1.2

Light

Light stress is not always a consequence of too much light; competition for light in the shade of a tree canopy is likewise stressful and can lead to complex morphological and physiological changes. One characteristic of shade leaves is a large leaf area but a low leaf weight. This feature increases the light-harvesting area, and also shades competing neighbouring plants. The genus *Cecropia* is particularly successful in adapting to light conditions in forming enourmous leaves. *Cecropia* trees also live in symbiosis with the ants that colonise the hollow stems. The ants chase damaging insects and provide the host with nitrogen and phosphate, nutrients which are limiting factors in tropical forests. La Carbonnera Reserve near Merida, Venezuela.
Photo E.-D. Schulze

Recommended Literature

- Dekker JP, Boekema EJ (2004) Supramolecular organization of thylakoid membrane proteins in green plants. Biochim Biophys Acta (Bioenergetics) in press
- Huner NPA, Öquist G, Sarhan F (1998) Energy balance and acclimation to light and cold. Trends Plant Sci 3:224–230
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Sunlight, by far the dominating energy source for all life on earth, varies from place to place, time of day to time of day, and season to season, in intensity and also in spectral composition (UV light will be discussed in a separate chapter). As primary producers, plants need to adapt to different light environments. Too little light means a negative energy balance, i.e. gain from photosynthesis does not cover energy require-

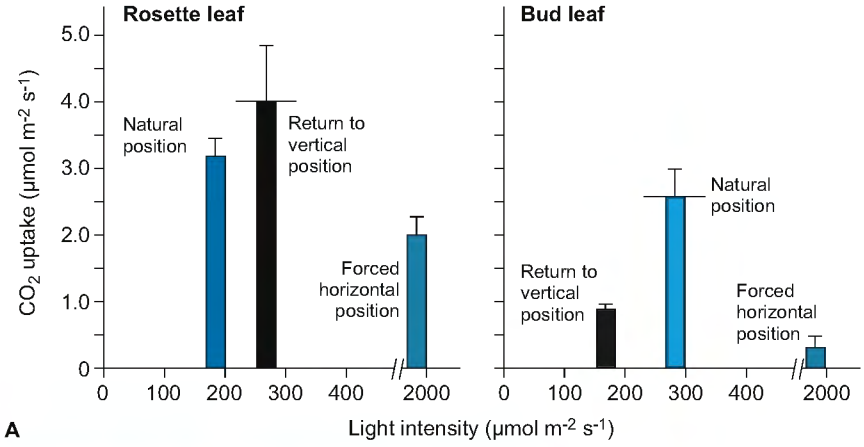


Fig. 1.2.1. Demonstration of photoinhibition in *Lobelia rhynchopetalum* of high mountains in East Africa [Sanetti Plateau (3900 m), Ethiopia]. A Net photosynthetic rate of a leaf from the rosette and from the bud in natural positions, when held horizontally (excess radiation) and after their return to the natural position. Whilst the rosette leaves are only marginally affected by the high light intensity and even have a higher rate when returned to the normal position, the photosynthetic rate of the outer leaves of the leaf bud is inhibited by more than 90% by the excess light and returning them to the normal position results in only a small recovery. B Structure of the terminal leaf rosette of *L. rhynchopetalum*. Photo E. Beck

ments. Excess light enters the chloroplast, is absorbed, but the energy is not used by assimilation, fluorescence or dissipated as heat, and so may lead to severe oxidative stress.

Adaptation to each light environment is present at all levels from the molecular to the morphological and may be permanent, e.g. formation of sun or shade leaves, or dynamic as observed in the movement of chloroplasts or leaves.

1.2.1
Visible Light

1.2.1.1
Morphological and Structural Adaptation

Lack of light is the main stressor for vegetation on the forest floor. At the edge of the forest, stems or shoots of plants of the forest floor bend so that the leaf area is able to absorb as much light as possible. This can be easily observed with bracken (*Pteridium*) that is often

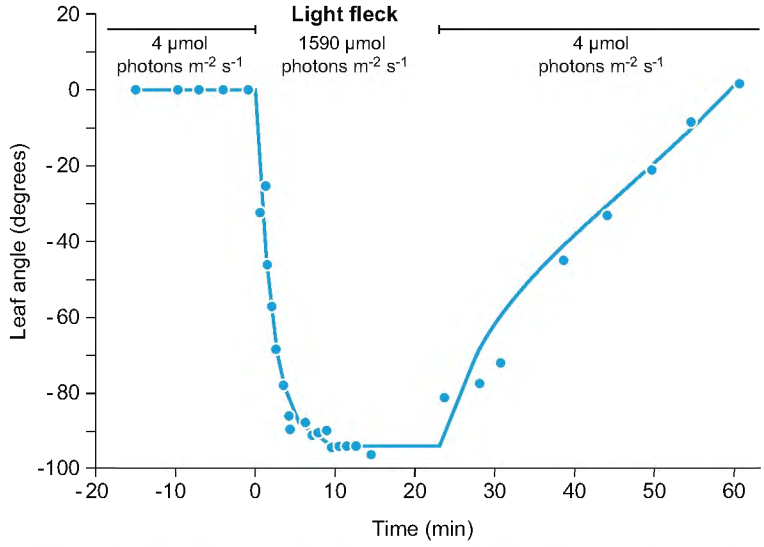


Fig. 1.2.2. Avoidance of light stress by changing the leaf angle. In the shade plant *Oxalis oregana*, the lowering of leaflets upon exposure to a light fleck, and then return to the normal day position after the light fleck moves away, are shown. (After Björkman and Powles 1981)

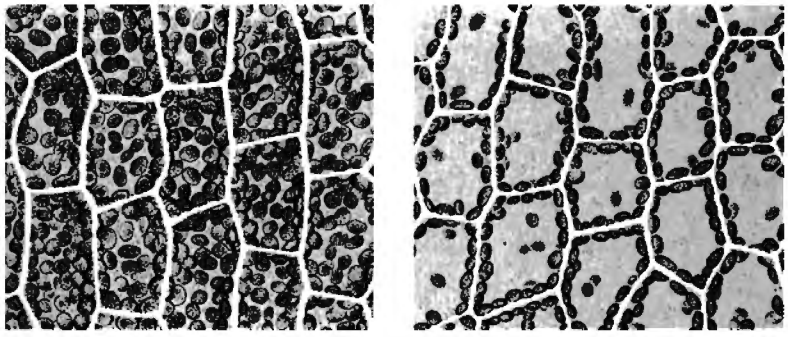


Fig. 1.2.3. Minimisation of light stress by chloroplast movement. Surface view of leaf cells of the moss *Funaria hygrometrica*. A Position of the chloroplast on the horizontal cell surface in weak light. B Position on the side walls of cells under high light. (Nultsch 1996)

found at the edges of clearings in a forest. Lianas and epiphytes are light parasites avoiding the scarcity of light within the stand by using trees as support, and thus capturing resources which are then available for the development of their own leaves and flowers in the canopy of the supporting plant.

It is different for **surplus of light**: leaves often avoid excessive radiation by adopting a parallel position to the incident light (see also Chap. 2.1, Fig. 2.1.14). The hanging leaves of *Eucalyptus* are well known, forming the "shade-less forests" in Australia. The vertical position of leaves has been shown to be an important avoidance strategy for tropical plants in high tropical mountains (Fig. 1.2.1).

The position of the leaves of many plants changes during the day, affecting the angle of the incoming light and thus the intensity of incoming radiation. Such leaves often have pulvini (particularly Leguminosae), which enable plants to change the position of their leaves or pinnules (see also Fig. 2.1.14). The reaction of the North American wood sorrel to a short-term light stress of strong intensity caused by a sun ray ("light fleck") is shown in Fig. 1.2.2. The sensor for the light intensity is possibly a pigment absorbing blue light.

Avoidance of light stress in shade plants can also be observed at the cellular level. In many algae and mosses, but also in some cormophytes, chloroplasts move to the lesser irradiated, vertical surfaces of the cell and turn their front side towards the incoming light. In weak light they are found in positions with their larger surface exposed to the incident light (Fig. 1.2.3).

Besides such short-term adaptations (reaction time within minutes), the development of leaves is also affected by the light environment, with the formation of sun and shade leaves (Fig. 1.2.4 and Table 1.2.1).

Sun leaves are usually small, but thick, because they possess a well-developed mesophyll, frequently with several layers of palisade cells.

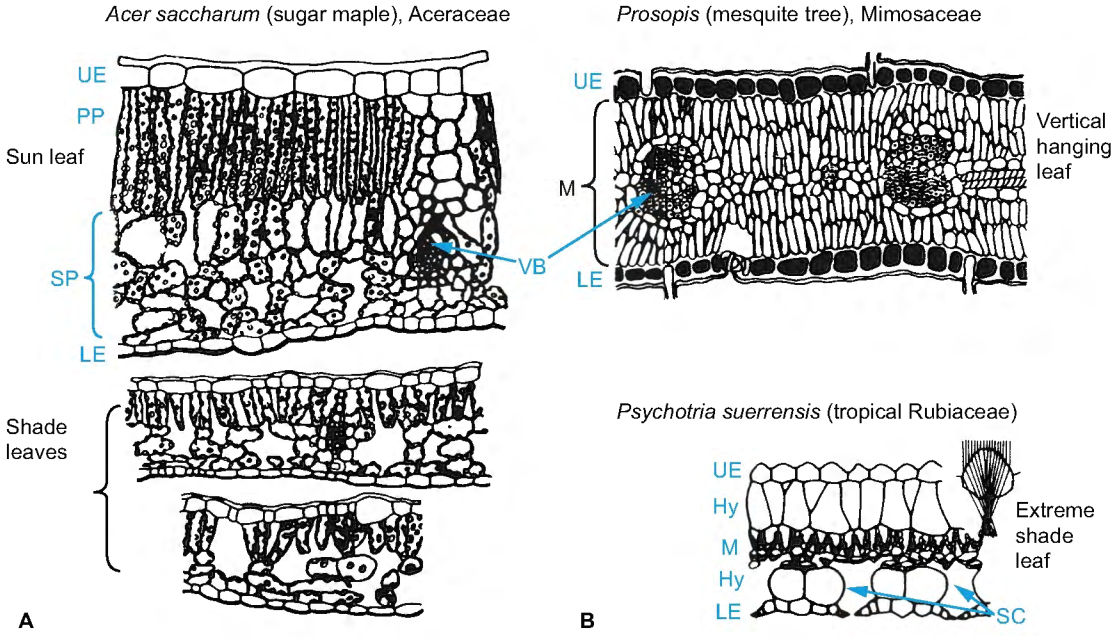


Fig. 1.2.4. Anatomical adaptation of leaves to the light environment. A The anatomical structure of leaves from light and shade positions in the crown of sugar maple is shown. The weaker the light intensity, the less developed is the photosynthetic parenchyma. B The mesquite tree has hanging leaves of equifacial anatomical structure which only absorb diffuse solar light when the sun is at its highest. The vacuoles of the thick-walled epidermal cells are filled with protective pigments. *Psychotria*, a relative of coffee, grows in deep shade in tropical rain forests in very low light. Lens-shaped cells in the upper epidermis are very characteristic and could gather light for the weakly developed mesophyll. *UE* Upper epidermis; *LE* lower epidermis; *PP* palisade parenchyma; *SP* spongy parenchyma; *M* mesophyll; *Hy* hypodermis; *VB* vascular bundle; *SC* substomatal cavity. (After Larcher 1994)

Table 1.2.1. Comparison of characteristics of sun and shade leaves of beech (*Fagus sylvatica*) and ivy (*Hedera helix*), and the range of adaptation of various characters. (Larcher 1994)

Characteristic		*Fagus sylvatica*		*Hedera helix*	
		Sun	Shade	Sun	Shade
Leaf surface	[cm^2]	28.8	48.9		
Thickness of leaf	[μm]	185	93	409	221
Density of stomata	[$dm^2 \cdot g^{-1}$ TS]			0.97	2.6
Stomatal conductance	[$N \cdot mm^{-2}$]	214	144		
Number of chloroplasts					
per surface of leaf	[$N \cdot 10^9 \cdot dm^{-2}$]			5.09	2.45
per volume of leaf	[$N \cdot 10^9 \cdot cm^{-3}$]			1.24	1.11
Chlorophyll concentration (a+b)					
Chl/leaf	[mg]	1.6	1.9		
Chl/surface	[$mg \cdot dm^{-2}$]			8.7	5.5
Chlorophyll a/b		3.9	3.9	3.3	2.8
RuBP-carboxylase activity	[μmol $CO_2 \cdot dm^{-2}\ h^{-1}$]			398	202
Net photosynthesis	[mg $CO_2 \cdot dm^{-2}\ h^{-1}$]	3.5	1.3	22.3	9.4
Light compensation point	[$W \cdot m^{-2}$]	2.5	1		
Light saturation	[$W \cdot m^{-2}$]	8.5	44		
of net photosynthesis	[$\mu mol \cdot m^{-2}\ s^{-1}$]			600	250
Dark respiration	[$mg \cdot dm^{-2}\ h^{-1}$]	0.5	0.16		

Leaves with predominantly vertical orientation are usually equifacial, i.e. palisade cells are found on both sides. **Shade leaves**, on the other hand, are large and tender with a rather weakly developed mesophyll and may possess additional facilities to trap light, e.g. with the epidermis or hypodermal cells acting like lenses (*Psychotria*, Fig. 1.2.4 B). Further differentiation of the epidermis, e.g. by production of dense hairs that reflect incident light, in contrast reduces the light intensity on chloroplasts (see also Chap. 2.1, Fig. 2.1.12). Protective pigments, called chymochromes, in epidermal cells are particularly effective against short-wave radiation. A typical example of that is the so-called juvenile anthocyanin, which protects not yet fully green leaves against the destructive effects of high light intensities (see Fig. 1.2.15).

1.2.1.2 Ultrastructural Adaptation

Adaptation at the subcellular level is, of course, also observed in chloroplasts (Fig. 1.2.5). Chloroplasts of sun leaves possess only small thylakoid grana and only a few individual stromal thylakoids, but plastids of shade leaves possess very large grana and many stromal thylakoids. The importance of the size of the grana and distribution of thylakoids becomes obvious when considering the molecular structure of thylakoid membranes (Box 1.2.1).

As photosystem II (PS II) has a larger antenna apparatus than photosystem I (PS I), over half of chlorophyll-a, almost all chlorophyll-b and most of the xanthophylls are associated with PS II. The largest part of a cross section of a photosystem consists of antenna. Thus a **shade chloroplast** possesses, because of the large proportion of appressed regions (contact areas of thylakoids), a very large proportion of antennae and very few reaction centres relative to the number of chloroplasts. **Chloroplasts of sun leaves**, in contrast, contain very small antennae, but many reaction centres. This can be interpreted as adaptation, as both combinations allow optimal utilisation of the incident light. Such differences are not only found in shade and sun leaves, but also within a single leaf. Chloroplasts on the upper side of the leaf, usually in better light, correspond to the sun type whilst those on the lower side correspond to the shade type. If a leaf is fixed with the upper side down, the thylakoid structures change accordingly.

The dimensions of the antenna complexes may change as well as the proportion of antennae to reaction centres. The so-called major antenna of PS II, **LHC II (light-harvesting chlorophyll protein)**, is subdivided into a smaller, inner complex, termed the **core antenna**, and into a larger, outer circle, the **peripheral or mobile antenna**. Excessive energy pressure on PS II leads to **"photoinhibition"**, a transient destruction of the D1 protein of the affected reaction centre (see Fig. 1.2.8). Permanent photoinhibition leads also to greater damage, resulting in destruction of the thylakoid. When the energy pressure on the reaction centre is high, a large phosphorylation potential is produced; protein kinases are activated which phosphorylate threonine residues in the mobile antenna proteins. The negative charges accumulated as a consequence of the added phosphate groups dissociate

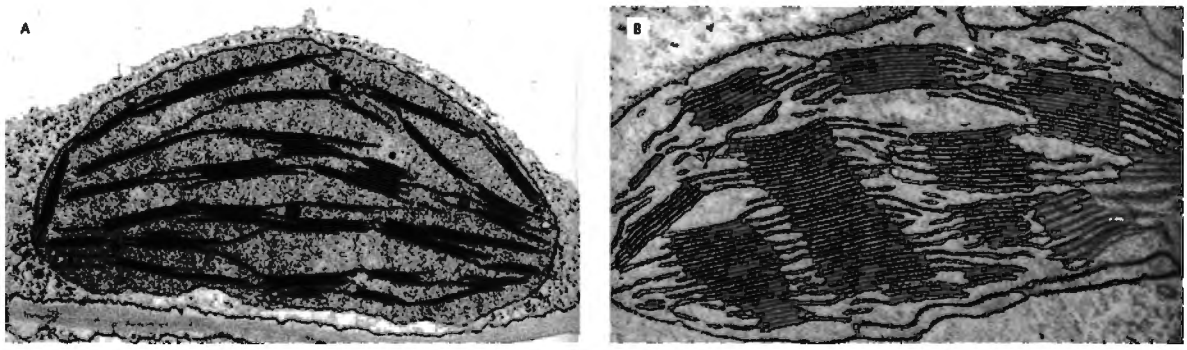
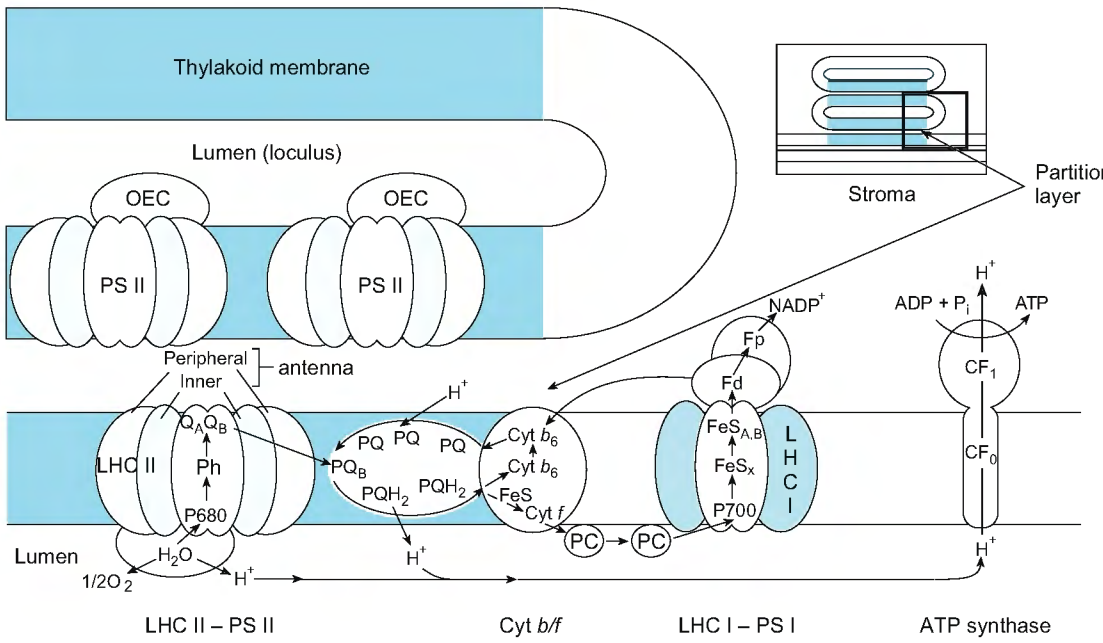


Fig. 1.2.5. Adaptation of chloroplast ultrastructure to the light environment. A Chloroplast from a sun leaf of tobacco. The small granal stacks are typical of chloroplasts from "high-light leaves" (Hall and Rao 1994). B Transverse section of a chloroplast from a shade leaf of antirrhinum (*Antirrhinum majus*) showing substantial thylakoid stacking. (Strasburger 1983)

Box 1.2.1 Ultrastructure of thylakoid membranes

Photosystem I and ATP-synthase occur predominantly in the non-appressed regions of the thylakoid and in those parts of the thylakoid system that have direct contact with the chloroplast stroma; in contrast, photosystem II is predominantly found in the appressed regions or the partition regions and also in the stacks (*blue*). The cytochrome-b_6/f complex is distributed in both regions of the thylakoid systems. The reason for this uneven distribution ("lateral heterogeneity") is not completely clear. As a rule, a chloroplast contains substantially more PS II than PS I. The ratio of PS II to PS I is variable not just between different plants but even within the same plant, and can also change, in the short term, in a single chloroplast (Fig. 1.2.6, from Anderson and Andersson 1988).



