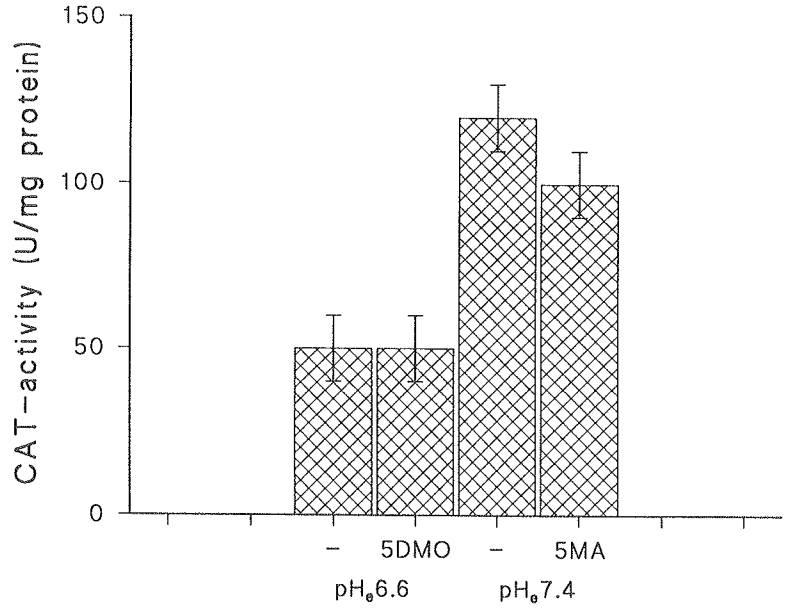

Figure 1. The effect of 5 mM DMO and 5 mM MA on the transient expression of the CAT gene fused to the 35S promoter. Transfected barley aleurone protoplasts (prepared as described in Wang *et al.*, 1990) were washed extensively with, and then resuspended (at 5 x 10^5 pp/mL) in 10 mM phosphate buffer (0.5 M Mannitol, 2% (W/V) glucose, 10 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 10 mM KH_2PO_4/Na_2HPO_4; approximately 900 mOsm) at pH 6.6 (5 mM DMO treatment and control) or pH 7.4 (5 mM MA treatment and control). The protoplast were incubated at 25 °C in the dark. After 16 h the protoplasts were collected and resuspended in 0.25 M Tris (pH 8.0). CAT activity was determined essentially as described by Skriver *et al.* (1990). Means ± S.E. of 5 experiments are presented.

acid DMO (both preventing ABA-induced pH_i increase) were previously shown to inhibit ABA induction of Rab mRNA levels in barley aleurone protoplasts. A pH_i increase, brought about with the weak base MA, did not significantly affect the ABA-induced gene expression (chapter 2: Van der Veen *et al.*, 1992). We used the Rab 16A promoter (-442 to +27) fused to the CAT-coding region+polyadenylation site described in the previous section, to study the effects of pH_i changes on ABA-induced gene expression. The induction of this Rab-CAT construct by ABA was affected by an intracellular pH decrease in a similar fashion as the native Rab-mRNA-levels in protoplasts (chapter 2: Van der Veen *et al.*, 1992); i.e. clamping

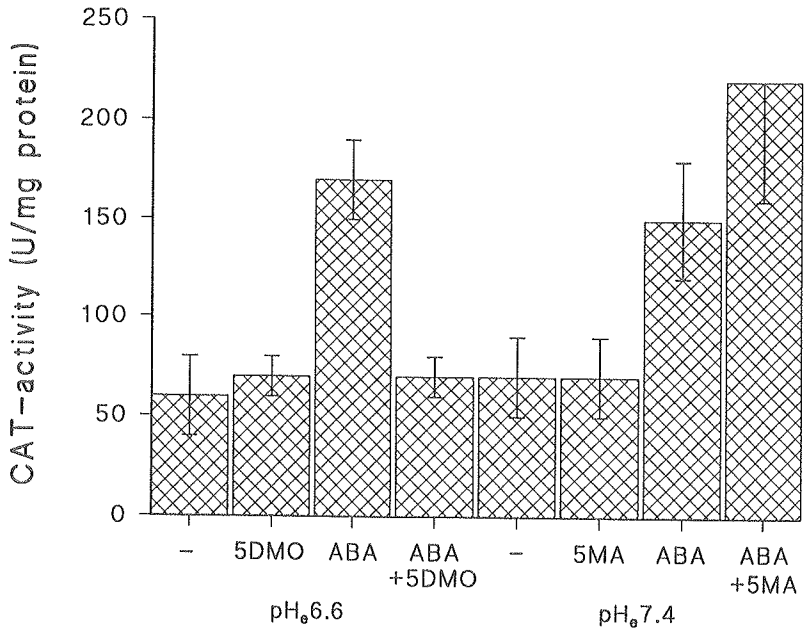

Figure 2. The effect of 5 mM DMO, 5 mM MA and 10 μM ABA on the transient expression of the CAT gene fused to the Rab promoter. The protoplasts were treated as described in figure 1. The protoplasts treated with 5 mM DMO, 10 μM ABA, 5 mM DMO and 10 μM ABA or without additions (control) were incubated at pH_e 6.6. Protoplasts incubated with 5 mM MA, 10μM ABA, 5 mM MA and 10 μM ABA or without additions (control) were incubated at pH_e 7.4. CAT activity was determined essentially as described by Skriver *et al.* (1990). Means ± S.E. of 7 experiments are presented.

of the pH_i at a low level inhibited the induction of the Rab promoter (Fig. 2). From this we conclude that the inhibition of Rab gene expression by DMO results from an inhibitory effect of low pH_i on the induction of the promoter and not by a decrease in mRNA stability. An increase in pH_i by MA, similar in magnitude to that caused by ABA, did in itself not cause induction of the Rab promoter. It seemed however able to enhance the promoter induction brought about by ABA (Fig. 2). Although a large spread in the level of induction in different experiments caused a large standard deviation, the same trend was observed in six independent experiments. These data further support the conclusion of Van der Veen *et al.* (1992, chapter 2), that a pH_i increase is important for Rab gene expression, but not sufficient for induction. These phenomena indicate that shifts in pH_i can

only work in concert with other signals of the ABA signal transduction cascade. Often, changes in pH_i are linked to changes in cytosolic Ca^{2+} concentrations (e.g Gehring *et al.*, 1990, Felle, 1988). Otherwise, a shift in pH might influence the interaction of DNA binding proteins with their target. Such interactions with (other) secondary messengers can probably not be evoked by artificial pH_i shifts in the absence of ABA.

The enhancing effect of MA on ABA induction of the Rab promoter is not reflected by increased native Rab mRNA levels in protoplasts (chapter 2: Van der Veen *et al.*, 1993). This can be due to a negative effect of high pH_i on mRNA stability, thereby overcoming its stimulating effect on promoter induction. In addition, the ABA-induced pH_i increase lasts only for less than 6 h (chapter 4: S. Heimovaara-Dijkstra *et al.*, 1994) and thus may have less effect on mRNA levels then the (continuous) artificial pH_i increase.

In contrast to the 35S promoter construct, the Rab promoter driven CAT expression seems to be unaffected by a change of pH_e from 6.6 to 7.4. The same was found for native Rab mRNA expression in protoplasts (chapter 2: Van der Veen *et al*, 1992).

Induction of the α-amylase promoter by GA is not affected by shifts of pH_i

To study pH_i effects on GA-induced gene expression, barley aleurone protoplasts were transfected with an α-amylase promoter construct (Skriver *et al.*, 1991). This construct consists of the -639 to +43 fragment of the barley amy1/6-4 promoter fused to the CAT coding region+polyadenylation site described earlier. To investigate the role of pH_i shifts in GA-induced gene expression is complicated as the induction of α-amylase gene expression takes at least 16 h and *de novo* protein synthesis is necessary (Muthnukrishnan *et al.*, 1979). GA is reported to rapidly induce a decrease of pH_i to around 6.8, lasting for less than 6 h (chapter 4: S. Heimovaara-Dijkstra *et al.*, 1994). Such temporary pH_i shifts could therefore only influence the steps leading to the onset of α-amylase expression. We are aware that artificially applied pH_i changes are continuous whereas the hormone-induced changes are not. However, if pH_i changes in the first 6 h are the trigger for induction of α-amylase transcription, we should be able to observe induction of the α-amylase promoter construct. We found that neither a decrease of pH_i with DMO nor an increase of pH_i with MA brought about a significant change in α-amylase promoter driven CAT

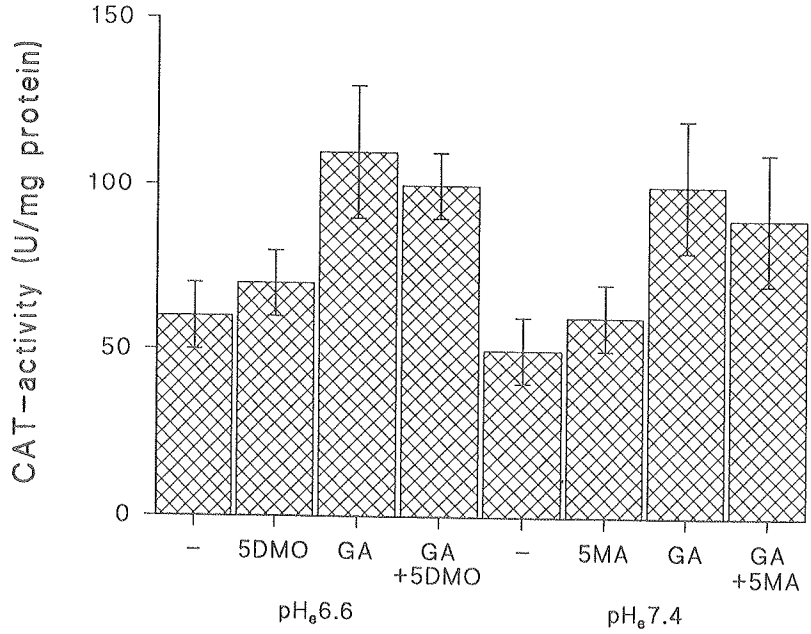

Figure 3. The effect of 5 mM DMO, 5 mM MA and 10 μM GA on the transient expression of the CAT gene fused to the α-amylase promoter. The protoplasts were treated as described in figure 1. The protoplasts treated with 5 mM DMO, 10 μM GA, 5 mM DMO and 10 μM GA or without additions (control) were incubated at pH_e 6.6. Protoplasts incubated with 5 mM MA, 10μM GA, 5 mM MA and 10 μM GA or without additions (control) were incubated at pH_e 7.4. CAT activity was determined essentially as described by Skriver *et al.* (1990). Means ± S.E. of 6 experiments are presented.

expression (Fig 3). Apparently, the induction of the α-amylase promoter by GA in this system was not sensitive for pH_i shifts between 6.8 and 7.2.

In addition to the complete α-amylase promoter, we tested a chimeric GA-responsive promoter, consisting of six GA_3-responsive elements (GARE's) inserted at position -90 of the -941 to +9 35S promoter (constructed and described by Skriver *et al.*, 1991). This promoter construct can be induced by GA_3 and is inhibited by ABA in the same manner as the complete α-amylase promoter, but the GA_3 induction is approximately twofold higher (Skriver *et al.*, 1990). In contrast to the complete α-amylase promoter, induction of the chimeric promoter is sensitive for changes in both pH_i and pH_e. Our results show that this construct is induced by a lowering of pH_i (5 mM DMO) both in the presence and absence of GA_3, but was not significantly affected by an increase of pH_i (5 mM MA; Fig.

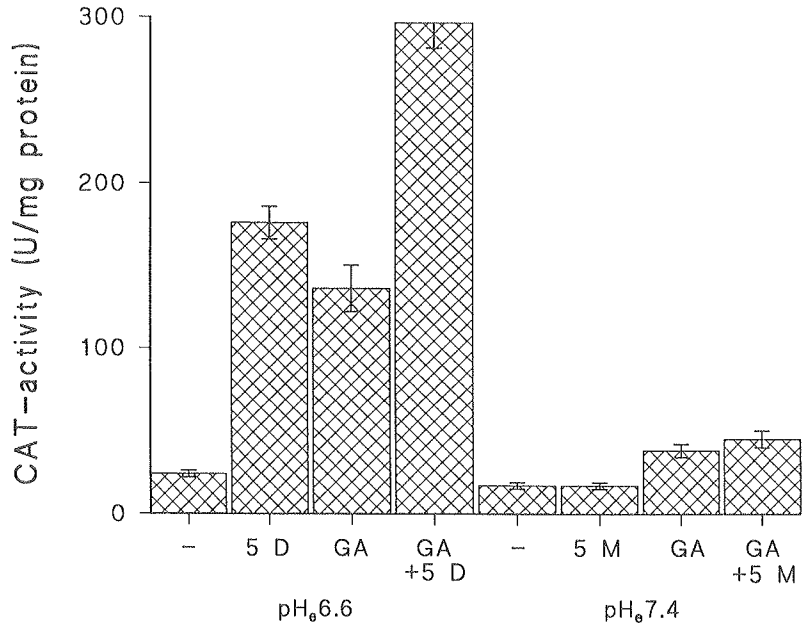

Figure 4. The effect of 5 mM DMO, 5 mM MA and 10 μM GA on the transient expression of the CAT gene fused to six GA-responsive elements from the α-amylase promoter (described by Skriver *et al.*, 1990). The protoplasts were treated as described in figure 1. The protoplasts treated with 5 mM DMO, 10 μM GA, 5 mM DMO and 10 μM GA or without additions (control) were incubated at pH_e 6.6. Protoplasts incubated with 5 mM MA, 10μM GA, 5 mM MA and 10 μM GA or without additions (control) were incubated at pH_e 7.4. CAT activity was determined essentially as described by Skriver *et al.* (1990). Means ± S.E. of 3 experiments are presented.

4). In addition, the chimeric promoter is sensitive for changes in pH_e, showing a decrease in GA-induced expression at pH_e 7.4. This indicates that the chimeric promoter is affected by shifts of pH_i in a different way than the complete α-amylase promoter. Besides, this artificial promoter is inhibited by high pH_e, although not completely. Further study may reveal what mechanisms are responsible for the pH sensitivity of the chimeric promoter and why these mechanisms do not affect the complete α-amylase promoter.

The fact that induction of the intact α-amylase promoter was not affected by a shift in extracellular pH from 6.6 to 7.4 (Fig 3), sheds some new light on previous data concerning the effect of pH_e on α-amylase secretion (Bush and Jones, 1988). These authors showed that α-amylase

secretion could not be induced by GA_3 at pH_e 7.5, whereas GA_3-induction of α-amylase secretion was optimal at pH_e 6.5. Our data show that induction of the α-amylase promoter was not inhibited when pH_e was shifted from 6.6 to 7.4. One can argue that no α-amylase secretion is observed at pH_e 7.4 due to instability of α-amylase at that pH. However, Sinjorgo *et al.* (1993) showed that when extracellular pH was increased from 3.7 to 6.0, secretion of α-amylase was inhibited, whereas the total amount of α-amylase produced was not significantly affected. Although these data do not extend to pH_e higher than 6.0, they support the hypothesis that increasing pH_e affects secretion of α-amylase, rather than synthesis.

By artificially changing pH_i in transfected protoplasts, we have studied the effect of pH_i shifts on the induction of different promoters. A chimeric promoter, consisting of 6 repeats of the GARE box, was induced by high pH_i. However, we observed that GA induction of the complete α-amylase promoter was insensitive for the changes in pH_i. It thus seems unlikely that intracellular pH plays an important role in GA signal transduction leading to α-amylase gene expression.

ABA-induced gene expression on the other hand, does seem sensitive for changes in pH_i. Clamping of the pH_i at a relatively low level inhibited ABA-induced Rab promoter induction, whereas an artificially applied high pH_i caused a increase in ABA-induced Rab promoter induction. However, a high pH_i was not sufficient in itself to induce Rab promoter driven expression. We conclude from the data above that pH_i does influence ABA-induced gene expression in a way in which a lowered pH_i inhibits, while a pH_i higher than 7.1 stimulates ABA induction of the Rab promoter. We were not able to clarify why GA does not inhibit native Rab mRNA levels, whereas it is able to bring about a decrease in pH_i, as was described by S. Heimovaara-Dijkstra *et al.* (1994, chapter 4). Several physiological mechanisms could underlie this phenomenon. As discussed earlier, interactions with other second messengers could well be necessary for induction, but could not be mimicked by these artificial pH_i changes.

Given the present data, we cannot suggest that hormone-induced pH_i changes act as a trigger in the signal transduction leading to gene expression. However, (basal) pH_i can have an influence on the regulation of the ABA-regulated gene induction. Intracellular pH measurements of aleurone protoplasts isolated from dormant (ABA-sensitive) and nondormant (GA-sensitive) barley plants, revealed that the first had a higher basal pH_i than the latter (van Beckum *et al.*, 1993). Our data indicate that the lower

pH_i of the nondormant aleurone layers is unbenificial for ABA-induced Rab gene expression of this tissue. It was indeed shown that ABA-induced Rab mRNA levels were much lower in nondormant layers than in dormant layers (Van Beckum *et al.*, 1993). According to our hypothesis, the basal pH_i can play a role in determining the ABA responsiveness of a certain tissues.

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Abscisic acid-induced gene expression requires the activity of (a) protein(s) sensitive to the protein-tyrosine phosphatase inhibitor phenylarside oxide*

Abstract

Evidence is now accumulating that phosphorylation and dephosphorylation of proteins play an important role in signal transduction cascades of plants, like in other organisms. To study the role of phosphorylation in ABA signal transduction, we used six different compounds which were reported to inhibit protein-phosphatase action. Three of these inhibitors: phenylarside oxide (PAO), Calyculin A (CA) and Okadaic Acid (OA) were capable of inhibiting ABA-induced gene expression. The same three inhibitors were shown to bring about hyperphosphorylation of two approximately 40 kDa proteins, present in the membrane-bound fraction of barley aleurone. We could not detect an effect of the other three inhibitors on *in vitro* phosphorylation of barley proteins. The hyperphosphorylation of the 40 kDa protein(s) is, at least in part, due to hyperphosphorylation of tyrosine residues, as was shown by two-dimensional western blots probed with anti-phosphotyrosine antibodies.

Introduction

Phosphorylation/dephosphorylation of proteins is an important step in many signal transduction pathways. Several well-studied examples of protein phosphorylation have been described that act as an essential component in hormonal regulation (e.g. in Badwey, 1991). Only recently evidence is accumulating that hormonal regulation acts via phosphorylation processes in plants. Many protein kinases and some protein phosphatases (for review see Verhey and Lomax, 1993) are now being identified, but the function of these proteins is largely unknown. To date, reports on the effect of phytohormones on protein phosphorylation is scarce. Indications that protein phosphorylation plays a role in ABA action are now appearing. Recently, a gene responsible for a wide spectrum of ABA responses in

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Arabidopsis was cloned and appeared to be a protein-phosphatase homologue (Leung *et al.*, 1994; Meyer *et al.*, 1994). Earlier, Koontz and Choi (1993) reported that ABA induces relatively rapid dephosphorylation of at least 3 acidic phosphoproteins in somatic carrot embryo's. In addition, a gene was identified whose expression was regulated by water stress and ABA and which appeared to be homologous to protein kinases (Anderberg and Walker-Simmons, 1992).

