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RESEARCH OPINION

Membranes and seed dormancy: beyond the anaesthetic hypothesis

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Abstract

The breaking of dormancy in seeds can be elicited by many factors, including temperature and short exposure to low molecular weight amphipathic molecules such as primary alcohols, monocarboxylic acids and anaesthetics. Their action has been suggested to be mediated through effecting changes to membranes. Paradoxically, though, these molecules can inhibit the germination of some non-dormant seeds. Here, we review the structure–activity relationships between amphipathic molecules and dormancy breaking and, based on the known responses of membranes to them and to temperature changes, offer an alternative interpretation of the data and a new hypothesis to explain their action. We suggest that amphipathic molecules break dormancy by partitioning into the membrane, thereby increasing and optimizing phospholipid headgroup spacing. This, in turn, facilitates the binding and activation of a peripheral membrane protein component of a signal transduction pathway that is essential for the completion of germination. In cases where amphipathic molecules inhibit germination, it is predicted that they cause the optimal headgroup spacing to be exceeded, thus preventing subsequent association of the membrane with the peripheral protein component. The hypothesis is extended to explain membrane changes that can lead to dormancy breaking during dry after-ripening.

Keywords: after-ripening, amphipathic molecules, anaesthetic hypothesis, germination, phospholipid headgroup spacing, seed dormancy, signal transduction, temperature

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Introduction

The publication of the 'fluid mosaic model' of biological membranes (Singer and Nicholson, 1972) and advances in electron microscopy in the 1970s focused attention on the cell membrane, its structure and properties. The recognition that membrane lipids are very responsive temperature-sensing molecules and that biological membranes consist of as much as 50% protein, largely of unknown function, indicated that membranes are dynamic structures with the potential to influence physiological processes. In seed germination studies, observations of the temperature dependence of solute loss during imbibition coupled this response to changes in membrane properties. The temperature dependence of germination was also inferred to depend on membrane properties. Similar findings in seeds of numerous species indicated the generality of these responses to temperature and implicated membranes in the regulation of seed dormancy and germination (Hendricks and Taylorson, 1976, 1979).

It is difficult to define precisely how biochemical and physical changes in membranes can affect dormancy, but it is well known that membrane lipids modulate the activity of their associated proteins. Dormancy is often broken by changes in environmental conditions such as temperature or light quality, or combinations of these (Hilhorst and Karssen, 1992). This suggests that a signal transduction pathway must be activated in order to release a seed from dormancy (Bewley, 1997). Such pathways, known to have one to several membrane-associated components in animal cells, have been more difficult to study in plant cells and, consequently, are not as well characterized.

The essential elements of a signal transduction pathway (e.g. the inositol–phospholipid signalling pathway) are the signal receptor, signal transducer, effector protein, one or more types of second messenger to amplify the signal and a target protein

(Scherer, 1996; Trewavas and Malhó, 1997). A signal culminates in a cellular response when these components are sequentially activated. The signal transducer and effector protein are typically peripheral membrane proteins. Peripheral proteins have various mechanisms for binding to the membrane. For example, the G protein signal transducers are anchored in the membrane by lipid tags (Casey, 1994). The γ-subunit of the heterotrimeric G protein is prenylated with a 15- or 20-carbon isoprenoid unit, while the α-subunit is acylated with either myristate or palmitate. A more tenuous binding mechanism is employed by protein kinase C (PKC), which equilibrates between the soluble and bound conformations, the latter being a requirement for activity (Gennis, 1989). PKC binding depends on certain physical properties of the membrane (Slater *et al.*, 1994). Since temperature directly affects membrane fluidity and phospholipid headgroup spacing, the temperature of the membrane environment may be a factor in the binding of peripheral proteins such as PKC.

The temperature-sensing ability of a membrane lies primarily within the hydrophobic acyl-chain region of the membrane bilayer. The sensitivity of the acyl chains to temperature is reflected in the transition temperature, T_m, between the gel and liquid crystalline phases of pure phospholipids, where small differences in the acyl-chain length and in the degree of unsaturation of the acyl chains cause substantial changes in T_m (Gennis, 1989). Although the physical properties of membranes in the gel phase are thought to be unsuitable for biological function, a more subtle effect of temperature on membrane lipids occurs within the liquid crystalline phase: as temperature decreases, both membrane fluidity and the area per phospholipid decrease (Heimburg, 1998). This is due to increased ordering among the acyl chains. In contrast, as temperature increases, thermal energy is translated into molecular motion, resulting in increased fluidity and cross-sectional area.

In some species, seed dormancy is broken during stratification, a moist incubation at a specific temperature, either higher (warm stratification) or lower (cold stratification) than the ambient temperature. It may be that at the stratification temperature, but not at other temperatures, a particular peripheral protein having a specific headgroup spacing requirement binds to the membrane, permitting the completion of a signal transduction pathway essential for germination. In this way, the temperature-sensing ability of membranes becomes an ideal mechanism for regulating physiological processes in poikilothermic organisms.

In the laboratory, dormancy breaking occurs in seeds of numerous species following pretreatment with ethanol or other anaesthetic-like substances during imbibition (Taylorson and Hendricks, 1979; Taylorson, 1988; Cohn *et al.*, 1989). These small amphipathic molecules are characterized by partition coefficients which predict their relative solubility in aqueous and lipid phases (Hansch and Leo, 1979). The logs of the partition coefficients for methanol and ethanol are negative, indicating a preference for the aqueous phase, while those for propanol and longer-chain alcohols are positive, indicating a preference for the lipid phase. The relative activity of these substances in dormancy breaking is directly correlated with their lipophilicity, suggesting that activity requires their binding to a hydrophobic site, e.g. the acyl-chain region of a membrane or the hydrophobic pocket of a protein (Hilhorst and Cohn, 2000). A membrane site is consistent with evidence from nuclear magnetic resonance (NMR) studies which localize ethanol within the headgroup region of a phospholipid bilayer (Barry and Gawrisch, 1994). The effect of ethanol on headgroup spacing and on membrane fluidity is similar to that caused by an increase in temperature.

Seeds of some species, particularly very small seeds such as those of *Barbarea verna* and *Lactuca sativa*, require red light during imbibition in order to break dormancy (Hendricks and Taylorson, 1979), indicating that a phytochrome signal transduction pathway must be activated before germination can occur. However, red light alone does not result in appreciable germination unless it is given within a specific temperature range. This, together with other reports linking photosensitivity with temperature and anaesthetics in *Amaranthus albus* (Chadoeuf-Hannel and Taylorson, 1985a, b), suggests that a component of the phytochrome signal transduction pathway is membrane linked.