OEC Oxygen-evolving complex; *LHC II* light-harvesting chlorophyll protein II; *PQ* plastoquinone; Q_A, Q_B quinones bound to PS II; *Ph* pheophytin; P_{680}, P_{700} chlorophyll dimers of the reaction centres of PS II and PS I; *FeS* Rieske protein; *FeSx*, $FeS_{A,B}$: iron-sulfur proteins in PS I; *Fd* ferredoxin; *Fp* flavoprotein; CF_o integral membrane proton channel of ATP-synthase; CF_1 coupling factor of ATP-synthase.

the phosphorylated external antennae from PS II. This, in turn, reduces their light-absorbing cross section (Fig. 1.2.6). The accumulation of negative charges is also thought to cause a "loosening" of the appressed regions, enabling the lateral movement of protein complexes. There are indications that mobile antennae partially associate with PS I and thus guarantee a more uniform excitation of PS II and PS I and avoid over-reduction of PS I. With decreasing light intensity, protein phosphatases are thought to cause dephosphorylation of LHC II and, subsequently, the mobile antennae return to PS II. This shift of mobile antennae triggered by the over-excitation of PS II, resulting in a higher efficiency of the total electron transport, was described in the early phases of photosynthetic research as state transitions or state I ⇒ state II transition without, however, the molecular mechanisms being understood.

1.2.1.3 Physiological Adaptation

Besides the morphological and structural adaptations of plants to the dominant light environment, physiological-biochemical mechanisms of

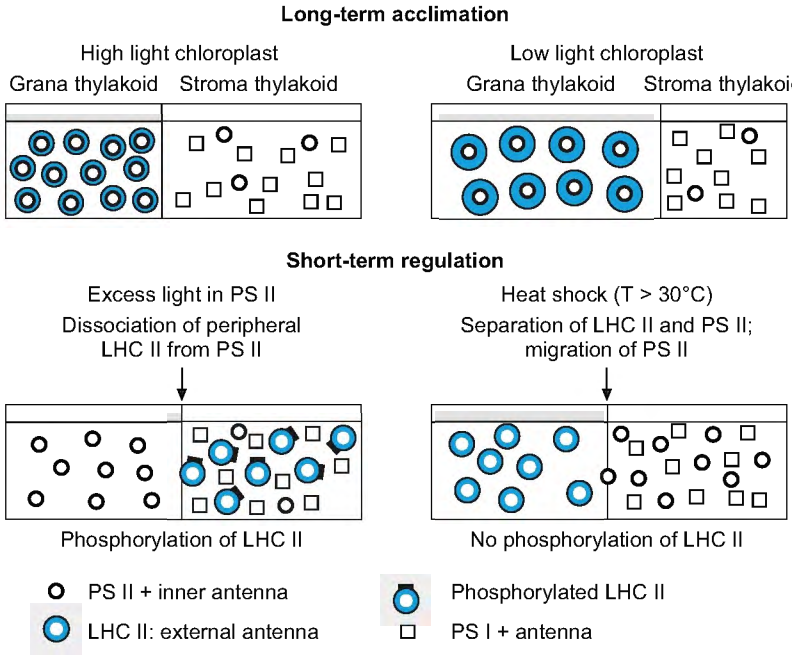


Fig. 1.2.6. Dynamics of the photosynthetic membranes in response to light climate and temperature. (After Anderson and Andersson 1988)

adaptation are known. These mechanisms are able to react even faster than the structural adaptations and thus always take place, regardless of whether structural or morphological changes occur or not.

Starting from the photosynthetic reductive pentose phosphate cycle (Calvin cycle), 2 mol NADPH is required for the assimilation of 1 mol CO_2, corresponding to 4 mol electron and 8 mol light quanta. The cycle also consumes 3 mol ATP. As four electrons produce about 2.7 molecules of ATP, the real requirements for photons or quanta are a little higher. Lowest measured values are 9.4 quanta per mol assimilated CO_2. Light must be the limiting factor to achieve such measured values, i.e. measurements must be in the linear range of the light response curve (see Fig. 1.1.1).

If it is CO_2 supply or activities of the photosynthetic enzymes (e.g. because of cold) rather than light intensity that limit CO_2 assimilation, then more light energy is absorbed than is required. However, what happens to the excess light energy? This energy must be safely dissipated. The plant has several ways of doing this:

- The **glycolate pathway** associated with photorespiration (Box 1.2.2). If the CO_2 supply limits its photosynthesis, photorespiration is able to keep the Calvin cycle running by making phosphoglycerate available and thus maintaining a certain consumption of reducing and energy equivalents.
- The **reduction of nitrite and sulphate** also consumes electrons and ATP. However, it plays a relatively minor role in photosynthetic electron flow.
- **Oxygen** may also be **reduced photosynthetically** (see Chap. 1.3.5 concerning reactive oxygen species). This is called the Mehler reaction. Chloroplasts require NADPH to detoxify the reduced oxygen.

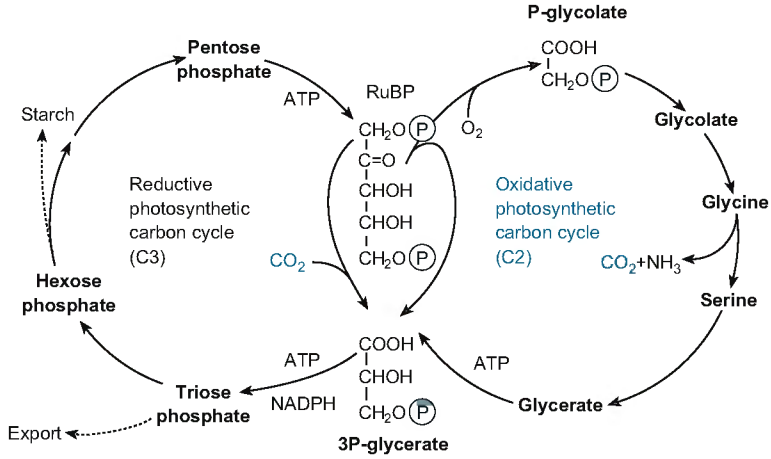
If sufficient electrons are able to flow from water to one of the acceptors, they do not accumulate in one of the components of the electron transport chain. If the rate of consumption falls below the rate of production of reducing power and energy equivalents, other mechanisms of energy dissipation (as yet unexplained) are activated.

- **Non-photochemical energy quenching (NPQ)** and **photoinhibition.**

Plotting the rate of photosynthesis in relation to increasing light, the initial slope of the curve is linear before the rate becomes constant as photosynthesis is saturated (Fig. 1.2.7). The difference between extrapolation of the initial straight line and the rates measured at higher light intensities shows the incident light energy which cannot be used photosynthetically. The higher this value, the more "closed" the reaction centres, i.e. they

Box 1.2.2 Schematic of the photosynthetic reactions determining gas exchange in a leaf

Even when the stomata are closed, CO_2 is produced via photorespiration from the oxidative photosynthetic carbon cycle, and the CO_2 is re-assimilated by the reductive photosynthetic carbon cycle. Consequently, under these conditions, at least part of the absorbed light energy is used. **A** Formal scheme, **B** schematic of gas exchange (after Tolbert 1994).



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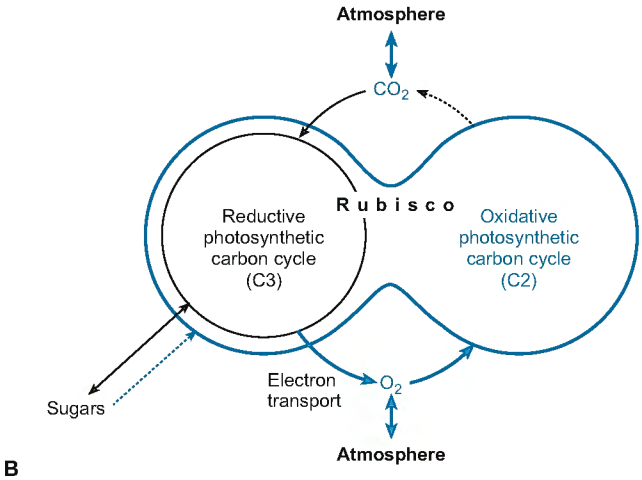


B

are unable to reoxidise by passing on the excited electrons because of the lack of acceptors: the excited reaction centres cannot become photochemically active. A small proportion of the excitation energy will be dissipated as fluorescence with an emission maximum at 685 nm (see Box 1.2.3, Fig. 1). At room temperature this fluorescence is derived exclusively from PS II; excited PS I fluoresces only at –70 °C or at even lower temperatures. Even though the proportion of fluorescent light emitted from the excited PS II is very low, it nevertheless can provide much information about the status of the photosynthetic electron transport chain and the CO_2 assimilation (Schreiber et al. 1994). The most important parameters measured in this way are the dynamics and the proportion of the absorbed light energy used and the proportion of accumulated electrons (over-excitation) dissipated as heat (thermal dissipation; non-photochemical fluorescence quenching: Box 1.2.3). Change

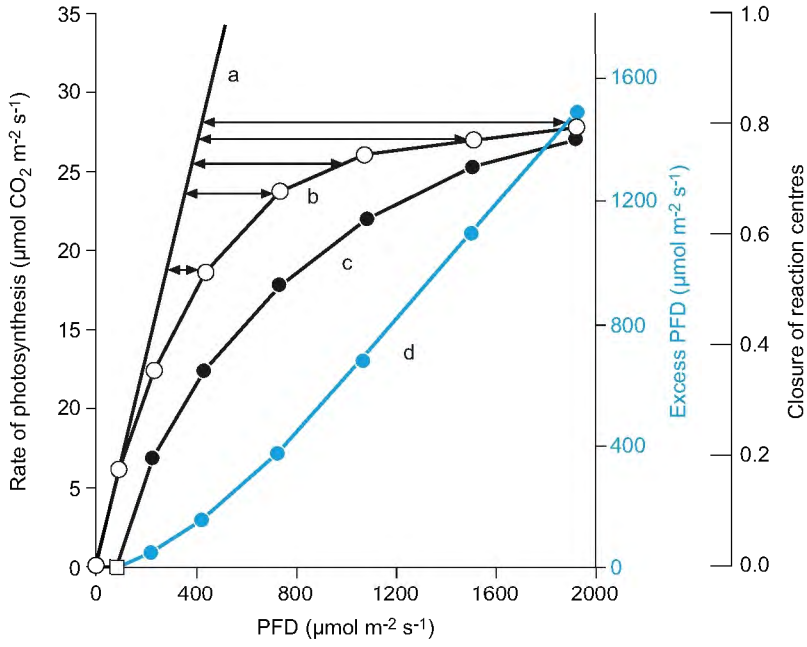


Fig. 1.2.7. Interpretation of a light response curve. *Curve a* Linear increase of the photosynthetic rate in weak light. *Curve b* Photosynthetic rate. *Curve c* Calculated closure of reaction centres of PS II with excess light. *Curve d* Excess light energy (corresponds to the *horizontal arrows* between *a* and *b*). *PFD* Photon flux density. (After Schäfer and Björkman 1989)

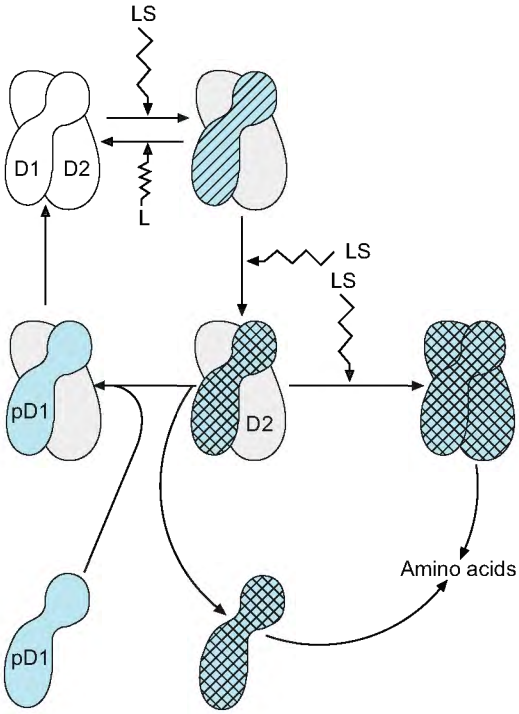


Fig. 1.2.8. Model of the sequence of reactions during damage and repair of the reaction centres of photosystem II. Light stress (*LS*) leads to transient or irreversible damage to the D1 protein (*hatched* or *cross-hatched*, respectively). Irreversible damage triggers cleavage and the subsequent degradation of the D1 protein (presumably to amino acids). A precursor (*pD1*) forms a new reaction centre with the persistent D2 protein which associates again with the antenna protein. In particularly high light stress the D2 protein is also attacked (*cross-hatched*). In the plant cell, the D1 protein has a high turnover rate; the D1 protein is coded in the chloroplast genome. *L* Light. (Schäfer and Schmid 1993)

in these proportions, the so-called rate constants, in favour of thermal energy dissipation is an efficient short-term de-energising reaction which operates during over-excitation of PS II.

Upon long-term exposure to high light, or during particularly strong light stress and during irradiation with UV-B light, this mechanism is not sufficient protection, however. In this case, the core protein, D1, of the PS II reaction centre is degraded, probably with the help of active oxygen species (Fig. 1.2.8) at a specific site and thus the photosystem is deactivated. This process is called **photoinhibition** (of photosynthesis) and is being investigated in many laboratories. It is assumed that

Box 1.2.3 Chlorophyll a fluorescence as an indicator of excitation and redox states

On illumination the chlorophyll molecule can be excited to both states, singlet 2 (absorption of blue light) and singlet 1 (absorption of red light) (Fig. 1). Singlet 2 is too short-lived to induce a photochemical reaction. The singlet 2 state can change to singlet 1 or to the ground state by dissipating energy as heat. Singlet 1 is produced by absorption of a quantum of red light and is sufficiently long-lived to donate an electron to an acceptor and thereby to initiate a photochemical process. Emission of fluorescence is also related to singlet 1. By losing energy as heat, singlet 1 transfers to the ground state but may also, to a lesser extent, convert to the triplet state. Production of the triplet state is dangerous because this excited chlorophyll molecule can react with the normal triplet oxygen, producing singlet oxygen which is a particularly strong oxidising molecule.

If a pre-darkened leaf is illuminated, the absorbed light energy can be used in three ways: by emission of heat (which is the major part), by "photochemistry" and by emission of fluorescence photons. Photochemistry and fluorescence change in opposite ways and in particular proportions, i.e. the distribution (the so-called rate constants) of energy flux to heat and [photochemistry + fluorescence] is constant within the leaf. Maximum fluorescence results when there are no photochemical processes, i.e. when there is no acceptor for the excited electrons in PS II.

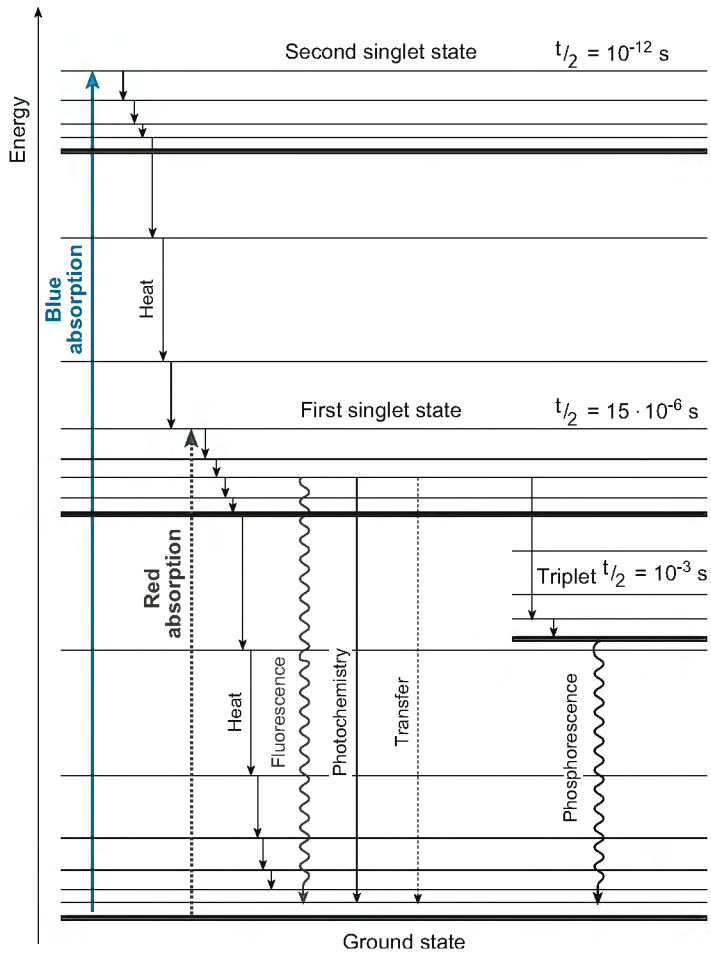


Fig. 1. Different excited states of chlorophyll a caused by light absorption and energy conversion into heat, fluorescence, photochemical work, state 1–state 2 transfer and phosphorescence. (After Heldt 1999)

Box 1.2.3 (continued)

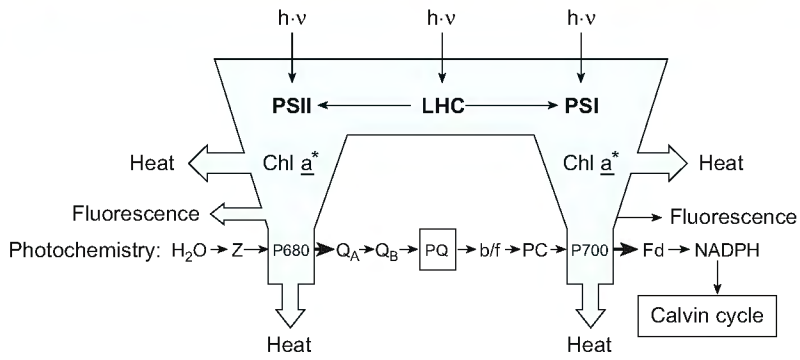
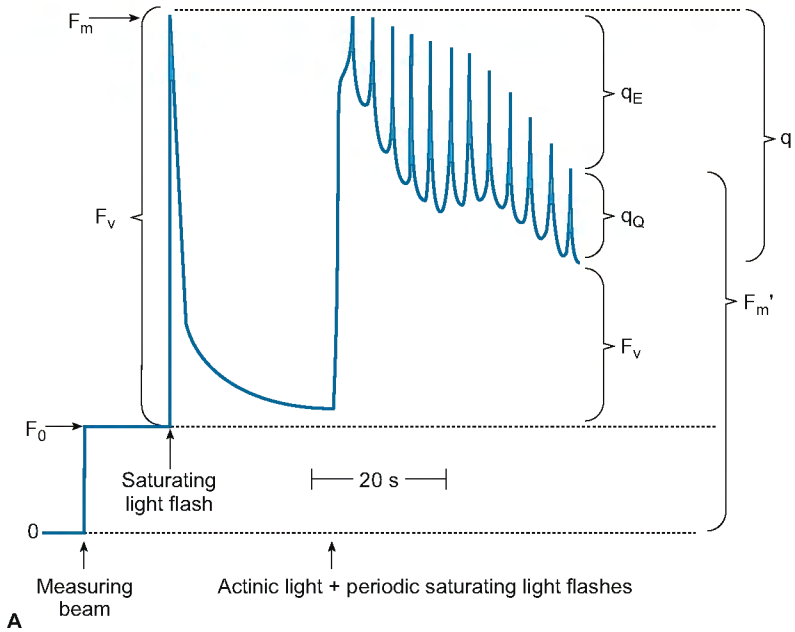


Fig. 2. Use and dissipation of light energy in the photosynthetic apparatus. Light energy absorbed by the photosynthetic apparatus is mainly lost as heat. A small proportion (<10%) is emitted as fluorescence. A variable proportion can be used for photochemical work. With strong illumination the external antenna of PS II can dissociate and, at least in part, associate with PS I. Thereby PS II absorbs less and PS I more light energy. This type of balancing is called "state 1–state 2 transition". The rate constants (thickness of the *arrows*) can be changed by over-energetisation. (After Schreiber et al. 1994)

The photosystem is then closed. The more photosystems that are "open", the more oxidised acceptor is available, the smaller is the fluorescence. From the rate constants of fluorescence, i.e. its intensity, it is possible to determine the rate (efficiency) of the actual photosynthetic process. It is interesting that with a long period of excitation during which electrons are not transferred completely to $NADP^+$ and further to CO_2 or another reducible substrate, a strong decrease in the maximum fluorescence occurs. This reduction of fluorescence with closed reaction centres shows the redistribution of the rate constants in favour of heat emission (thermal dissipation). This is called "non-photochemical quenching" (Figs. 2 and 3).