The role of protein phosphorylation in the action of other plant hormones, e.g. ethylene, is better documented. Raz and Fluhr (1993) have shown that ethylene induction of pathogenesis-related (PR)-proteins can be blocked by protein-kinase inhibitors and can be mimicked by protein-phosphatase inhibitors. Auxin has been shown to bring about both phosphorylation and dephosphorylation of certain proteins in *Avena* coleoptiles (Velumthambi and Poovaiaah, 1986).

The use of specific inhibitors of protein kinases or phosphatases has proven to be a valuable tool in the study of the involvement of these enzymes in several physiological processes. Many such inhibitors are well documented in animal literature, but their action in plants is largely unknown. Sofar, staurosporine analogues (e.g. Grosskopf *et al.*, 1990; Raz and Fluhr, 1993) and the phorbol ester TPA (Olah and Kiss, 1986) have been shown to affect the phosphorylation status of plant proteins. Several phosphatase inhibitors were shown to have various effects on physiological processes. Okadaic acid seems to be the most widely used phosphatase inhibitor in plants: it was shown to affect PR-gene induction (Raz and Fluhr, 1993), pollen tube growth in *Brassica* (Rundle *et al.*, 1993) and it mimics elicitor action in tomato cell suspensions (Felix *et al.*, 1994; Vera-Estella *et al.*, 1994). The latter effect could also be brought about by another phosphatase inhibitor: Calyculin A.

Here we report on the use of several inhibitors of protein phosphatases to study the role of protein (de)phosphorylation in ABA signal transduction leading to gene expression in barley aleurone layers. Besides studying their effect on ABA-induced gene expression we also investigated their specific effects on phosphorylation of barley aleurone proteins.

Materials and Methods

Materials

All phosphatase inhibitors were purchased from Salomon labs LTD (Jeruzalem, Israel). [γ-^{32}P]ATP (>5000 Ci/mmol) was from Amersham

(Buckinghamshire, UK). Monoclonal anti-phosphoserine and anti-phosphothreonine antibodies, (±)cis-trans ABA and ATP were from Sigma (St. Louis, MO, USA). Anti-mouse or rabbit-IgG AP conjugate, 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro-blue-tetrazolium (NBT) were from Promega (Madison, USA). All other chemicals were from Merck (Darmstadt, Germany).

Isolation of protoplasts

Aleurone protoplasts from barley (*Hordeum vulgare* cv Himalaya) were prepared essentially as described before (Wang *et al.*, 1991): aleurone layers were isolated from 3-day H_2O-imbibed sterile half seeds and treated overnight with an enzyme solution (cellulase). The protoplasts were harvested and washed three times with washing buffer (0.5 M mannitol, 10 mM KCl, 1 mM $MgCl_2$, 1.1 mM $CaCl_2$, 0.1 mM EGTA, 0.5 mM K_2HPO_4, 10 mM PIPES-HCl (pH 6.8), subsequently the osmolarity was brought to approximately 800 mOsm with mannitol).

Gene expression studies

Barley aleurone protoplasts were incubated at a density of 4 $x10^5$pp/mL in washing buffer with or without ABA and/or phosphatase inhibitors at 25°C in the dark for 2 h. RNA isolation, electrophoresis, blotting and hybridization were performed as described in chapter 3 (Heimovaara-Dijkstra *et al.* 1994). Quantification was performed by density scanning of autoradiograms with an LKB ultrascan (Sweden).

Protein isolation and phosphorylation reactions

Barley-aleurone protoplasts were resuspended in 40 mM HEPES (pH 7.7), 0.5 mM EDTA, 50 mM Sucrose at 0°C in a density of 1 $x10^6$ pp/mL. The suspension was pressed through a Nuclearpore membrane filter (pore ∅ 5μM) and centrifuged 10 min, 14,000 rpm at 0°C. The supernatant, containing soluble proteins, was kept on ice until use. The pellet was washed twice in 10 mM Tris.Cl (pH 8.2), 2 mM $MgCl_2$, 1 mM ATP, 20 mM DTT at 0°C. Subsequently, the pellet was resuspended in a buffer containing 20 mM Tris.Cl (pH 7.5) and proteinase inhibitors (described by Das and Henderson 1983) at a density equivalent of 5 $x10^6$pp/mL (approximately 1 μg/μL). This suspension, consisting of membrane and cytoskeleton-bound proteins, was kept on ice until use.

Phosphorylation reactions were carried out by incubating 40 µL of these protein suspensions in 50 µL solution of 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 50 µM ATP, 10 µCi [γ^{32}P]ATP (facultative) and the indicated amounts of ABA and/or inhibitors. Reactions were performed at room temperature and were stopped by heating 2 for min at 100°C.

Protein analysis

Protein samples, isolated and treated as described in the previous section, were solubilized and then separated on 12.5% SDS-PAGE (Leammli, 1970). The gel was dried (for autoradiography) or the proteins were transferred electrophoretically to a nitrocellulose membrane as described by Klein *et al.* (1987). The nitrocellulose blots were blocked in phosphate-buffered saline containing 68.5 mM NaCl and 0.05% (v/v) Tween-20 and 1% (w/v) BSA for 1 h and incubated overnight at 4°C with 1:500 diluted rabbit antiserum against phosphorylated tyrosine-residues. Subsequently they were incubated for 1 h with 1:7.500 diluted goat-anti-rabbit IgG conjugated to alkaline phosphatase. Specific bands were visualized using 5-bromo-4-chloro-3-indolyl-phosphate/nitro-blue-tetrazolium as substrate.

For two-dimensional electrophoresis as described by O'Farell (1977), protein samples were first separated according to their isoelectric point on tube gels containing 0.8% ampholines pH 5-7 and 0.8% ampholines pH 3.5-10. After isoelectric focussing (16.5 h at 375 Volts and 1 h at 800 Volt) the tube gels were equilibrated in 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 62.5 mM Tris.Cl (pH 8) for 1 h and then mounted on a 12.5% SDS-PAGE gel and separated as described in the previous section.

Results

The effect of phosphatase inhibitors on ABA-induced gene expression

We used 6 protein-phosphatase inhibitors known from animal literature to study the role of protein dephosphorylation in ABA signal transduction: Okadaic Acid (OA) (Haystead *et al.*, 1989) and Calyculin A (CA) (Biolan and Takai, 1988), both being potent inhibitors of serine/threonine-specific protein phosphatases 1 and 2A; Phenylarside oxide, a putative inhibitor of tyrosine phosphatases (PAO) (Garcia-Moralez *et al.*, 1990); Sodium fluoride, a potent inhibitor of acid, alkaline, P-2 and P2A phosphatases (NaF) (Gruol and Wolfe, 1990); Pyrophosphate (Damuni, 1990) and vanadate, both general protein phosphatase inhibitors (Dayani *et al,* 1990).

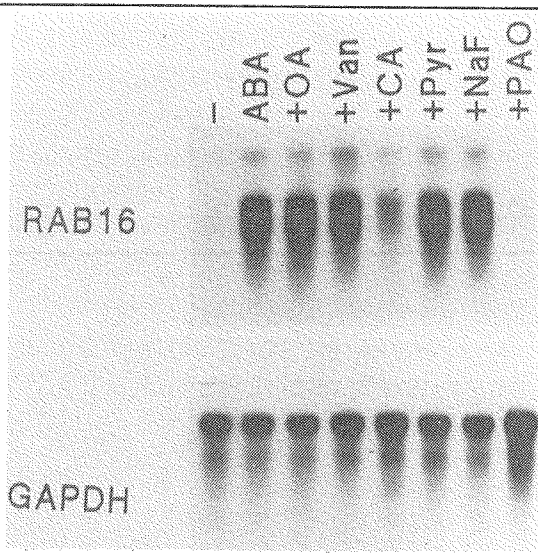

Figure 1. Barley aleurone protoplasts were incubated in the presence of the following compounds: "_", control; "ABA", 5x 10^{-6}M ABA: "OA", ABA+ 1x 10^{-7}M Okadaic acid; "Van", ABA+ 1 mM Vanadate; "CA", ABA+ 1x 10^{-7}M Calyculin A; "Pyr", ABA+ 1 mM Pyrophosphate; "NaF", ABA+ 1 mM Sodium fluoride; "PAO", ABA+ 1 mM Phenyl arside oxide. After 2 h, cells were harvested, RNA was isolated and hybridized with the Rab 16-cDNA probe. The blot was then stripped and rehybridized with the GAPDH-cDNA probe. One representative example of three experiments is shown.

We tested the effect of these inhibitors on ABA-induced Rab-gene expression. Upon induction with ABA, at least three mRNA's that are homologous to the rice Rab-16 gene are expressed in barley aleurone (Fig 1 and 2). Using the inhibitors at concentrations suggested in the literature, we found that CA significantly inhibited ABA-induced Rab-gene expression in aleurone protoplasts, whereas in the presence of PAO no Rab-mRNA was induced at all (Fig 1). None of the other inhibitors we used had an affect on Rab-mRNA levels. To test whether the observed inhibition by CA and PAO was specific for ABA-induced Rab-gene expression rather than an overall effect on transcription, we rehybridized the blot with a cDNA probe corresponding to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a non-ABA-regulated gene (Cojecki, 1986). Figure 1 shows that none of the compounds altered the level of GAPDH expression.

We subsequently tested the concentration dependency of the inhibition of ABA-induced Rab-gene expression by PAO, CA and OA. The effect of OA was studied as it was expected to give similar results as CA. Both compounds are reported to affect the same classes of phosphatases, although

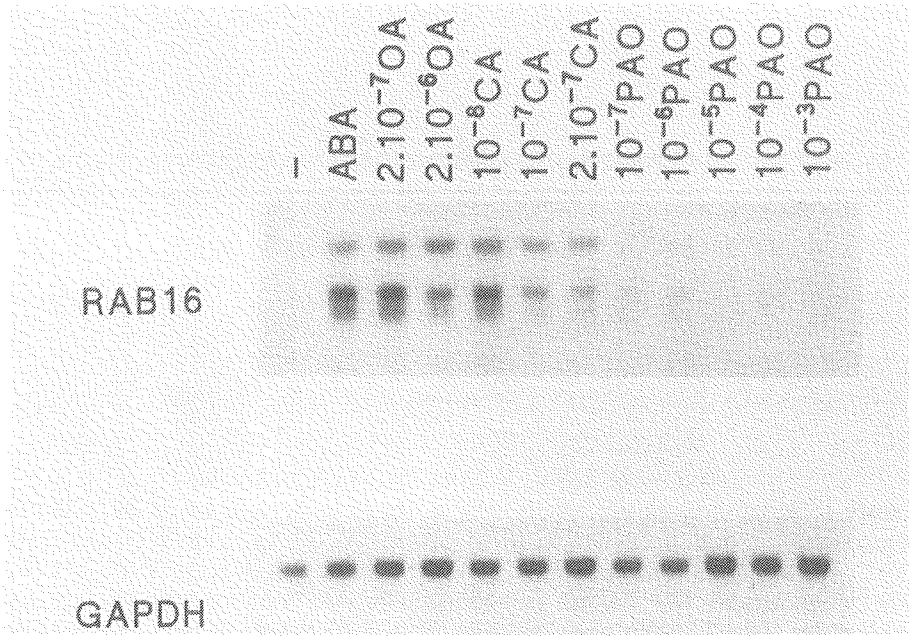

Figure 2. Barley aleurone protoplasts were incubated in the presence 5x 10^{-6}M ABA (except for the control "_") and the inhibitors OA; CA; and PAO in the concentrations indicated (in M). After 2 h, cells were harvested, RNA was isolated and hybridized with the Rab 16-cDNA probe. The blot was then stripped and rehybridized with the GAPDH-cDNA probe. One representative example of two is shown.

OA is considerably less active (e.g. Felix *et al.*, 1994). It appeared that PAO was able to completely inhibit Rab-mRNA induction at a concentration of 10^{-7}M (Fig. 2). CA and OA partly suppressed expression of the Rab genes (Fig 2). One species of Rab-mRNA band (the upper band in Fig. 2) seemed rather insensitive for inhibition by either CA or OA (Fig 1 and 2). This suggests that the corresponding Rab gene is driven by a signal transduction pathway that is less sensitive for these inhibitors than that of other Rab-genes. The maximum effect of CA (approximately 60% reduction as measured by density scan) was reached at 10^{-7} M, whereas it required at least 2 x10^{-6} M OA to suppress expression Rab genes (the lower two bands in Fig. 2) with approximately 30% (Fig. 2). The above results suggest that protein phosphatases with different sensitivities for the inhibitory compounds PAO, CA and OA may be involved in the induction

of Rab-gene expression by ABA. Rehybridization with the GAPDH-cDNA clone showed that these compounds did not affect overall transcription levels in the concentrations used.

Effect of phosphatase inhibitors on phosphorylation of barley aleurone proteins

The inhibitors we used are reported to affect dephosphorylation processes in several organisms. However, there are no data available on their precise action in plants. We, therefore, investigated the effect of these compounds on phosphorylation of proteins from barley aleurone. To this end we used two approaches: studies involving ^{32}P-labelled phosphorus and studies using antibodies against phosphorylated amino-acid residues.

Barley-aleurone proteins were isolated and roughly separated into a 'membrane'-fraction (consisting predominantly of membrane and cytoskeleton-bound proteins) and a soluble fraction as described in Materials and Methods. Both fractions were incubated with ^{32}P γ-labelled ATP in a solution of 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 50 μM ATP (pH 7.5) in the absence or presence of the inhibitors. Phosphorylation of the proteins in the membrane fraction was not visibly affected by OA, NaF, pyrophosphate or vanadate (Fig. 3A). The other two inhibitors, PAO and CA, brought about hyperphosphorylation of a protein band with a molecular mass of approximately 40 kDa, PAO being the most effective (Fig 3A). This could be confirmed by quantitative analysis of the relative intensity of the bands (Fig. 3B). The hyperphosphorylation brought about by PAO and CA was consistently observed in separate experiments. However, other effects that were observed, like the decrease in intensity of an approximately 43 kDa protein band in the PAO-treated membrane-bound fraction, and of an approximately 75 kDa protein band in the pyrophosphate-treated soluble fraction, both shown in figure 3A, was not reproducible.

The effect of PAO on hyperphosphorylation of the 40 kDa protein(s) was clearly visible after 5 min incubation and increased up to 15 min, and then decreased after longer periods of incubation (data not shown). A similar transient nature of the inhibitory action of PAO on dephosphorylation processes was described earlier (Medema *et al.*, 1991).

In the soluble protein fraction no effects of NaF, pyrophosphate and vanadate could be observed. PAO, CA and OA on the other hand brought about a decrease in phosphorylation of (an) soluble protein(s) with a molecular mass of approximately 42 kDa. No hyperphosphorylation was

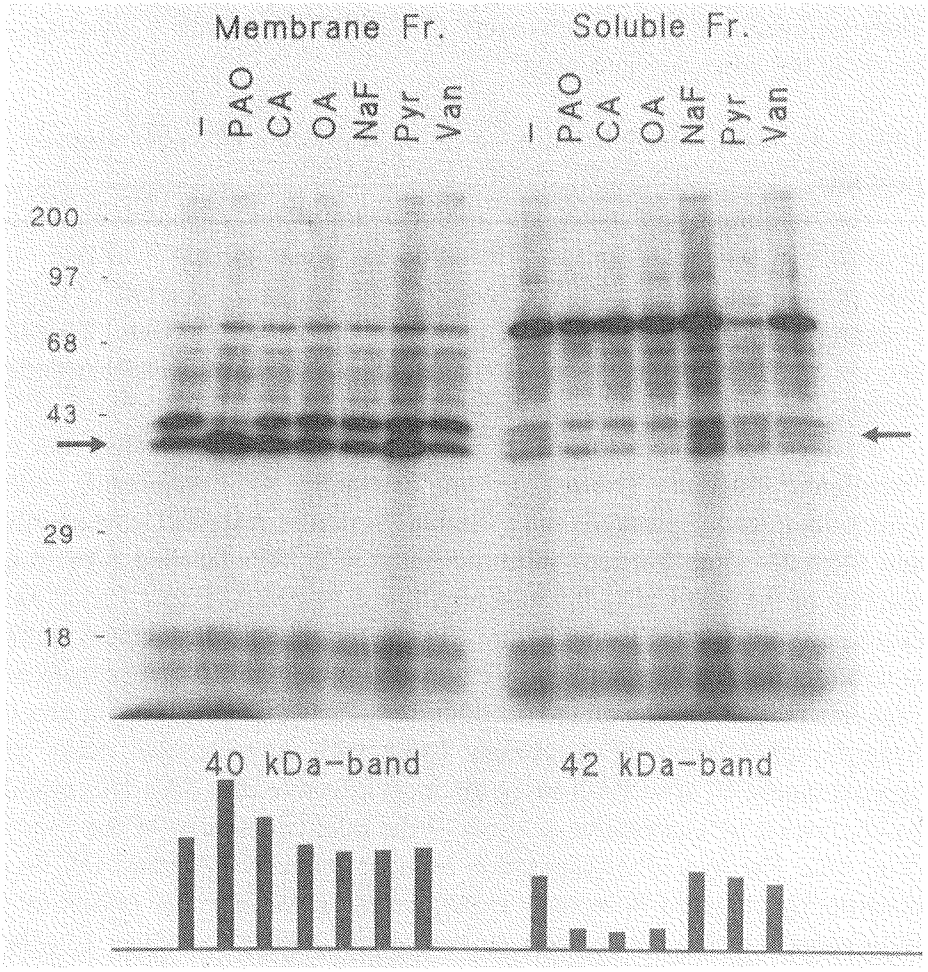

Figure 3. A (upper panel): Barley aleurone proteins were isolated and separated into a fraction containing mainly membrane- and cytoskeleton-bound proteins "Membrane Fr" and a fraction containing soluble proteins "soluble Fr", as described in Materials and Methods. Per sample, 40 µl of the protein fractions was incubated in 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 50 µM ATP, 20 mM Tris.Cl (pH 7.5) in the presence of 10 µCi [γ^{32}P]ATP and the following inhibitors: "_", control; "PAO", 1 mM Phenylarside oxide; "CA", 10^{-7}M Calyculin A; "OA", 10^{-7}M Okadaic acid; "NaF", 1 mM Sodium fluoride; "Pyr", 1 mM Pyrophosphate; "Van", 1 mM Vanadate. After 15 minutes reactions were terminated by heating for 2 minutes at 100 °C, separated by SDS-PAAGE, dried and exposed to an X-ray film. One representative example of 3 experiments is shown.
B (lower panel): relative intensities of the 40 kDa band (membrane fr.) and the 42 kDa band (soluble fr.) as compared to the total intensities of the samples. Intensities were determined with a LKB ultrascan.

observed. The inhibitory effect on the relative intensity of the 42 kDa band seemed similar in magnitude for PAO, CA and OA (Fig. 3B).

We also studied the effect of ABA on phosphorylation of the protein fractions. No clear effect of ABA was observed under our experimental conditions. Earlier attempts to visualise an effect of ABA on phosphorylation of proteins from intact aleurone protoplasts had been unsuccessful as well.