In *Arabidopsis*, a family of phytochrome isoforms (PhyA–E), which regulate numerous physiological responses to light in the red/far-red spectral range, has been identified (Whitelam and Devlin, 1998). Phytochromes A–E are specifically expressed in various tissues and at different developmental stages. Phytochrome A and phytochrome B independently regulate the photoinduction of seed germination (Shinomura *et al.*, 1996). PhyB is expressed constitutively and, although synthesized in the inactive form, may occur as either P_R (the inactive red light-absorbing conformer) or P_{FR} (the biologically active far-red light-absorbing conformer) at seed dispersal, depending on the light environment during seed development. PhyB mediates the reversible red/far-red low fluence response, while light-labile PhyA, induced by 48 h of dark incubation, mediates the very low fluence response. This redundancy permits germination under different light conditions.

In order to understand the role, if any, of

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membranes in the regulation of seed dormancy and germination by phytochromes A and B, the sites where the phytochrome signal transduction pathways intersect with membranes must be identified. The interactions at these sites may be temperature/ethanol sensitive. We have identified two sites of interaction, described below, and expect that, as phytochrome signalling pathways become better defined, additional sites will be identified.

Unlike most receptors, the photoreceptor phytochrome is a soluble, cytosolic protein (Pratt, 1994). The subcellular localization of phytochrome has been studied in transgenic plants overexpressing PhyA–green fluorescent protein (GFP) or PhyB–GFP (Kircher *et al.*, 1999). In dark-adapted plants, PhyA–GFP and PhyB–GFP localize in the cytosol; in response to red light, both PhyA–GFP and PhyB–GFP translocate to the nucleus. A functional nuclear localization signal (NLS) has been identified in the C-terminal region of the PhyB apoprotein, and amino-acid sequence homology indicates the presence of multiple weak nuclear localization signals in PhyA (Sakamoto and Nagatani, 1996). Nuclear import consists of two distinct steps: binding of the NLS to a nuclear pore complex, followed by translocation of the protein across the nuclear envelope (Raikhel, 1992). The second step is reported to be temperature sensitive (Garcia-Bustos *et al.*, 1991) and may account for the temperature dependence of red light-induced germination (Hendricks and Taylorson, 1979).

Following stimulation with red light, PhyB–GFP fluorescence becomes completely localized in the nucleus, but PhyA–GFP fluorescence shows partial retention in the cytosol (Kircher *et al.*, 1999). This indicates that both PhyA and PhyB directly regulate the transcription of light-regulated genes, but that PhyA has an additional signalling function in the cytosol. The microinjection of putative signalling intermediates into cells of a PhyA-deficient mutant of tomato has identified several PhyA signalling intermediates, e.g. heterotrimeric G proteins, calcium/calmodulin and cGMP (Neuhaus *et al.*, 1993; Bowler *et al.*, 1994). A positive response following microinjection with GTPγS, a non-hydrolysable analogue of GTP, provides indirect evidence for a heterotrimeric G protein. Although this putative G protein has not been purified, heterotrimeric G proteins are well documented in plants. Furthermore, G proteins are activated by the same series of short-chain alcohols (Munnik *et al.*, 1998) which are effective in dormancy breaking. The (in)ability of this G protein, or perhaps another unidentified peripheral protein in the phytochrome signal transduction pathway, to bind to the membrane may be responsible for the effect of temperature and anaesthetics on photosensitivity.

The interaction of phytochrome with growth regulators has been suggested by reports correlating active phytochrome with increases in gibberellins (GAs) and cytokinins (Bewley and Black, 1994). In microinjection studies of the above-mentioned PhyA-deficient mutant of tomato, a positive phytochrome response was defined as anthocyanin biosynthesis and chloroplast development, suggestive of a response to GAs and cytokinins. It may be that active phytochrome modulates the amounts of growth regulators, or that their signalling pathways intersect, due to common elements (Roux, 1994).

The antagonistic action of abscisic acid (ABA) and the GAs has been a recurring theme in the study of seed dormancy (Hilhorst and Karssen, 1992; Léon-Kloosterziel *et al.*, 1996). While ABA is associated with the development of desiccation tolerance and the induction of dormancy, GAs are associated with vegetative growth. Each ligand is expected to have its own receptor and signal transduction pathway, including some membrane-associated components. Factors affecting responsiveness to these phytohormones include concentration, 'free' versus 'bound' forms and sensitivity, i.e. presence of a receptor.

The intention of this paper is to develop the hypothesis that the dynamic and structural properties of membrane lipids regulate the binding of a peripheral protein component of a signal transduction pathway that is essential for the completion of germination. It is not necessary to delineate this pathway in order to test the hypothesis.

Seed dormancy experiments establish a link to membranes

Hendricks and Taylorson (1979) investigated the effect of temperature on germination of seeds of *B. verna* and lettuce. They observed a narrow temperature range for germination in the region of 20–28°C, as well as a marked increase in solute loss during imbibition at temperatures above this range. A crude membrane preparation from each species was labelled with an anilinonaphthalene sulphonate (ANS) molecular probe, known to fluoresce when bound to membrane lipids and to fluoresce even more strongly when bound to proteins. Fluorescence data showed an inverse correlation with temperature in the region of 15–40°C. Although the ANS probes are predicted to orient at the membrane interface (Slavík, 1982), it is not clear from these data whether the probe is reflecting a change in the ordering of membrane lipids, a conformational change in the membrane proteins, or both. Nevertheless, data from these experiments were thought to support the role of certain undefined 'membrane transitions' in seed physiological processes.

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Of particular interest in these experiments is the synergistic interaction of temperature and red light on the germination of seeds of *B. verna* and lettuce. Although a membrane phase-change is unlikely in the temperature range (20–28°C) that promotes germination in these species, the thermal expansion of lipids in the lateral plane can result in a subtle, but perhaps significant, increase in headgroup spacing. For example, a synthetic membrane composed of DMPC (dimyristoylphosphatidylcholine) has a measured thermal area expansion coefficient of 0.0042 T^{-1} (Heimburg, 1998). For this lipid in the liquid crystalline phase, the area per phospholipid increases by 0.42% with each 1°C increase in temperature. The effect of a 10°C increase in temperature on such a membrane will be a 4.2% increase in area. This is relevant to imbibed seeds of *B. verna* which are dormant at 15°C and reach maximum germination at 25°C. The effect of headgroup spacing on the binding of peripheral proteins to the membrane (to be developed below) is known to be a critical factor for certain components of signal transduction pathways. Evidence suggests that one or more components of the phytochrome signal transduction pathway is membrane linked (Bowler and Chua, 1994). Therefore, it is possible that phospholipid headgroup spacing is the key to understanding the synergistic action of temperature and red light.