A number of parameters can be derived from the analysis of chlorophyll fluorescence (Fig. 3). The maximum fluorescence upon illumination of a pre-darkened leaf: F_m. The reduced maximum fluorescence after a saturating light flash: F_m'. The minimal fluorescence of the antenna pigments of a pre-darkened leaf in which photosynthesis does not occur: F_0; in an illuminated leaf, this is generally slightly reduced; it is then called F_0'. The difference between F_m and F_0 is the theoretical maximal useful energy available for photochemistry: $F_v=F_m-F_0$. The actual fluorescence F, which is between F_0' and F_m', shows the portion of energy which is not used for photochemistry, although in theory this could be used. The maximum quantum efficiency of PS II (Φ_{II}) is shown through the relation of F_v and F_m $\Phi_{II}=F_v/F_m$. The effective quantum efficiency is $\Phi'=(F_m'-F)/F_m'$. The fluorescence quenching by photochemistry ("non-photochemical quenching"): $q_p=1-(F_m'-F_0')/F_v$ is a measure of increased thermal dissipation.

Box 1.2.3 (continued)



A

F_0: Fluorescence from the antenna

F_m: maximum fluorescence of a predarkened leaf

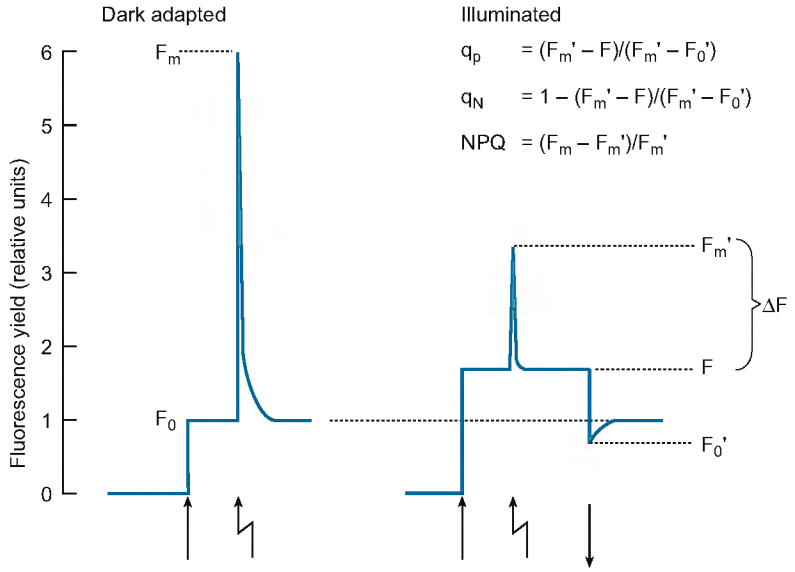
F_m': maximum fluorescence of an illuminated leaf (saturating light flash)

F_v: variable fluorescence during illumination with actinic light

q_Q: (new q_p) photochemical quenching

q_E: (new q_{NP} or q_N) non-photochemical quenching at great ΔpH (increase in the rate constant for thermal dissipation)

q: total quenching under continuous light



Optimal quantum efficiency: $(F_m' - F_0)/F_m = F_v/F_m$

Actual quantum efficiency: $(F_m' - F)/F_m' = \Delta F/F_m'$

B

Fig. 3. Analysis of chlorophyll fluorescence from photosystem II. A Kinetics of fluorescence and explanation of the parameters (after Walker 1992). B Nomenclature used for characteristic fluorescence parameters, definition of quenching coefficients and calculation of the quantum efficiency of photosystem II. (After Schreiber et al. 1994)

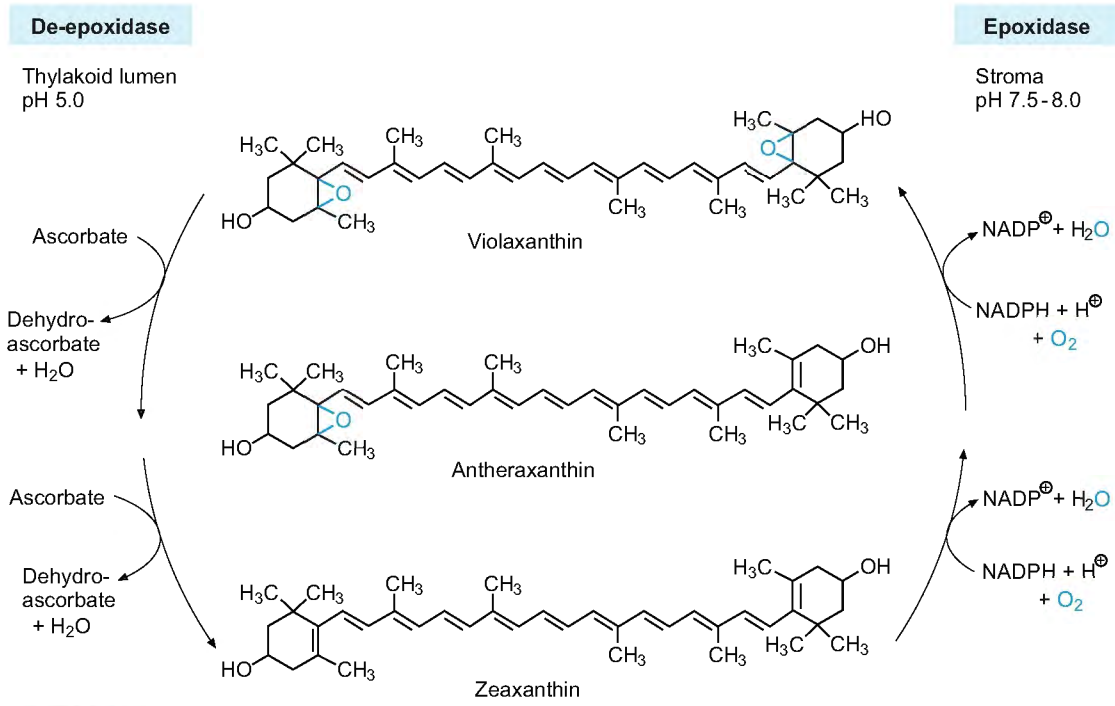


Fig. 1.2.9. The xanthophyll cycle in chloroplasts. In high light violaxanthin is converted into zeaxanthin by the de-epoxidase; in darkness re-oxidation is catalysed by the epoxidase. (Heldt 1999)

defence against light stress developed during evolution in the form of the extremely easily degradable D1 protein. A special metabolic cycle for the replacement of the damaged D1 protein via an undamaged D1 precursor, followed by processing of the preprotein, is associated with this repair cycle (Fig. 1.2.8). Photoinhibition is reversible, as the D1 protein has a very fast turnover (it is exchanged after 10^6–10^7 excitation cycles). **Photodestruction** occurs if the capacity for replacement of D1 is overstretched, leading to loss of photosynthetic capacity.

Photoinhibition of PSI has also been described (Sononike 1996a, Jiao et al. 2004). It is particularly strong for algae and higher plants during cold. The primary damage probably occurs at the acceptor side of PS I, upon which the B protein (which corresponds to the D1 of PS II) is degraded. This photoinhibition is, surprisingly, also observed during weak light and is also attributed to reactive oxygen species (Sonoike 1996b). It is thought that PS I is more robust, with photoinhibition or damage only occurring under extreme conditions.

- **Dissipation of excitation energy in the xanthophyll cycle.** Non-photochemical energy quenching requires a large proton gradient across the thylakoid membranes and thus an acid pH in the lumen of thylakoids. An acid environment of the thylakoid membrane activates an enzyme in the membrane, **violaxanthin de-epoxidase** (maximum activity at pH 5, Fig. 1.2.9).

In dark or low light the pH value of the lumen increases and another enzyme, **zeaxanthin-epoxidase**, is activated. Both reactions result in a light-dependent reaction cycle involving the xanthophylls violaxanthin, antheraxanthin (as an intermediary compound) and zeaxanthin (Fig. 1.2.9).

These xanthophylls are in the antenna complexes of PS II and probably also in PS I. Although the physiological importance of the xanthophyll cycle may be different in various species, there is no doubt that this cycle plays an important role in quenching excess photosynthetic energy.

- In the short term, in excess light, violaxanthin is converted into zeaxanthin, which de-energises the triplet excitation state of the chlorophyll which can form easily from excess energy. Zeaxanthin is probably able to react also with the first singlet state of chlorophyll and take up its energy. However, this has not yet been proven conclusively. Excitation energy

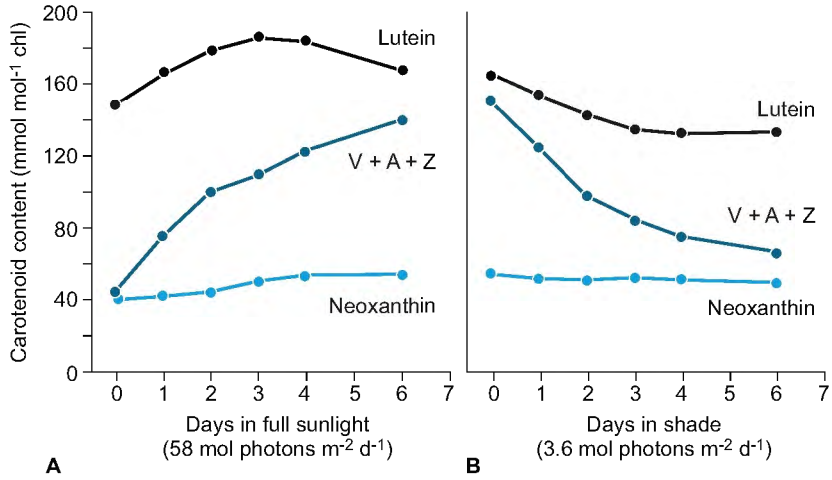


Fig. 1.2.10. Dynamic changes in the xanthophyll pools of young leaves of cotton in strong and weak light. The *left-hand diagram* shows the effects of transferring plants from shade into full sun light; the *right-hand diagram* the transfer from sunlight into shade. Note that the pools of lutin and also neoxanthin change relatively little in comparison with the total pool of violaxanthin, antheraxanthin and zeaxanthin. (Björkman and Demming-Adams 1994)

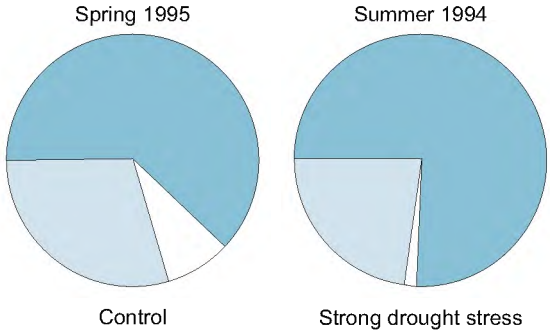


Fig. 1.2.11. Use of absorbed light energy in leaves of the Pacific madrone (*Arbutus menziesii*) in full sunlight. *Blue* Dissipation as heat; *white* photosynthetic CO_2 assimilation; *light blue* all additional uses including photorespiration and the Mehler reaction. (After Osmond et al. 1997)

transferred to zeaxanthin is released as heat; it is still an open question whether the formation of zeaxanthin from violaxanthin is the cause of the changes in membrane leading to NPQ, or whether the changes run in parallel. The physiological importance of the xanthophyll cycle is also shown in the selective and reversible accumulation of the three xanthophylls involved in the photosynthetic membranes during longer exposure to excess light (Fig. 1.2.10).

If one considers all physiological reactions for adaptation to, and avoidance of, excess light, and all the possibilities for repair in PS II, it is not surprising that many reactions and mechanisms are more or less attuned to each other. However, not all plants have all these possibilities available to the same extent; there are plants in which, for example, the xanthophyll cycle does not play an important role because these pigments only occur in very small concentrations and do not accumulate even after longer stress. That the removal of excessive energy can become a problem is shown by the various paths for the de-activation of absorbed light energy in the chloroplast (Fig. 1.2.11).

During drought stress (closed stomata and therefore secondary light stress through lack of CO_2), only a very small proportion of the light energy is used for photosynthesis and almost all available energy must be disposed of safely. It is obvious that one single mechanism is not sufficient for this.

Another argument for the use of many mechanisms for adaptation is the large variation in light intensities to which plants are subjected; e.g. with changing cloud cover or with light flecks on forest floors, light intensity can vary by a factor of 10^3 within seconds and plants must be able to cope with this stress (see Fig. 1.2.2 and Chap. 2.4, Fig. 2.4.9).

Summary

1. Light is the indispensable precondition for all plant life. Plants adapt to light intensities at all levels of organisation, from the molecular to the morphological, as light environments vary on earth not only in regions, but also in time. Continuously changing light conditions, or extremely low or high light intensities, together with other stressors, for example, lack of water, heat or cold, are particularly challenging to plants. Plants in those environ-

ments display very dynamic, adaptive behaviour.

2. The stressor "weak light" means lack of energy. Plants have adapted to this situation by developing different life forms (lianas, epiphytes as light parasites) and by adjusting the positions of their leaves (positive phototrophic reaction). A weakly developed mesophyll, but very large stacks of thylakoids in chloroplasts, are features of the anatomical and ultrastructural design of shade leaves. Shade leaves are characterised at the molecular level by a relatively low density of PS II reaction centres, but a correspondingly large area of antenna complexes.
3. Stress through high light intensity damages plants acutely because of over-excitation and formation of chemical radicals. These radicals easily react with oxygen to form reactive oxygen species (ROS). ROS destroy membranes, including proteins and other components (e.g. chlorophyll). Plants possess detoxification systems for ROS (see Chap. 1.3.5.3).
4. Because of the very substantial damage arising from over-excitation, there are many different mechanisms for avoidance and adaptation. Adaptation at the morphological level is explained in Chapter 2 "Autoecology". Many plants deal with high light energy by reactions which alter the position of the leaf. These positions may be permanent or dynamic. Sun leaves have a highly developed mesophyll, often with several layers of palisade cells. Chloroplasts are able to position themselves on the sides of the cell according to the light intensity, moving from the position with the stronger to the weaker radiation, and vice versa. The thylakoid system of sun leaves is not well developed, but consists of many photosynthetic reaction centres and a smaller antenna area (particularly the LHC II is more weakly developed). Dissociation of peripheral antenna from PS II, and a partial association with PS I, is often observed, resulting in a better balanced electron flow.
5. At the biochemical level strong light produces a change in the rate constant of energy flow in favour of heat (non-photochemical quenching). If this mechanism does not suffice, the absorbed light energy is disposed of via zeaxanthin, which is produced in the xanthophyll cycle from violaxanthin. At the same time, particularly because of closed stomata in heat or drought, light energy is dissipated with the help of photorespiration. This process produces CO_2 which is then photosynthetically assimilated.
6. If energetisation still exceeds the capacity of these quenching systems, D1, one of the core proteins of PS II, is degraded. The resulting inhibition of photosynthesis is reversible as long as the capacity of the specific repair system, the "D1 cycle", is sufficient. This reversible inhibition is called photoinhibition. If strain on the D1 cycle is persistent, "photodestruction" occurs with strong bleaching of the chloroplast.
7. Light energy can also be absorbed by pigments in the epidermis, as well as by the photosynthetic apparatus. Light can be reflected by the surface of the epidermis. A hairy tomentum can increase the proportion of reflected light.

1.2.2

UV Radiation

Recommended Literature

- Gyula P, Schäfer E, Nagy F (2003) Light perception and signalling in higher plants. Curr Opinion Plant Biol 6:446–452
- Jansen MAK, Gaba V, Greenberg BM (1998) Higher plants and UV-B radiation: balancing damage, repair and acclimation. Trends Plant Sci 3:131–135
- Short FT, Neckles HA (1999) The effects of global climate change on seagrasses. Aquatic Botany 63:169–196
- Stratmann J (2003) Ultraviolet-B radiation co-opts defense signaling pathways. Trends Plant Sci 8:526–533

Ultraviolet radiation coming from the sun is divided into three ranges: The long wave **UV-A** (315–400 nm), the biologically effective **UV-B** (280–315 nm) and the even shorter wavelength and therefore particularly energy-rich **UV-C** (100–280 nm). The atmospheric ozone layer absorbs very little UV-A, a considerable amount of UV-B and all the UV-C. Therefore, the population of the world is currently very concerned about the **ozone hole**, i.e. the decrease in stratospheric ozone concentration particularly in the polar and subpolar regions (Fig. 1.2.12).

It should, however, be realised that, because of the sun's position above the equator, solar radiation crosses the atmosphere in regions of high geographical latitude at an angle and thus

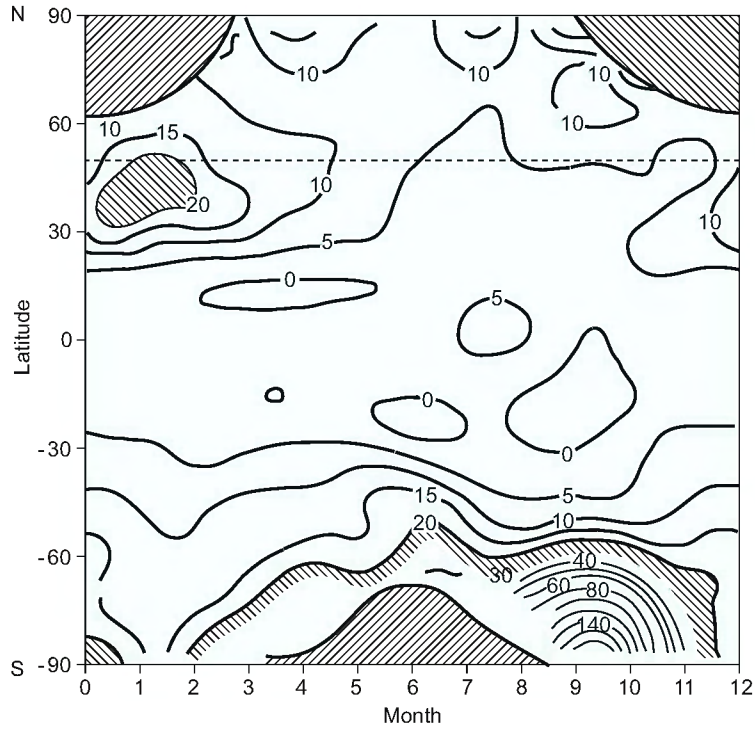


Fig. 1.2.12. UV-B radiation and the "ozone hole". The trends in daily UV dose (in DNA destruction units) in % for a 10-year period in different geographical latitudes and throughout the year are shown. (Zellner 1996)

encounters a considerably "thicker" ozone layer than at the equator, where it takes the shortest path through the atmosphere. The UV radiation is thus relatively high in the tropics and relatively low in polar regions. The higher intensity of UV radiation with increasing elevation above sea level is also known. This is caused by an attenuation of the tropospheric ozone layer as well as by a decrease in the intensity of haze.