As an alternative approach to investigate the action of especially PAO and ABA on the phosphorylation of aleurone proteins, we used antibodies against phosphorylated amino-acid residues. According to Garcia-Moralez (1990), PAO inhibits tyrosine phosphatases. Increased phosphorylation due to inhibitory action of PAO is therefore expected to be found on tyrosine residues. Both intact protoplasts and isolated membrane fractions were treated with either PAO or ABA. The proteins of the isolated membrane fraction were immunoblotted and incubated with an antibody against phosphorylated tyrosine residues kindly provided to us by Dr Maassen, Leiden University. It was clearly visible that two 40 kDa proteins from the membrane-bound protein fraction showed a stronger reaction with the ^{p}tyr antibodies if the fraction was incubated in the presence of PAO (Fig. 4). Proteins extracted from intact protoplasts which were incubated for 20 min with PAO showed tyrosine hyperphosphorylation of two seemingly identical 40 kDa proteins on two dimensional western blots (data not shown).

Treatment of either isolated proteins or protoplasts with ABA did not reveal any changes in tyrosine phosphorylation (two-dimensional analysis). Incubation of identical immunoblots with anti-phosphoserine or anti-phosphothreonine antibodies revealed no effect of PAO on the phosphorylation status of these amino-acid residues. Apparently PAO mainly affects tyrosine phosphorylation, as was reported in the literature (Garcia-Moralez *et al.*, 1990). Tyrosine phosphorylation of cytosolic proteins was not visibly affected by PAO (data not shown).

Discussion

We have shown that PAO inhibited ABA-induced Rab-gene expression and brought about hyperphosphorylation of (an) 40 kDa membrane- or cytoskeleton-bound protein(s). Another protein phosphatase inhibitor, CA, was less effective than PAO in inhibiting ABA-induced Rab-gene expression and in bringing about hyperphosphorylation of the 40 kDa protein(s). OA only had a small effect on ABA-induced Rab-gene

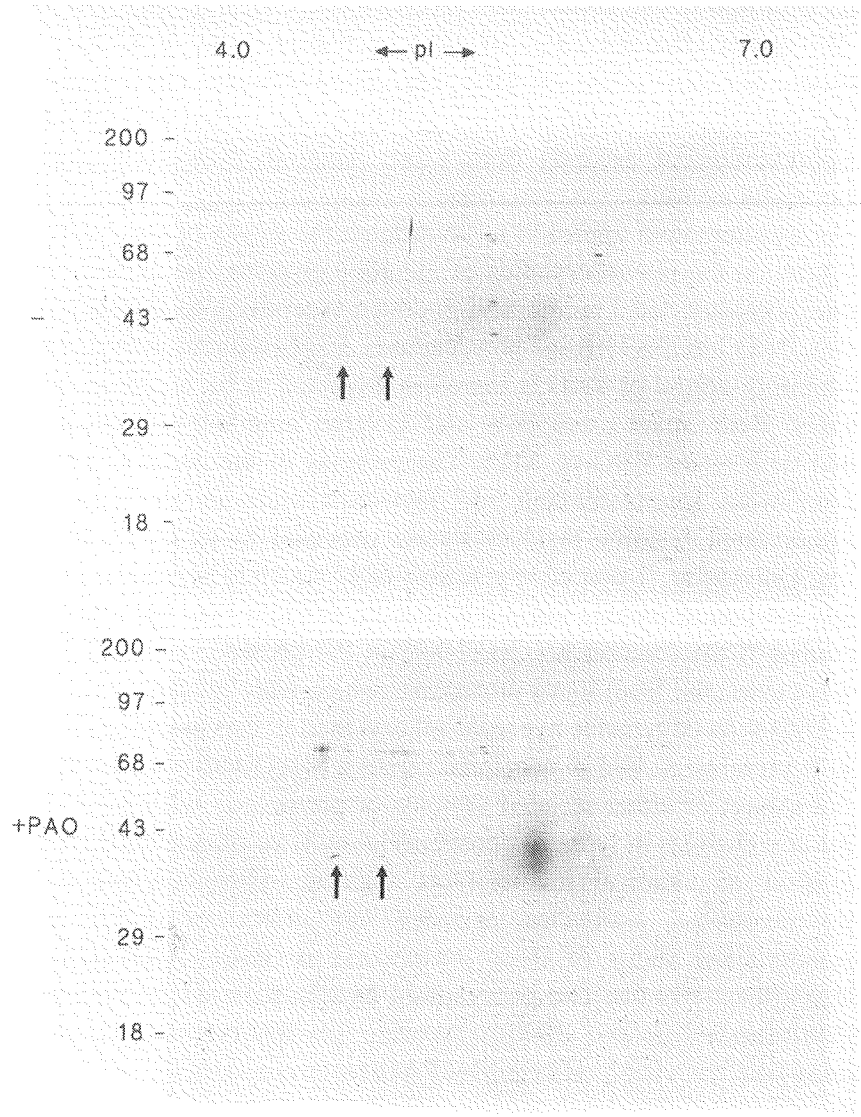

Figure 4. Membrane-bound protein fraction, isolated as described in the Materials and Methods section, were incubated in 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 50 μM ATP, 20 mM Tris.Cl (pH 7.5) in the absence ("_") or presence ("+PAO") of 100 μM PAO. After 20 minutes reactions were terminated by heating for 2 minutes at 100 °C. Proteins were separated by 2-dimensional electrophoresis, blotted and incubated with anti-phosphotyrosine antibodies. Arrows indicate spots that were hyperphosphorylated in the presence of PAO. One representative example of five experiments is shown.

expression at a relatively high concentration (2 x 10^{-6}M). The other inhibitors that were tested (NaF, pyrophosphate and vanadate) had no effect on either ABA-induced Rab-gene expression or on phosphorylation of the 40 kDa protein(s). The observed correlation between the degree of inhibition of ABA-induced Rab-gene expression by protein phosphatase inhibitors and the degree of hyperphosphorylation of the 40 kDa protein(s), suggests that these protein(s) may play a role in the induction of Rab-gene expression by ABA.

Using antibodies against phosphorylated tyrosine residues, we could demonstrate that PAO brought about hyperphosphorylation of two 40 kDa proteins both *in vitro* and *in vivo*. This result confirms that PAO acts as an inhibitor of tyrosine phosphatases as was described by Garcia-Moralez *et al.* (1990). Whether or not these particular proteins play a role in ABA signal transduction needs further study. Until now, we could not demonstrate that ABA had any effect on phosphorylation of either isolated proteins or proteins from intact protoplasts. However, modulation of phosphorylation of particular proteins by ABA may be a subtle process, the elucidation of which may require more specific tools. As a first attempt to obtain such tools, further experiments are aimed at isolation and characterisation of the two membrane- or cytoskeleton-bound proteins and PAO-sensitive phosphatases.

The fact that PAO induces hyperphosphorylation in disrupted cells and in the absence of soluble proteins suggests that the affected phosphatase(s) are themselves membrane or cytoskeleton bound, and that they are in close contact with their substrate. The (two) 40 kDa protein(s) in the membrane fraction may themselves be phosphatases, exhibiting autodephosphorylation. The molecular weight of these proteins is comparable in size to the above-mentioned *Arabidopsis* phosphatase (Leung *et al.*, 1994; Meyer *et al.*, 1994) and to members of the family of MAP kinases, which are signal transduction components known to exist in plants (Jonak *et al.*, 1994).

We did not find a clear correlation between phosphorylation of proteins from the soluble fraction and inhibition of Rab-gene expression. PAO, CA and OA (at 1 x 10^{-7}M) all brought about a quantitatively similar dephosphorylation of (a) 42 kDa protein(s) (Fig 3). However, at this concentration OA had no effect at all on ABA-induced Rab-gene expression (Fig. 2). Apart from the fact that we are probably dealing with an indirect effect, for example indirect inhibition of a protein kinase, apparently dephosphorylation of the 42 kDa protein(s) as such had no effect on ABA-

induced Rab-gene expression. We have therefore given less priority to further characterisation of these proteins.

Acknowledgements

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Identification of a protein disulphide isomerase-like protein that might act in abscisic acid signal transduction*

Abstract

A polyclonal antibody, raised against ABA-binding proteins from maize (Wan *et al.*, 1991; Wan and Hasenstein, 1993), was shown to specifically inhibit ABA-induced Rab gene expression in barley aleurone protoplasts. This antibody recognized several proteins in barley aleurone or embryo, among these a predominant protein of approximately 60 kDa. Screening of a cDNA library of developing barley grains resulted in the selection of 10 positive clones, 7 of which contained identical cDNA inserts. Sequence analysis of a matching full-length clone revealed an 1583 bp long open reading frame, encoding a polypeptide with a deduced length of approximately 58 kDa. Both nucleotide and deduced amino-acid sequence showed high similarity with protein disulphide isomerase (PDI)-like proteins, especially with PDI from alfalfa (Shorrosh and Dixon, 1991). The gene was expressed predominantly in the embryo and aleurone layer. Expression in the rest of the grain was low, expression in roots was hardly detectable. During grain development, expression of the gene was positively correlated with the amount of ABA present in the grains. In embryo, expression could be induced by ABA. Induction of gene expression was also achieved by treatment with Tunicamycin, a glycosylation inhibitor. Aleurone tissue with increased level of expression, due to Tunicamycin pretreatment, appeared to be more responsive to ABA than untreated aleurone.

Introduction

The physiological role of ABA in plant growth and development is well established (e.g. Davies and Jones, 1991) and signal transduction mechanisms of ABA, especially those involved in ABA-inducible gene expression, are among the best studied of all phytohormones (e.g. Verhey and Lomax, 1993; Ho and Hagen, 1993; Skriver *et al.*, 1991). Knowledge of ABA perception, however, is rather scarce. Recently, both Gilroy and

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Jones (1994) and Anderson *et al.* (1994) concluded from microinjection of hormones in either aleurone or guard cells, that ABA is perceived at the external face of the plasma membrane of the cell. Allan *et al.*. (1994), however, found that the perception of ABA leading to stomata closure occurred intracellular. Allan and Trewavas (1994) propose that perception at the plasmalemma deals with rapid responses whereas cytoplasmic receptors might mediate more prolonged action of ABA.

In spite of many efforts to characterise ABA perception, hardly any successful attempts to identify ABA-binding proteins have been published. Hornberg and Weiler (1984) reported the presence of ABA-binding sites in *Vicia faba* guard cells but their results could not be reproduced since then. Studies concerning other phytohormone receptors have proven more successful, resulting in the characterisation of for example three binding sites for auxins: one on the endoplasmatic reticulum (site I), one on the tonoplast (site II) and one on the plasmalemma (site III) (Dohrmann *et al.*, 1978). The site I auxin-binding protein, later named ERabp (endoplasmatic reticulum auxin binding protein) has been studied most thoroughly, resulting in the cloning of the corresponding gene(s) (Tillmann *et al.*, 1989; Hesse *et al.*, 1989). In addition, a 60 kDa cytosolic auxin-binding β-glucosidase was identified in maize coleoptiles (Campos *et al.*, 1992). In oat aleurone, a 60 kDa GA-binding site was identified by Hooley *et al.* (1993). A 150-200 kDa (native MW) soluble GA-binding protein was partially purified from mung bean hypocotyls (Nakajima *et al.*, 1993). These putative receptor proteins were all identified using one of the following approaches: fotoaffinity labels or affinity chromatography.

Wan *et al.* (1991) isolated ABA-binding proteins from *Zea mays* root tips utilising ABA immobilized on Sephadex 4B. These authors were able to show that the isolated protein fraction competed for ABA binding with ABA monoclonal antibodies (Wan *et al.*, 1991). The ABA-binding protein(s) were later used to obtain polyclonal antibodies, which were affinity purified with immobilized ABA-binding proteins. We describe here the identification of a gene encoding for (one of the) protein(s) that are recognized by this antibody and discuss its possible relevance for ABA action.

Materials and Methods

Materials

Barley (*Hordeum vulgare* cv Himalaya) of the 1985 harvest from Pulmann and cv Triumph, grown in phytotrons as described by Schuurink *et al.* (1992) was used. Polyclonal ABA-Binding Protein (ABABP) antibody, affinity purified, was a kind gift from professor X. Zhou (Nanjing University, Peoples Republic of China). DNA-modifying enzymes, DNA-sequence kit and restriction enzymes were from Pharmacia (Brussels, Belgium), "*In vitro* Express" Transcription and Translation kits were from Stratagene (La Jolla, Ca, USA). ABA-ELISA-kit was from Idetec (Sunnyvale, CA, USA). Goat anti-rabbit IgG coupled to alkaline phosphatase was from Promega (Madison, WI, USA). PIPES from Janssen Chimica (Tilburg, the Netherlands). Cellulase Onozaka R-10 was purchased from Yakult Honsha (Tokyo, Japan). Gamborg B5 was obtained from Flow Laboratories (Irvine, Scotland). All other chemicals were from Merck (Darmstadt, FRG).

Library screening

The cDNA expression-library was constructed in the Uni-ZAP XR vector (Stratagene, La Jolla, CA) from pooled poly(A)$^+$ RNA of different stadia of developing barley Triumph grains. The library was expressed in XL1Blue *E. coli* strain and nitrocellulose-prints were screened with the ABABP-antibody. Ten plaques remained positive in 2 successive rounds of screening. All ten were *in vivo* excised as described by Stratagene, yielding a Bluescript KS$^+$-vector containing the original λ phage cDNA-inserts. The clones were compared by restriction analysis and cross-hybridization, revealing 7 identical clones. A full length clone was obtained by screening the same λ ZAP-library with the insert of the originally obtained clone.

DNA-sequence analysis

Sequencing was performed with deoxynucleotide chain termination (Sanger *et al.*, 1977), using Exo/Mung bean deletion-subclones. Computer analysis of nucleotide and amino acid sequence data were carried out with the Sequence Analysis Software Package of the Wisconsin Genetics Computer Group.

Isolation of protoplasts

Barley (*Hordeum vulgare* cv Himalaya) aleurone protoplasts were prepared essentially as described by Wang *et al.* (1991). The obtained protoplasts were washed three times with washing buffer (0.5 M mannitol, 10 mM KCl, 1 mM $MgCl_2$, 1.1 mM $CaCl_2$, 0.1 mM EGTA, 0.5 mM K_2HPO_4, 10 mM PIPES-HCl (pH 6.8), the osmolarity was brought to approximately 800 mOsm with mannitol).

RNA analysis

Total cellular RNA was isolated and purified as described by Van der Meulen *et al.* (1993). Northern blots were made by separating 10 μg RNA samples in a glyoxal/DMSO 1 % agarose gel (Sambrook *et al.*, 1989) followed by blotting onto a nylon membrane and hybridization with a random-labelled cDNA probe following the instructions by Genescreen. Blots were hybridized with cDNA-probes from Rab 16 (Mundy and Chua, 1988) or Glyceraldehyde-3-phosphate dehydrogenase, GAPDH (Cojecki, 1986). The intensities of the autoradiographs of these northern blots were semi-quantitatively analyzed with an Ultrascan Kl densitometer.

***In vitro* translation and immunoprecipitation**

One μg linearized DNA was transcribed in 1x transcription buffer (40 mM Tris.Cl (pH 7.5), 50 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine), 400 μM rNTP's, 2.5 μL CAP-analogue (5' 7meGppp 5'G, Stratagene), 30 mM DTT and 20 units T3 RNA polymerase at 37°C. The RNA was purified by phenol extraction and alcohol precipitation. Approximately 2 μg RNA was translated in rabbit reticulocyte lysate (Stratagene *in vitro* express translation kit) in the presence of [^{35}S]methionine at 30 °C (total volume 25 μL)

Protein isolation and western analysis

For protein extraction, barley aleurone layers were ground under liquid nitrogen and dissolved in 100 volumes of 0.25 M Tris.Cl (pH 7.5). The slurry was sonicated 2 x 10 sec. and protein concentration was determined with BCA-colorimetric Reaction (Pierce, Rockford Il., USA). Approximately 50 μg protein per lane was separated on vertical SDS-PAGE as described by Leammli (1970).

Proteins were transferred electrophoretically to a nitrocellulose membrane as described by Klein *et al.* (1987). The nitrocellulose blots were blocked in phosphate-buffered saline containing 0.1% Tween-20 and 1%

(w/v) BSA for 1 h and incubated overnight at 4°C with 1:4,000 diluted ABABP-antibody and subsequently for 1 h with 1:7,500 diluted goat anti rabbit IgG conjugated to alkaline phosphatase. Specific bands were visualized using 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium as substrate.

ABA detection using monoclonal ABA-antibodies:

ABA was quantified using ELISA (enzyme-linked immunosorbent assay), utilizing ABA-monoclonal antibody. The procedure as described by Walker-Simmons and Abrams (1992) was followed

Results

A putative ABA-binding protein antibody affects ABA-induced gene expression

A polyclonal antibody, raised against maize ABA-binding protein (ABABP, Wan *et al.*, 1991) was tested for its effects on ABA action in barley aleurone protoplasts. Protoplasts were incubated in the presence of ABA with or without the putative ABABP antibody or its pre-immune serum. It was clearly shown that the antibody partly suppressed ABA-induced Rab gene expression, whereas incubation with pre-immune serum had no effect (Fig. 1). The inhibition was not caused by general transcriptional deactivation, as shown by rehybridization of the RNA with the non-ABA-induced gene GAPDH (data not shown). In addition, GA-induced production and secretion of α-amylase by aleurone protoplasts was not affected by the ABABP antibody or its pre-immune serum (Table I). This indicates that the inhibitory effect of the ABABP antibody is probably specific. The inhibitory effect of the antibody on ABA-induced Rab gene

Table I. *Effect of ABABP-ab and its pre-immune serum on GA-induced α-amylase expression.*

1 x 10^5 pp/ml were incubated for 16 hr at 25 °C with or without 1 x 10^6 M Ga_3 in the presence of 1:100 diluted ABABP-ab ("ABABP-ab") or 1:100 diluted pre-immune serum ("PS"). α-Amylase was measured as described in materials and methods. Units/100µl are given.

	Control	ABABP-ab	PS
-	191	188	195
GA	290	281	304

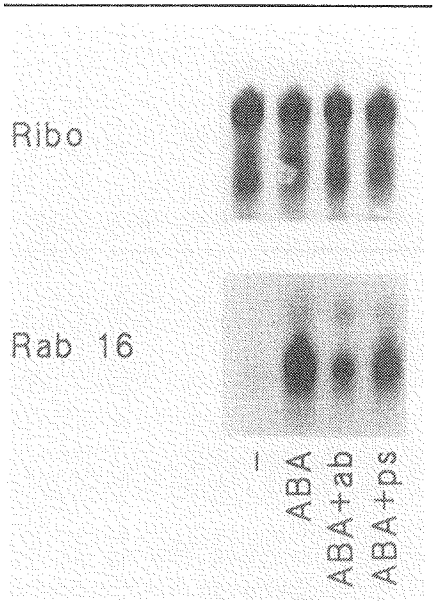

Figure 1. 2x 10^6 pp were incubated in washing buffer without "-" or with 10^{-7}M ABA "ABA" and with ABA and ABABP-antibody "ABA+ab" or its preimmuno serum "ABA+ps" (both 1:100 diluted). After 2 h at RT in the dark protoplasts were harvested and RNA isolated. A northern blot was hybridized with Rab 16 from rice and successively with GAPDH (data not shown) and ribosomal probe.

expression was not brought about by a decrease in the level of free ABA by high-affinity binding of the hormone to the ABABP antibody: i.e. (pre)-incubation of free ABA with the ABABP-antibody did not affect the amount of ABA as detected by monoclonal ABA-antibodies (Table II).