A role for membranes in dormancy is further supported by the demonstrated effect of ethanol and other anaesthetics on dormant caryopses of fall panicum, *Panicum dichotomiflorum* Michx. (Taylorson and Hendricks, 1979). This species has a complex dormancy, requiring imbibition at 35°C for 7 d, followed by red light irradiation (5 min), and germination at alternating temperatures (20/30°C). This procedure results in 25% germination. Treatment with 0.5 M ethanol during imbibition replaced the red light requirement, resulting in 97% germination. Ethanol pretreatment also permitted high germination at other temperatures (20, 25, 30 and 35°C), with or without red irradiation, unlike the control seeds imbibed in water. Thus, ethanol treatment during imbibition not only bypassed the requirement for subsequent activation of phytochrome and for alternating temperatures, but resulted in a much higher germination as well. Several other weedy grass species, but not all of those tested, had a similar response to ethanol.

Experiments on seeds of other species and with other small organic molecules, known to partition into membranes and which often have an anaesthetic response in animals (Taylorson and Hendricks, 1980/81), led to the so-called 'anaesthetic hypothesis'. Although membranes were now implicated in the regulation of dormancy in many species, the mechanism was not understood.

Structure–activity studies using amphipathic solutes

Red rice

Many small organic molecules, e.g. alcohols, aldehydes and monocarboxylic acids, break dormancy in seeds of different species. Cohn and co-workers (1989), studying dormant caryopses of red rice (*Oryza sativa* L.), found a direct correlation between the activity of a dormancy-breaking compound and its lipophilicity, as measured by the octanol/water partition coefficient, $K_{o/w}$. In a series of primary alcohols or monocarboxylic acids of increasing chain length, the concentration of a substance which elicits 50% germination decreases in a linear manner (see Fig. 1).

Although the dormancy-breaking substances belong to chemically distinct classes of organic molecules, e.g. alcohols, aldehydes and monocarboxylic acids, they have in common the property of being amphipathic, as are membrane lipids molecules lacking this property, e.g. the alkanes propane and butane, also lack dormancy-breaking activity when applied to dormant caryopses of red rice (Cohn *et al.*, 1989). The requirement for amphipathicity suggests a mechanism for the binding of these substances to membranes and favours the membrane phospholipid, rather than the hydrophobic region of a protein, as the binding site leading to dormancy-breaking activity.

The monocarboxylic acids and their corresponding primary alcohols do not have significantly different partition coefficients, but the former are somewhat more active germination stimulants (Cohn *et al.*, 1989). For example, 7 mM propionic acid is as effective as 41 mM propanol in promoting germination in red rice. This finding suggests that, in addition to lipophilicity and amphipathicity, the ability of a dormancy-breaking substance to form a more stable association with a membrane contributes to its relative activity. The carboxyl group of a monocarboxylic acid at its pK_a is both a hydrogen-bond donor and a hydrogen-bond acceptor, thereby forming two hydrogen bonds, whereas the hydroxyl group of a primary alcohol participates in only one hydrogen bond. Thus, the greater hydrogen-bonding ability of the monocarboxylic acids may explain their enhanced dormancy-breaking activity.

The effect of a more stable association with a membrane is further illustrated by a study of certain chemical 'pairs', e.g. 1-pentanol with 2-pentanol, 1-hexanol with 2-hexanol, or 2-cyclohexylethanol with 1-cyclohexylethanol (Cohn *et al.*, 1991). Although 1-pentanol and 2-pentanol have similar values for $K_{o/w}$, the former results in 60–85% germination when applied

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to dormant caryopses of red rice, while the latter is inactive. Similar data on other primary/secondary alcohol pairs establishes that linearly well-separated hydrophilic and hydrophobic regions are an additional requirement for dormancy-breaking activity.

The partition coefficient for ethanol ($K_{o/w}$ = 0.49) predicts that it is distributed by a 1:2 ratio between the lipid and aqueous phases, respectively. As alcohol dehydrogenase (ADH) activity in the cytosol removes ethanol from the aqueous phase, the concentration of ethanol in the lipid phase will decrease proportionately, and eventually ethanol will be removed from the system. Thus, ADH activity establishes a rather narrow window for dormancy breaking by the alcohols, consistent with the notion that dormancy occurs when one step in a sequence of events between imbibition and germination is blocked. Reports that the ADH inhibitor, 4-methylpyrazole (4-MP), prevents the germination of ethanol-treated dormant caryopses of red rice and oat (Corbineau *et al.*, 1991; Lin and Cohn, 1997) are not surprising. These reports suggest that the sustained exposure to dormancy-breaking levels of ethanol is sufficiently toxic that germination is prevented. Dose–response data for the ethanol-induced germination of dormant lettuce seeds identified reduced radicle length as the first indication of ethanol toxicity, followed by reduced germination at higher ethanol concentrations (Taylorson and Hendricks, 1980/81). The authors comment that the optimal concentration of a dormancy-breaking substance is 'actually a balance between promotive and toxic effects'.

Barnyard grass

Taylorson (1988), studying germination of dormant *Echinochloa crus-galli* (barnyard grass) caryopses, found a similar structure–activity relationship for a series of primary alcohols. In this case, dormancy-breaking activity was correlated with the relative lipophilicity, measured by the membrane/buffer partition coefficient. A membrane localization of the dormancy-breaking substances was further indicated by two other observations: the reversal of the effect when pressure was applied simultaneously with the chemical treatment, similar to reports of pressure reversal on anaesthetic action in animals; and increased solute loss correlated with dormancy-breaking activity, e.g. *n*-propanol causes greater leakage than 2-propanol, a substance which has little effect on dormancy.

By analogy to anaesthetic action in animals, Taylorson (1988) suggested that the membrane is the site of action for these small, amphipathic molecules and that the membrane plays a critical role in dormancy.

Lettuce

Paradoxically, in non-dormant seeds of lettuce (*Lactuca sativa*), the series of primary alcohols and monocarboxylic acids, which promote germination in red rice and barnyard grass, inhibit germination (Reynolds, 1975, 1977). Figure 1 shows a direct correlation between activity, either the promotion of germination in red rice or the inhibition of germination in lettuce, and the lipophilicity of a series of short-chain primary alcohols. The remarkable similarity in the slopes of the regression lines, representing data sets for the two species, suggests that the primary alcohol series from methanol to pentanol affects both the promotion of germination in red rice and the inhibition of germination in lettuce by the *same* mechanism.