UV radiation may damage cells and so is dangerous to organisms (Table 1.2.2); its intensity is therefore often not given as the flux of quanta, but in units expressing the damaging effects, e.g. as **DNA thymine dimerisation** or erythema formation (reddening of skin, sunburn). There is consensus that plants may suffer UV damage; thus the debate is how much UV radiation is tolerated by plants, or the extent to which damage can be repaired. Scientists vary considerably in their opinions. The phytoplankton of cooler seas appears to be particularly susceptible and significant reduction in their mass has been repeatedly shown, despite the shallow depth to which UV light penetrates into a body of water (Fig. 1.2.13). The effect of increased UV-B radiation on the growth and yield of food plants has been extensively studied. Significant differences in sensitivity of various cultivars, e.g. of soya bean or maize, have been shown. Decreased yields have been statistically established occasionally, but the reductions were relatively small (usually <10%). For sensitive species, the onset of flowering was delayed. However, by harvest, this delay was usually balanced out. For a long-day plant (*Hyoscyamus niger*, henbane), UV even stopped the induction of flowering.

Table 1.2.2. Physiological effects of increased UV-B radiation

DNA damage	Dimerisation of thymine; breakage of strands
Biomembranes	Lipid peroxidation
Photosynthetic apparatus	Inactivation of PS II; acceleration of the turnover of D1 (and D2?); damage to thylakoid membranes; bleaching of pigments; decrease in the activity of photosynthetic enzymes (particularly Rubisco); inactivation of photosynthetic genes
Phytohormones	Photooxidation of auxin
UV avoidance:	
Secondary metabolism	Activation of the UV-B/blue light receptors; activation of expression of the key genes of phenylpropane metabolism and accumulation of flavonoids; accumulation of alkaloids, waxes and polyamines
Radical scavengers	Increased capacity of the antioxidative system (ascorbate peroxidase, SOD, glutathione reductase, and others)

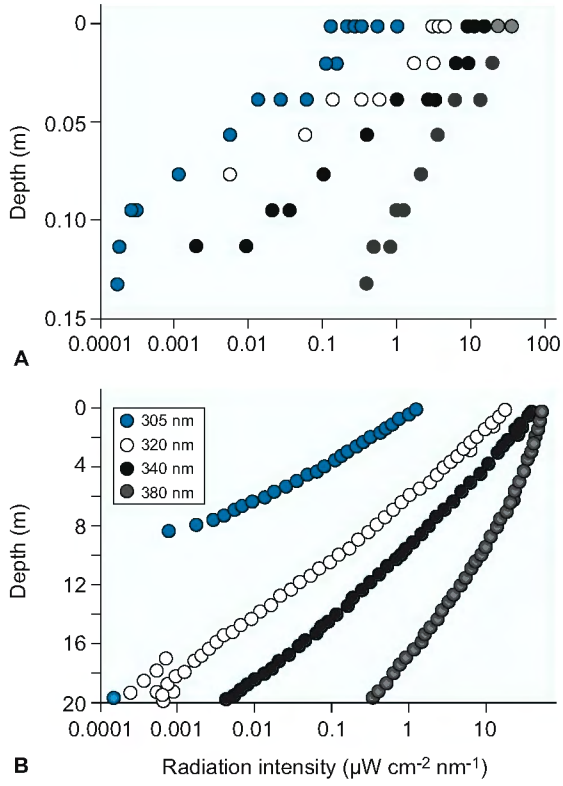


Fig. 1.2.13. Decrease in UV radiation with depth of water. A In the humin-rich Lake Neusiedler (Austria) and B in the northern Adriatic. Both measurements were made on a cloud-free August day. The intensity of UV radiation at a depth of 5 cm in Lake Neusiedler corresponds approximately to a depth of 5 m in the Adriatic. (Herndl 1996)

1.2.2.1
UV-B Damage and Its Repair

Recommended Literature

- McCullough AK, Dodson ML, Lloyd RS (1999) Initiation of base excision repair: glycosylase mechanisms and structures. Annu Rev Biochem 68:255–285

Morphological-anatomical symptoms of a UV-B stress that is still tolerated are swollen but shorter internodes, smaller leaves with the edges usually rolled in at the top surface, and increased growth of lateral buds (Ballaré et al. 1996). Leaves tend to be succulent, have a particularly thick, usually pigmented epidermis and low density of stomata. Tolerated UV stress often results in accumulation of vitamin C and soluble sugars in fruits, thus increasing the quality of foods.

The damage caused to organisms by UV radiation is undisputed, but there is considerable discussion about if – and to what extent – damage is caused by this radiation in the open field (Searles et al. 2001). Contradictory results could have methodological causes, e.g. the relative proportions of UV-B and PAR or the use of plant species differing in sensitivity. The main reason for different scientific statements is the type and dosage of UV radiation. Often, because of the experimental limitations, the increased UV radiation is applied as a pulse, with unnaturally high intensity, to compensate for the short duration of exposition. The same stressful radiation may be applied using long-term, slightly increased amounts of radiation, but completely different physiological reactions (strain) result. With the UV stress applied in short pulses, the damage, and its possible repair, are the dominant effects, whilst continuous but low-intensity stress leads to hardening and avoidance (e.g. by protective pigments). Table 1.2.2 shows the various physiological reactions of plants to UV-B stress. The effects of UV-B on DNA have been particularly well studied, namely the dimerisation of thymine, where cyclobutyl pyrimidine dimers or 4,6-thymine dimers are formed.

Higher doses of UV lead to breakage of single or double strands of DNA and finally to loss of DNA. Naked DNA of mitochondria and probably also of the chloroplasts is particularly sensitive. UV radiation of yeast cells caused a 10% loss of nuclear DNA, but at the same time a 50–60% loss of mitochondrial DNA.

There are several mechanisms to repair such damage, of which two are mentioned here: photolyase, and base excision and recombination reactions. DNA of mitochondria can also be repaired if the damage is not too severe. Repair usually takes only a few hours. Strong UV stress, however, leads directly to irreparable chromosome breakage and deletions; this is the basis for sterilisation of rooms and instruments with intense UV light.

In addition to the damage to DNA, photooxidation of UV-absorbing pigments is known: Yellowing and complete bleaching of leaves of house plants after abrupt transfer to the open air are frequent phenomena. Here, the protective effect of photosynthetic pigments which absorb short-wave radiation like proteins can be seen. Photodestruction of thylakoid pigments leads to a significant reduction of the Rubisco content.

1.2.2.2
UV Detection

In plants and animals UV radiation triggers protective reactions which means that these organisms must possess photoreceptor systems for detecting this radiation. It is known that plants have a sensor for UV-B and two further sensors (cryptochrome or phototropin 1 and 2) for UV-A/blue light, which cooperate with the phytochrome system, i.e. they determine the sensitivity of the plant for visible and UV light (Ballaré 1999; Fig. 1.2.14).

Signal transduction chains from the UV-A/blue light and UV-B receptors possibly react only with the signal from phytochrome A, i.e. from that phytochrome form which accumulates in seeds and controls germination, for example. In addition to the morphogenetic effects mentioned above, the effects of UV radiation on metabolism, particularly on **secondary metabolism**, have also been studied in detail. Products of **phenylpropane metabolism** exhibit strong absorbance of short-wave irradiation and are thus able to function as effective UV filters. Also, the anthocyanins (Fig. 1.2.15), mentioned above, are protective pigments because they absorb visible as well as UV light.

The key enzyme of the phenylpropane metabolism (Box 1.2.4, Fig. 2) is chalcone synthase (CHS), whose formation is strongly induced by UV-B as well as UV-A/blue light. Both wavebands of light achieve their effects via the Ca^{2+}-dependent signal transduction pathways. Upon UV stress, calcium is released from cellular stores. However, the transduction pathways differ in their degrees of dependence on calcium.

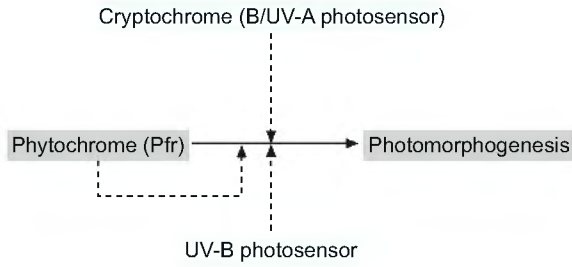


Fig. 1.2.14. Model of the interaction between phytochrome and the blue/UV-light receptor. Activation of the blue/UV-light sensor regulates the sensitivity of the photoresponse to activate phytochrome (Pfr). Pfr itself can autocatalytically increase the efficiency of the phytochrome system. (Mohr 1996, see also Elliott et al. 2004)

1.2.2.3
Avoidance of UV-B Stress and Development of UV-B Resistance

Research with pine needles and beech leaves has shown that, in addition to the various flavonoids formed constitutively and independently of UV, there are other related compounds in the epidermis. For example, after UV-B radiation, the concentration of 3″, 6″-DCA and 3″,6″-DCI (=3″,6″-di-*para*-coumaroylastragalin and 3″,6″-di-*para*-coumaroylquercetin) for pines, as well as 2″,2″-di-*para*-coumaroylkaempferol-3α-D-arabinoside for beech had risen five-fold (Table 1.2.3; Figs. 1.2.16 and 1.2.17).

Compounds which can be induced by UV are exclusively located in the epidermis, while those

Fig. 1.2.15. Anthocyanins in the young leaves of the tropical Sapotacee *Inhambanella henriquesii* in the coastal forests of Kenya. The anthocyanin is contained in the vacuoles of the leaf epidermis and protects the leaves, which are not yet fully green, from radiation damage. The close-up (*right*) clearly shows the mixture of colours between the red anthocyanins and the chlorophyll. (Photo E. Beck)

Box 1.2.4 Plant phenols

A large number of the phenolic compounds formed by plants are synthesised by the so-called shikimate pathway named after the characteristic intermediate shikimic acid. The second aromatic ring of the C_{15}-derived phenylpropane derivatives (flavonoids) results from the condensation of three acetyl residues from malonyl-CoA (Fig. 1).

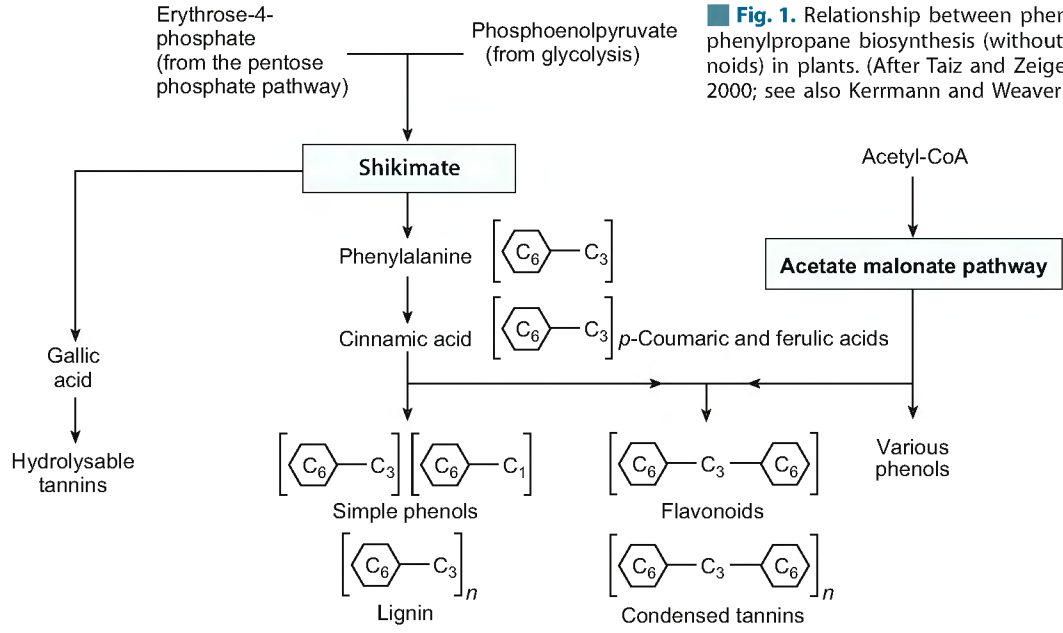


Fig. 1. Relationship between phenol and phenylpropane biosynthesis (without terpenoids) in plants. (After Taiz and Zeiger 2000; see also Kerrmann and Weaver 1999)

Table 1.2.3. Calculated concentrations and shielding effects of UV-B-absorbing pigments in the epidermis of needles of pine seedlings. The volume of the epidermis (ca. 104 µl/g fresh weight) was calculated from the needle surface und the mean thickness of the epidermis. The absorption at 300 nm, and the corresponding value of transmission, were calculated using the Lambert-Beer law. (After Schnitzler et al. 1996)

	Control needles			UV-irradiated needles		
	Concentration (mM)	Total absorption at 300 nm (%)	Transmission at 300 nm (%)	Concentration (mM)	Total absorption at 300 nm (%)	Transmission at 300 nm (%)
Cell wall-bound pigments						
p-Coumaric acid	13.4	7.9	30.8	13.0	6.1	31.8
Ferulic acid	1.6	1.2	83.9	1.9	1.1	81.5
Astragalin	16.7	10.7	20.1	19.1	9.8	15.9
Lignin	6.5	1.2	83.6	6.5	0.9	83.5
Sum	–	21.0	4.3	–	17.9	3.45
Soluble pigments (vacuole)						
Diacylated flavonoids	35.4	79.0	0.0007	46.1	82.1	0.00002
All pigments	–	100.0	0.00003	–	100.0	0.0000009

that are formed constitutively are distributed throughout the mesophyll. The non-conjugated (*p*-coumaric acid) phenylpropanes are localised in the epidermal cell wall, while the conjugates are mainly sequestered into the vacuoles of these cells (Fig. 1.2.17). The anthocyanin accumulation induced during leaf flushing by tropical trees (see Fig. 1.2.15) takes place in the vacuoles of the epidermal cells.

Box 1.2.4 Plant phenols (continued)

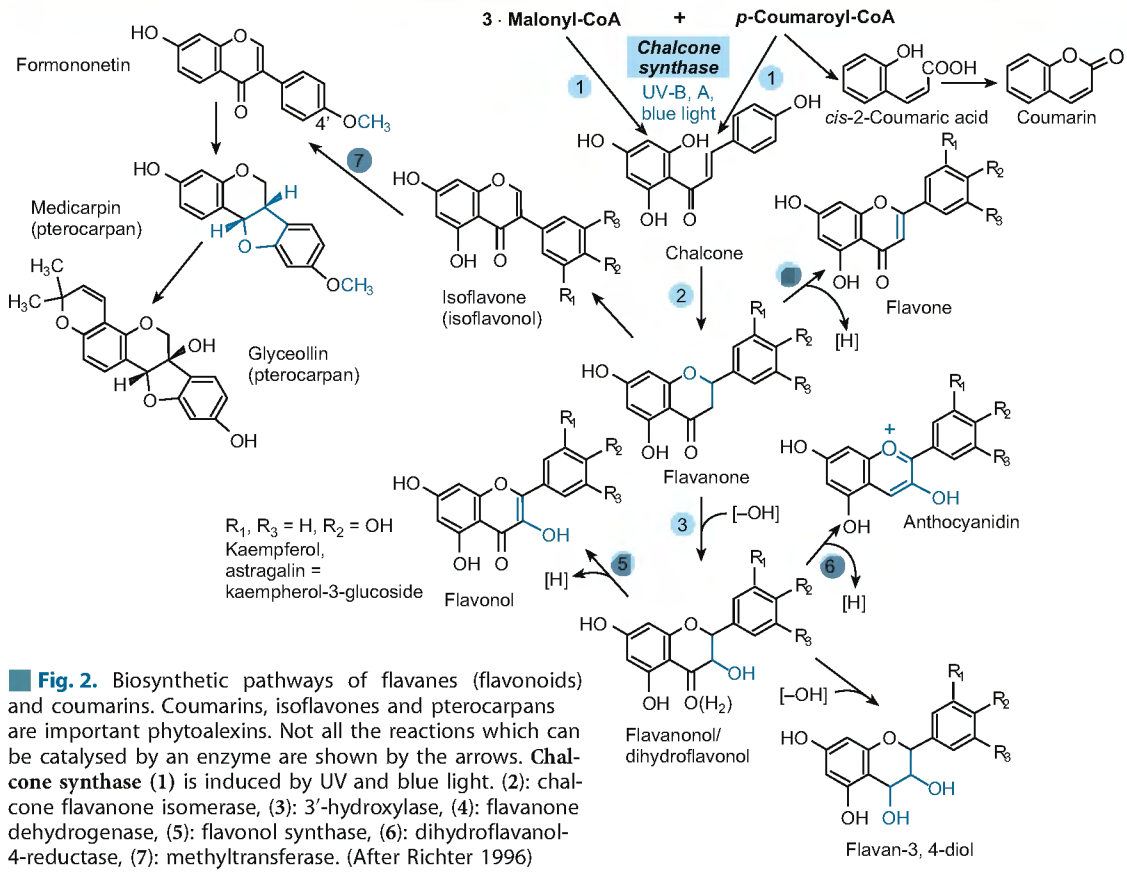
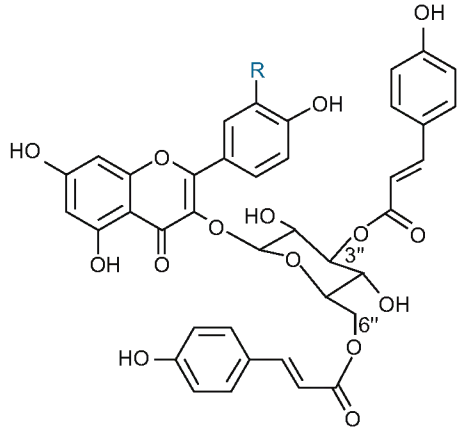


Fig. 2. Biosynthetic pathways of flavanes (flavonoids) and coumarins. Coumarins, isoflavones and pterocarpans are important phytoalexins. Not all the reactions which can be catalysed by an enzyme are shown by the arrows. **Chalcone synthase (1)** is induced by UV and blue light. (2): chalcone flavanone isomerase, (3): 3′-hydroxylase, (4): flavanone dehydrogenase, (5): flavonol synthase, (6): dihydroflavanol-4-reductase, (7): methyltransferase. (After Richter 1996)



3″,6″-di-*p*-cumaroylastragalin (DCA; R=H) and 3″,6″-di-*p*-cumaroylisoquercitrin (DCI; R=OH)

Fig. 1.2.16. 3″,6″-Di-*p*-coumaroylastragalin (R=H) and 3″,6″-di-*p*-coumaroylisoquercitrin (R=OH) as examples of diacylated flavonoids (see Table 1.2.3)

However, the particularly effective UV absorbers *p*-coumaric acid and astragalin are deposited in the cell wall. It is estimated that the outer cell walls of the epidermis allow about 4% of the UV-B to pass through. Diacylated flavonoids, dissolved in the cell, are able to filter out the residual UV-B radiation because of their greater absorbing capacity.

It is likely that plants with low or even no capability of synthesising such protective pigments are UV-sensitive. Other types of protective pigments, belonging to the class of mycosporines, are found in phytoplankton and other marine algae; they are not degraded in the nutritional chain and provide organisms of the next trophic level with a certain degree of UV protection.

In addition to flavanoid metabolism, other enzymes producing or activating radical acceptors are formed: Glutathione reductase, ascorbate- and glutathione-peroxidase.