The putative ABABP-antibody recognizes barley aleurone proteins

To gain more insight in the effect of the ABABP antibody on ABA action, we tried to answer the question whether or not the antibody recognises specific barley proteins. A western blot of barley aleurone proteins (cv Himalaya) showed that several proteins were recognized, with two predominant bands around 60 kDa (Fig. 2). The proteins were not aleurone specific since bands of similar size were also recognized in western blots of barley-embryo proteins (data not shown).

Screening of a cDNA library with the ABABP-antibody

To further elucidate the identity of the protein(s) which interact with the antibody, we used the antibody to select clones from a cDNA expression-library from developing barley grains. Ten positive clones were selected out of 100,000 plaques screened. Seven appeared to have identical inserts. A matching full-length clone was isolated from the same library, using the originally cloned cDNA fragment as a probe.

This clone, "A", was sequenced using Exo/mung deletions and appeared to be 1833 bp long. The nucleotide sequence contains a putative open reading frame of 1538 bp (Fig. 3), starting with an ATG-triplet at bp 29, surrounded by a consensus sequence as proposed by Kozak (1981) and later

specified for plants by Lüttcke *et al.* (1987), A/C A/G C/A A/C A U G G C. The open reading frame ends at bp 1565 with a TGA stop-codon. The 3' untranslated region contains a polyadenylation signal (AATAAATG) at bp 1802 (Fig. 3A).

From the open reading frame, a 512 amino-acid long polypeptide can be deduced with a calculated molecular mass of 57,622 daltons. The polypeptide contains a hydrophobic N-terminal signal peptide of approximately 24 amino-acids (Fig. 3B), predicted following the rules for signal peptide sequences by Von Heijne (1985). This signal peptide will probably cause targeting of the protein to the lumen of the endoplasmatic reticulum. None of the other hydrophobic regions have average hydrophobicity characteristics of transmembrane proteins (Kyte and Doolittle, 1982). In addition to the signal peptide the protein has a C-terminal Lys-Asp-Glu-Leu (KDEL)-sequence (Fig. 3A), commonly recognized as being an endoplasmic reticulum retention signal (Denecke *et al.*, 1990), and a predicted glycosylation site at amino-acid 281.

Both nucleotide sequence and deduced amino-acid sequence show high similarity with protein-disulphide isomerases. Protein-disulphide isomerases are multifunctional enzymes which can also act as the β-subunit of prolyl 4-hydroxylase and of the T_3BP-thyroid-hormone-binding protein. All currently characterised members from these three groups of enzymes therefore share high similarity. Figure 4 shows an amino-acid comparison between some members of these families and the aleurone clone A. The aleurone clone shows strongest homology, both in nucleotide as in amino-acid sequence, with alfalfa protein disulphide isomerase (Shorrosh and Dixon, 1991): 66% and 63% identity, respectively. Homology with vertebrate protein disulphide isomerase is around 54% for nucleotide sequence and 35% on amino-acid identity (Fig. 4). The putative active sites

Table II. *ABA-binding properties of ABABP-binding antibody*
Three given amounts of (+/-) cis-trans ABA were detected with monoclonal ABA-antibody as desribed in Materials and Methods, in the absence or presence of 1:100 diluted ABABP-antibody. OD readings (405 nM) of the samples are given.

pg ABA	control	+1:100 ABABP
1000	0.254 ± 0.000	0.254 ± 0.008
500	0.294 ± 0.007	0.282 ± 0.004
250	0.360 ± 0.005	0.353 ± 0.017

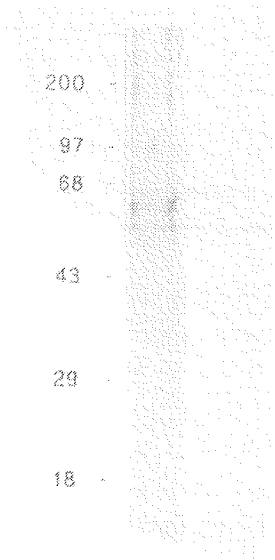

Figure 2. Total protein fraction of Himalaya aleurone protoplasts, incubated with 1:100 diluted ABABP-ab. The western blot was treated as described in Materials and Methods.

of protein disulphide isomerase, a Cys-Gly-His-Cys sequence, are conserved for 100% in clone A. The aminoacid sequences flanking the active sites are also very conserved in the different genes. Other common features of the family of protein disulphide isomerase (PDI)-like proteins, like the signalling sequences responsible for targeting and retention in the E.R. and a glycosylation site, are shared by clone A.

In-vitro transcription and translation of the cDNA clone A yielded three products of about 58 kDa, 56kDa and 30 kDa (Fig. 5). The molecular mass of the predicted amino -acid sequence is 58 kDa and fits well with the longest translational product. The smaller proteins probably represents products of premature termination of translation or *in vitro* degradation. The two longest translation products could be imunnoprecipetated with the ABABP-antibody (Fig. 5), confirming that the barley clone encodes for a protein that is recognized by this antibody, which was raised against putative ABA-binding proteins.

Expression of the putative ABA-binding barley protein mRNA

We studied developmental expression and tissue specificity of the cDNA clone A. Genomic blots, probed with clone A revealed that the corresponding gene is present in low copy number in barley (data not shown). Northern hybridizations show that transcript(s) of approximately 2 Kb were recognized by cDNA-clone A at moderate hybridization stringency. Clone A was expressed in mature grains, mainly in the embryo and aleurone. Expression in embryos was significantly higher than in aleurone or scutellar tissue. Clone A was expressed at relatively low levels in roots (Fig. 6).

To gain some insight in the function of the clone A transcript and to study its possible involvement in ABA action, we compared the levels of clone A mRNA in two batches of barley which differ in ABA sensitivity. These batches were obtained by growing barley at different climatological

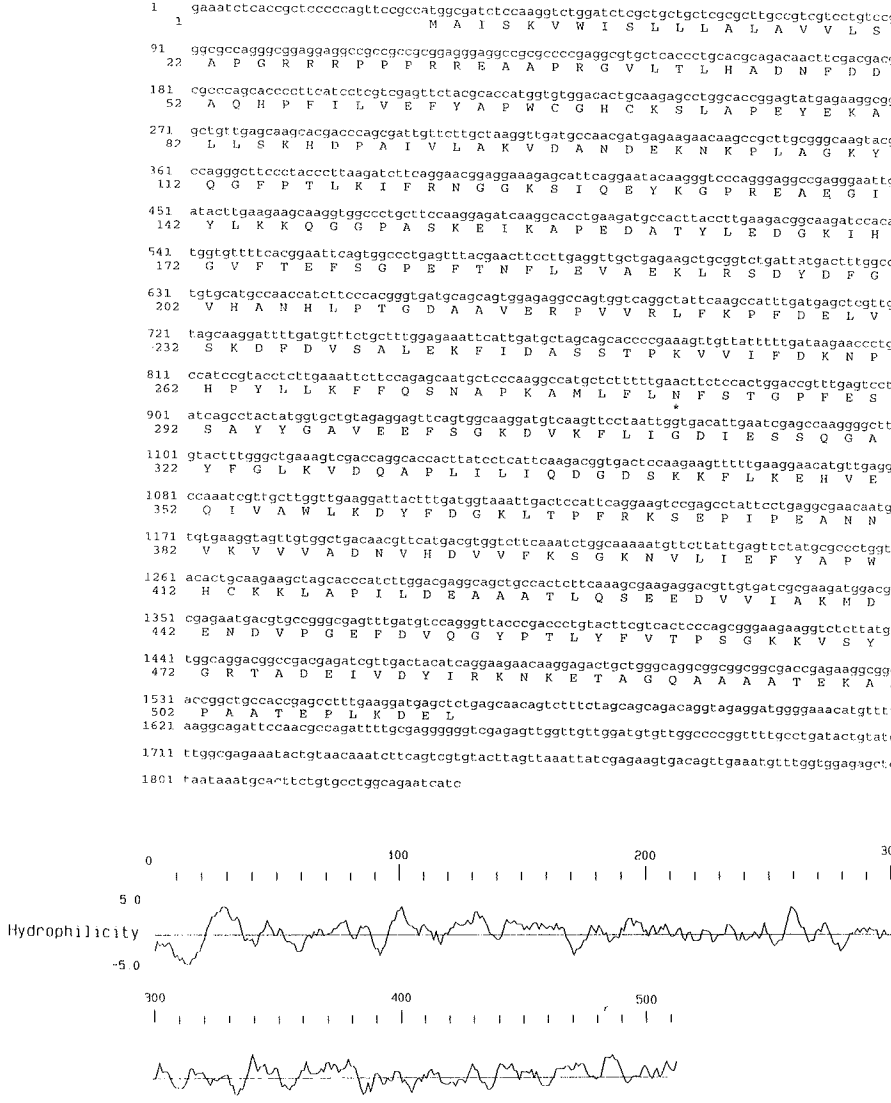

Figure 3. **A**: Nucleotide and deduced amino acid sequence of cDNA clone A. The nucleotide sequence includes a putative open reading frame and its immediate 5' and 3' flanking sequences. "*" Indicates a potential N-linked glycosylation site.
B: Hydrophaty plot of the deduced polypeptide encoded by clone A (Kyte and Doolitlle, 1982).

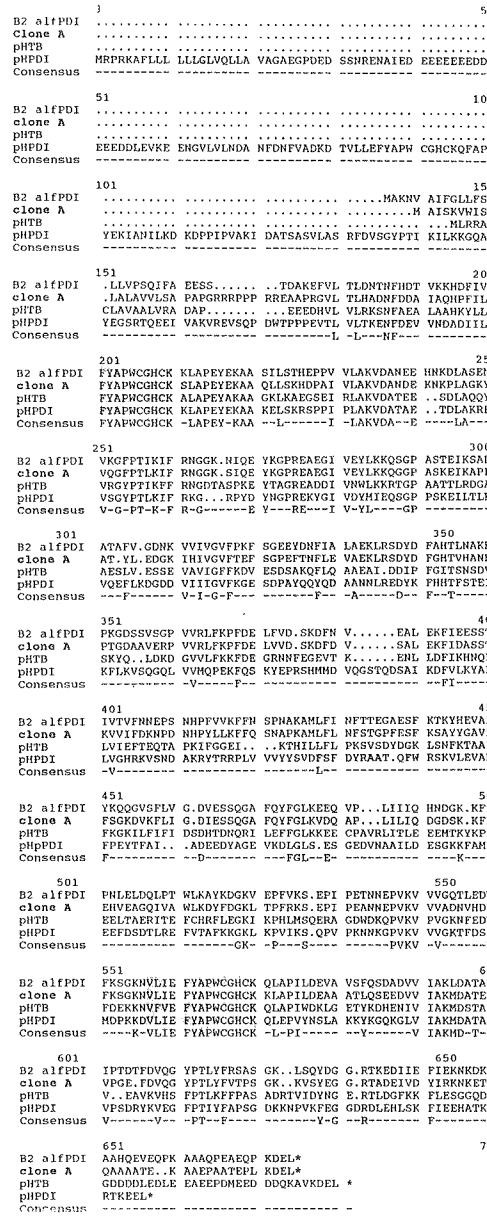

Figure 4. Comparison of the deduced amino acid sequence of clone A with alfalfa Protein disulphide isomerase ("B2 alfPDI"), human thyroid binding protein ("pHTB") and human protein disulphide isomerase ("pHPDI"). Conserved aminoacids are denoted as "consensus". Two repeat sequences corresponding to the predicted active sites of PDI are shaded.

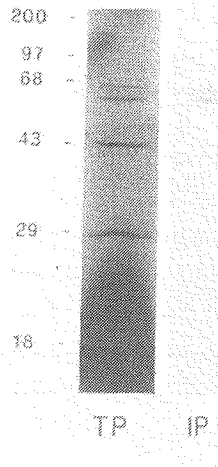

Figure 5. "TP": In vitro translation Products of clone A. the 45 kDa protein is a methionine-binding protein present in the reticulocyte-lysate. Clone A cDNA was transcribed and translated as described in Materials and Methods. The approximately 30 kDa protein is probably a product of premature translational termination.
"IP": Immunoprecipitated translation products of clone A. The [^{35}S]-Methionine labelled translation products were incubated with preimmune serum and a surplus protein A-coated sepharose for 6 h (4°C). Sepharose was removed by centrifugation and the supernatant was incubated with ABABP-antibody and protein A coated sepharose for approximately 16 h (4°C). The protein A-sepharose was collected by centrifugation and repeatedly washed until no ^{35}S could be detected in the supernatant. The immunoprecipitated protein was recovered by heating the sepharose to 100°C in 1x protein Sample buffer and separated on SDS-PAGE. The gel was dried and an autoradiograph is shown.

conditions (Schuurink *et al.*, 1992). Both embryos and aleurone layers of barley that was grown at high temperature/long photoperiod (long day plants, "LDP"), were less sensitive to ABA than grains of barley grown at a lower temperature/short photoperiod (short day plant, "SDP") as described by Van Beckum *et al.* (1993). We found that aleurone layers of SDP plants contained more clone A mRNA than those of LDP plants. The levels of clone A mRNA were not significantly affected by exogenous ABA in these tissues (Fig. 7).

Levels of clone A mRNA rapidly increased during grain development until approximately 20 days after pollination (DAP) and then decreased (Fig. 8A). This expression pattern positively correlates with the presence of ABA during grain development (e.g. Walker-Simmons and Sesing, 1990). As clone A mRNA levels in aleurone layers were unaffected by exogenous ABA, we studied whether expression of the clone A-gene was ABA-responsive in other tissues. We found that clone A mRNA could be induced by ABA in embryos (Fig. 8B). Apparently, transcriptional regulation of the clone A gene in embryos differs from that in aleurone tissue.

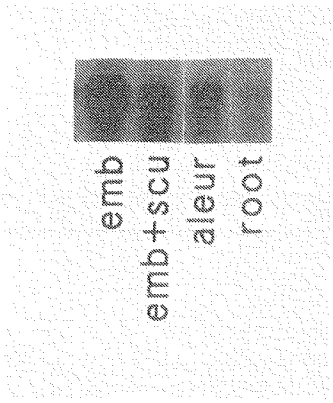

Figure 6. Expression of clone A in embryo (emb), embryo+scutellum (emb+scu), aleurone layer (aleur) and root (root) of Triumph barley.

The expression of clone A mRNA could be enhanced by prolonged incubation of aleurone layers with Tunicamycin (Fig. 9), as was described earlier for PDI-genes from e.g. alfalfa (Shorosh and Dixon, 1991). Aleurone layers with increased expression of Clone A (due to Tunicamycin induction) showed increased ABA responsiveness as compared to aleurone layers in which Clone A mRNA levels were not increased (Fig. 10). ABA responsiveness was determined by Rab-gene induction. When aleurone layers were incubated with Tunicamycin for a short period, during which clone A mRNA levels were not yet increased, ABA-induced Rab gene expression was not affected (data not shown). The increased responsiveness was not due to increase in general transcription level, as was determined by rehybridization with GAPDH, a non-ABA induced gene (data not shown).

Discussion

To date, very few data have been published on ABA-binding proteins. To our knowledge only two publications appeared: one on ABA-binding proteins in guard cells (Hornberg and Weiler, 1984) and one on ABA-binding proteins from maize (Wan *et al.*, 1991). The latter publication reports on a protein fraction which was able to compete for ABA-binding with antibodies raised against ABA itself (Wan *et al.*, 1991). These protein(s) were used to obtain a polyclonal antibody. The polyclonal antibody was affinity-purified with the original ABA-binding protein fraction (pers. commun. prof. X. Zhou). We used this antibody, raised against putative ABA-binding proteins from maize, and identified a barley-gene encoding a protein-disulphide isomerase (PDI) homologue.

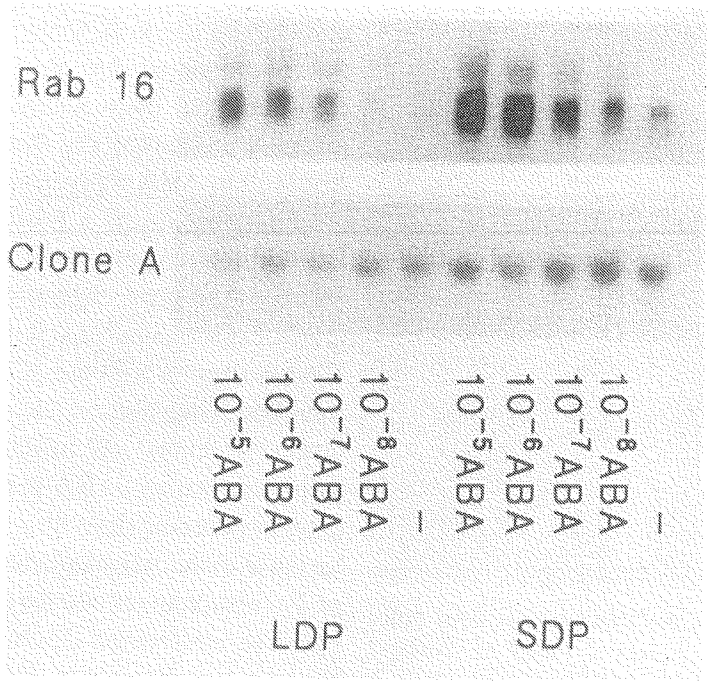

Figure 7. Expression of clone A mRNA and Rab 16 in ABA-treated aleurone layers of barley grown at high temperature/long photoperiod ("LDP") or grown at lower temperature/short photoperiod ("SDP"). Isolated aleurone layers were incubated in the indicated concentrations of ABA for 2 h at RT.

Is the protein corresponding to clone A involved in ABA-action?

We have found that the ABABP antibody inhibited ABA-induced gene expression in barley aleurone protoplasts, whereas pre-immune serum did not. This suggests that this polyclonal antibody recognizes particular protein(s) which are involved in ABA signal transduction. By screening an expression library with the ABABP antibody we have isolated a barley cDNA clone corresponding to an approximately 60 kDa protein. Immunoprecipitation of the *in vitro* transcription/translation product of clone A showed that this protein was indeed recognized by the ABABP antibody. One should however bear in mind that the antibody is polyclonal and was raised against a fraction of proteins. It could, therefore, interfere with several different (ABA-binding) proteins. The protein(s), responsible for ABA-signal transduction and apparently inhibited by the antibody, need not

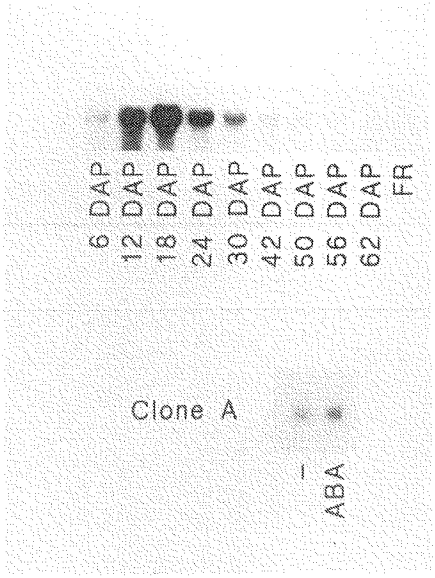

Figure 8. A: RNA samples from different developmental stages of Triumph barley grains, hybridized with clone A dna. DAP=Days After Pollination; FR= Fully Ripe.
B: ABA-responsiveness of clone A in embryos incubated for 2 h in water ("_") or with 10 μM ABA ("ABA").

necessarily be the same protein as encoded by clone A, even if they are both recognized by this antibody. However, we have several lines of evidence suggesting that this protein is involved in ABA signal transduction.