A new interpretation and hypothesis

The non-specific interaction of ethanol and other anaesthetic-like molecules with membranes was indicated initially by the correlation of lipophilicity, as measured by one or more partition coefficients, with anaesthetic potency (Seeman, 1972). Later, Goldstein and Chin (1981) reported that membrane fluidization, a well-known effect of ethanol, could be attributed to

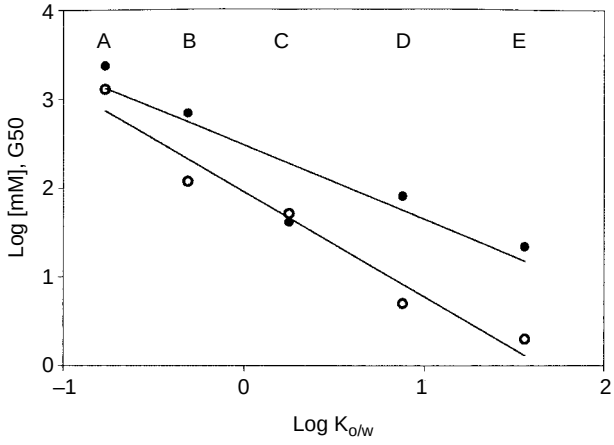

Figure 1. Correlation between the relative lipophilicity, $K_{o/w}$, of a series of primary alcohols and their biological activity, G50, the concentration eliciting 50% germination, in dormant red rice caryopses (●) or non-dormant lettuce seeds (○). A, Methanol; B, ethanol; C, propanol; D, butanol; E, pentanol. Values for $K_{o/w}$ from Hansch and Leo (1979). Data on red rice from Cohn *et al.* (1989). Data on lettuce from Reynolds (1975, 1977). Solid lines are linear regressions to the data sets.

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the acyl-chain disorder caused by the partitioning of ethanol into membranes. More recently, NMR studies, in conjunction with several other lines of evidence, have localized ethanol binding to the interfacial region of membranes (Barry and Gawrisch, 1994). In this region, it is proposed that the non-polar ethyl moiety interacts with the glycerol backbone and uppermost methylene group of an acyl chain, while the polar hydroxyl group hydrogen-bonds to oxygen of a carbonyl or phosphate group. Other amphipathic molecules are predicted to bind in a similar manner, those with longer hydrocarbon chains penetrating farther into the acyl-chain region of the membrane. This orientation is consistent with the structure–activity studies cited above. The intercalation of ethanol between headgroups results in an increased area per lipid, permitting an increase in acyl-chain disorder which results in membrane 'fluidization'. The increase in area per lipid is estimated to be 6.3% for a 1:1 ratio of lipid to ethanol. Thus, within the liquid crystalline phase, the effect of ethanol is comparable to that of a small increase in temperature. The significance of headgroup spacing is that it determines the spatial arrangement of dipoles and charge on the surface of the membrane. This is a critical factor for the binding and subsequent activation of certain peripheral proteins.

This evidence leads us to suggest a new hypothesis: amphipathic molecules, such as ethanol, break dormancy by partitioning into the membrane, thereby increasing the phospholipid headgroup spacing which, in turn, promotes germination by optimizing the binding and activation of an essential peripheral membrane protein (Fig. 2). In those cases where ethanol has been found to inhibit germination, as in non-dormant seeds of lettuce and in some grasses, it is predicted that the amphipathic molecules cause the optimal headgroup spacing to be *exceeded*, thereby preventing the binding of this peripheral membrane protein. Similarly, temperature may either promote or inhibit germination via an effect on headgroup spacing. For example, the germination of *B. verna* with respect to temperature is described as a biphasic curve from 15–35°C, with optimal germination occurring at 25°C (Hendricks and Taylorson, 1979). In some species, dormancy breaking may be more complex, requiring lipid segregation at low temperatures or lipid mixing at high temperatures, with or without ethanol, in order to achieve optimal headgroup spacing. This may be the case in oats, where freshly harvested caryopses germinate readily at low temperatures, but require ethanol for germination at high temperatures (Corbineau *et al*., 1991).

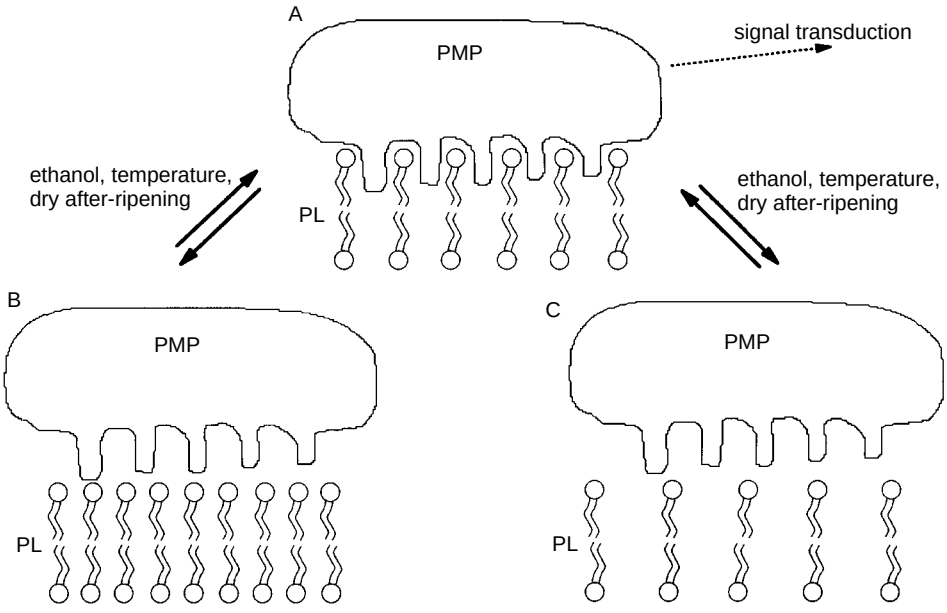

Figure 2. A model to explain the relationship between membrane phospholipid (PL) headgroup spacing and the binding of a peripheral membrane protein (PMP) component of a signal transduction pathway, essential for germination. (A) An optimal PL headgroup spacing permits the binding and activation of a PMP which completes a signal transduction pathway preceding germination; (B) a suboptimal PL headgroup spacing prevents PMP binding, thereby interrupting signal transduction and causing dormancy; (C) an above-optimal PL headgroup spacing also prevents PMP binding, resulting in dormancy. The transition between dormancy and germination can be manipulated by factors that affect phospholipid headgroup spacing, e.g. temperature, amphipathic molecules and dry after-ripening.