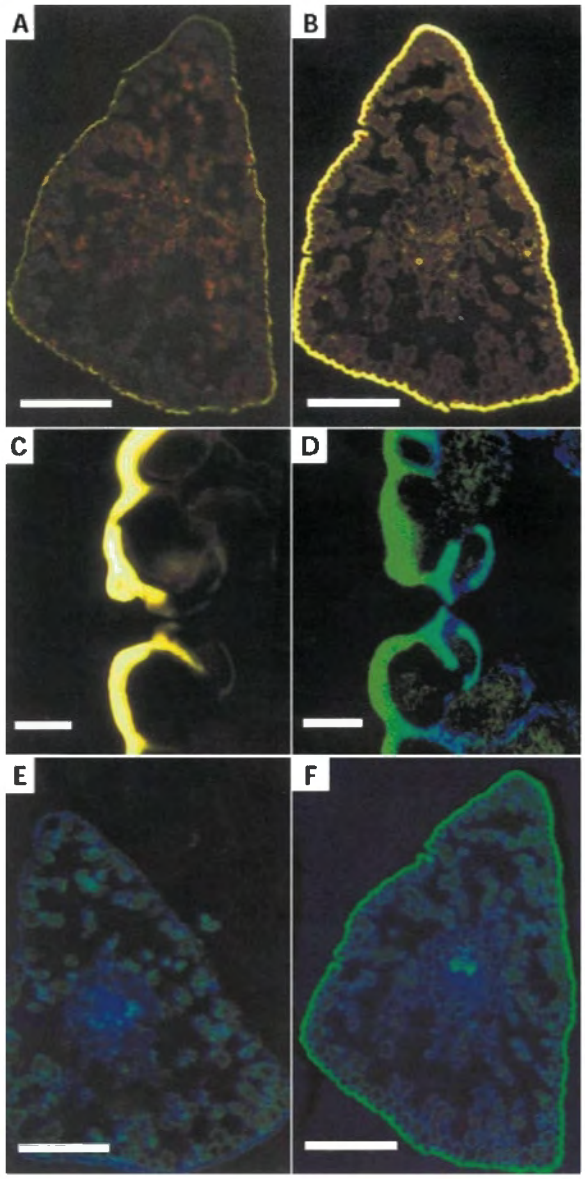


Fig. 1.2.17. Histochemical evidence for UV-absorbing substances in the epidermis of pine needles. A–C Fluorescence (excited by light of 450–490 nm wavelength, determined at $\lambda > 520$ nm); A control, B and C after treatment with a reagent which intensifies flavonoid fluorescence. D–F Observation with a confocal laser microscope (which shows the cells and their contents more clearly). E control, D and F after staining as above. A, B, E, F Transverse section of a needle, C, D stomata. (After Schnitzler et al. 1996)

Summary

1. The ultraviolet spectrum is divided according to the energy content of the radiation (wavelength); UV-C (100–280 nm), UV-B (260–315 nm) and UV-A (315–400 nm). The shorter the wavelength of the UV radiation, the stronger is its destructive effect, but the more it is absorbed by atmospheric ozone. UV-C is completely absorbed when passing through the atmosphere; UV-A does not damage most organisms. The biologically effective UV light is UV-B.
2. Strong UV-B radiation damages plants, particularly DNA (breakage of strands and deletions) of nuclei, chloroplasts and mitochondria, and also damages the photosynthetic apparatus (bleaching of photosynthetic pigments, destruction of photosynthetic enzymes). The strength of UV radiation required to produce such damage probably does not occur in nature or only in extreme cases. Organisms have very efficient repair systems, particularly for damaged DNA.
3. Tolerable UV-B radiation is morphologically effective: thickening and shortening of internodes, reduced growth in area but increased thickness of leaves, increased growth of side shoots.
4. At the biochemical level, UV leads to increased phenylpropane metabolism and the formation of UV-absorbing protective pigments in the epidermis, and to increased detoxification systems for ROS.
5. Plants possess a UV-B receptor and at least two UV-A/blue light receptors (cryptochromes). These receptors cooperate with the phytochrome system.

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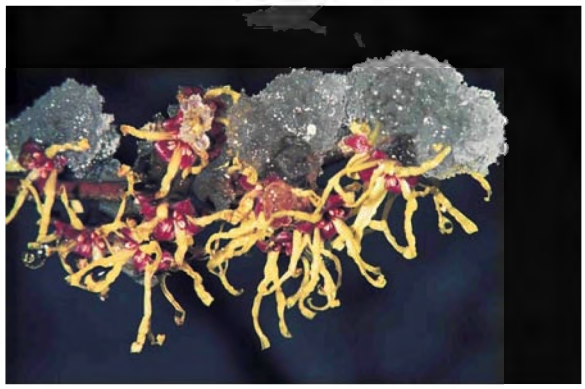
1.3

Temperature



Winter flowering: The shrivelled petals of the witch hazel (*Hamamelis virginiana*) clearly show the effects of water loss from the cells caused by frost. As a consequence of the low average temperatures, the flowers of the North American or Japanese (*H. mollis*) witch hazel persist for many months. Witch hazel does not tolerate extreme cold and dryness, but is well adapted to the moderate winters of central Europe, where it is much prized as an ornamental shrub. Its green leaves in summer are like those of hazel. Photo K. Liedl, Munich

Recommended Literature

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1.3.1

Temperature Ranges and Temperatures Limiting Life

For many organisms temperature limits for active life can be determined, as well as those beyond which life is no longer possible. Before an organism reaches lethal temperatures of heat or cold, it usually falls into a "rigid" or "stiff" condition in which the active processes of life only occur at minimal, hardly measurable rates without lethal damage. This is termed the "condition of latent life". The stages of cold death, cold rigidity, range of active life, heat rigidity and heat death are designated as the **cardinal points of temperature tolerance**. These differ in their totality from species to species (see Fig. 1.1.3), whereby different cardinal points can be exhibited even among the individual tissues and organs of a single plant. Active life is quite generally dependent on the temperature range of liquid water. Hydrated plant tissues thus have cardinal points which are closer together than those of dry plant tissues (e.g. seeds or plants resistant to drying out – poikilohydric plants). Tables 1.3.1 and 1.3.2 show some examples. Limiting temperatures, however, are not decisive for the growth and development of a plant, but

Table 1.3.1. Maximum temperature resistance of microorganisms and poikilohydric plants in the turgescent state and in the rigid states caused by drought. (Larcher 2003)

Plant group	Cold damage[a] at °C		Heat damage[b] at °C	
	Moist	Dry	Moist	Dry
Bacteria				
Archaebacteria			100–110	
Cyanobacteria and other photoauthotropic bacteria			55–75	
Saprophytic bacteria			60–70	
Thermophilic bacteria			Up to 95	
Fungal fruiting bodies				
Bacterial spores		LN_2[c]	80–120	Up to 160
Fungi				
Plant pathogenic fungi			45–65 (70)	
Saprophytic fungi	0 to below –10		40–60 (80)	75–100
Fungal fruiting body	–5 to –10 (–30)			
Fungal spores		LN_2	50–60 (100)	Above 100
Algae				
Marine algae				
Tropical seas	+14 to +5 (–2)		32–35 (40)	
Temperate seas				
Eulitoral	–2 to –8		25–30	
Tidal zones	–8 to –40		30–35	
Polar seas	–10 to –60		(15)20–28	
Fresh water algae	–5 to –20 (–30)		35–45 (50)	
Airborne algae	–10 to –30	LN_2	40–50	
Eukaryotic thermal algae	+20 to +15		40–50	
Lichens				
Polar regions, high mountains, deserts	–80	LN_2		
Temperate climate zones	–50	LN_2	33–45	70–100
Mosses				
Humid tropics	–1 to –7			
Temperate zone				
Humid locations	–5 to 15		40–45	
Ground mosses in forests	–15 to –25		40–50	80–95
Epiphytic and epipetric mosses	–15 to –35	LN_2		100–110
Polar regions	–50 to –80	LN_2		
Poikilohydric ferns	–20	LN_2	47–50	60–100
Seed plants				
Ramonda myconi	–9	LN_2	48	56
Myrothamnus flabellifolia		LN_2		80

[a] After at least 2 h of exposure to cold
[b] After 30 min of heat treatment
[c] Temperature of liquid nitrogen (–196 °C)

rather the temperature range of active life is. Plants are much more similar in this respect than they are with regard to their limiting temperatures. Day temperatures (air temperatures) of between 15 and 25 °C and night temperatures of about 10 K lower are, on average, optimal for plant growth. Organisms inhabiting extreme locations, such as snow and ice algae (*Haematococcus pluvialis*, and various Diatomeae) with a life range of between +5 and –5 °C or cyanobacteria and bacteria living in geysers or in the black smokers of the deep sea with optimal temperatures of between +80 and above 100 °C, are exceptions.

Types of organisms constitutively adapted to cold are called **psychrophiles** and those requiring high temperatures are called **thermophiles** (or extreme thermophiles), but most organisms are **mesophiles.**

Not all life processes of a plant have the same optimal temperatures. The temperature requirements of these processes must be adapted to the

Table 1.3.2. Temperature resistance of the leaves of cormophytes from various climate zones. The data quoted correspond to the limiting temperatures causing 50% damage (TL_{50} in °C) after at least 2 h of exposure to cold or after 30 min of heat treatment. (After Larcher 2003)

Plant group	Cold damage in hardened state	Heat damage during vegetative period
Tropics		
Trees	+5 to −2	45–50
High mountain plants	−5 to −15 (−20)	about 45
Subtropics		
Evergreen woody plants	−8 to −12	50–60
Palms	−5 to −14	55–60
Succulents	−5 to −10 (−15)	58-67
C4 grasses	−1 to −5 (−8)	60–64
Winter annual desert grasses	−6 to −10	50–55
Temperate zones		
Evergreen woody plants of mild winter coastal areas	−7 to −15 (−25)	46–50 (55)
Dwarf shrubs of Atlantic heathlands	−20 to −25	45–50
Summer green trees and shrubs	(−25 to −35)[a]	about 50
Herbaceous plants		
Sunny sites	−10 to −20 (−30)	47–52
Shady sites	−10 to −20 (−30)	40–45
Halophytes	−10 to −20	
Water plants	−5 to −12	38–44
Homeohydric ferns	−10 to −40	46–48
Winter cold areas		
Evergreen conifers	−40 to −90	44–50
Boreal deciduous trees	(to LN_2)*	42–45
Arctic-alpine dwarf shrubs	−30 to −70	48–54

[a] Vegetative buds

conditions under which the individual steps of development take place. This can be illustrated by the example of germination in contrast to growth and development. Seeds which germinate in the autumn or winter (such as those of winter cereals) do so at temperatures that are considerably lower than those relevant for germination in the spring or summer. Independently of the temperature range in which germination is possible, the rate of germination itself shows the typical temperature dependence of biological processes (Q_{10} between 1.5 and 2.5; see Chap. 1.3.2). Such germination behaviour can be modified by inhibitory mechanisms (e.g. the requirement for a prolonged exposure of imbibed seeds to cold – **stratification** – or the fire-dependent opening of seeds in **pyrophytes**), which prevent premature germination at unfavourable times.

Frost represents a long-term stress situation in contrast to, for example, fire, and can last for hours, days and even months. Frost acts not only as a temperature stress, but primarily as a drought stress due to the freezing of tissue water. Plants undergo a process of hardening during the advent of the colder season or in the presence of permanent frost during their juvenile development. This enables them to withstand even the sometimes extremely severe frosts which occur in nature (air temperatures as low as −70 °C have been recorded in Siberia).

Temperature stress does not occur only in extreme locations, and plants can be subjected to large ranges of temperature even during the course of a "normal" day (see Chap. 2.1, section on radiation climate). Temperatures of above +50 °C occur frequently at the immediate surface of the soil during extended periods of solar irradiation, particularly if there is no plant growth and the soil is dark and thus reflects little of the incident sunlight. However, air temperature decreases rapidly with the distance from the soil surface, and the temperature gradient within the soil itself is even steeper (see Figs. 4.3.2 and 2.1.6). Whereas an air temperature of +40 °C was measured about 10 cm above soil exhibiting a surface temperature of +50 °C, the soil temperature at a depth of 10 cm was below 25 °C (see Fig. 2.1.6). Temperature gradients within the soil and within the surface-near air layer also occur on clear nights, whereby their direction is reversed and their amplitude is of a lesser magnitude. Cooling of surfaces to tempera-

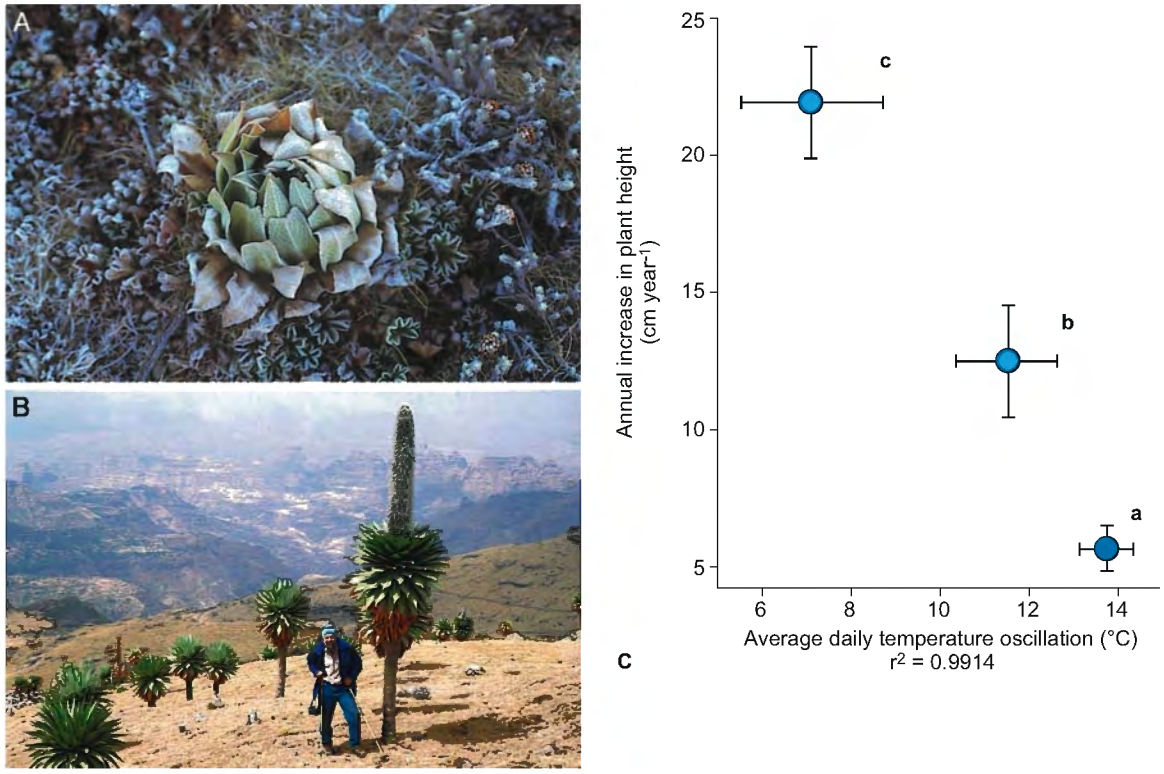

Fig. 1.3.1. Influence of the microclimate on the growth of *Lobelia rhynchopetalum* in the Ethiopian Highlands. A Night ground frosts (hoar frost on the leaves) stimulate the plant to form "night buds" (cf. Chap. 1.3.6.7). B The leaf crowns of the specimens which have already developed a trunk are subjected to a considerably milder microclimate, i.e. they are exposed to significantly smaller temperature fluctuations. The rate of growth therefore increases the further the leaf rosette is removed from the soil on the growing trunk (C). (After Fetene et al. 1998; photos E. Beck)

tures below that of the air is caused by radiation from all horizontal and sloping areas. This type of heat radiation also occurs during the daytime; the heat gain via absorption of the sun's rays is then, however, considerably greater than the heat lost through radiation. Young plants situated directly on the soil surface are understandably subjected to greater temperature stress than plants which have formed a stem, and they therefore grow only slowly. As soon as a stem is formed and leaves have grown away from the severe microclimate near the soil surface, the plant's rate of growth increases considerably (Fig. 1.3.1).

1.3.2

The Temperature Dependence of Biochemical Processes, Q_{10} and Activation Energy

Recommended Literature

- Nelson DL, Cox MM (2005) Lehninger Principles of Biochemistry, 4th edn. Freeman, New York

The rate of reaction of uncatalysed reactions, as well as of enzymatic reactions, depends on temperature.

The measure for the temperature dependency is the so-called Q_{10} (quotient of the rates of reaction at two temperatures differing by 10 K):

$$Q_{10} = \frac{v_{T+10}}{v_T}$$

If the rate constants k_{T1} and k_{T2} are inserted instead of the rate of reaction v, the activation energy E_a can be calculated with the following equation:

$$\ln\frac{k_{T_1}}{k_{T_2}} = \frac{E_a}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

The Q_{10} is a readily illustrative measure of the activation energy of a reaction. For enzymatic reactions it is between 1.4 and 2.5, for biophysical processes between 1.03 and 1.3.

An increase in temperature leads to a speeding up, and a decrease in temperature to a slowing down of biological processes.

1.3.3

Temperature and the Stability/ Functionality of Biomembranes

1.3.3.1

Influence of Temperature on the Lipid Composition of Biomembranes

In addition to its influence on the rate of metabolism, temperature affects the stability and accordingly also the chemical structure of biomembranes. Active life is absolutely dependent on functional biomembranes. The lipids of these extremely complex structures must be present in a viscous-fluid state for the proteins and protein complexes anchored within them to fulfil their function. The melting point and viscosity of the lipids depend on the length of the component fatty acids and the degree of their unsaturation. The longer the chain length of the fatty acids and the lower the number of double bonds, the higher the melting point is (Box 1.3.1).

Box 1.3.1 Chemical structures and melting points of the fatty acids most commonly found in plant biomembranes

Carbon skeleton	Structure	Common name	Melting point (°C)
16:0	$CH_3(CH_2)_{14}COOH$	Palmitic acid	63.1
18:0	$CH_3(CH_2)_{16}COOH$	Stearic acid	69.6
20:0	$CH_3(CH_2)_{18}COOH$	Arachidic acid	76.5
16:1 (Δ^9)	$CH_3(CH_2)_5CH{=}CH(CH_2)_7COOH$	Palmitoleic acid	–0.5
18:1 (Δ^9)	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$	Oleic acid	13.4
18:2 ($\Delta^{9,12}$)	$CH_3(CH_2)_4CH(=CHCH_2)_7COOH$	α-Linoleic acid	–5.0
18:3 ($\Delta^{9,12,15}$)	$CH_3CH_2CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$	α-Linolenic acid	–11
20:4 ($\Delta^{5,8,11,14}$)	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_3COOH$	Arachidonic acid	–49.5

Table 1.3.3. The effect of growth temperature on the lipid composition of the biomembranes of the cyanobacterium *Synecchocystis*. Increasing growth temperature results in a lesser degree of desaturation of the fatty acids. The chemical structures of the individual fatty acid species are shown in Box 1.3.1. *X* denotes a phospho-component ("phospholipid"). (Murata et al. 1993; Gombos et al. 1996)

Cultivation temperature (°C)	Fatty acid species					
	16:0 16:0 X	18:1 16:0 X	18:2 16:0 X	18:3γ 16:0 X	18:3α 16:0 X	18:4 16:0 X
	Total fatty acid species (%)					
22	2	4	16	42	16	16
34	16	16	24	34	0	0

Table 1.3.4. Changes in the composition of the lipids of a defined biomembrane (chloroplast envelope membrane of spruce needles) during the course of frost hardening ("winter") or dehardening ("summer"). (Senser and Beck 1992)

Lipid	Fatty acids (%) 16:0+18:0 (+18:1^{tr})	Fatty acids (%) 16:3+18:1+18:2+ 18:3+18:4+18:2-hydroxy+20:3
Phospholipids		
Summer	55.4	41.2
Winter	25.6	66.0
Galactolipids		
Summer	33.4	64.4
Winter	9.4	84.8

Organisms are able to adapt the viscosity of their biomembranes to temperature to a certain extent by exchanging certain types of fatty acids for others. This enables metabolism to proceed in an orderly manner upon temperature changes. For example, more saturated fatty acids are incorporated into the membranes upon an increase in temperature (Table 1.3.3).

Changes in lipid composition also take place during frost hardening and dehardening, which can be related to the maintenance of optimal viscosity and functionality of the biomembranes. Here, the viscosity is decreased by increasing the degree of unsaturation of the membrane lipids (Table 1.3.4).

1.3.4

Heat (Hyperthermy)

Recommended Literature

- Scharf K-D, Höhfeld I, Nover L (1998) Heat stress response and heat stress transcription factors. J Bioscience 23:313–329
- Sun W, Van Montagu M, Verbruggen N (2002) Small heat shock proteins and stress tolerance in plants. BBA 1577:1–9

Heat should be understood here as the upper temperature range in which active life is possible. In this range strain increases with increasing temperature, and there is a direct correlation between the stressor and strain. In addition to this obvious relationship, however, biphasic relations, i.e. discontinuity between stressor and strain, have also been observed. Figure 1.3.2 shows such a stress response for poplar leaves in the month of September. Similar observations have been made for privet and for bindweed. In all three cases, the damage within two different temperature ranges was only observed in late summer, possibly an indication of the transition into the frost-hardening phase. During this stage, there are evidently two temperature ranges in which metabolic flows and metabolic pools are relatively well balanced and therefore no damage occurs. However, this phenomenon must still be studied in more detail.