First, we found a positive correlation between the level of ABA-induced Rab-gene expression and the level of clone A mRNA. Treatment of aleurone layers with the glycosylation inhibitor Tunicamycin increased the level of clone A mRNA. Dose-response curves of ABA-induced Rab-gene expression showed a significant increase in the ABA-induced response of Tunicamycin-treated layers as compared to non-treated layers (Fig. 10). We did not observe a difference in ABA sensitivity. Of course, we have to interpret these results with care, since we cannot exclude that Tunicamycin altered the Rab-gene expression via its effects on the glycosylation of certain proteins.

In addition, we found a positive correlation between clone A mRNA-levels and ABA-responsiveness in aleurone layers isolated from SDP and LDP barley plants (Fig. 7). The SDP and LDP plants show a slightly different ABA sensitivity and a clear difference in the level of ABA responsiveness with respect to the induction of Rab-gene expression (Van Beckum *et al.*, 1993). The layers which are more responsive to ABA, exhibited a higher level of clone A mRNA than the less responsive batch.

Although our results only provide circumstantial evidence for a role of the clone A protein in ABA signal transduction, the different approaches all appear to support the hypothesis that clone A is involved in ABA action. We feel that the results are interesting enough to justify further research based on this hypothesis. ABA-binding experiments with *in vitro* tran-

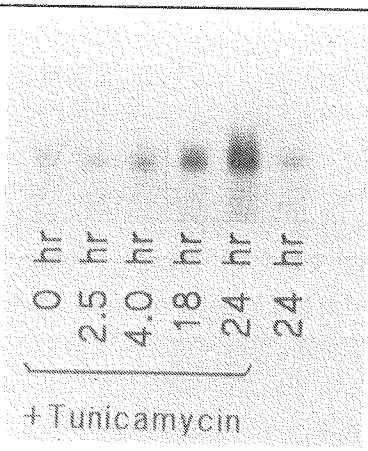

Figure 9. Tunicamycin induced expression of clone A. Barley aleurone layers were isolated as described in Materials and Methods and incubated in H_2O at 25 °C in the dark in the presence (+Tunicamycin) or absence of 10 µg/mL Tunicamycin. At indicated times aleurone layers were harvested and RNA was extracted.

scribed/translated clone A protein, aimed at establishing a receptor function, were as yet unsuccessful.

Expression of proteins which play a role in signal transduction pathways is often regulated by the signal involved. In this respect it is interesting to note that expression of clone A mRNA correlated positively with ABA peaks that occur during grain development (see Walker-Simmons and Sesing, 1990). Since exogenous ABA had no effect on clone A mRNA in aleurone layers, the correlation may be explained by the induction of clone A mRNA by ABA in the embryo. Apparently, regulation of the homologous gene(s) is different in these two tissues. Further study is required to establish if the transcripts that are recognized by clone A cDNA in embryo and aleurone, are encoded by identical or by different genes.

Possible biochemical functions and localization of clone A

The high homology of clone A with PDI-like proteins may suggest a multitude of possible actions of the protein. PDI is a multifunctional protein in vertebrates. In addition to its action in protein secretion by catalysing the formation of disulphide bonds, it can act as β-subunit of prolyl-4-hydroxylase (Pihlajaniemi *et al.*, 1987) and it is suggested to be needed to maintain the post-transcriptional glycosylation processes (Bulleid and Freedman, 1990). All PDI-like proteins possess dehydroascorbate reductase and thioredoxin activity (Lundström *et al.*, 1990; Wells *et al.*, 1990). The thioredoxin activity is not surprising as the active sites of PDI, a CGHC-sequence, are identical to those of the small redox protein thioredoxin (Edman *et al.*, 1985). In addition, strong homology exists with form I phosphoinositide-specific phospholipase C (PIPLC) (Bennet *et al.*, 1988). Interestingly, several thyroid hormone binding proteins identical to PDI, denoted T_3BP, have been identified in vertebrates (e.g. Fliegel *et al.*, 1990;

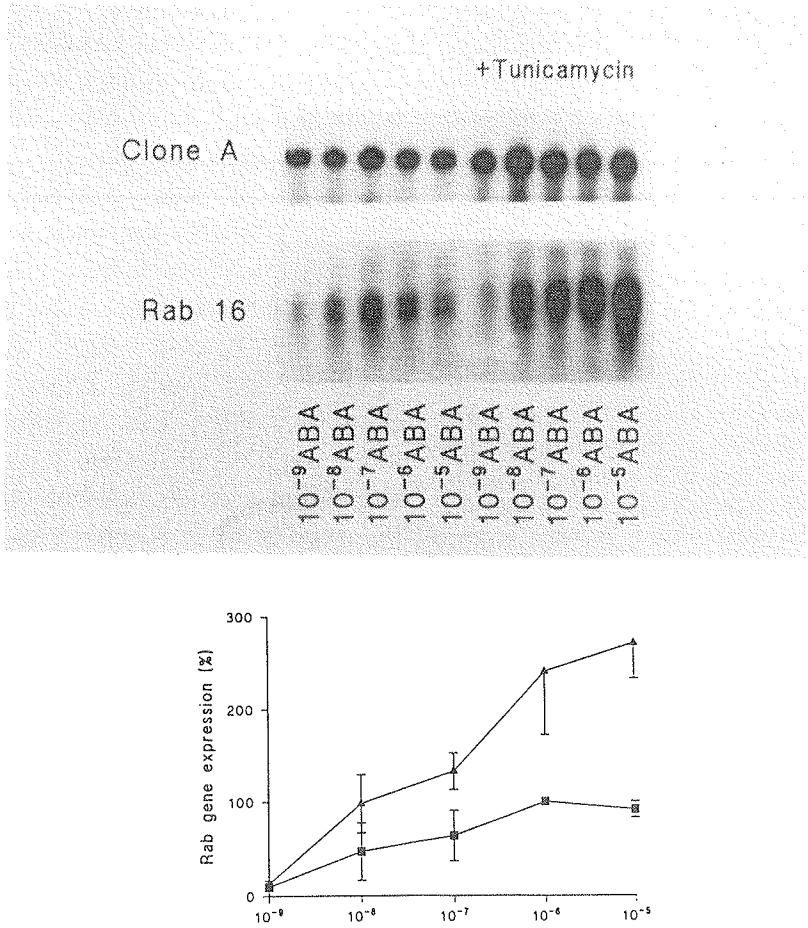

Figure 10. Aleurone layers were incubated either in the absence, or presence (+Tunicamycin), of 10μg/mL Tunicamycin for 18 h. The layers were then washed and incubated in fresh H_2O with several concentrations of ABA for 2 h. RNA was extracted and hybridized with Clone A and Rab 16. The upper panel shows the autoradiogram of one such experiment (concentrations of ABA are indicated in M.). Loading of RNA was determined by rehybridization with Ribosomal cDNA, indicating that the 3th and 7th lane from the left were slightly overloaded (data not shown). The lower panel shows the mean values ± S.E. of three such experiments. Triangles indicate Rab gene induction in Tunicamycin-treated layers, squares the results from non-treated layers. Autoradiograms were semiquantitatively scanned and densities were corrected for loading differences as determined with a ribosomal probe. Densities are expressed as percentages, with Rab-gene expression in non-treated layers incubated with 10^{-6} M ABA as 100 %.

Cheng *et al.*, 1987). The abundance of clone A mRNA in barley embryo tissue, which, as distinct from aleurone tissues, does not have a major secretory function, suggests that the clone A protein is not (solely) involved in protein secretion. A study of the effect of ABA on the above described activities may reveal the possible mode of action of the protein and ascertain its involvement in ABA-responses.

If indeed the clone A protein is involved in ABA-signal transduction, some questions arise concerning its localization. The fact that ABABP-antibody is able to inhibit ABA-induced gene expression by simple co-incubation with protoplasts, strongly suggests that the protein acts at the outside of the cell. However, the deduced amino-acid sequence reveals a C-terminal KDEL-signal, suggesting localization in the lumen of the endoplasmatic reticulum. Studies on the Auxin-binding protein ABP1, which shares the KDEL-sequence (Tillmann *et al.*, 1989), reveal that this protein can be secreted from the cell and can act extracellularly (Jones and Herman, 1993). Barbier-Brygoo and coworkers (1989) showed that antibodies raised against ABP1 from Maize are able to suppress auxin-induced stimulation of the plasma membrane H^+-ATPase in tobacco protoplasts. This in analogy with our experiments that show that ABABP-antibody from Maize inhibits ABA-induced Rab-gene expression in barley aleurone protoplasts. The auxin binding protein I shows more similarity with clone A protein. Besides their protein-targeting signals (and thus probably localization) they also share a putative glycosylation site.

None of the recently identified phytohormone-binding proteins exhibit classical-transduction characteristics such as protein-kinase activity or G-protein coupling, on basis of their homology. Rather, they may play a role in the mobilization of pools of inactive hormone-conjugates. One such example is an Auxin-binding protein with β-glucosidase activity. Auxin was shown to inhibit β-glucosidase activity (Campos *et al.*, 1992). Another alternative may be that hormone-binding simply modulates kinetic properties of certain target enzymes. This has been suggested for a cytokinin binding protein from tobacco, which shows high homology with S-adenosyl-L-homocystein hydrolase (Mitsui *et al.*, 1993). It thus seems that some of the phytohormone-induced activities could be controlled via regulation of enzyme-activities different from those known from animal signal transduction.

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General discussion

Tissue specificity of ABA signal transduction mechanisms

This thesis discusses six cellular responses that might act in ABA signal transduction. These are: changes in intracellular pH (pH_i, chapter 2&4), in membrane potential (E_m, chapter 3), in K^+ concentration (chapter 3), in L-malate concentration (chapter 4), in extracellular pH (pH_e, chapter 4) and changes in (de)phosphorylation of proteins (chapter 6). Some of these signals were also found to be related to ABA action in other cell types, especially pH_i changes and K^+ fluxes. The concentration of another putative ABA messenger, Ca^{2+}, was found to change upon ABA addition in several cell types, including barley aleurone (e.g. McAinsh *et al.*, 1990; Wang *et al.*, 1991). The ion Ca^{2+} is probably the best studied putative second messenger of ABA.

Although identical second messengers are found in different cell types, the effect of ABA on these signals varies. This is best illustrated by comparison of some of these signals in two of the best studied ABA-responsive cell types: Guard cells, in which ABA mainly regulates ion and water transport, and aleurone cells, in which ABA is known to regulate the expression of several genes. The result is summarized in Table I.

For guard cells, reasonable consensus has now been reached on the effect of ABA on pH_i and intracellular Ca^{2+}. Upon addition of ABA, both pH_i and the concentration of cytosolic Ca^{2+} increase (e.g. Blatt and Armstrong, 1993; McAinsh, 1992; Gilroy *et al.*, 1991). The pH_i rise occurs in approx. 3 minutes (Blatt and Armstrong, 1993). The Ca^{2+} increase takes approximately 2 minutes, although more rapid transient increases in Ca^{2+}

Table I. *Putative second messengers of ABA in guard cells and aleurone cells*
References are given in the text.

	Guard Cells	Aleurone cells
$[Ca^{2+}]$	increase	decrease
pH_i	increase	increase
$[K^+]$	decrease	increase
[L-malate]	increase	increase

have been observed (McAinsh *et al.*, 1992). In aleurone cells, ABA affects the intracellular Ca^{2+} concentration in an opposite manner. ABA causes a decrease of Ca^{2+} within 5 seconds after addition of the hormone (Wang *et al.*, 1991). pH_i on the other hand, is increased by ABA in aleurone cells as it is in guard cells, both with approximately 0.2 pH-units. The alkalinization in aleurone cells takes about 30 minutes (chapter 2).

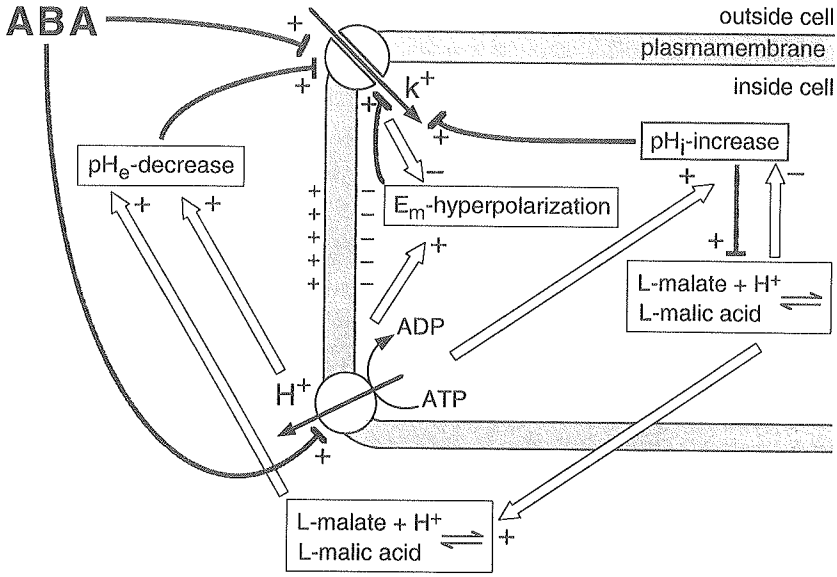
The effect of ABA on K^+ channels in guard cells seems to be the opposite of that in barley aleurone as well. ABA is reported to stimulate a K^+ outward rectifier and to inhibit the K^+ inward rectifier in guard cells (e.g Blatt and Armstrong, 1993), thus resulting in a decrease in intracellular $[K^+]$. The stimulation of the K^+ outward-rectifying channels in guard cells is directly mediated by the pH_i increase (Blatt and Armstrong, 1993). In aleurone, ABA seems to stimulate opening of K^+ inward rectifiers (chapter 3 and B. van Duijn, personal communication). Opening of these channels could be brought about directly by ABA, through membrane hyperpolarization, or it could be the result of the pH sensitivity of the channels. It will be of interest to study the effect of ABA on K^+ concentration in barley aleurone layers and in their immediate environment.

Few data have been published concerning second messengers of ABA in other tissues. Maize-coleoptile and parsley-hypocotyl tissue showed similar responses to ABA as guard cells with respect to pH_i and intracellular Ca^{2+} concentration, both increasing within comparable timespans (Gehring *et al.*, 1990).

The different, sometimes opposite effects of ABA on putative second messengers could play a role in the tissue-specific responses to ABA. In aleurone, ABA-induced Ca^{2+} decrease is supposed to be involved in the regulation of hydrolase secretion (e.g. Bush *et al.*, 1989). In guard cells, however, Ca^{2+} increase is a direct mediator in the inhibition of a K^+ inward rectifier (Wan *et al.*, 1993), thus influencing turgor. Notably, the role of Ca^{2+} increase as a second messenger of ABA leading to stomata closure is as yet disputed, since not all guard cells that close upon addition of ABA appear to show the ABA-induced Ca^{2+} increase (Gilroy *et al.*, 1991).

Interconnection of the putative second messengers of ABA

Five of the six putative messengers which are described in this thesis are clearly interconnected as depicted in figure 1. Both the membrane-potential hyperpolarization and pH_i increase, brought about by ABA, are sensitive to the plasma-membrane proton-pump-inhibitor DES (chapter 2 and 3). We


ABA
outside cell
plasmamembrane
inside cell
k+
pHi-increase
pHe-decrease
Em-hyperpolarization
L-malate + H+
L-malic acid
ADP
ATP
H+
L-malate + H+
L-malic acid

Finally, the decrease of pH_e caused by addition of ABA to aleurone layers is in good agreement with the hypothesis of ABA-activated H^+-ATPases. Stimulated secretion of protons will generally result in extracellular acidification. Moreover, part of the ABA-induced pH_e decrease can be inhibited by the plasma membrane proton pump inhibitor DES (data not shown). The part of the decrease of pH_e which is not affected by DES, can be accounted for by an increase in malate release (chapter 4). This increase in malate release, could be the result of the pH_i increase brought about by ABA, as is suggested by the pH-stat model of Davies *et al.* (1991). This model, in which cytosolic malate concentration acts as a buffer system to stabilize pH_i, is supported by our results. Introducing an artificial decrease of pH_i resulted in a decrease in intracellular malate concentration and vice versa (chapter 4).

Relevance of the putative ABA second messengers for ABA action

ABA-induced Rab-gene expression as a model system for ABA action

In order to gain more insight into the role of putative second messengers in ABA signal transduction, we examined the effect of artificial manipulation of the given response on ABA-induced Rab-gene expression. We choose ABA-induced Rab-gene expression as a model system because of its relatively reproducible response to ABA. The response can be quantified and it is relatively rapidly induced by ABA. Rab-mRNA levels increase within 15 min after ABA addition. This relatively fast response discriminates it from most other known ABA responses, like inhibition of germination or inhibition of hydrolase secretion. It can therefore be more easily related to the relatively fast modulation of putative second messengers. Finally, ABA-induced Rab-gene expression can be studied in either isolated aleurone layers or in protoplasts.

Rab-mRNA induction cannot be considered a model system for all ABA actions. As discussed in the General Introduction, ABA is responsible for the regulation of several developmental and stress responses. These different responses to ABA can be mediated by different signals.

Intracellular pH as a second messenger

We investigated the relevance of ABA-induced modulation of pH_i for ABA induced Rab-gene expression. Changes in intracellular pH have been widely recognized as second messengers in animal systems (Busa and

Nuticelli, 1984). In plants, a rather reserved attitude is taken towards this type of transduction mechanism. Nevertheless, both auxin and ABA have now been shown to bring about changes in pH_i, both with consequences for further hormone action. The pH_i decrease induced by auxin is a direct mediator for auxin-induced calcium increase (Felle, 1988) and ABA induced pH_i increase in guard cells is responsible for the activation of K^+ efflux (Blatt and Armstrong, 1993).

In this thesis, we show that prevention of the ABA-induced pH_i increase (by inhibiting H^+ATPase activation or with the introduction of an artificial pH_i decrease) results in a clear inhibition of ABA-induced gene expression (chapters 2, 3 and 5). This inhibition takes place at the level of Rab-promoter induction, as shown in chapter 5. The pH_i increase can, however, not act as a direct inductor of ABA-induced gene expression (chapter 2, 3, 5). An increase of pH_i was shown to stimulate Rab-promoter induction by ABA. However, such stimulative effect of high pH_i was not observed on native Rab-mRNA induction by ABA. This could, however, be due to a negative effect of high pH_i on Rab mRNA stability.

Since a change in pH_i is a very common response of cells, and might result from a variety of (metabolic) actions, it is hard to think of it as the only effector or mediator of intracellular events. Busa and Nuticelli (1984) therefore suggested a model in which changes in pH_i act as *synergistic* messengers; i.e. they act in concert with other intracellular signals. Several reports suggest a synergistic action of intracellular Ca^{2+} and pH_i changes in signal transduction cascades (e.g. Felle, 1988; Gehring, 1990; Blatt, 1993) We suggest that these signals play a role in ABA signal transduction in barley aleurone as well (chapter 2; Wang *et al* 1991). It has been suggested that some of the synergistic action of Ca^{2+} and pH_i may be brought about by alteration of calcium-binding properties of particular proteins (e.g. Felle, 1988). Synergism may also exist between pH_i and other ions (K^+). On the other hand it should be recognized that pH can act independently as a regulator of e.g. enzyme activity. Many enzymes have narrow pH optima and can therefore be drastically influenced by small changes in pH_i.

Relevance of other putative second messengers

The pH_e decrease and malate increase brought about by ABA seem not to be related to the induction of Rab-mRNA: neither one could be shown to affect ABA-induced Rab-gene expression (data not shown).