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The report of qualitative and quantitative changes in proteins extracted from plasma membranes isolated from dormant and *n*-propanol-treated dormant caryopses of *E. crus-galli* (Di Nola *et al.*, 1990) provides direct support for the headgroup-spacing hypothesis. The germination protocol for this species requires imbibition and dormancy breaking at 35°C, followed by germination at alternating (20/30°C) temperatures, making it possible to isolate and define the plasma membrane proteins unique to the dormant, non-dormant and germinating caryopsis. In this study, the membrane proteins were separated by one-dimensional SDS-PAGE and characterized by molecular mass. The major changes in the plasma membrane protein profiles during the transition from the dormant to the non-dormant state were an increase in the 20, 22 and 23 kDa proteins, a decrease in the 15 and 18 kDa proteins, and the appearance of a <22 kDa protein. Following 24 h of germination, the changes in the 20, 22, 23 and <22 kDa proteins were *reversed*, consistent with the catabolism of propanol and the restoration of pretreatment headgroup spacing. This reversal would not be expected to occur in the presence of the ADH inhibitor, 4-MP, and we suggest that this accounts for the inhibition of germination by 4-MP in ethanol-treated dormant caryopses of red rice (Lin and Cohn, 1997).

A similar study of secondarily dormant cucumber (*Cucumis sativus* L.) seeds identified changes in the plasma membrane proteins of the embryonic axes from ethanol-treated dormant seeds, relative to untreated dormant seeds (Sreenivasulu and Amritphale, 2000). Here, the dormancy-breaking chemical was applied to the dry seed (rather than to the imbibed seed as in the study cited above). After 21 h of imbibition, both dormant and non-dormant cucumber seeds have completed Phase II of water uptake and non-dormant seeds will then germinate. A comparison of the plasma membrane protein profiles from 21-h-imbibed, untreated dormant seeds and 21-h-imbibed, ethanol-treated dormant seeds showed several differences. The ethanol-induced transition to the non-dormant state was characterized by an increase in the 20 and 36 kDa proteins and the decline of a 14 kDa protein.

Unlike the dormancy in barnyard grass, the secondary dormancy in cucumber seeds, induced by far-red treatment of partially imbibed seeds, is controlled by phytochrome. Therefore, it is not surprising to find that different changes occur in the membrane protein profiles which accompany dormancy breaking in these two species. While the data from cucumber and barnyard grass do not permit correlations between specific changes in the plasma membrane protein composition and the transition from the dormant to the non-dormant state, they do establish that such changes occur, both in primary and in secondary dormancy. Further, in companion studies from two laboratories (Di Nola *et al.*, 1991; Sreenivasulu and Amritphale, 1998), DPH (1,6 diphenyl-1,3,5-hexatriene) fluorescence anisotropy data provide empirical evidence that these short-chain alcohols, known to partition into the lipid–water interface of membranes, increase membrane fluidity at dormancy-breaking concentrations, coincident with the changes in membrane protein composition described above.

In the absence of perturbing molecules, the area per lipid is determined by the headgroup and by the degree of unsaturation of the acyl chains. One parameter by which phospholipids are characterized is molecular shape, e.g. cylinder, cone or inverted cone (Cullis *et al.*, 1986). Phospholipids having headgroup and acyl-chain regions which occupy similar volumes, e.g. phosphatidylcholine (PC), are described as cylinders and are predicted to form stable bilayers. However, phospholipids with small headgroups, e.g. phosphatidylethanolamine (PE), are described as cone-shaped and, under certain conditions, form the hexagonal II (H_{II}) phase, while those having small acyl-chain regions, e.g. lysophospholipids (LPLs), are described as inverted cones and tend to form micelles. Due to their geometry, cone- and inverted cone-shaped lipids create packing stresses within a bilayer. A measure of the packing stress is given by the intrinsic curvature of the membrane, a property which may be either positive or negative. Membranes having a negative intrinsic curvature, due to lipids such as PE and diacylglycerol, are said to have a *propensity* to form the inverted hexagonal phase, H_{II}. When such lipids are confined to a stable bilayer, this propensity is not expressed. Instead, lipids such as PE adjust their molecular shape to approximate a cylinder: the headgroup region enlarges due to an increase in the interstitial hydrogen-bonded water and the acyl-chain region compresses due to increased ordering (Slater *et al.*, 1994; Gawrisch and Holte, 1996). Such an arrangement is described as being energetically 'frustrated' (Stubbs and Slater, 1996).

Lipids with an H_{II} propensity are linked to the attachment and activation of a number of peripheral proteins. Researchers studying PKC, a peripheral protein common to many animal signal transduction pathways, have correlated its activation with the intrinsic curvature of the membrane and with phospholipid headgroup spacing (Slater *et al.*, 1994). When PKC activity was assayed in mixed PE/PC vesicles, maximum activity occurred at 40% PE and declined sharply above and below this concentration of PE, thus defining a narrow range for optimal activity. PKC activity was also assayed in PC vesicles of different diameters, ranging from large (400 nm) vesicles to very small (25 nm) vesicles; the very small

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diameter (25 nm) vesicles had the highest PKC activity. As vesicle diameter decreases, the headgroup spacing in the outer leaflet increases. A similar headgroup spacing effect occurs when PE is confined to a bilayer stabilized by PC. In both cases, the interstitial hydrogen-bonded water in the headgroup region increases, thereby decreasing the density of the headgroup region. This may facilitate the insertion of PKC into the membrane. These experiments confirm that the effect of PE is due to a change in headgroup spacing and not due to a specific interaction with PKC.

Daunomycin, an anthracycline used in the treatment of leukaemia, induces the dissociation of two types of peripheral proteins involved in signal transduction from the plasma membranes of rat cortical cells (Escriba *et al.*, 1995). Enrichment of these membranes with PE, but not PC, increases the binding of G proteins and PKC, even in the presence of daunomycin. Experiments on synthetic PE membranes show that daunomycin raises the lamellar to hexagonal phase-transition temperature (T_H), thus reducing the propensity of PE to form non-bilayer structures. Although it was once thought that maximizing bilayer stability was important for cell function, researchers are becoming increasingly aware of the importance of non-lamellar lipids in cell physiology (Dowhan, 1997). The effect of daunomycin on signal transduction suggests a mechanism by which inhibitors of germination may operate, and that such dormancies may be overcome by changes in the phospholipid composition of the membrane.