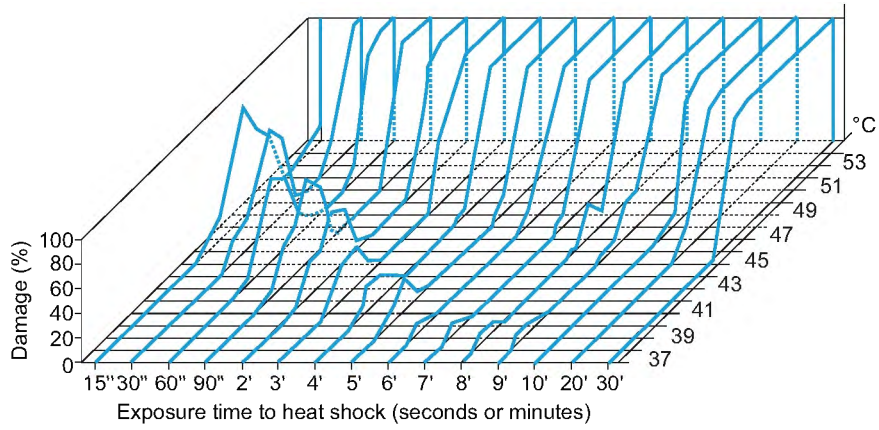

Fig. 1.3.2. Course of heat damage in poplar leaves (*Populus deltoidis* x *simonii*) in dependence on the amount of heat (stress duration×exposure time) during September. The heat treatment corresponded to, in principle, the cold treatments described in Box 1.3.6. Damage was quantified by determining the frequency of necroses (compare Box 1.1.1). (After Kappen and Zeitler 1977)

1.3.4.1
Heat: Metabolism and Growth

Temperature above the optimal range not only accelerates, but also redirects metabolic processes and leads to changes in metabolite pools and to growth anomalies. This can be seen by the example of the heat-sensitive potato cultivar 'Up-to-Date' and the heat-tolerant cultivar 'Norchip' (Box 1.3.2). The potato plant comes from the medium-altitude mountain areas of the South American Andes and requires a relatively cool climate. Potato can tolerate neither very warm weather nor frost. Not only the enzyme activities and metabolite pools of the potato are altered under heat stress, but also morphogenesis, particularly the way in which the distribution of biomass between the green shoot and the tubers results in a change of sink strengths.

Although only growth and tuber formation and some parameters of carbohydrate metabolism were measured in this experiment, the complete redirection of the physiology of these plants becomes evident. One explanation may be the different Q_{10} values of the affected reactions which lead to changes in the steady states of the reactions. However, the redirection may also be a consequence of the heat shock reaction of the plants.

1.3.4.2
Heat Shock

Recommended Literature

- Nover L, Scharf KD (1997) Heat stress proteins and transcription factors. Cell Mol Life Sci 53:80–103
- Nover L, Scharf KD, Gagliardi D, Vergne P, Czarnecka-Verner E, Gurley WB (1996) The Hsf world: classification and properties of plant heat stress transcription factors. Cell Stress Chaperones 1:215–223
- Vierling E (1991) The roles of heat shock proteins in plants. Annu Rev Plant Physiol Plant Mol Biol 42:579–620

Heat shock is a short- or longer-term increase of temperature within the range of temperature which can still be tolerated.

Table 1.3.5 shows various reactions of normal metabolic functions (the so-called **housekeeping reactions**) in leaf tissue to a heat shock treatment lasting for 5 min. Instead of a short – but sharp – heat pulse, less heat can be applied for a longer time, i.e. the product of $+\Delta T \times t$ is the stress dosage.

The example of the potato plant mentioned earlier (Box 1.3.2) demonstrates that changes already take place at temperatures of around +30 °C. These changes result to a large extent,

Table 1.3.5. The effect of a 5-min heat pulse at the specified temperatures on various housekeeping functions of leaves of *Tradescantia fluminensis*. (Nover and Höhfeld 1996)

Tissue type	Change	Temperature range (°C) in which the change was observed
		37 39 41 43 45 47 49 51 53 55 57 59 61 63 65
Epidermis	1. Anthocyanin loss from the vacuole	
	2. Loss of plasmolysis	
	3. Loss of plasma streaming	
	4. Increase in viscosity	
Parenchyma	5. Loss of photosynthesis	
	6. Loss of chloroplast phototaxis	
	7. Loss of chlorophyll fluorescence	
All tissues	8. Uncoupling of oxidative phosphorylation	
	9. Loss of electrolytes	
	10. Loss of respiration	

Box 1.3.2 The effect of heat stress on the growth, tuber formation, carbohydrate level and enzyme activities of a heat-tolerant potato (*Solanum tuberosum*) variety ("Norchip") and a heat-sensitive variety ("Up-to-Date") (Lafta and Lorenzen 1995)

Both varieties were grown initially at 19/17 °C day/night temperatures and a daily 14-h light period. In experiment 1 the heat stress was applied at the onset of tuber formation (Tables 1 and 2) and in experiment 2 (Table 3) after the induction of the tubers. The heat stress consisted of raising the temperature from 19/17 °C to 31/29 °C in experiment 1 and to 29/27 °C in experiment 2. The duration of exposure to the heat treatment is given in the tables

Table 1. Biomass production and growth of the potato plants after a 4-week heat stress. 19/17 °C: control; 31/29 °C: growth at increased day/night temperatures. The variety "Norchip" is considered to be heat-tolerant, "Up-to-Date" is a temperature-sensitive variety. (Lafta and Lorenzen 1995)

Cultivar	Temp. (°C)	Shoot (g/plant)	Tuber (g/plant)	Total weight (g/plant)	Plant height (cm)
"Norchip"	19/17	244±6	134±3	378±4	35±1
	31/29	253±4	5±3	258±4	45±2
"Up-to-Date"	19/17	197±17	131±14	328±13	42±1
	31/29	96±2	0.6±0.6	96±3	57±0.5

Table 2. The influence of temperature on the carbohydrate level in the fully developed leaves of the heat-tolerant and -sensitive potato plants. The carbohydrate pools are filled by photosynthesis at the end of the day; at the end of the night they have been depleted. (Lafta and Lorenzen 1995)

Cultivar	Temp. (°C)	Carbohydrate	3 Days after temperature increase		8 Days after temperature increase	
			End of day	End of night	End of day	End of night
Norchip	19/17	Glucose	3.3±0.2	2.8±0.1	5.2±0.9	3.8±0.3
	31/29	(mg g^{-1} dry wt.)	5.3±1.1	2.5±0.0	6.2±0.9	3.0±0.1
Up-to-Date	19/17		3.0±0.1	2.9±0.3	3.5±0.0	2.9±0.2
	31/29		4.2±0.2	2.6±0.2	6.2±0.6	3.1±0.7
Norchip	19/17	Sucrose	10.6±0.1	6.4±0.8	10.1±1.0	3.4±0.2
	31/29	(mg g^{-1} dry wt.)	13.1±1.4	3.8±1.0	17.6±0.9	4.8±0.6
Up-to-Date	19/17		9.8±0.3	5.2±0.8	12.5±0.8	4.6±0.3
	31/29		12.9±1.3	3.6±0.3	17.0±1.2	4.5±0.9
Norchip	19/17	Starch	294±2	195±9	290±9	180±12
	31/29	(mg g^{-1} dry wt.)	202±14	92±9	129±2	72±3
Up-to-Date	19/17		222±32	117±19	242±14	126±12
	31/29		168±16	64±1	99±12	50±6

however, solely due to an acceleration of the normal housekeeping reactions.

The so-called **heat shock reaction** of the cell is quite a different matter. For plants with a temperature optimum between 15 and 25 °C, this reaction takes place at temperatures above +35 °C and consists of:

- the slowing down of housekeeping reactions, particularly of the expression of the housekeeping genes;
- an almost exclusive synthesis of so-called **heat shock proteins** (HSPs);
- cessation of the production of the HSPs after 6–8 h; and
- a gradual resumption of the expression of the **housekeeping genes**.

Box 1.3.2 (continued)

Table 3. Enzyme activities in the potato tuber tissue after 2 weeks of exposure to the day/night conditions quoted in column 2. The increased temperature treatment began 10 days after the initiation of tuber formation. (Lafta and Lorenzen 1995)

Cultivar	Temp. (°C)	Sucrose synthase (mg starch g^{-1} h^{-1})	AGPase (µmol g^{-1} min^{-1})	UGPase (µmol g^{-1} min^{-1})	Sucrose-P-synthase (mg sucrose g^{-1} h^{-1})	
					V_{max}	V_{lim}
Norchip	19/17	33.4±11.9	1.2±0.11	40.2±5.1	21±1[a]	6±0.9[a]
	29/27	13.7±3.8	0.93±0.05	39.9±5.2	15.2±1.5[a]	5±0.5[a]
Up-to-Date	19/17	15.0±4.2	1.17±0.12	27.8±3.1		
	29/27	4.2±2.3	0.84±0.08	24.4±3.1		

[a] In these cases potatoes grown under the 31/29 °C treatment were used; there was no statistical difference between the two cultivars. V_{max}: in the enzyme assay the substrates UDP glucose and fructose-6-P were saturating; V_{lim}: the concentrations of both substrates were reduced to about one-third, i.e. they were limiting in relation to the amount of enzyme used. Sucrose-synthase: sucrose + UDP ↔ UDP glucose + fructose; sucrose-phosphate-synthase: UDP glucose + fructose-6-P ↔ sucrose-6-P + UDP; AGPase = ADP glucose pyrophosphorylase: glucose-1-P + ATP ↔ ADP-glucose + PP_i; ADP glucose → → starch; UDPase = UDP glucose pyrophosphorylase: glucose-1-P + UTP ↔ UDP-glucose + PP_i; UDP glucose → → sucrose

From the selection of data presented in Tables 1, 2 and 3 the following can be derived:

1. Biomass production (growth) is inhibited at temperatures above the optimum. This inhibition was significantly less in the heat-tolerant than in the heat-sensitive variety.
2. Tuber formation was almost completely suppressed in both varieties.
3. Heat-stressed plants exhibited greater growth length than the controls.
4. The total levels of carbohydrate in the leaves of the heat-stressed plants were drastically reduced, primarily due to the starch contents. The comparatively low levels of soluble carbohydrates were not (glucose) or only slightly (sucrose) affected.
5. The sucrose-phosphate synthase activity (SPS) in the leaves was significantly increased at elevated temperature, whereby there was no difference in the responses of the two cultivars.
6. In the rudimentary tubers of the high-temperature plants the activities of both the enzymes involved in starch metabolism sucrose synthase (active phloem unloading: sucrose+UTP ↔ UDP glucose+fructose+P_i) and ADP glucose-pyrophosphorylase (supplies the substrate for starch synthase: glucose-1P+ATP ↔ ADP glucose+PP_i) were clearly decreased, while UDP glucose-pyrophosphorylase (sucrose synthesis) showed no noticeable differences in activity. SPS activity of the tubers was also reduced in the high-temperature plants. Thus neither starch nor sucrose synthesis takes place in these plants: the tubers have no sink strength.

The total redirection of protein synthesis in favour of HSPs occurs, in principle, only a few K above the optimal temperature of the organism. In other words: Many organisms already live near the upper temperature limit at which the stress reaction occurs, i.e. where activation of the heat shock genes starts. For example, *E. coli* has a optimum temperature of 37 °C versus 42 °C for the heat shock reaction; baking yeast: 23/37 °C; human beings: 37/42–45 °C; *Euglena*: 25/37 °C; tomato: 25/37 °C.

At present ten families of HSPs are known, which are ordered according to their molecular weight (Box 1.3.3). They occur in all compartments of cells.

Most HSPs are constitutively expressed proteins, so-called **molecular chaperones** ("governesses"), and are present under "normal" condi-

Box 1.3.3 Overview of the heat shock protein families (After Nover and Höhfeld 1996)

HSP family / Group of organisms	Cellular localisation: Cytoplasm/nucleus	ER/Golgi	Mitochondria (m)/ chloroplasts (c)	Comments
HSP100 family (Ec-ClpA/B)				
Mammals	**HSP110**	?	?	ATP-binding proteins with chaperone activity which are part of a protease complex in bacteria; in mammalian cells HSP110 is in the nucleolus
Plants	ClpB, HSP101	?	ClpCc	
Yeast	**HSP104**	?	**ClpB (HSP78)**	
HSP90 family (Ec-HtpG)				
Mammals	**HSP90** α, β	GRP94	–	ATP-binding, autophosphorylating proteins; interact with Ca-calmodulin; cytoplasmic representatives form complexes with steroid hormone receptors, protein kinases and HSP70
Plants	**HSP80**	GRP94	HSP90c	
Yeast	**HSP90**, HSC90	–	–	
HSP70 family (Ec-DnaK, Ec-Hsc66)				
Mammals	**HSP70, A, B** HSC72, p73	GRP78 (BIP)	HSC70m	ATP-binding autophosphorylating proteins; bind to denatured/ unfolded proteins; part of a chaperone machinery together with representatives of the HSP40 and HSP23 families
Plants	**HSP70**, HSC70	GRP78	HSC70c **HSP68m**	
Yeast	**SSA 1–4**, SSB1,2 **MSI3**, SSE1/2	KAR2	SSC1m	
HSP40 family (Ec-DnaJ, Ec-CbpA)				
Mammals	**HDJ-1, HDJ-2**	?	?	Always closely associated with representatives of the HSP70 family; responsible for recognition of unfolded protein substrates by HSP70; ANJ1 with farnesyl side chains integrated into the glyoxysome membrane
Plants	**ANJ1**	+	?	
Yeast	YdJ1, SIS1	SEC63	**SCJ1**, Mdj1	
HSP60 family (Ec-GroEL)				
Mammals	TCP1	–	**Cpn60m**	ATP-binding, oligomeric protein complexes; interact with representatives of the HSP10 family; essential role in protein folding; various TCP1-type proteins in the cytoplasm form complexes of ~ 900 kDa which interact with actin and tubulin systems
Plants	TCP1	–	HSP60c	
Yeast			**HSP60m**	
	TCP1 α, β BIN2/3 ANC2	–	HSP60m	
HSP10 family (Ec-GroES)				
Mammals	?	–	Cpn10m	Heptameric complexes which work together with the HSP60 complexes; presence of corresponding subunits for TCP1 complex is still not clear
Plants	?	–	Cpn10m Cpn12c	
Yeast	?	–	Cpn10m	
HSP23 family (Ec-GrpE)				
Mammals	?	?	?	Little studied subunits of the HSP70 chaperone machinery; binds to SSC1 in yeast mitochondria; essential for protein import into mitochondria; nucleotide exchange factor
Plants	?	?	?	
Yeast	?	?	Mge1p	

Box 1.3.3 (continued)

HSP family / Group of organisms	Cellular localisation: Cytoplasm/nucleus	ER/Golgi	Mitochondria (m)/ chloroplasts (c)	Comments
HSP20 family (Ec-lbp A/B)				
Mammals	**HSP 25/27, α,β-Crystallin**	?	?	More variable in number and sequence than other HSP families; sequence relationship to eye lens proteins (α,β-crystallin); frequently forms oligomeric aggregates of 200–500 kDa; protective function for mRNA in plants
Plants	**HSPs 15–18**	**HSP22**	**HSP 21/22c**	
Yeast	**HSP26**	–	–	
HSP8,5 family (Ec not present)				
Mammals	Ubiquitin	–	–	Small protein which is considered to act as a marker for protein breakdown via isopeptide links with Lys side chains (substrate for proteosome complexes)
Plants	Ubiquitin	–	–	
Yeast	Ubiquitin	–	–	
Immunophilins (Ec-cyclophilin/-FKBP)				
Mammals	FKBP 12, 25 CYP 18, 22, 40 **HSP56**	FKBP 13 CYP 22	?	Proteins with prolyl-*cis,trans* isomerase activity with ubiquitous distribution; activity is inhibited by immunosuppressants (cyclosporin A, FK 506)
Plants	**CYP**	–	CYP, FKBP	
Yeast	**CYP 1, 2**	**FKBP 13**	CYP 20	

The multiprotein families are summarised on the basis of sequence homologies and functional similarities. The representatives that are induced by heat stress are shown in **boldface type**. The family name is followed by the designation of the proteins which have been identified in *Escherichia coli* (Ec). Designations which deviate from the standard nomenclature for heat stress proteins (e.g. HSP70) often originate from the identification of the corresponding gene defects in microorganisms

tions only in very small amounts. During stress, the housekeeping genes are "switched off" and subsequently only HSPs are produced.

Every stress that leads to a denaturing of proteins and to the formation of "false" proteins, i.e. which attacks at the level of proteins (heavy metals, insertion of amino acid analogues into proteins, inhibitors of protein biosynthesis, alcohol), triggers heat shock reactions, which are thus a general and not a specific stress reaction. Heat shock reactions are currently one of the best-investigated reactions to stress and confer a number of highly interesting insights into cell physiological aspects at the molecular level. These are, in particular:

- the switching off of the expression of the housekeeping genes;
- the reaction of existing proteins to heat shock;
- the switching on of the heat shock genes: what is the molecular signal and how does the switch function?
- the function of the HSPs;
- the function of the HSPs in the unstressed cell;
- the return of the cell to "normal" life after the heat shock and the switching off of the heat shock response.

These aspects are discussed in the following sections. At times it will be necessary to consider results from microorganisms and animals, when research in plants has not yet progressed far enough in itself.

How is the Expression of Housekeeping Genes Switched Off During Heat Shock?

After its synthesis, mRNA is normally packed with small nuclear ribonucleoproteins (snRNPs), which together with small nuclear RNA molecules (snRNAs) form the spliceosome. This excises the introns from the pre-mRNA, and thus plays an essential part in mRNA processing. This splicing reaction does not occur after heat stress, so that no functional mRNA of the housekeeping genes is formed. The processing of ribosomal precursors in the nucleolus also ceases at the same time, i.e. no new ribosomes are formed.

Translation is also interrupted: Heat stress leads to the inactivation of initiation factors. In biochemical terms this happens via the dephosphorylation of an initiation factor (the eukaryotic IF-4Fα) which binds to the 5′ cap [7mG(5′)ppp(5′)nucleotide] of the mature mRNA and thus contributes to the formation of the initiation complex. Protein synthesis, which is still ongoing, ceases after a few minutes, as the still-active polysomes can no longer bind to new mRNA molecules and thus dissociate into their subunits.

Heat shock genes do not possess introns; they need not be spliced and thus form functional mRNA (or functional mRNPs) even when the splicing reaction is inhibited. This can then form functional ribosomes and polysomes with the dissociated ribosomal subunits. The formation of the HSmRNA-ribosome complex evidently does not require the formation of an initiation complex with "capped" mRNA.

Most of the no longer active housekeeping mRNA is conserved and deposited in so-called **heat shock granules**, where it is associated with two heat shock proteins, an HSP70 and an HSP17. These granules are held in the immediate vicinity of the cell nucleus by the cytoskeleton.

How Does Heat Shock Affect Proteins?

Heat causes denaturation of proteins. Denaturation means partial or total unfolding of the tertiary structure and the dissociation of subunits, in short the production of non-functional polypeptides. Partial unfolding can explain how hydrophobic side chains are no longer "shielded" within the protein (hydrophobic interactions), but are exposed. As a consequence, several proteins associate via hydrophobic interactions: they aggregate and form insoluble complexes, which can only be removed by proteolysis. Heat shock thus causes a disturbance of the protein homeostasis of the cell via denaturation; the "internal equilibrium" of the cell is disrupted.

Switching On Heat Shock Genes: What Is the Molecular Signal and How Does the Switch Function?

The promoters of the heat shock (HS) genes are of prime importance for switching on the expression of these genes. These promoters exhibit conserved motifs, so-called **heat shock elements** (HSEs), which display a mirror sequence in the two complementary strands (a so-called **palindrome**):

- 5′.... AGAA n n TTCTAGAA n n TTCT ....3′
- 3′.... TCTT m m AAGATCTT m m AAGA ....5′

... n and m are variable and not fixed.