The activity of protein phosphatases seems to be essential for Rab-gene induction as described in chapter 6. The protein phosphatase inhibitor PAO could completely block Rab-gene expression. The precise mechanism that is affected by this inhibitor has yet to be elucidated. A doublet of membrane-bound proteins of approximately 40 kDa that was hyperphosphorylated in the presence of PAO may perhaps provide a clue.

One should recognize that the action of ABA in barley grain is certainly not restricted to the regulation of gene expression when considering the relevance of putative second messengers. ABA has a pivotal role in the control of seed development and germination, together with its antagonist GA. Wang *et al.* (1994), showed that Rab-gene expression does not mediate inhibition of germination by ABA. This emphasises that induction of Rab-gene expression cannot be considered a model system for all other ABA actions. Changes in pH_i, K^+, and Malate-concentration can play an important role in the regulation of processes involved in seed development or germination by ABA.

The role of second messengers in the cross talk between ABA and its antagonist GA

ABA and GA are the two main regulators of seed development and germination in plants. ABA is important during seed development, being involved in storage protein synthesis, desiccation tolerance and prevention of precocious germination (see Introduction). GA, on the other hand, plays a stimulative regulatory role during germination. It induces embryo germination and the production and secretion of proteases and hydrolases (e.g. α-amylase and β-glucanase), necessary for the mobilization of nutritional reserves for growth of the embryo. GA is present in developing seeds, but does not seem essential for the developmental processes (Barendse *et al.*, 1992). At the onset of germination, the time the seed has to switch from (quiescent) developing mode to further growth, ABA and GA appear to perform antagonistic actions. As shown in Table II, ABA and GA have opposite effects on most of the responses of barley aleurone to these hormones. Induction of Rab-gene expression appears to be the only response known until now that is only affected by one of the two hormones.

Best studied until now is the interaction of ABA and GA in induction of gene expression. In both ABA- and GA-induced promoters hormone-responsive boxes have been identified (e.g. Skriver *et al.*, 1991). Induction of GA-responsive elements -GARE's"- of for example α-amylase genes, can

be inhibited by ABA, whereas induction of ABA-responsive elements - "ABRE's"- (from e.g. the Rab-promoter) is unaffected by GA. Note that protein synthesis is required for α-amylase gene expression, but not for Rab-gene expression (Skriver *et al.*, 1991). Although ABRE-binding proteins (Leucine zipper-like; Guiltinan *et al.*, 1990) and putative GARE-binding proteins (e.g. Rushton *et al.*, 1992) have been found, no data are as yet available on the interaction of ABA and GA on either production or of binding of these transcription factors.

The changes in ion concentrations induced by ABA and GA, such as listed in Table II, may act as second messengers. The interaction of the two hormones on Ca^{2+}, H^+, K^+ and malate may implicate that ABA and GA share (part of their) signal transduction mechanisms. None of these four ions has yet been proven to act as second messenger. It is however generally believed that Ca^{2+} plays a role in the regulation of α-amylase production by ABA and GA. Ca^{2+} is considered an important ion for the stability and secretion of α-amylase (Bush *et al.*, 1989). GA is known to stimulate hydrolase production and causes an increase in Ca^{2+}-levels in the cytoplasm and ER (Gilroy and Jones, 1991; Bush *et al.*, 1993). ABA counteracts the effect of GA on Ca^{2+}-levels, bringing about a decrease in Ca^{2+}-concentration both in the ER (Bush *et al.*, 1993) and in the cytosol, probably due to stimulation of Ca^{2+}-ATPases (Wang *et al.*, 1991). This effect of ABA on the Ca^{2+} levels might be one of the mechanisms by which the ABA inhibits GA-induced hydrolase secretion.

The role of pH_i, K^+ and malate in the counteraction between ABA and GA is not clear. Malate is considered an important messenger in the control of stomatal closure by ABA (Hedrich *et al.*, 1994). But no evidence is yet

Table II. *Antagonistic actions of GA and ABA on some responses in barley aleurone*

	ABA	GA	ABA+GA
α-amylase expression	inhibition	induction	intermediate
Rab-gene expression	induction	no effect	induction
pH_i	increase	decrease	intermediate
[L-malate]	increase	decrease	decrease
[cytosolic Ca^{2+}]	decrease	increase	intermediate
[E.R. Ca^{2+}]	decrease	increase	ND
K^+-flux	influx	efflux	ND

reported on a role of malate as a second messenger in either aleurone or embryo. The effect of the two hormones on malate concentrations is probably mediated by their effect on pH_i, as described in chapter 4. The ABA-induced pH_i increase seems to trigger the increased production of malate. GA is able to decrease pH_i and thereby inhibits malate production. ABA and GA are suggested to have an opposite effect on intracellular K^+ concentrations. GA is reported to stimulate K^+ efflux from barley aleurone (Bush *et al*, 1988). ABA on the other hand seems to induce an influx of K^+ ions by the opening of K^+ inward rectifiers, as suggested by our results, described in chapter 3. This was later confirmed by patch-clamp studies by dr. B. van Duijn (personal communication). At present there is no evidence that changes in K^+ concentration influence signal transduction cascades underlying seed development or germination.

ABA perception

The slow progress in the field of ABA perception does not reflect the amount of research dedicated to this subject. Several approaches have been used in an attempt to reveal the identity of (the) ABA receptor(s).

One of the key questions in the study of ABA receptors has been the site of perception. Several studies have now been published which try to determine whether ABA receptors are localized either inside or outside the cell. Gilroy and Jones (1994) have used microinjection of ABA in aleurone cells to study inhibition of GA-induced α-amylase production. They found that intracellular ABA was less effective than ABA present at the outside of the cell. Anderson *et al.* (1994) used a similar approach to study closure of stomata. They reported that microinjected ABA was less effective in inducing closure than extracellular ABA. They concluded that ABA (mediating stomata closure) was perceived extracellularly. This conclusion was contradicted by the results of Allan *et al.* (1994) who used "caged" ABA. They found that release of caged ABA inside guard cells induced closure of stomata. Intracellular perception of ABA by guard cells is supported by the finding that ABA is more active on guard cells at pH 5.5 than at pH 7 (Patterson *et al.*, 1988). ABA is a small molecule and can easily penetrate the plasma membrane in its protonated form. At pH 5.5 considerably more ABA would be trapped in the guard cell, thus suggesting an (additional) intracellular site of perception. Within the cell, ABA will be redistributed over (acidic) compartments. In guard cells an ABA transporter has been identified in the vacuolar membrane (Baier and Hartung, 1988).

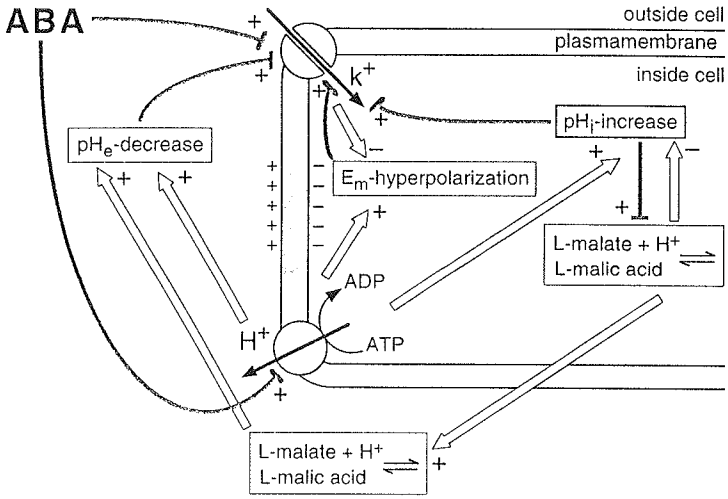


Figure 1. Interconnection of ABA-induced H^+-ATPase activation; E_m hyperpolarization; K^+-influx; pH_i-increase; L-malate increase and pH_e-decrease. ⟶ indicates stimulation, ⟹ indicates resultant negative effect, ⟹ indicates resultant positive effect. For simplicity ABA is drawn as if acting directly on de H^+-ATPase, this might very well involve intermediate (intracellular) messengers.

therefore suggest that both are the result of ABA-induced activation of a plasma-membrane proton pump.

Our data indicate that a potassium influx occurs upon ABA stimulation (chapter 3). This influx is possibly brought about by the opening of voltage or pH-gradient gated K^+-channels. This assumption was later supported by work of Dr. B. van Duijn. He performed patch-clamp experiments on barley-aleurone protoplasts and found that ABA brought about the opening of K^+ inward rectifiers (personal communication). In addition, Bush *et al.* (1988) describe the existence of voltage-gated K^+-inward rectifiers in barley aleurone, showing that hyperpolarization of the plasma-membrane potential to values more negative than -45 mV (dependent on $[K^+]$ concentrations in and out of the cell) would result in K^+ influx.

Allan and Trewavas (1994) and Slovik and Hartung (1992) hypothesize that cells react upon certain signals (especially stress) with a redistribution of intracellularly stored ABA. The redistribution would be brought about by simply influencing pH gradients over the separate (organelle) membranes involved. In this way the cell could be able of a prolonged response to certain signals.

Apart from localization of putative receptor sites, it has been attempted to identify the protein(s) involved. The most straightforward approach is to characterise ABA-binding proteins. The only publication to date reporting successful ABA binding was published in 1984 (Hornberg and Weiler), but unfortunately these results could not be confirmed. Another approach uses affinity chromatography with immobilized ABA to isolate putative ABA-binding proteins. Wan *et al.* (1991) used this approach to purify ABA-binding proteins from *Zea maize* coleoptiles. The isolated protein fraction was able to compete for ABA binding with monoclonal antibodies against ABA. Subsequently, antibodies were raised against this protein fraction. We have used these antibodies to screen a barley cDNA expression-library and have isolated a barley cDNA clone. We were not able to show that the (*in vitro* translated) protein encoded by this gene specifically binds ABA. However, until now no successful and reproducible protocol for ABA binding has been reported. Further research is now in progress to reveal whether this protein is involved in ABA action and to determine its localization.

The different approaches in studies concerning ABA perception have not yet been able to elucidate the mechanisms or identify the proteins that are involved. However, it has become likely that ABA is perceived by more than one receptor and that different responses will be mediated by separate receptors.

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Intracellular microelectrode membrane potential measurements in tobacco cell-suspension protoplasts and barley aleurone protoplasts: interpretation and artifacts*

Abstract

Intracellular microelectrode measurements in plant cell protoplasts have been widely used to study hormone signal transduction processes. However, the interpretation and reliability of such measurements are largely dependent on a detailed evaluation of the measurement conditions, as investigated in the present paper. Upon microelectrode penetration of tobacco cell suspension protoplasts and of barley aleurone protoplasts a fast negative going impalement-induced potential transient of less than a few msec duration could be observed. After reaching a steady-state potential at the msec time scale the measured potential hyperpolarized again and, in most cases, subsequently depolarized to a new steady-state value. Analysis of the electrical equivalent circuit of the measurement configuration showed that the occurrence of the impalement-induced potential transient indicates that these measurements suffer from a microelectrode-induced shunt resistance which loads the measurement. In addition, it is shown that the peak-value of the potential transient is the most reliable indicator of the true membrane potential and of true membrane potential changes of the protoplast, since this value is rather membrane resistance independent. For correct interpretation of steady-state measurements of membrane potential and stimulus-induced membrane potential changes data on membrane and shunt resistance are essential. As an example of the measurement of membrane potential changes the effects of 1-NAA on measured potential values in tobacco protoplasts and the effect of extracellular pH changes on barley aleurone protoplasts are analyzed with regard to the above described conclusions.

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Introduction

The plasma membrane potential and plasma membrane potential changes may play an important regulatory role in different cellular processes and in the functioning of plant cells. In addition, the membrane potential can be seen as an indicator for ion fluxes across the plasma membrane, and can be used to assay the sensitivity of cells for compounds affecting the electrophysiological properties of the membrane, such as plant hormones (e.g. Eprithine *et al.*, 1987; Barbier-Brygoo *et al.*, 1989; Felle *et al.*, 1991; Mathieu *et al.*, 1991).

In general the membrane potential of plant cells in a tissue can be relatively easily measured with intracellular microelectrodes. However, since many experimental approaches require the use of a protoplast system, e.g. when antibodies against plasma membrane proteins are used, it has been shown useful to perform membrane potential measurements in plant cell protoplasts (Eprithine *et al.*, 1987; Barbier-Brygoo *et al.*, 1989; Venis *et al.*, 1991; Barbier-Brygoo *et al.*, 1991; Venis *et al.*, 1992). Since membrane potential values of around -50 mV for tobacco suspension cells and protoplasts have been reported (Mathieu *et al.*, 1991; Briskin and Leonard, 1979), the relatively small potential values (in the order of 0 to -10 mV), reported more recently for tobacco protoplasts with the use of intracellular microelectrodes (e.g. Eprithine *et al.*, 1987; Barbier-Brygoo *et al.*, 1989; Venis *et al.*, 1990; Venis *et al.*, 1992), may cause some scepticism about the reliability of this method in protoplasts. Therefore, a careful interpretation of results obtained with intracellular microelectrodes in protoplasts and a detailed analysis of such measurements is essential to draw valid conclusions. In contrast to intracellular microelectrode measurements in whole plant cells, these measurements are technically difficult in protoplasts. Especially the introduction of a (in time variable) shunt resistance upon microelectrode penetration complicates interpretation and analysis of this type of measurements.

The patch-clamp technique in the whole-cell configuration provides an alternative method to measure the membrane potential without interference of a shunt resistance. However, several disadvantages in the use of this technique for routine membrane potential measurements exist (e.g. Neher, 1992; Neher and Sakmann, 1992; van Duijn *et al.*, 1993a). For example, only just after obtaining the whole-cell configuration by breaking the membrane under the patch-electrode, the membrane potential of the undisturbed protoplast can be measured. Perfusion of the cytoplasm with the

patch-pipette content and diffusion of cytoplasmic compounds out of the protoplast into the pipette will influence cellular properties and likely affect signal transduction cascades. So, in fact only a single time point measurement of the membrane potential of the undisturbed cell can be made with the patch-clamp technique, unless the "slow whole-cell" configuration can be used (e.g. Schroeder, 1988). Other alternative methods for membrane potential measurements in protoplasts include e.g. the use of potential sensitive fluorescent indicators and measurements of the distribution of lipophilic cations. These methods, however, have many disadvantages as well (e.g. calibration problems, compartmentalization of the indicators etc.) and only are indirect indicators of the membrane potential.

In view of the above mentioned complications of different methods to measure membrane potentials in plant cell protoplasts and the fact that a number of "key" studies in plant hormone signal transduction research are based on intracellular microelectrode measurements a detailed analysis of such measurements is necessary.

In this paper we present data obtained from intracellular microelectrode measurements on tobacco cell suspension protoplasts and barley aleurone protoplasts. These data are analyzed and interpreted with regard to the electrical behaviour upon microelectrode impalement as predicted from electrical equivalent circuit simulations of the measurement configuration. The possible pitfalls, essential checks and precautions for performing microelectrode measurements in protoplasts and reliable interpretation of such measurements are discussed.

Glossary

C_e = microelectrode capacitance (after capacitance compensation)
C_m = membrane capacitance
E_d = diffusion potential across the ion unselective microelectrode induced shunt
E_m = true membrane potential across undisturbed plasma membrane
E_{max} = most negative potential value reached after E_p
E_p = most negative value of the microelectrode-induced potential transient
E_s = initial steady-state potential value reached just after E_p
E_{ss} = final steady-state potential value reached during the measurement
R_e = microelectrode resistance
R_i = input resistance of impaled protoplast
R_m = membrane resistance

R_s = microelectrode-induced shunt resistance
V_e = potential value measured by microelectrode
V_m = shunted membrane potential

Materials and Methods

Protoplast isolation

Tobacco protoplasts

Tobacco suspension cells (*Nicotiana tabacum* L. cv Bright Yellow) were grown in Linsmaier and Skoog medium (Linsmaier and Skoog, 1965) as described before (Van Duijn *et al.*, 1993b). Protoplasts were prepared from the cells by incubation for 4 h in a solution containing 0.4 M mannitol, cellulase and pectolyase as elsewhere described (Boot *et al.*, 1993). Protoplasts were collected after three times washing with enzyme free solutions by centrifugation (3 min, 100*g*).

Barley aleurone protoplasts.

Barley (*Hordeum vulgare* L. cv. Himalaya, harvest 1985; Department of Agronomy, Washington State University, Pullman, WA) aleurone protoplasts were prepared as described by Jacobsen and Chandler (Jacobsen and Chandler, 1987), except for the imbibition of the half grains which was carried out in H_2O and incubations with enzymes which were carried out at 25°C in the dark for 16 h. Protoplasts were sieved (100 μm sieve) and washed with B-ECS (see solutions).

Solutions

Tobacco protoplasts were incubated in a standard extracellular solution (T-ECS) consisting of 10 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM KOH, and 10 mM MES (pH 5.5). The osmolarity of ECS was adjusted with mannitol to about 570 mOsm.

The standard extracellular solution (B-ECS) for barley aleurone protoplasts consisted of 0.5 mM K_2HPO_4, 10 mM KCl, 1 mM $MgCl_2$, 1.1 mM $CaCl_2$, 0.1 mM EGTA, 10 mM PIPES-HCl (pH 6.8) and adjusted to about 830 mOsm with mannitol.

Intracellular microelectrode measurements

Fine tipped, wide taper, 3 M KCl filled intracellular microelectrodes with a resistance between 30 and 70 MΩ were used for membrane potential measurements essentially as described before (Van Duijn *et al.*, 1988). A

microelectrode amplifier with capacitance compensation (WPI Series 700 Micro Probe Model 750, WP Instruments, New Haven, CT) was used. Microelectrode capacitance was compensated to obtain rise times (=time to reach 66% of the potential response upon a current pulse) faster than 0.05 ms. All potential values were measured with respect to the microelectrode tip potential. To ensure rapid (4 μm/0.1ms) and reproducible impalements of protoplasts with minimal lateral vibration, a piezo-stepper device (Piezo-stepper P-2000, Physik Instrumente (PI) Gmbh Co., Waldbronn-Karlsruhe, F.R.G.) was used. This device proved to give minimal variation in the impalement-induced shunt resistance. For microelectrode measurements protoplasts were kept in a glass bottom Teflon culture dish (Ince *et al.*, 1985) with 2 mL extracellular solution and observed with 40x or 100x objective magnification.

Statistics

Data are presented as means ± S.E.M., with n the number of measurements. Differences between values were tested with Student's t-test.