Red rice has a coat-imposed dormancy, i.e. removal of the hull and pericarp relieves dormancy (Cohn and Hughes, 1981). Hull, testa and pericarp tissues may contribute to dormancy by impeding gas exchange or water uptake, by providing mechanical resistance to germination, or by restricting the loss of inhibitors via diffusion during imbibition (Bewley and Black, 1994). In the case of red rice, by invoking an inhibitor to germination it is possible to accommodate the coat-imposed dormancy with the known effect of ethanol and dry after-ripening on the release of the intact or dehulled seed from dormancy; it is plausible, but unsubstantiated, that in red rice an inhibitor to germination is deposited in the pericarp during development and that during imbibition it becomes solubilized and diffuses into the embryo. While there is no empirical evidence either for or against germination inhibitors in red rice, dormancy in cereal caryopses is thought to be related to high concentrations of free phenolic acids and oxidized flavonoid polymers (Debeaujon *et al.*, 2000, and references therein). The mechanism of this inhibition is not known, but the structures of these substances suggest that they interact with membranes in a manner similar to daunomycin. If this is so, the dissociation of such an inhibitor from the membrane may be induced by the application of dormancy-breaking substances which, like ethanol, bind to the lipid–water interface, or by changes in the phospholipid composition of the membrane, suggested to occur during dry after-ripening.

Dormancy in *O. sativa* (red rice) can be broken by dry after-ripening for 60 d at 22°C (Footitt and Cohn, 1992). The process of after-ripening is not understood, although it is known to be a function of temperature (Bewley and Black, 1994). The rate of dry after-ripening could depend on either diffusion, a linear function of temperature, or enzyme activity, in which case the log(rate) is a negative linear function of the inverse of temperature. Arrhenius plots prepared from germination data on four varieties of *O. sativa* (Roberts, 1965) are shown in Fig. 3A. The strong linear relationship between the log(rate) and the inverse of temperature predicts that dry after-ripening is an enzymatic process and not a diffusion-limited process. The slopes of these plots, and therefore the activation energies derived from these slopes, are very similar (Robyt and White, 1990). This indicates that the rate-limiting step is the same in these varieties of *O. sativa*. When the data from Roberts (1965), for temperatures ranging from 27 to 47°C, are compared with a smaller set of data for red rice at temperatures of 30, 20 and 5°C (Cohn and Hughes, 1981), a discontinuity in the linear nature of the Arrhenius plot is seen to develop as temperature decreases to 5°C (Fig. 3B). This is suggestive of a change in state with cooling (Raison, 1980). Phospholipids characteristically undergo a phase change, from liquid crystalline to gel, with decreasing temperature. Similarly, biological membranes, i.e. mixed lipid systems, become increasingly 'ordered' as temperature decreases. This observation suggests that phospholipids and, perhaps, the enzymatic modification of phospholipids are key components of the process of dry after-ripening. Several membrane-bound enzymes (including phospholipase A_2, phospholipase C, *N*-acylphosphatidylethanolamine synthase and acyl-chain desaturases) are candidates for the *in situ* modification of phospholipids in the dry state. It is possible that the selective activity of such enzymes optimizes the intrinsic curvature of the membrane, permitting an event critical to germination to proceed, thus breaking dormancy.

Recently, the enzymatic modification of phospholipids by phospholipase A_2 (PLA_2) and phospholipase C (PLC) has been reported to occur at low water contents. PLA_2 and PLC activities have been documented in unilamellar phosphatidylcholine (PC) vesicles, freeze-dried in the presence of trehalose and then rehydrated at various relative humidities (Oliver *et al.*, 1997). These partially rehydrated liposomal membranes are analogous to the

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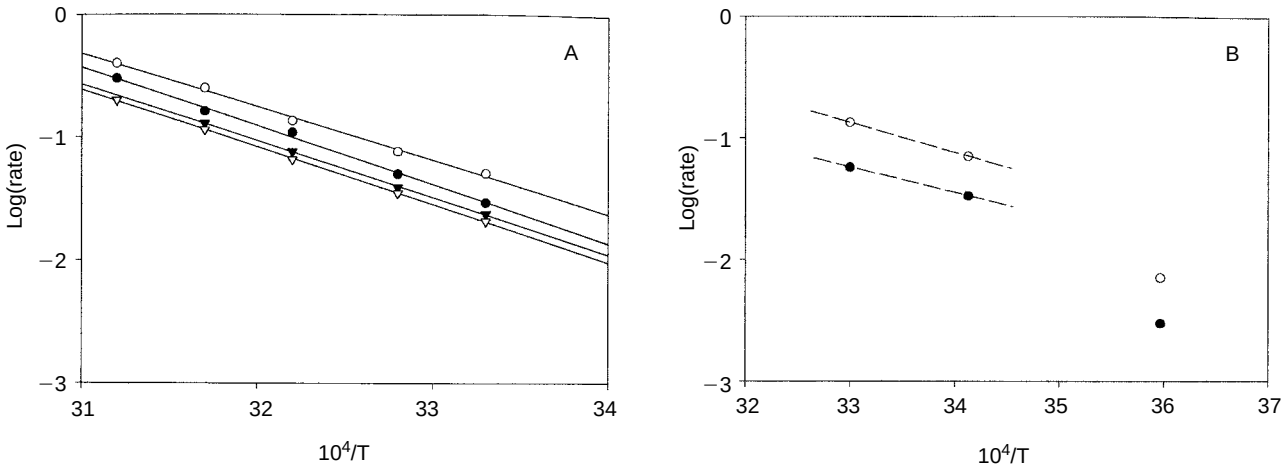

Figure 3. Arrhenius plots showing the relationship between the rate of dry after-ripening and temperature in caryopses of *Oryza sativa* L. The rate, d^{-1}, is derived from the number of days of dry after-ripening required for 50% dormancy breaking. Temperature, T, corresponds to the Kelvin scale. (A) Effect of dry after-ripening at 27, 32, 37, 42 and 47°C on four varieties of *O. sativa* (Mas 2401, ○; Nam Dawk Nai, ●; India Pa Lil, ▼; and Lead 35, ▽). Germination tests on intact caryopses were conducted in darkness at 32°C for 7 d. (Data from Roberts, 1965.) (B) Effect of dry after-ripening at 5, 20 and 30°C on red rice [*O. sativa* L. 'strawhulled (SH)']. Germination tests on dehulled (○) and intact (●) caryopses were conducted in darkness at 30°C for 7 d. (Data from Cohn and Hughes, 1981.)

membranes in the dry orthodox seed, where part of the bound water has been removed and replaced by a mixture of sucrose and oligosaccharide during the maturation drying phase of development. Oliver and co-workers (1997) reported that PLA_2 activity occurs at PC hydration as low as two water molecules per lipid molecule, whereas fully hydrated PC binds 10–12 water molecules. This level of hydration is consistent with that of red rice caryopses (which have a moisture content of 11–12%, indicative of the partial removal of bound water), lending credibility to the suggestion for enzyme activity within the lipid phase during dry after-ripening. PLA_2 and PLC are of particular interest because their products are non-bilayer-forming lipids which have opposing effects on membrane intrinsic curvature (Cornell and Arnold, 1996). Lysophospholipids (LPLs) and fatty acids, the products of PLA_2 activity, prefer the micellar (H_I) phase, thereby contributing to positive intrinsic curvature, while diacylglycerol (DAG), the lipid product of PLC activity, prefers the inverted hexagonal (H_{II}) phase, contributing to negative intrinsic curvature.