Heterologous insertion of these HSEs into other promoters makes these promoters responsive to heat stress.

The simplest type of HS promoter contains a TATA box for association with the transcription apparatus about 30 base pairs upstream of the start of transcription, and one to several HSEs a further 20–30 base pairs upstream. Such promoters could be of great importance in biotechnology: They enable genes to be switched on in a specific manner.

Which Factors Activate Heat Shock Promoters By Reaction with Heat Shock Elements (*cis*-Responsive Elements)?

These are proteins which interact with the DNA of HS elements, the so-called **HS-transcription factors (HSFs)**. These proteins exhibit similar sequencing motifs associated with particular functions in all eukaryotes. Their expression is activated by heat. They are divided into families according to their size. Any one plant will contain HSFs from different families.

Ubiquitous functional motifs of heat shock factors are (Fig. 1.3.3):

- a DNA binding domain of about 100 amino acids near the N-terminus which binds to the *cis* elements (HS elements);
- an adjacent region in the C-terminal direction with repetitive hydrophobic heptapeptides which is important for the association of several monomer HSFs via hydrophobic interactions (the so-called oligomerisation domains: HR 1, HR 2 and sometimes also HR 3). Only

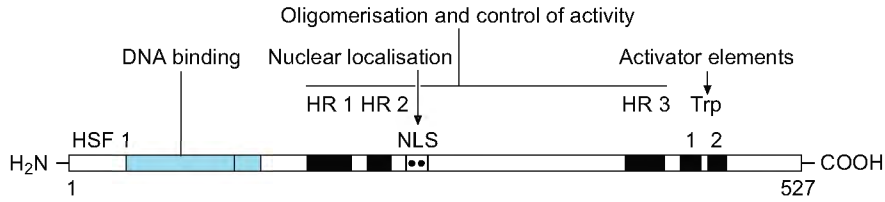

Fig. 1.3.3. Structure of a heat shock (transcription) factor from tomato. H_2N- N-terminus; *DNA-binding* binding domain on the HSE; *HR 1,2,3* regions for oligomerisation via hydrophobic interactions; *NLS* nuclear localisation sequence; *Trp* central tryptophan of the activator element. (After Nover and Höhfeld 1996)

the oligomerised HSF (trimer) is active, i.e. binds to DNA and HSPs;

- a cluster of alkaline amino acids C-terminal to HR 1, 2: the signal peptide for import into the nucleus (NLS: nucleus localisation signal);
- peptide motifs with a central tryptophan (Trp) as activator element which can interact with the transcription apparatus and thereby activate it. In larger HSFs the activator region contains a further hydrophobic heptade.

A Heat Shock Factor Must First Be Activated

Monomeric HSFs are inactive, i.e. they cannot bind to a heat shock element. The pool of HSFs is probably kept small in the normal state of the cell because of binding to the free HSPs (HSP70 and probably also HSP90). After dissociation from the HSP70/90 (and perhaps due to heat), the HSF changes its form so that it is able to form trimers. The trimer formed in the cytoplasm must now be imported into the nucleus to bind to the heat shock element of the promoter. The activator region becomes active because of binding to the HSE, and transcription can start.

What is the "actual signal" in the reaction chain leading to the activation of HSFs? The prerequisite for the activation of the HSFs is the dissociation of the HSF-HSP70/90 complex. Such dissociation would, of course, be favoured by a decrease in the free pools of HSP70/90. But how can the free pool of these HSPs decrease as a result of heat shock? These proteins bind as a consequence of the heat shock to other, denatured, proteins. It may be readily assumed that heat-denatured housekeeping proteins bind the free HSP70.

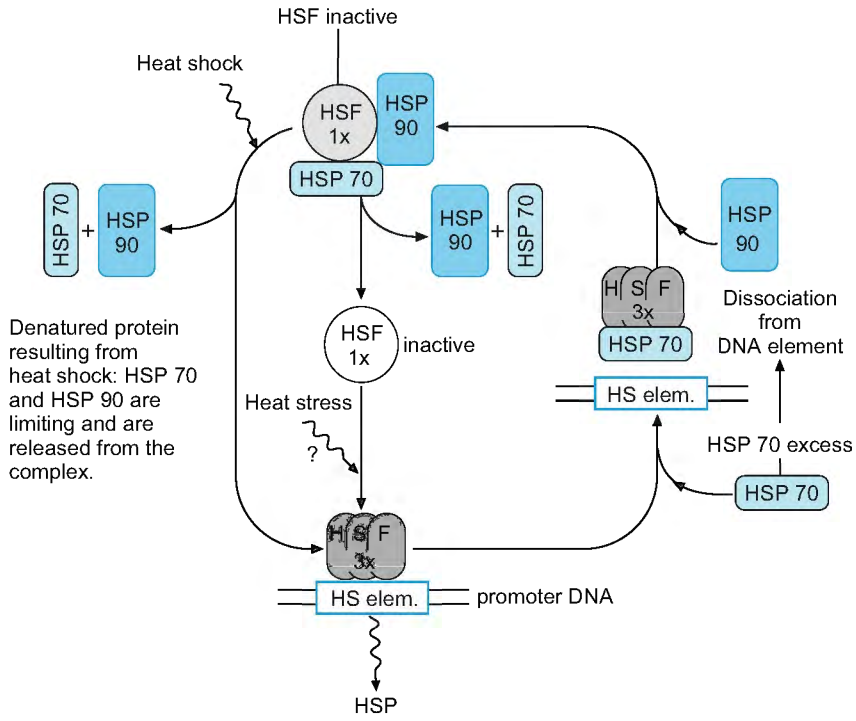

Fig. 1.3.4. The heat shock factor (HSF) cycle. (After Nover and Höhfeld 1996)

Box 1.3.4 Do HSPs have functions in non-stressed cells?

Most heat shock proteins (HSPs) are produced in small quantities even in non-stressed cells. This is the basis for the genesis of the signal for the activation of the HSF described in the text by the lowering of the homeostatic HSP level due to binding to denatured or partially denatured housekeeping proteins. It can be asked whether the HSPs also have other functions in cellular processes. The answer comes from protein biochemistry: Nascent polypeptides already tend to fold while still attached to the ribosome during their synthesis. The structural elements of the α-helix and the β-folded sheets arise more or less spontaneously in this way via H-bonds, van der Waals forces and hydrophobic interactions between the adjoining amino acid residues. Further folding, and in particular the formation of domains, requires, however, a fully synthesised polypeptide. This is because the active form of a protein is not always that possessing the lowest energy, but is rather a metastable form which is more flexible. Two phenomena must thus be counteracted: the formation of a basically incorrect domain during the synthesis of the polypeptide and (inappropriate) folding into the lowest energy state.

First, the nascent protein must be protected. A chaperone of the HSP70/DnaK family therefore associates with the growing polypeptide during its synthesis and prevents misfolding under ATP consumption (see Fig. 1.3.5). In order to fold correctly, the complete protein is transferred to HSP60/GroEL-ES (TCP-1). These chaperones form two rings, in the central cavity of which the folding proceeds; this once again requires the consumption of ATP.

The cell thus requires an effective system of molecular chaperones to ensure the correct folding of its proteins. These chaperones present in the non-stressed cells are considered to be related to the HSPs and have been termed HSCs, i.e. heat shock cognates, by some authors.

A second important function of chaperones relates to **topogenesis**: Most of the proteins of a cell are synthesised in the cytosol. Many of them, however, are part of the protein complement of organelles, i.e. the proteins must first find their way into the correct organelle. This takes place with the help of additional sequences, so-called signal peptides, which are mostly N-terminal sequences but can also be localised within polypeptides or at the C-terminus. The organelles possess receptors for the relevant signal peptides, which bind to the protein to be imported and are mostly also involved in the transport of the protein across the membrane. The signal peptides are usually removed upon import into a specific organelle or incorporation into a specific membrane, a process termed protein maturation. A complicated pathway can be dependent on several signal sequences, e.g. the incorporation of a protein into the thylakoid membrane requires the sequential removal of four such signal peptides.

In order to pass through a biomembrane a polypeptide (pre-protein) must unfold, i.e. it must be stretched out as linearly as possible. It must thus depart from its energetically favourable tertiary structure, for which chaperones are once again required. The movement through the membrane is furthermore favoured in that the polypeptide assumes a different configuration on the inner side; for this HSCs are also necessary. When it has arrived at its destination, the protein must be brought into its functional form and in some cases be incorporated into the membrane; these processes also require HSCs. Since the function of the HSP/HSCs is usually coupled with energy consumption, it is not surprising that up to 100 ATP molecules must be used up to ensure that a newly synthesised protein arrives at its final destination. The large number of HSP/HSCs in an organism (Hartl et al. 1994) is quite understandable when one assumes that these polypeptides are also specific for particular organelles and are correspondingly widely distributed.

This process may be illustrated as the HSF cycle (Fig. 1.3.4), which provides a fine example of a self-regulating system.

The question as to why HSPs bind to denatured proteins goes far beyond the aspect of "heat shock" itself and points to the function of constitutively expressed heat shock or stress proteins (Box 1.3.4).

What Is the Function of Heat Shock Proteins in the Cell Stressed by Heat?

Heat shock proteins have at least three different functions in relation to the stresses which denature proteins:

- chaperone function,
- conservation of housekeeping mRNA in HS granules,
- catalysing proteolysis of irreversibly denatured, aggregated proteins.

Heat Shock Proteins as Chaperones

The system of the HSP70 chaperones is that having been best investigated. It consists of at least three HSPs – HSP70, HSP40 and HSP23; in the bacterial system they are called DnaK, DnaJ and GrpE. The mechanism of chaperone operation is best understood for prokaryotes and the prokaryote system will therefore be explained here as a model (Fig. 1.3.5).

HSP70 (DnaK) has an N-terminal ATP- or ADP-binding domain and a C-terminal domain for interaction with proteins. HSP40 (DnaJ) binds to the partially unfolded protein ("U" in Fig. 1.3.5) via a cysteine-rich C-terminal zinc finger domain. After hydrolysis of the bound ATP (→ ADP-DnaK), the ADP-DnaK binds to the N-terminal J domain of DnaJ-U. Denatured proteins (probably in the form of HSP40 complexes) stimulate the ATPase activity of HSP70/DnaK. A ternary complex is formed, in which the partially unfolded protein U is bound to both HSPs. The ternary complex interacts with HSP23 (GrpE: nucleotide exchange factor), which leads to the dissociation of ADP from the complex and the weakening of the interaction of U with HSP70/DnaK. The complex dissociates completely upon renewed binding of ATP to HSP/70/DnaK, and U is now able to fold back into its native state or to enter into the cycle again if it possesses other denatured components. How many and how often HSP70 molecules react with U is not known. The energy required to completely fold a medium-sized protein corresponds to around 100 ATP.

HS Granules

Since it is non-functional upon heat stress, housekeeping mRNA would be predestined for rapid degradation by RNAses. The return to normal cell metabolism, however, requires large amounts of housekeeping mRNA, and it is therefore advantageous to conserve this mRNA during the heat stress response. Association of released mRNA with HSPs protects the housekeeping mRNA from degradation. This association leads to the formation of heat stress granules of 40 nm in size with a molecular mass of about 500 kDa, which are held in the vicinity of the nucleus by the cytoskeleton and contain mainly proteins of the HSP70 and HSP20 types. The HS granules dissociate again after the heat stress response subsides, whereby it is not clear how this dissociation is triggered.

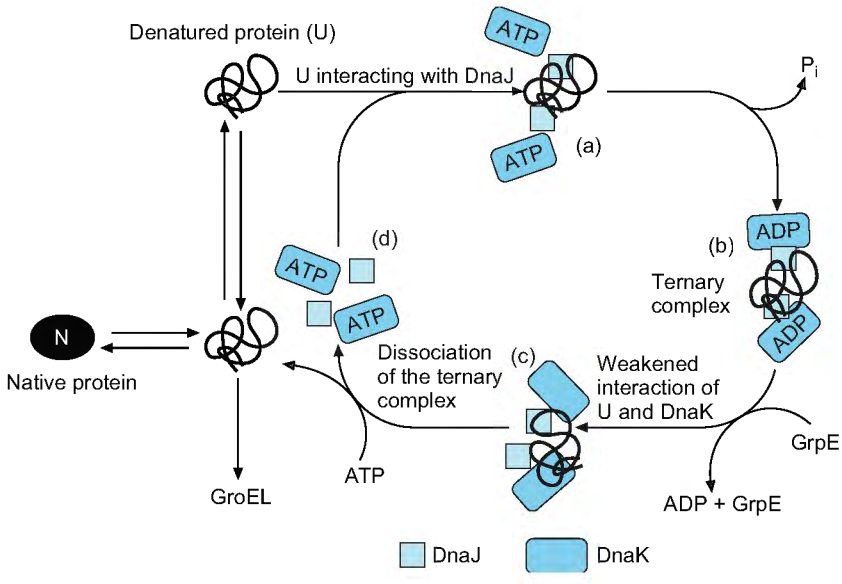

Fig. 1.3.5. Model of the HSP cycle using the bacterial DnaK, DnaJ, GrpE and GroEL system as an example. Explanations in the text. (After Hartl et al. 1994; see also Georgopoulos 1992; Nover and Höhfeld 1996)

Catalysis of Proteolysis of Irreversibly Denatured and Aggregated Proteins

Some proteins cannot be rescued after a heat pulse: They are irreversibly denatured, i.e. incorrectly folded, and have already formed aggregates with each other to some extent. Incorrectly or irreversibly denatured proteins must be removed from the cell rapidly and efficiently; this also costs energy. First, these proteins must be recognised and marked. This takes place upon cleavage of the N-terminal methionine by the protein **ubiquitin**, which exhibits enhanced expression in stressed cells. The expression of the helper enzymes of the ubiquitin conjugation reaction pathway, which are required for marking, is also enhanced. The protein marked repeatedly by ubiquitin is now broken down in **26S proteasomes** under consumption of ATP. The manner in which the HSPs are involved in the proteolytic degradation of "protein rubbish" has not yet been convincingly elucidated. Mutants which cannot form HSP70/DnaK also have a defect in ATP-dependent, rapid protein degradation.

How Does the Cell Return to "Normal Life" After Heat Shock, i.e. How Is the Heat Stress Response Switched Off?

If the depletion of the free pool of HSP70 is the signal for triggering the heat shock response, the growth of this pool, e.g. after the removal of defective protein, should lead to normal protein synthesis again. A higher concentration of free HSP70 leads to inactivation of the heat protection factors, and this would also stop the synthesis of HSPs or at least reduce it to the normal level. At the same time, the transiently immobilised, but conserved housekeeping functions, which also include the processing of the large precursor rRNPs accumulated in the nucleolus, could be activated again. Many of the questions as to the switching on of the housekeeping functions are not understood at present. This applies particularly to the other stressors which disturb protein homeoeostasis, such as in heavy metal stress, etc.

As mentioned earlier, long-term exposure of plants to temperatures 10 K above the optimum suffices to induce heat stress reactions. This means that plants already live close to the verge of this reaction. There is a relatively great danger that overheating during the day can progress to the point where this complete rearrangement of metabolism can occur. Figure 2.1.15 shows that irradiated leaves can rapidly reach such temperatures. Avoidance mechanisms to reduce overheating are therefore of great importance.

1.3.4.3 Avoidance of Heat Stress

Avoidance mechanisms may be morphological (see also Chap. 2.1): Slit leaves heat up less than do undivided leaves, and hairy leaves reflect more radiation than do those without hair. The position of the leaf, and thus the extent of direct (solar) radiation it absorbs, also plays an important role. Transpiration cooling is an important physiological avoidance mechanism which can result in considerable lowering of leaf temperatures (see Fig. 2.1.15). The combination of heat and drought is problematic for plants in this regard, in which transpiration is substantially limited (see Chap. 2.2).

1.3.4.4 Hardening

In addition to heat avoidance, there is, naturally, also heat tolerance acquired through hardening. The changes in membrane lipids which take place during the course of the year have already been mentioned (Chap. 1.3.3.1). It is not known to what extent heat shock proteins (HSPs) play a role in hardening. The mechanism of the heat shock response would suggest that a certain degree of hardening occurs within the period of the accumulation of HSPs – hours or days, at most. It is difficult to envisage long-term hardening occurring via HSPs.

Hardening based on damage repair was shown by Larcher (1987) for fir needles (Fig. 1.3.6). In this example, recuperation from the initial heat stress takes 5–6 days, i.e. it took this much time to effect repair and to switch from heat stress reactions to normal housekeeping photosynthesis. It can be assumed that a higher level of HSPs was still present at the time of the second heat stress. On the other hand, there are also so-called **chemical chaperones** which can increase the heat resistance of cells and shift the triggering of the HS response toward higher temperatures. These are the compatible solutes, such as glycerol, proline and betaine, which stabilise the native folding of mature proteins. It is not known to what extent such effects play a role in heat stress hardening.

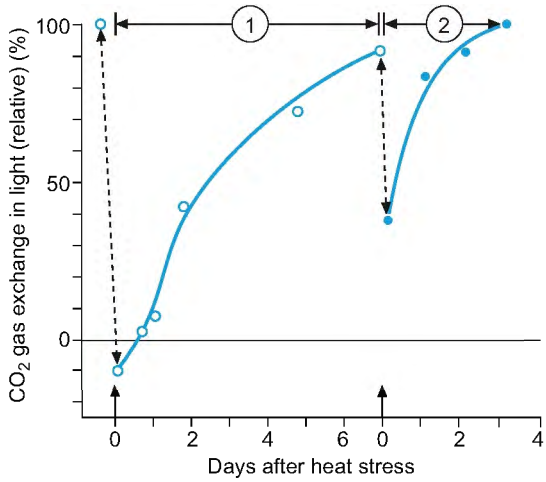

Fig. 1.3.6. Hardening of fir needles after a heat stress treatment. Damage and hardening were measured by the photosynthetic activity of the needles. *1* Recovery phase after the initial heat stress treatment; *2* after the second heat stress treatment (44 °C, 30 min). The extent of damage and the length of the recovery phase (repair) are clearly less after the second stress treatment than they were upon the first heat pulse. (Larcher 1987)

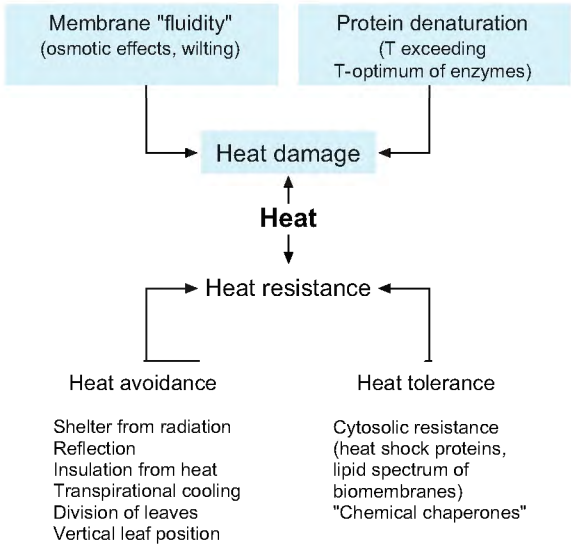

Fig. 1.3.7. The effects of heat on plants and the ways in which they react to the heat stress

The aspects of heat damage, heat avoidance and heat tolerance described above are summarised in Fig. 1.3.7.

1.3.5
Cold

Recommended Literature

- Bigras FJ, Colombo SJ (2001) Conifer cold hardiness. Kluwer, Dordrecht
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On about two-thirds of the land mass of the earth the annual minimum temperatures fall to below 0 °C, on half of the land mass to −10 °C. Thus it is not surprising that cold is the best studied of any abiotic stressor for plants and animals, and it is of enormous economic importance.

In comparison with the stressor heat, where the primary reaction affects membranes and metabolic and protein homeostasis, the environmental factor cold additionally causes stress by desiccation of tissues with accompanying ice formation at temperatures below the freezing point (freeze-desiccation). Distinction is therefore made between cold stress above the freezing point, so-called **chilling**, and stress by frost, i.e. at temperatures below the freezing point, which always includes a temperature component and a desiccation component caused by ice formation (Fig. 1.3.8).