Results and Discussion

Response upon microelectrode impalement

Impalement of free protoplasts with a microelectrode is not easy, but in clean preparations protoplasts tend to adhere to a carefully cleaned glass surface which makes microelectrode penetration possible with the methodology described (see Materials and Methods). Upon penetration of both tobacco protoplasts and barley aleurone protoplasts a potential transient could be observed within the first milliseconds upon impalement (Fig. 1A, B). The occurrence of such an impalement-induced transient in animal cells has first been described by Lassen *et al.* (Lasse *et al.*, 1971) and was thereafter experimentally and theoretically analyzed in more detail in human monocytes and *Dictyostelium discoideum* amoeba (e.g. Ince *et al.*, 1986; Van Duijn *et al.*, 1988). The impalement with a microelectrode can be simulated with the use of an electrical equivalent circuit of the measurement configuration, by instantaneous introduction of R_s (the microelectrode-induced shunt resistance) and R_e (the resistance of the microelectrode) in the electrical equivalent circuit of the protoplast, resulting in the circuit in figure 2A. In first instance we have to look at the steady-state behaviour of this circuit. In this circuit the measured potential, V_e, in the steady-state condition is given by the following equation:

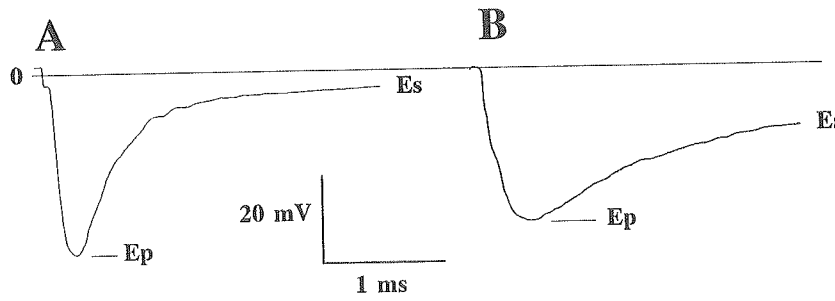

Figure 1. Microelectrode impalement-induced potential transients observed upon penetration of a tobacco protoplast (A) and a barley aleurone protoplast (B). Upon touching the protoplasts with the microelectrode a small positive prepotential was seen in many cases. The time-course of depolarization after reaching the peak-value, E_p, is mainly determined by the time constant of the penetrated protoplasts membrane. E_s indicates the steady-state level reached at a millisecond timescale.

$$V_e = V_m = \frac{E_m \cdot R_s + E_d \cdot R_m}{R_m + R_s} \qquad [1]$$

in which E_m is the true membrane potential, E_d is the diffusion potential across the microelectrode-induced shunt, V_m is the shunted membrane potential, R_m is the membrane resistance and R_s is the microelectrode-induced shunt resistance. From equation 1 it can be seen that in the steady-state condition V_e depends on the ratio between R_m and R_s, and only will be close to the value of the real membrane potential, E_m, if $R_s >> R_m$.

When this is not the case, e.g. in cells with a relatively high R_m or when the membrane does not seal off around the microelectrode (R_s will be small), V_e will only be a fraction of E_m (i.e. V_m decays). When we look at the dynamic behaviour of the circuit upon introducing microelectrode impalement we see that, due to the presence of different capacitors in the circuit, the decay of V_m is not stepwise, but follows a time-dependent decrease. Under favourable conditions (a.o. a small microelectrode rise-time, see Ince *et al.* (1986) the introduction of R_s can be observed in the occurrence of a fast peak-shaped potential transient in V_e and a decay of V_m which reflects the discharge of the membrane capacitance, C_m, to the new steady-state potential (as seen in a simulation with circuit parameter values

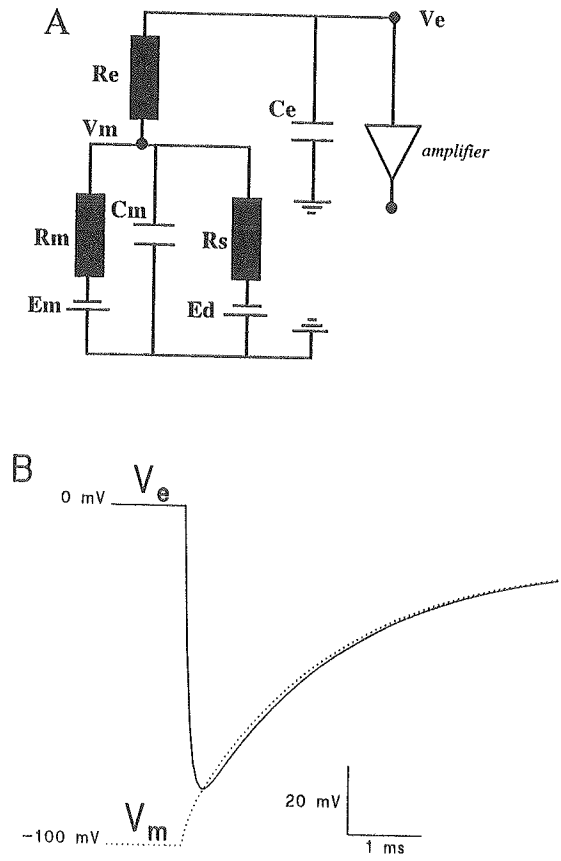

Figure 2. A Electrical equivalent circuit of the intracellular microelectrode measurement configuration. The membrane resistance R_m and membrane capacitance C_m determine the electrical membrane properties, and describe together with the membrane potential E_m the electrical equivalent circuit of the undisturbed protoplast. Upon microelectrode penetration a shunt resistance R_s in parallel with R_m and C_m is introduced. E_d represents the diffusion potential across the ion unselective shunt. R_e and C_e represent the microelectrode resistance and capacitance (after capacitance compensation), respectively. V_m is the shunted membrane potential and V_e is the potential that is actually measured. **B** Simulation of the potential response of both V_e and V_m upon penetration of the protoplast membrane with the electrical equivalent circuit shown in (A). Upon introducing the microelectrode, and the microelectrode-induced shunt resistance in the circuit, V_m decays to a new, depolarized, steady-state level (described by Eq. 1), while V_e follows a transient potential change. The used circuit parameters in the shown simulation are in the same order of magnitude as the estimated or measured parameter values in real measurements: C_m= 20 pF, C_e= 1 pF, R_m= 1 GΩ, R_s= 100 MΩ, R_e= 80 MΩ, E_m=-100 mV, E_d=-5 mV.

in the same order of magnitude as found in the real measurements, figure 2B). The most negative value of the microelectrode-induced potential transient, E_p, is determined by all circuit components (Ince *et al.*, 1986), while the value of the steady-state potential reached thereafter, E_s, is described by equation 1. Most importantly, the value of E_p is very *un*sensitive for variations in R_m, whereas E_s is very sensitive for R_m variations (Ince *et al.*, 1986). The occurrence of such a peak-shaped potential transient upon impalement already shows that a shunt-resistance is introduced which loads the measurement. Hence, in measurements where this transient can be observed, the value of E_p is considered to be the best estimate of the membrane potential of the protoplast before impalement. This was, for instance, shown in whole-cell patch-clamp measurements in the current clamp mode in combination with intracellular microelectrode measurements in human monocytes (Ince *et al.*, 1986) and in measurements in *D. discoideum* cells of different sizes (Van Duijn *et al.*, 1988).

From the above we conclude that the occurrence of the peak-shaped potential transient in both tobacco and barley aleurone protoplasts indicates that a shunt resistance, which is loading the measurement, is introduced upon the impalement in both types of protoplasts. The mean value of E_p in tobacco protoplasts was about -28 mV, and about -45 mV for barley aleurone protoplasts (Table I). The mean E_s values were -10 mV and -16 mV for tobacco and barley aleurone protoplasts, respectively (Table I). The

Table I. *Different membrane potential indicators measured with intracellular microelectrodes in tobacco cell suspension protoplasts and barley aleurone protoplasts*

E_p is the peak value of the impalement-induced potential transient. E_s is "steady-state" value at the millisecond time scale after the impalement-induced potential transient. E_{max} is the maximal potential value reached during the measurement after the impalement-induced potential transient and E_{ss} is the final steady-state potential value reached during the measurement (stable value for > about 2 min). In a few cases E_{max} and E_{ss} were equal (see Fig. 3B).

	Tobacco protoplast	Barley aleurone protoplast
E_p (mV)	-27.7±2.6 (44)	-45.3±4.7 (31)
E_s (mV)	-10.5±0.8 (69)	-16.1±1.5 (49)
E_{max} (mV)	-14.9±0.9 (69)	-32.6±4.3 (49)
E_{ss} (mV)	-3.8±0.6 (69)	-11.9±2.9 (49)

occurrence frequency of a peak-shaped potential transient upon impalement was somewhat higher than 60% for both type of protoplasts. The other penetrations resulted in an instantaneous measurement of values around E_s. At these penetrations the impalement-induced decay of the potential is too fast to reach a peak-value close to the true resting membrane potential, e.g. due to a too small R_s value (i.e a too large impalement-induced leak), or penetration was into the vacuole. The more negative E_p and E_s values found in barley aleurone protoplasts as compared to tobacco protoplasts is in agreement with E_m measurements with the patch-clamp technique. Whole-cell patch-clamp measurements showed that the membrane potential of tobacco protoplasts in a similar extracellular solution is about -40 mV (Van Duijn *et al.*, 1993b), and in barley aleurone protoplasts around -60 mV (-58.3± 4.9 mV, n=8). Patch-clamp experiments performed as described before (Van Duijn *et al.*, 1993b).

Steady-state behaviour

After reaching E_s, the measured potential, V_e, often hyperpolarized to more negative values in both tobacco and barley aleurone protoplasts. This hyperpolarization was accompanied by an increase in the measured input resistance of the impaled protoplast, R_i, and most likely is due to a sealing of the membrane around the microelectrode, thereby increasing R_s. After this hyperpolarization, V_e depolarized again to a steady-state level a little less negative than the value of E_s (Fig. 3A). In a few cases, mostly in barley aleurone protoplasts, a much more negative potential could be maintained for a longer period (Fig. 3B). The measurement of steady potentials for a longer time could not be improved by using electrodes without Cl^- by filling them with 4M K-acetate (data not shown). The most negative potential value reached at this slower time scale, E_{max}, and the final steady-state potential, E_{ss}, were measured for both tobacco protoplasts and barley aleurone protoplasts (Table I). From table I it is clear that the mean value of E_{max} is less negative than the measured E_p in the same cells. The same is true for E_{ss} and E_s.

An increase in R_s may lead to the measurement of a constant potential V_e close to the value of E_m. In this case a hyperpolarizing response is measured which is accompanied by a R_i increase. On the other hand a similar effect on V_e may be obtained by opening of ion channels leading to a decrease of R_m. In this case the hyperpolarization is accompanied by a decrease of R_i (e.g. Ince *et al.*, 1987). Leak of chloride from the

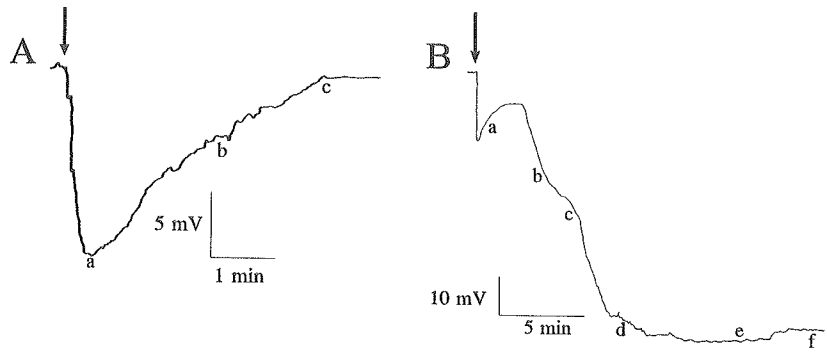

Figure 3. Slow time-scale recording of membrane potential measurement with intracellular microelectrode. Note the differences in time scale as compared to figures 1A,B. **A** Recording from a tobacco protoplast. The arrow indicates the moment of microelectrode impalement. The impalement-induced potential transient is not visible in this figure due to the low cut-off frequency of the chart recorder. Simultaneous recording on an oscilloscope showed that the impalement-induced potential transient had an E_p of -26.7 mV and an E_s of -1.5 mV. After reaching the E_s value the measured potential hyperpolarized again rapidly, as shown in the figure, to a maximal potential value indicated by (a). Thereafter a slower depolarization was recorded to a steady-state level of about -1.5 mV (c). At different time points the resistance of the penetrated protoplast was measured by application of -48 pA current pulses. R_i values at the indicated points were at (a) 57 MΩ, (b) 41 MΩ and (c) 29 MΩ. Microelectrode resistance was 52 MΩ. **B** Recording from a barley aleurone protoplast. As in (A) the impalement-induced potential transient is not visible in this figure. E_p of this protoplast was -62.0 mV, and E_s was -23.1 mV. After reaching E_s the measured potential hyperpolarized to a steady-state level which could be maintained for about 30 min. In this case E_{max} was equal to E_{ss}. The R_i values measured at the different indicated points were at (a) 5 MΩ, (b) 30 MΩ, (c) 53 MΩ, (d) 183 MΩ, (e) 239 MΩ, (f) 208 MΩ. Microelectrode resistance was 40 MΩ.

microelectrode into the cytoplasm may as well cause changes in E_m leading to changes in V_e, as was shown for non-animal cells (Van Duijn *et al.*, 1988; Blatt and Slaymann 1989). The potential increase (hyperpolarization) in both tobacco protoplasts and barley aleurone protoplasts was accompanied by a R_i increase, and the potential decrease (depolarization) by a R_i decrease (Fig. 3).

From equation 1 it is clear that the measured steady-state potential is strongly dependent on the ratio of R_s and R_m. In the given measurement

configuration (Fig. 2A), it is not possible to distinguish between R_m and R_s. Both R_m and R_s are recorded as part of the input resistance, R_i, which is the resistance of R_m and R_s in parallel:

$$R_i = \frac{R_m.R_s}{R_m + R_s} \qquad [2]$$

Combination of Eqs. 1 and 2 results in:

$$V_e = (E_m - E_d) \cdot \frac{R_i}{R_m} + E_d \qquad [3]$$

This predicts a linear relationship between V_e and R_i with a slope of $(E_m - E_d)/R_m$, assuming that variations in R_i are only due to variations in R_s. The V_e value for $R_s=0$ is equal to E_d (this is for $R_i=0$), while V_e is equal to E_m when $R_i=R_m$ (this is for $R_s \rightarrow \infty$). When we plot the measured potentials as a function of the measured R_i values for both tobacco protoplasts and barley aleurone protoplasts we, indeed, find a relationship as predicted by equation 3 (Fig. 4A,B). From these figures the estimated values of E_d are about -8 mV for both type of protoplasts. From the slopes of these linear relationships we can estimate the minimal value of R_m if we use the E_p values as the best (under)estimation of E_m. This results in minimal R_m values of about 310 MΩ and 160 MΩ for tobacco cell suspension and barley aleurone protoplasts, respectively.

From the above (Equation 3) it can be concluded that V_e will only change proportional with E_m changes when R_m and R_s are constant. However, since E_m changes in many cases are caused by or lead to changes in R_m this will not be the case in many experiments. Some examples of these situations are illustrated in Figure 5, which shows that the measured response at the microelectrode (V_e) on E_m changes is strongly R_m and R_s dependent. We conclude that for a correct estimation of true membrane potential values from steady-state intracellular microelectrode measurements, information about the ratio between R_m and R_s is required. In addition, for correct interpretation of membrane potential changes measured as changes in V_e data on R_m and R_s values are essential.

From the above we conclude that E_s, E_{max} and E_{ss}, in most cases, are not very reliable indicators for E_m changes as they are strongly R_i dependent.

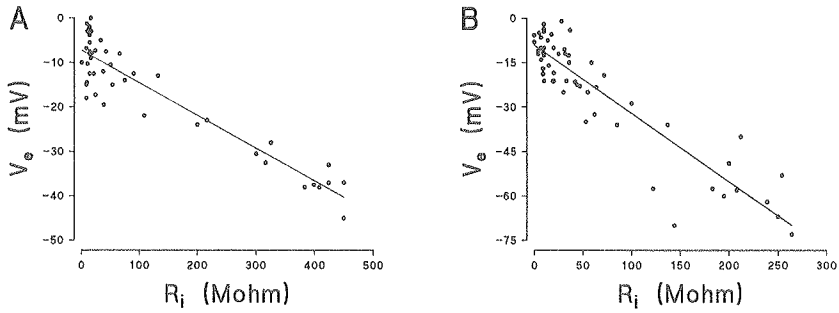

Figure 4. Measured potential values, V_e, as a function of the measured resistance of the impaled protoplast, R_i. The R_i was measured by application of a small current pulse (-48 pA) and recording of the potential change. R_i values are corrected for electrode resistance. The solid lines are the linear regression fits to the data points.
A Measurements in tobacco protoplasts. The linear regression line is: $V_e = -0.073R_i - 7.26$ (with V_e in mV, and R_i in MΩ), with a correlation coefficient of -0.92.
B Measurements in barley aleurone protoplasts. The linear regression is: $V_e = -0.23R_i - 9.01$, with a correlation coefficient of -0.91.

Measurement of membrane potential changes

For the measurement of stimulus-induced membrane potential changes of plant cell protoplasts with intracellular microelectrodes different strategies can be applied. The membrane potential of a number of protoplasts can be measured before addition of the stimulus. Subsequently the stimulus is added and the membrane potential of a number of stimulated protoplasts is measured (e.g. Eprithine *et al.*, 1987; Barbier-Brygoo *et al.*, 1989). Although technically most easy, the great disadvantage of this approach is that time-dependent changes are hard to measure, and may interfere with the measurements (i.e. only measurements can be done in steady-state conditions). In an other approach the membrane potential of one protoplast is measured continuously in time and the response upon addition of the stimulus is measured in time. This approach requires stable membrane potential measurements in a single protoplast over a long period, which are technically much more demanding. Both time dependent processes and membrane resistance can be monitored in this type of measurements, which are important for a correct interpretation of the measurements (see analysis above).

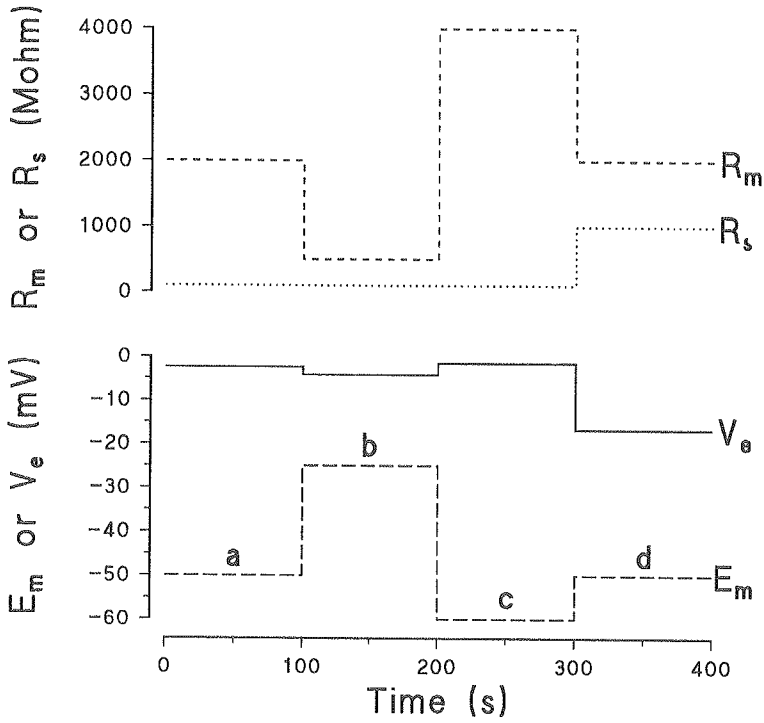

Figure 5. Simulation of measured potential values, V_e, in the steady-state condition (Eq. 3) for different given membrane potential E_m, membrane resistance R_m and shunt resistance R_s conditions. In the starting condition (a) R_s and R_m determine the measured potential V_e which is a fraction of the true membrane potential E_m. A membrane depolarization (condition b) accompanied by a membrane resistance decrease (e.g. due to opening of ion channels with a less negative reversal potential), results in the measurement of hyperpolarization of V_e. A membrane hyperpolarization (condition c) accompanied by a membrane resistance increase (e.g. due to closure of ion channels with a less negative reversal potential) leads to the measurement of depolarization of V_e. If in the starting condition (a) the shunt resistance is increasing (condition d), while the other parameters do not change, then a membrane hyperpolarization is measured at V_e.