The activity of the porcine pancreas PLA_2 used in the experiments reported by Oliver *et al.* (1995, 1997) is known to depend on the physical state of the membrane. In partially hydrated vesicles, similar to those described above, factors which increase the area per lipid, e.g. charge, hydration state, and degree of unsaturation of the fatty acyl chains, are correlated with increased PLA_2 activity (Oliver *et al.*, 1995). It is thought that these factors enhance PLA_2 access to the *sn*-2 bond of phospholipids and that water bound to the headgroup region is utilized in the subsequent hydrolysis of this bond. It is probable that the physical state of the membrane also regulates the activity of other phospholipid-modifying enzymes acting *in situ*. The discontinuity observed in the Arhennius plot of dry after-ripening in *O. sativa* (Fig. 3B), which arises with cooling, may reflect reduced access to the phospholipid substrate, due to increased ordering of membrane lipids at lower temperatures. This interpretation is supported by the substantial decrease in membrane surface area known to occur in the gel to liquid crystalline phase transition. For example, DMPC vesicles cooled through the phase transition are reported to have a 22% decrease in surface area (Needham and Evans, 1988).

Although PLA_2s from animal sources have been well studied, perhaps due to the abundance of the secretory forms from the pancreas and from bee and cobra venoms, convincing evidence for PLA_2 activity in plant tissue has lagged behind. In 1994, Kim and co-workers reported the partial purification and initial characterization of two PLA_2s from *Vicia faba* leaves. More recently, Stahl and co-workers (1998) reported what may be the first purification to homogeneity of a plant PLA_2. The enzyme was isolated from the soluble protein fraction extracted from developing embryos of elm (*Ulmus glabra*). This elm PLA_2 is similar in many respects to animal secretory PLA_2. It has a low molecular mass of

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13.9 kDa, requires Ca^{2+} for activity, contains 12–14 Cys residues, is inactivated by the disulphide-bond-reducing agents dithiothreitol (DTT) and β-mercaptoethanol, and exhibits optimal activity at alkaline pH. In addition, the N-terminal region of this elm PLA_2 shows a high degree of homology to the expressed sequence tag (EST) of a putative rice PLA_2. This evidence predicts that elm PLA_2 is a secreted protein and suggests that its substrate is the outer leaflet of the plasma membrane. If this is so, the plasma membrane will be modified to the exclusion of internal membranes, i.e. organelle membranes. The slow 'flip-flop' of phospholipids between the inner and outer leaflets of the membrane will then equilibrate the products of PLA_2 activity between the leaflets. The function of this secretory PLA_2 in elm embryos is unknown, but one can speculate about its impact on membrane properties.

Because PLA_2 and PLC activities have been demonstrated in the dry state, it is plausible that other membrane-bound enzymes, relying only on diffusion in the lipid phase, are also active. *N*-acylphosphatidylethanolamine (NAPE) synthase, studied in the microsomal membrane fraction from cottonseed cotyledons, acylates the headgroup of PE with free fatty acids to form NAPE (Chapman and Moore, 1993). Since this changes the molecular shape of PE, it has been suggested that NAPE functions to stabilize the membrane structure (Sandoval *et al.*, 1995). However, the quantity of PE in cottonseed membranes (20–25 mol%) is not considered to be a threat to bilayer stability. Further, when mixed NAPE/PC vesicles are subjected to a hypotonic stress similar to imbibition, permeability (measured by solute loss) is directly correlated with NAPE content (Domingo *et al.*, 1993). An alternative interpretation is that the ratio of NAPE:PE functions to regulate the intrinsic curvature of the membrane and, therefore, the binding of certain peripheral proteins. This interpretation is supported by the observation that cottonseed is non-dormant and maintains a relatively constant NAPE:PE ratio during 4 h of imbibition, even though the total phospholipid content of a seed increases.

Acyl-chain desaturases (Harwood, 1996) are also candidates for the *in situ* modification of membrane lipids in the dry state. The C_{18} fatty acids oleic, linoleic and α-linolenic constitute 85% of the membrane fatty acids in plants. They are synthesized sequentially from stearate by Δ^9-, Δ^{12}- and Δ^{15}-desaturases located on the endoplasmic reticulum. The relative activity of these lipid-linked desaturases is inversely correlated with temperature, suggesting that membrane fluidity regulates their activity. With increasing unsaturation, the molecular shape of a phospholipid changes. The increased volume of the acyl-chain region causes a curvature stress which then induces a headgroup spacing effect at the lipid–water interface (Stubbs and Slater, 1996). The magnitude of this headgroup spacing effect depends on the specific headgroup and on the degree of unsaturation of the acyl chains.

Testing the hypothesis

Dormant seeds can be separated into one of two categories, depending on whether dormancy is naturally broken in the imbibed seed or in the dry seed. This distinction suggests that in the imbibed seed, dormancy-breaking activity requires diffusion in the aqueous (cytosolic) phase, whereas in the dry seed, dormancy-breaking activity requires diffusion in the lipid (membranous) phase. This observation would seem to indicate that two very distinct mechanisms of dormancy breaking are operational in imbibed seeds versus dry seeds. However, the fact that ethanol pretreatment and/or temperature changes are effective in breaking dormancy in both groups suggests a common mechanism.

The dry seed

The hypothesis that the intrinsic curvature of the plasma membrane regulates the binding and activation of a peripheral protein component of a signal transduction pathway preceding germination can be tested by comparing analyses of the plasma membrane fraction prepared from the embryos of freshly harvested (dormant) and dry after-ripened (non-dormant) *O. sativa*, or other species behaving in a similar manner. If a compositional change occurs during dry after-ripening, it will be necessary to consider how this change might affect the dynamic and structural properties of the membrane. It is well documented that the intrinsic curvature, a property related to the binding of PKC and other peripheral proteins, will be affected by changes in the mole fraction of non-bilayer-forming lipids such as PE. However, smaller compositional changes may also affect the activation of peripheral proteins. For instance, PKC activity in mammalian cells is promoted by phosphatidylserine and diacylglycerol, while a plant protein kinase is activated by lysophospholipids, fatty acids and phosphorylated phosphatidylinositols (Scherer, 1996). Therefore, the accumulation of products of phospholipid metabolism and the covalent modification of phospholipids *in situ* are of particular interest with regard to the process of dry after-ripening.

The imbibed seed

The headgroup-spacing hypothesis has a critical requirement for a peripheral membrane protein which equilibrates between the soluble and bound

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conformations. Such a protein would be soluble and, therefore, not detectable in the protein profiles of the plasma membranes from dormant seeds. Following the transition from the dormant to the non-dormant state, it would appear as a 'new' protein in the profiles from non-dormant seeds. The <22 kDa protein from *E. crus-galli* (Di Nola *et al.*, 1990) meets these criteria. This band then decreases during the first 24 h of germination, consistent with the catabolism of propanol and the concomitant reduction of headgroup spacing. Further, the absence of a <22 kDa band on a fluorogram of plasma membrane proteins following a 24 h incubation with [^{35}S]methionine during dormancy breaking indicates that it is *not* a newly synthesized protein. We interpret this to mean that the <22 kDa protein was present in the cytosol of the dormant seed, but did not bind to the membrane until dormancy breaking occurred subsequent to *n*-propanol treatment. Technology now permits the isolation of such a protein by separation using two-dimensional polyacrylamide gel electrophoresis, and elucidation by microsequencing of internal and terminal peptide sequences. These can be matched to known proteins using database searches, with the possibility of identifying the nature and function of the protein.

Secondary dormancy in cucumber is artificially induced by far-red irradiation of partially imbibed seeds, obtained by imbibition in –1.8 MPa PEG (Sreenivasulu and Amritphale, 2000). This treatment renders PhyB inactive, while preventing the development of adequate turgor for cell expansion. This $PhyB_R$-imposed dormancy can be circumvented by ethanol treatment sufficient to increase membrane fluidity, permitting dark germination in the absence of $PhyB_{FR}$. Although it is puzzling that a small molecule like ethanol can replace P_{FR}, permitting the germination of these dormant seeds, the recent finding by Nagy *et al.* (2000) of nuclear import of *strongly* overexpressed PhyA–GFP and PhyB–GFP in dark-grown tobacco seedlings challenges our concept of phytochrome behaviour, particularly that the P_R to P_{FR} conformational change induced by absorption of red light is necessary for nuclear import. Their interpretation of this finding is that the NLS is not masked in the P_R confomer, that both the P_R and P_{FR} confomers are actively translocated to the nucleus, but that a cytosolic retention mechanism specifically binds P_R. When P_R is strongly overexpressed, the capacity of the retention mechanism is exceeded, permitting nuclear import of the P_R confomer. This interpretation is applicable to the P_R-induced dormancy in cucumber, where a dormancy-breaking treatment with ethanol completely bypasses the requirement for red light, suggesting that ethanol may act either by disrupting the cytosolic retention mechanism or by increasing nuclear membrane permeability. Further investigation of nuclear-localized P_R, i.e. whether nuclear P_R is competent to regulate the expression of light-controlled gene complexes, and, also, the identification of the cytosolic P_R retention mechanism may explain the dormancy-breaking effect of ethanol in phytochrome-controlled seed dormancies.

Discussion

The Meyer–Overton hypothesis of anaesthetic action, based on the direct correlation between the anaesthetic potency of a chemical and its lipophilicity, promoted the membrane as the site of anaesthetic action (Seeman, 1972). Initial reports of the fluidizing effect of anaesthetics in synthetic membranes supported the membrane theory. However, changes in membrane fluidity could not be demonstrated at clinical concentrations of general anaesthetics, e.g. chloroform and halothane, in biologically relevant model membrane systems containing at least 10 mol% cholesterol (Franks and Lieb, 1982). Recognizing that certain anaesthetics bind to proteins and that this binding is also correlated with the octanol/water partition coefficient, a protein site for anaesthetic action was proposed. According to this model, anaesthesia is induced by the binding of anaesthetics to the hydrophobic region of sensitive proteins, causing a loss of function.

Debate between the membrane theory and protein theory of anaesthesia has created uncertainty regarding the site of dormancy breaking in seeds, by ethanol and other small amphipathic molecules (Hilhorst and Cohn, 2000). There is, however, a fundamental structural difference between the general anaesthetics, e.g. chloroform and halothane, and the series of short-chain primary alcohols that are effective in dormancy breaking. Unlike the alcohols, general anaesthetics cannot hydrogen bond to phospholipid headgroups. And, furthermore, unlike the alcohols, general anaesthetics do not impart a fluidizing effect on membranes at biologically active concentrations. Although the potency of anaesthetics and the activity of dormancy-breaking chemicals are both correlated with their non-aqueous/aqueous partition coefficients, they do not necessarily act at the same site. It is plausible that anaesthetics act on specific proteins, but that dormancy-breaking chemicals act on membranes.

Ethanol has been described as a 'promiscuous' molecule, i.e. one that binds indiscriminately to virtually all biological materials (Komatsu *et al.*, 2000), making it difficult to designate either membrane lipids or the hydrophobic region of a protein as the binding site leading to dormancy breaking. In the absence of direct experimental evidence for a

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membrane site, the anaesthetic hypothesis has relied on certain types of indirect evidence for validation, e.g. the pressure reversibility of the effect, changes in membrane properties, and the correlation of leakage with dormancy-breaking treatments. Although a large body of data supports the membrane theory, some anomalous data have cast doubt, and the viability of the anaesthetic hypothesis has been questioned (Cohn and Hilhorst, 2000). Since the initial publications in the 1970s, which established that membranes may play a role in the dormancy of many species, there has been little progress in defining that role. It has been an experimentally difficult problem to approach. The extension of the anaesthetic hypothesis by the headgroup-spacing hypothesis, developed herein, defines a specific role for membranes in seed dormancy and germination, one that is supported by a new interpretation of existing data. The headgroup-spacing hypothesis may not explain all seed dormancies, but it can be applied to those in which small amphipathic solutes, changes in temperature and, perhaps, dry after-ripening are effective as dormancy-breaking treatments.

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