As an example of measurements of membrane potential changes we investigated in tobacco protoplasts the membrane potential response upon stimulation with the auxin analogue 1-NAA. The above discussed membrane potential indicators (E_p, E_s, E_{max}, E_{ss}) were measured before and after addition of 1-NAA (Table II). After addition of 1-NAA at least 15 min was waited to allow the membrane potential to reach a new steady-state

value, to avoid interference with the kinetics of the response (Felle *et al.*, 1991). Only the E_p values show a significant ($p<0.025$) change after 1-NAA stimulation. The E_{max} and E_{ss} values suggest a membrane hyperpolarizing effect of stimulation with 1-NAA, whereas the E_s values suggest a membrane depolarizing action of 1-NAA. In contrast to other reports (Barbier-Brygoo *et al.*, 1989; Barbier-Brygoo *et al.*, 1991) the change in E_{ss} values is not significant. This may be due to different R_s values and the number of measurements. Interpretation of the action of 1-NAA on E_m from E_s, E_{max} and E_{ss} values must be done carefully, since variations in R_i may complicate the measurements as was shown in the analysis described above. With regard to the above, E_p is the most reliable membrane potential indicator, (as E_p is almost R_m independent (Ince *et al.*, 1986) and also shows the largest potential change after 1-NAA stimulation (Table II). Therefore, it is concluded from these measurements that, at more than 15 min after addition, 1-NAA hyperpolarizes the membrane potential.

Intracellular microelectrode measurements in maize coleoptile cells showed that 1-NAA induces a membrane depolarization which is followed by a membrane hyperpolarization (Felle *et al.*, 1991). This initial depolarization could, however, not be measured in maize coleoptile protoplasts as a change in membrane current during whole-cell patch-clamp experiments, whereas the hyperpolarization was seen as in increase in outward directed H^+-current (Rück *et al.*, 1993). Maize suspension cells did not show a hyperpolarizing response upon stimulation with 1-NAA as measured with intracellular microelectrodes (Felle *et al.*, 1991). These differences found between different cell types may be due to existing

Table II. *Effect of 1-NAA on membrane potential indicators in tobacco protoplasts*
Microelectrode measurements were performed before (control) and more than 15 minutes after addition of 1-NAA to the bath solution. Only the change in E_p values is significant ($p<0.025$).

	Control	10 μM 1-NAA
E_p (mV)	-24.7±2.2 (n=12)	-32.8±2.6 (n=15)
E_s (mV)	-7.0±1.5 (n=14)	-4.9±1.1 (n=16)
E_{max}(mV)	-12.3±1.0 (n=14)	-14.9±1.4 (n=16)
E_{ss} (mV)	-2.7±0.6 (n=14)	-3.7±0.8 (n=16)

differences in response between different cell types, differences between cells and protoplasts, and due to different measuring techniques. We conclude that both intracellular microelectrode measurements and patch-clamp measurements may be necessary to measure the complete electrophysiological response of plant cells and protoplasts to a stimulus.

In addition, the effect of an extracellular pH change on the membrane potential of barley aleurone protoplasts was investigated. The membrane potential indicators E_p, E_s, E_{max} and E_{ss} were measured in the control condition, pH 6.8, and subsequently at a more acid pH of 6.3. Table III shows that this decrease in extracellular pH induces a significant decrease of E_p and E_{max}. This change in E_p (about 34 mV/decade) might indicate that E_m is partly determined by the proton diffusion potential (about 59 mV/decade). However, pH dependent proton pump activity changes may lead to similar pH dependent membrane potential changes around pH 6.5 (e.g. Beilby, 1984).

The above examples show that, although the E_p value is an underestimation of E_m, it can not only be used as an estimate of the resting membrane potential but also as a reliable indicator of stimulus-induced membrane potential changes.

Concluding remarks

In both tobacco suspension protoplasts and barley aleurone protoplasts a peak-shaped potential transient was observed upon microelectrode impalement. The theoretical analysis of the measurement configuration showed that the occurrence of such a transient indicates that a shunt-resistance is introduced which loads the potential measurement. In addition, these peak potential transients showed that the membrane potential of both type of protoplasts is much more negative than the final steady-state potential, E_{ss}, which is measured. The difference in E_p values between tobacco protoplasts and barley aleurone protoplasts is in agreement with a more negative membrane potential of barley aleurone protoplasts than tobacco protoplasts as measured with the patch-clamp technique. The small potential values reported frequently (e.g. Barbier-Brygoo *et al.*, 1989; Barbier-Brygoo *et al.*, 1991) for tobacco mesophyll protoplasts are very likely also due to the introduction of a microelectrode-induced shunt resistance rather than to Cl^- leak from the electrode (Barbier-Brygoo *et al.*, 1991), although a cell type dependent difference cannot be ruled out at the moment. The fact that the true membrane potential of the used protoplasts

Table III. *Effect of an extracellular pH change on the membrane potential indicators E_p, E_s, E_{max} and E_{ss} as measured in barely aleurone protoplasts*

The differences between E_p values, and the differences between E_{max} values at the respective pH values are significant ($p<0.025$)

	Control (pH 6.8)	pH 6.3
E_p (mV)	-40.9±6.2 (n=20)	-23.8±4.7 (n=15)
E_s (mV)	-12.5±1.2 (n=20)	-11.5±2.0 (n=15)
E_{max} (mV)	-17.6±1.6 (n=28)	-11.4±1.6 (n=20)
E_{ss} (mV)	-5.6±1.9 (n=28)	-2.8±0.6 (n=20)

is much less negative than the usually reported values for intact cells suggests that the procedures (enzyme treatments) to prepare protoplasts affects the electrophysiological properties of the membrane. Hence, conclusions derived from studies on protoplasts are not necessarily valid for intact cells.

In addition, the theoretical analysis of the measurement configuration showed that in membrane potential measurements that suffer from a shunt-resistance the correct interpretation of potential changes is difficult. The steady-state potential is very sensitive for changes in R_m and R_s. Therefore, data on the membrane resistance and the shunt resistance are essential for drawing valid conclusions. A good alternative is the measurement of the value of E_p since E_p is very unsensitive for variations in R_m. Measurements of the effect of 1-NAA on the membrane potential of tobacco protoplasts showed that membrane potential changes can indeed be measured as changes of E_p values. Besides being the most reliable indicator, E_p showed also the largest potential change upon stimulation with 1-NAA as compared to E_s, E_{max}, and E_{ss}.

It turned out to be difficult to perform longer lasting membrane potential measurements at potential values close to the real membrane potential. Specially in tobacco protoplasts stable potential measurements could only occasionally be performed and never longer than for about 10 minutes. Barley aleurone protoplasts were better suitable for this type of measurements, possibly due to absence of a large vacuole and the presence of a relatively larger cytoplasmic compartment.

Intracellular microelectrode measurements on protoplasts may provide an alternative for patch-clamp measurements when giga-seal formation turns

out to be difficult or when large populations of protoplasts have to be examined. However, since membrane potential measurements with intracellular microelectrodes in plant cell protoplasts suffer from an impalement-induced shunt resistance (as can be observed by the occurrence of a peak-shaped potential transient upon impalement) one should use the value of E_p rather than the E_s, E_{max} E_{ss} values as an indicator of true membrane potential changes. For direct measurement of the true absolute membrane potential of the investigated protoplasts the patch-clamp technique has to be used as E_p provides only an underestimation of E_m.

Acknowledgments

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Samenvatting

Dit proefschrift beschrijft enkele signalen en signaalmoleculen die een rol zouden kunnen spelen bij de respons van gerstzaad op abscisinezuur (ABA). ABA is een plantehormoon dat een belangrijke rol speelt bij de ontwikkeling en kieming van zaden. Tijdens de zaadontwikkeling zorgt ABA er onder andere voor dat bepaalde reserve-eiwitten worden aangemaakt, dat het embryo de uitdrogingsfase van het zaad doorstaat en dat het niet voortijdig kiemt. In het rijpe zaad zorgt ABA, samen het hormoon gibberilline (GA), ervoor dat het zaad kiemt als de omstandigheden gunstig lijken. Bovendien zorgt ABA er in sommige gewassen voor dat de kieming gespreid plaatsvindt, hetgeen de kans op overleving vergroot.

Om deze regulerende taken uit te kunnen voeren, moet ABA door de verschillende weefsels van het zaad worden herkend en moeten deze weefsels, al naar gelang het ontwikkelingsstadium van het zaad, met een bepaalde respons reageren. In dit proefschrift zijn enkele snelle responsen op ABA beschreven die mogelijk een rol spelen in de signaaloverdracht van ABA die leidt tot ondermeer inductie van genexpressie. De meeste van deze responsen zijn bestudeerd in aleuron, een weefsel in het gerstzaad dat een belangrijke rol speelt bij het vrijmaken van reservevoedsel voor het kiemende embryo. Aangezien ABA in de aleuronlaag een andere reactie teweeg brengt dan in bijvoorbeeld het embryo, kunnen de resultaten die hier beschreven zijn voor aleuroncellen niet klakkeloos worden geëxtrapoleerd naar andere weefsels.

Het meest uitgebreid wordt ingegaan op de verandering van de intracellulaire pH (pH_i) die ABA veroorzaakt. Binnen een half uur na toediening van ABA aan aleuronprotoplasten (aleuroncellen zonder celwand) steeg de pH_i van ca. 7.0 naar ca. 7.2. Door onderzoek naar het effect van ABA op de membraanpotentiaal van aleuronprotoplasten werd de vermoedelijke oorzaak van de pH_i-stijging achterhaald. Enkele seconden na toediening van ABA aan deze cellen trad een (tijdelijke) daling op van de potentiaal van de plasmamembraan, d.w.z. dat de membraanpotentiaal meer negatief werd. Deze zogenaamde hyperpolarizatie kon worden geremd door remmers van de protonpomp en lijkt derhalve het gevolg te zijn van activering van protonpompen in de plasmamembraan. Het is waarschijnlijk dat deze activering van de protonpompen verantwoordelijk is voor de pH_i-stijging die ABA teweeg brengt.

ABA speelt tijdens de zaadkieming een antagonistische rol ten opzichte van het hormoon GA. Ook het effect ABA op pH_i is tegengesteld aan dat van GA. GA veroorzaakte een verzuring tot ca. pH_i 6.8. ABA en GA hadden bovendien invloed op de mate waarin aleuronlagen hun omgeving verzuren. In aanwezigheid van ABA werd deze verzuring gestimuleerd. Deze stimulatie werd waarschijnlijk voor een deel veroorzaakt door activering van de aan de plasmamembraan gebonden protonpompen door ABA. Een ander deel lijkt voor rekening te komen van een verhoogde produktie en uitscheiding van appelzuur. Het effect van ABA op appelzuurproduktie kon volledig teniet gedaan worden door GA. Hierdoor was GA in staat om een deel van de extracellulaire verzuring door ABA tegen te gaan. Dat GA niet de volledige verzuring remde, wijst erop dat GA geen effect heeft op de activering van de protonpompen door ABA.

Eén van de langere-termijn responsen die ABA in het gerstzaad teweegbrengt, is (verhoogde) transcriptie van het Rab-gen, zogenaamde Rab-genexpressie. De pH_i-stijging die door ABA wordt veroorzaakt lijkt van belang te zijn voor inductie van de Rab-genexpressie. Indien de stijging van de pH_i kunstmatig werd tegengegaan, werd de Rab-genexpressie geremd. Wanneer de pH_i kunstmatige werd verhoogd bleek dat dit alléén niet genoeg was voor inductie van het Rab-gen.

Dat de hoogte van de pH_i effect heeft op de inductie van de Rab-promoter en niet zozeer op bijvoorbeeld mRNA stabiliteit, blijkt uit proeven waarbij de Rab-promoter, gekoppeld aan het reporter-gen Chloramphenicol Acetyltransferase (CAT), in aleuronprotoplasten tot expressie werd gebracht. Verlaging van de pH_i veroorzaakte een remming van de door de Rab-promoter aangedreven genexpressie. Bovendien bleek dat in dit geval kunstmatige verhoging van de pH_i de ABA-geïnduceerde genexpressie stimuleerde. Vergelijkbare proeven, waarin de promoter van een door GA geïnduceerd gen (α-amylase) was gekoppeld aan het CAT-gen, lieten zien dat deze promoter niet gevoelig is voor kleine veranderingen in pH_i.

Naast pH_i-veranderingen zou ook fosforylering of defosforylering van bepaalde eiwitten een rol kunnen spelen in de signaaloverdracht van ABA. Bepaalde remmers van eiwitfosfatases waren in staat de inductie van Rab-genexpressie door ABA verhinderen. Diezelfde fosfataseremmers gingen defosforylering van (een) eiwit(ten) van ca. 40 kDa tegen. De meest effectieve remmer (fenylarside oxide) bleek defosforylering van tyrosineresiduen van twee 40 kDa eiwitten te remmen. Dit resultaat

suggereert dat tyrosine-defosforylatie een belangrijke rol speelt in de ABA signaaloverdracht die leidt tot de inductie van Rab-genexpressie.

Er is nog zeer weinig bekend omtrent de herkenning van ABA door een cel. Wellicht speelt een eiwit dat lijkt op eiwit-disulfide-isomerase een rol bij de inductie van Rab-genexpressie door ABA. Wij konden aantonen dat een dergelijk eiwit uit gerstzaad werd herkend door antilichamen tegen ABA-bindende eiwitten uit mais. Een copie-DNA fragment dat codeert voor dit eiwit werd gekloneerd. Verschillende proeven hebben aangetoond dat er een samenhang bestaat tussen de hoeveelheid RNA die voor dit eiwit codeert in een bepaalde aleuronlaag, en de mate waarin die laag responsief is voor ABA.

Nawoord

Op deze plaats wil ik enkele mensen die de afgelopen jaren waren betrokken bij dit werk met name noemen, te beginnen met mijn collega's van TNO. Rob, Jan, Norbert en vooral René hebben met name de eerste jaren op "Lab 5" op zeer positieve wijze verlevendigd. Jolanda en Norbert hebben over mijn veiligheid gewaakt en waren samen met alle andere huidige en vroegere collega's een bron van informatie en gezelligheid.

Manuela van Alphen-Copier, Peter van Leerdam en Thomas Nieland hebben alledrie minimaal een half jaar bijgedragen aan het hier beschreven onderzoek in het kader van hun studie. Van en door hen heb ik veel geleerd en met hen heb ik veel pret gehad.

Een zeer grote bijdrage aan dit proefschrift is geleverd door de heer en mevrouw van Duijn. Zij hebben mij veel geleerd en vooral ook groot enthusiasme voor de wetenschap bijgebracht. Hen is nooit een moeite teveel geweest om mij te helpen.

Joosje wil ik memoreren voor haar geduld en het stellen van een duidelijke dead-line. Timo ben ik zeer erkentelijk voor zijn opbouwende kritiek, onvoorwaardelijke steun en heel veel hulp.

Curriculum vitae

Sjoukje Heimovaara-Dijkstra werd geboren op 12 juni 1965 te Tilburg. In 1983 behaalde ze haar Atheneum B diploma aan de Rijksscholengemeenschap Thorbecke te Zwolle, waarna ze in hetzelfde jaar begon met de studie plantenveredeling aan de toenmalige Landbouwhogeschool te Wageningen. Deze studie onderbrak ze één jaar om in de senaat van de Wageningse Studentenvereniging "Ceres" plaats te kunnen nemen. Tijdens haar studie werkte zij zes maanden bij de vakgroep Moleculaire Biologie van prof. dr. A. van Kammen aan de identificatie van het nemathoden-resistentie-gen in tomaat. Hierna bracht zij een half jaar door bij het Carlsberg Research Laboratory in Kopenhagen waar ze aan de moleculair-genetische karakterisering van anthocyaan-mutanten in gerst werkte in de afdeling van prof. dr. D. von Wettstein. Tot slot werkte ze zes maanden bij de vakgroep Genetica (prof. dr. M. Koorneef) waar ze de mogelijkheid onderzocht om asymmetrische fusies tussen verschillende plantenrassen te maken. Ze behaalde haar ingenieurs-bul in november 1989. Vanaf september 1989 werkt ze als onderzoeker bij TNO in het Centrum voor Fytotechnologie. In mei 1990 is het hier beschreven onderzoek gestart onder de bezielende leiding van dr. Wang Mei. Een belangrijk deel van dit onderzoek is verricht in het kader van het ABIN-project (Adaptation of Barley for Industrial Needs). In dat kader werd samengewerkt met het Carlsberg Research Laboratory, hetgeen ondermeer uitmondde in het onderzoek naar het effect van intracellulaire pH op promoter inductie, wat begin 1993 in Kopenhagen werd uitgevoerd met de groep van dr. J. Mundy. Vanaf 1994 werd een deel van het onderzoek voortgezet in het kader van de samenwerking tussen TNO en de Rijksuniversiteit Leiden. Als onderdeel hiervan werd ondermeer onderzoek gedaan naar de membraanpotentiaal van gerst in samenwerking met dr. B. van Duijn van de afdeling Moleculaire Plantkunde en het Laboratorium voor Fysiologie en Fysiologische Fysica.

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aleurone protoplasts. 6th International Symposium on Preharvest Sprouting Idaha, USA

STELLINGEN

behorende bij het proefschrift "Signals mediating ABA action in barley grain" van Sjoukje Heimovaara-Dijkstra

1. Malaat kan in planten functioneren als tweede boodschapper
   -Hedrich et al. (1994) Plant journal 6: 741-748

2. Opslag van hormonen in de vorm van inactieve conjugaten lijkt een belangrijke rol te spelen in de hormonale regulatie van planten. Enzymen die betrokken zijn bij dit metabolisme verdienen daarom aandacht.
   o.a. -Brzobohaty et al. (1993) Science 262: 1051-1054
   -Delbarre et al. (1994) Planta 195: 159-167

3. De conclusie van Macnicol en Jacobsen, dat organische zuren verantwoordelijk zijn voor de helft van de verzuring van het endosperm tijdens de ontwikkeling van gerstzaad, is gebaseerd op de foutieve veronderstelling dat de pH-schaal lineair is.
   -Macnicol and Jacobsen (1992) Plant Physiol 98: 1098-1104

4. Van een gerstras mag worden verwacht dat het fenotypisch, maar niet dat het genotypisch homogeen is.

5. Er is behoefte aan regelgeving voor het benoemen van plante-eiwitten en genen .
   o.a. -Mundy (1989) Plant Mol Biol Rep 7: 247-254

6. Zonder fundamenteel onderzoek heeft toegepast onderzoek geen toekomst.

7. Zonder toegepast onderzoek heeft fundamenteel onderzoek geen toekomst

8. Voor een wetenschappelijke carriere is het van belang kennissen te vergaren.

9. De richtlijn van het college van dekanen van de Rijksuniversiteit Leiden voor het afkorten van de titel doctor is in strijd met de Nederlandse wet. (art 7.22, lid 3)

10. Het is inconsequent sporters te veroordelen voor het gebruik van spierversterkende middelen, en kunstenaars niet voor het gebruik van geestverruimende middelen.