The phenomena of chilling (see Allen and Ort 2001), rather than those of freezing, are thus analogous to heat stress. In heat stress the physiological strain occurs through the (dissimilar) speeding up of metabolic processes culminating in the specific heat stress response. While metabolic processes also get out of equilibrium during cold stress due to the different activation en-

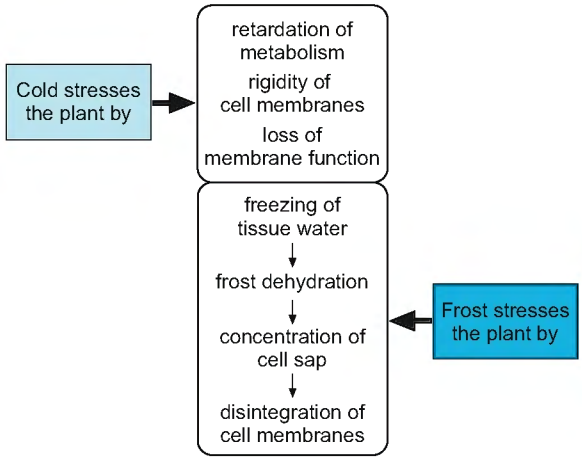


Fig. 1.3.8. Stress from cold and frost

ergies of individual reactions, the strain is altogether less because all of the reactions are slowed down. A rapid cold stress response like that of heat shock in the form of heat shock proteins is therefore not to be expected, even though the term "cold shock proteins" is used at times in the literature. These are not proteins with chaperone functions, but rather proteins whose names indicate their various functions: antifreeze proteins, cold acclimation proteins or proteins of the group of "dehydrins".

A problem specific to cold stress is the increase in viscosity of the membranes. An organism which cannot react rapidly due to low temperatures and possibly also due to severe tissue desiccation is much more prone to secondary

Box 1.3.5 Measurement of cold and frost damage

In order to determine damage due to cold or frost or to determine the limits of resistance, a standardised stress treatment is required. In contrast to the case with heat shock, a comparatively slow change in temperature is preferred when analysing the effects of cold, in order to allow for the slower rate of biochemical reactions at lower temperatures.

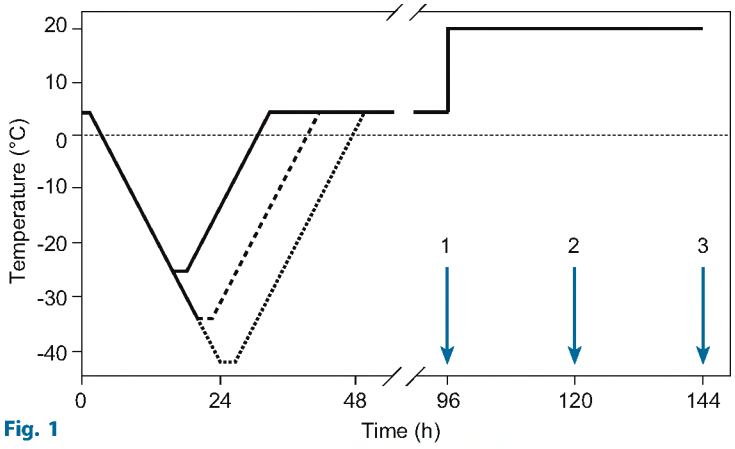
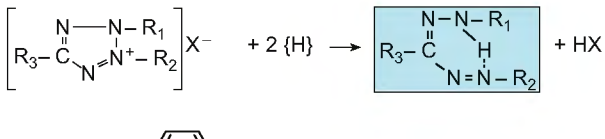
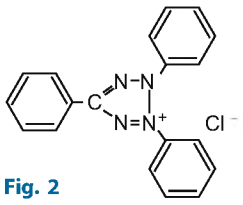


Fig. 1

Fig. 1. shows the temperature course during the entire test. First, the sample is slowly cooled (1–2 K/h). It is then exposed to a defined final temperature for 2 h before being slowly warmed (1–2 K/h) again. The sample is then held at +5 °C for 48 h to enable any incurred damage to develop. It is then placed in a solution of TTC. Dehydrogenases now reduce the tetrazolium to coloured formazan (Fig. 2, formazan is shown in blue). After 24 h this is extracted (for ca. 12 h) and quantified photometrically.





2,3,5,-Triphenyltetrazolium chloride (TTC)

Fig. 2

In addition to this test, the vitality tests presented in Box 1.1.1 can also be used.

stressors. Particularly interesting in this regard is the stress combination of cold and high light energy, which leads to considerable damage in non-hardened plant organs, especially in the photosynthetic apparatus (see Chap. 1.2.1.3). Frost hardening and frost dehardening are, therefore, classical topics for the study of adaptation reactions. The response of plants to cold takes the form of both specific adaptations and general stress reactions (strain). The latter relate to changes in membranes, as well as to strengthening of systems for the detoxification of radicals and reactive oxygen species (see Fig. 1.3.32).

Because of the slower rate of metabolism in the cold, experiments to measure cold tolerance and cold damage must be very carefully designed. Plants must not be cooled too rapidly, nor should they be warmed up too quickly at the end of the experiment. Box 1.3.5 shows an appropriate procedure.

1.3.5.1 Stress by Cold Above the Freezing Point (Chilling): Cold Damage Is Predominantly Membrane Damage

Damage due to temperatures which are low, but above the freezing point, occurs practically only in tropical and subtropical plants which are genetically adapted to a temperature range of between +5 and about 50 °C (see Table 1.3.1). Palms, mangroves, coffee plants and some tropical herbs known to us as houseplants, such as *Peperomia* and the African violet (*Saintpaulia ionantha*), have been studied particularly intensively. As is the case for temperature stress in general, the individual organs of a plant show different sensitivities. A good example is the coffee plant (*Coffea arabica*, Fig. 1.3.9). It is typical of chilling that the old leaves (as well as the cambium in the root) are most sensitive to cold, not the young leaves. Individual metabolic processes also exhibit different sensitivities. Among the most sensitive are membrane-bound processes, such as ion homeostasis and photosynthesis. Certain stages in the development of a plant are also particularly cold sensitive, namely germination and fruit ripening. In general, those stages are most sensitive in which the system for the detoxification of radicals and aggressive oxygen species is not yet, or no longer, very efficient. Pollen ripening is, at any rate, the most sensitive stage.

The actual amount of chilling stress is, as far as is known, the product of intensity and the duration of the stress, i.e. a 4-h period of cooling to +1 °C results in approximately the same degree of damage as lowering the temperature to 3.5 °C over a 20-h period (Chap. 1.1.3, Fig. 1.1.6). At the subcellular level, chilling damage

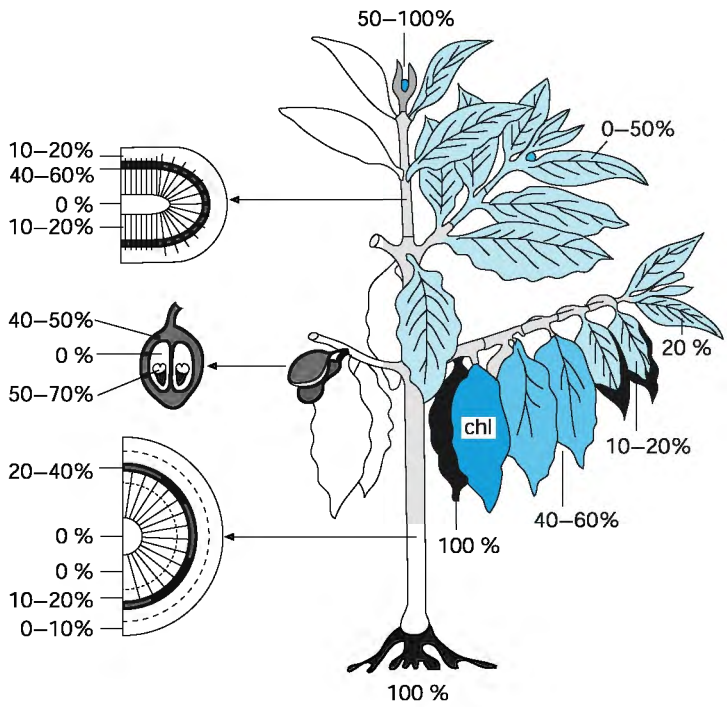


Fig. 1.3.9. Differences in sensitivity to cold in organs of the coffee tree. Percentual damage after 3 days of continual cooling to +1 °C. *Black* Complete damage; *chl* chlorotic. (Larcher 2003)

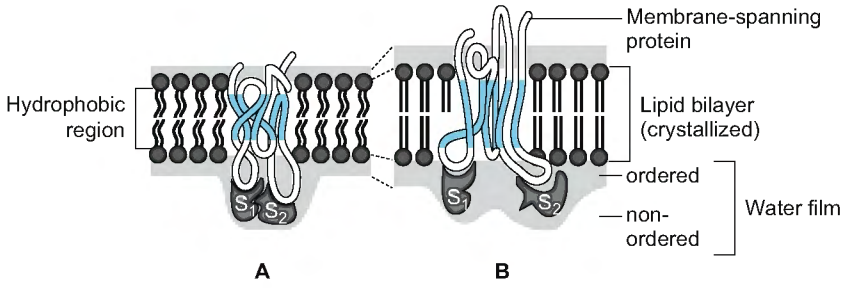


Fig. 1.3.10. Model of a biomembrane in cross section, in a fluid (active A) and in a rigid (inactive B) state. S_1, S_2 substrates. (After Wolfe 1978)

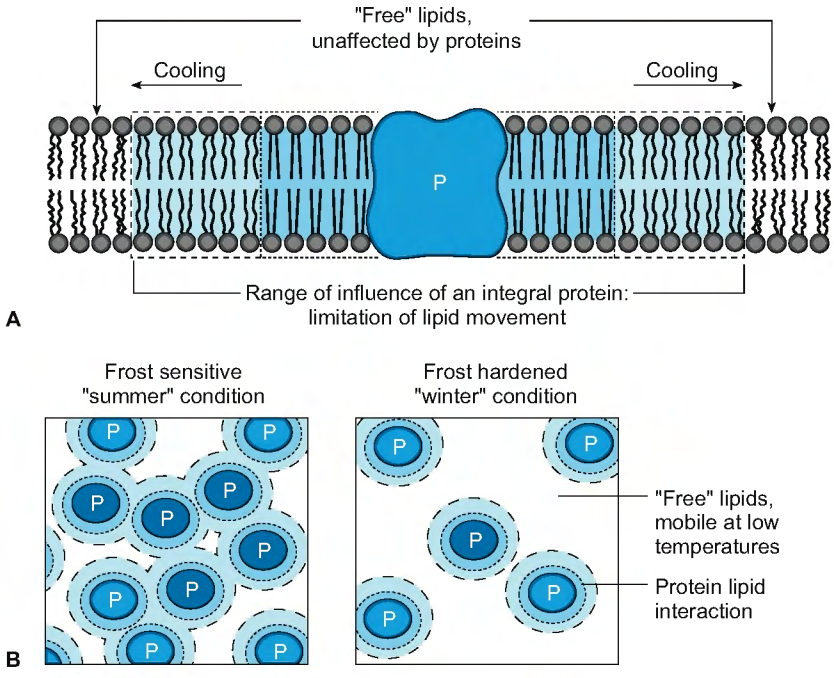


Fig. 1.3.11. A Schematic representation of the interactions between proteins and lipids in thylakoid membranes during cooling. B Influence of frost hardening on the processes taking place during cooling of the thylakoid membranes of *Pinus sylvestris*. (After Vogg et al. 1998)

occurs through metabolic imbalances and membrane "stiffening".

How does cold inactivate membranes? The classical conception is that when cells which are not cold resistant are cooled, the biomembrane lipids change from the fluid aggregation state to the crystalline, inflexible state. This is accompanied by a thickening of the biomembrane and a deformation (inactivation) of integral membrane proteins (**thermotrophic phase transition**). This explanation is plausible, but is certainly oversimplified (Fig. 1.3.10).

It is much more likely that a zone of lipid molecules with limited mobility surrounds the proteins spanning the membrane. This zone expands during cooling; at dense protein packing, i.e. at high membrane protein content, such zones can aggregate and form larger areas with high viscosity (Fig. 1.3.11).

In such regions membranes appear to be no longer "tight", making it difficult to form and maintain concentration gradients. Furthermore, ion pumps are inactivated by cold, which also results in a progressive breakdown of compartmentation. This applies particularly to the proton gradients which are required for the supply of ATP in the cell. It is still unresolved as to whether the water loss which is often observed from damaged cells is merely a consequence of the loss of solutes from the cell (passive flux) or whether aquaporins play a role (see Box 1.5.2).

The sensitivity with which particular membranes react to cold appears to depend on the type of plant involved. In some cases the plasma membrane has been identified as the site of primary damage (e.g. for *Eucalyptus* cell cultures), in other cases the tonoplast is probably particularly cold sensitive (e.g. in spruce).

In contrast to the annual cycle of frost hardening and dehardening, there is almost no acclimation to chilling. No appreciable shifts to lower LD50 values could be observed with either mangroves or palms. However, there are genotypes with different cold sensitivities within the same plant species. The less cold-sensitive genotypes are usually found in the subtropics (10 to 23 °N and S of the equator), and the more sensitive ones in the tropics (10 °N and S of the equator). Molecular mechanisms effecting hardening in the more resistant genotypes are usually based above all on a higher degree of unsaturation of the membrane lipids.

It is of particular interest in this connection to modify the membrane lipids of important crop plants to confer greater cold tolerance. One way of achieving this is to lower the proportion of saturated fatty acids (see Box 1.3.6 and Table 1.3.4) in favour of unsaturated fatty acids. In principle, this concerns the group of the glycerol lipids which can be either phosphatides or glycolipids. Initial genetic engineering attempts to increase the amount of unsaturated fatty acids

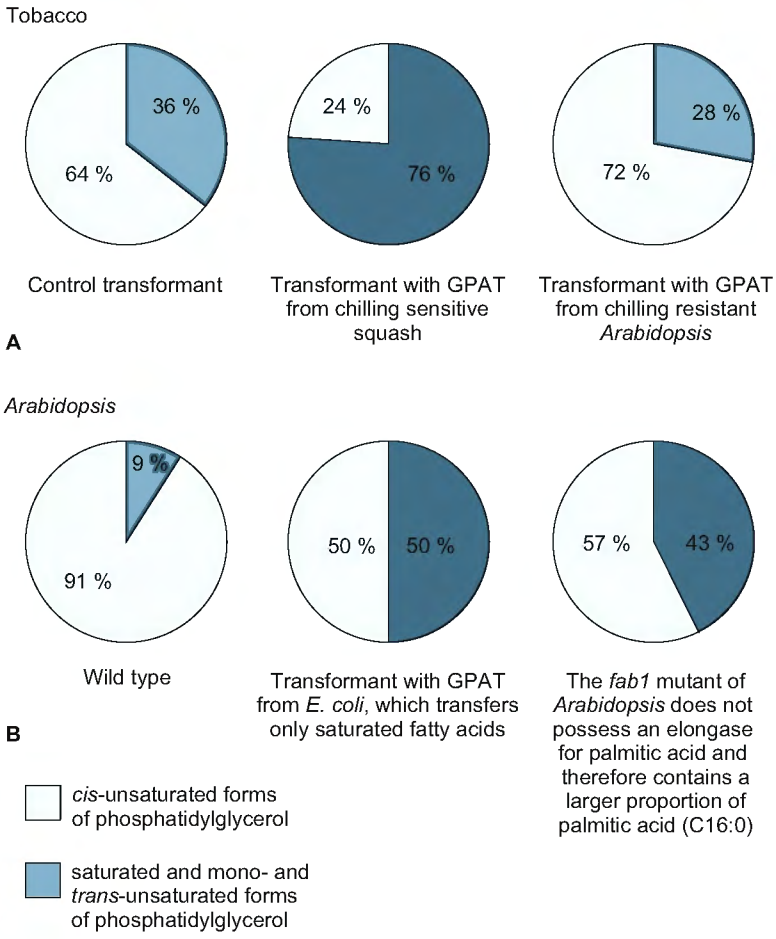
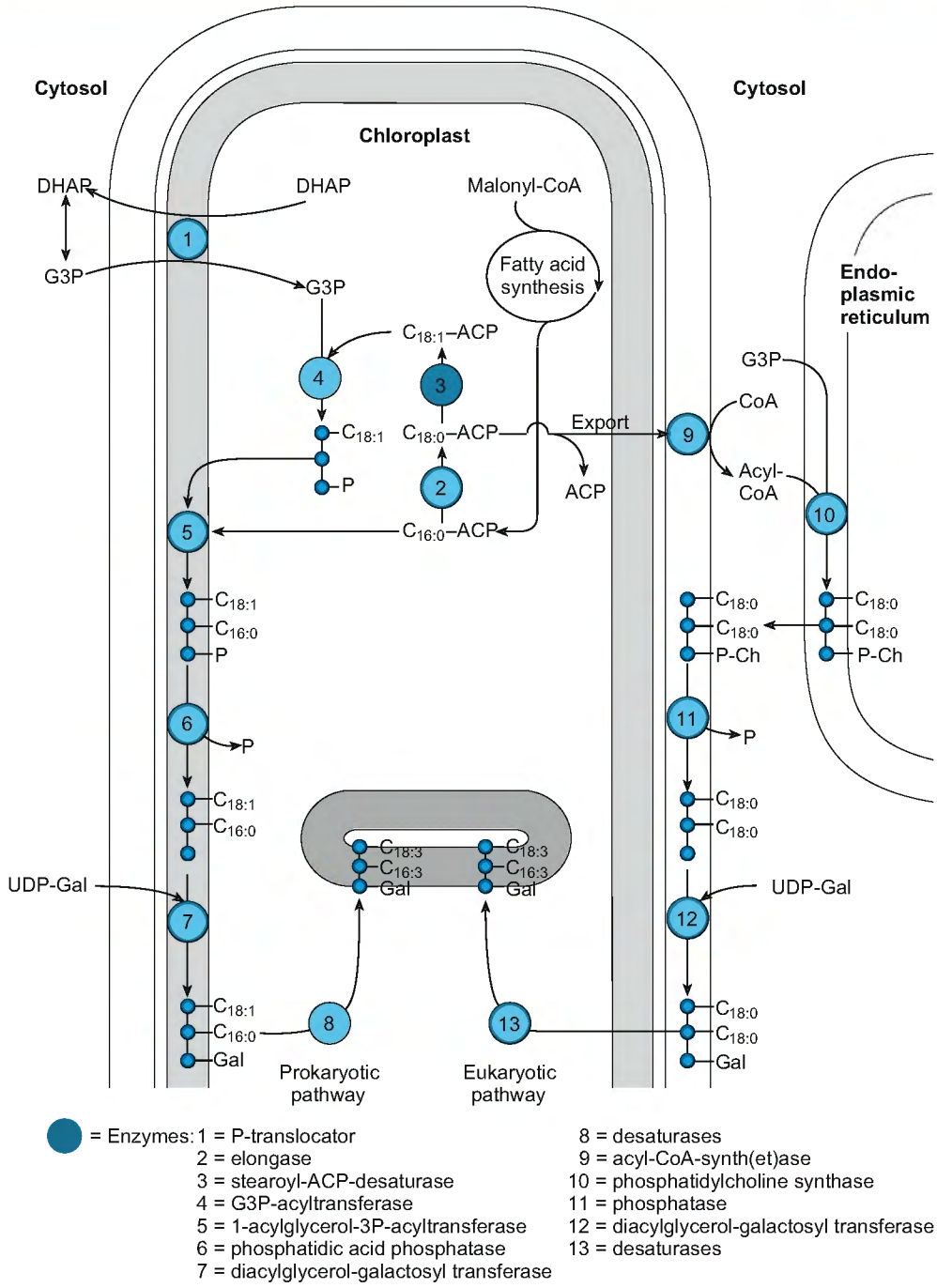


Fig. 1.3.12. Changing chilling sensitivity by altering the degree of unsaturation of the fatty acids in phosphatidylglycerol. Chilling-resistant and -sensitive plants are available for gene transfer. It is important that chloroplastic glycerol-3P-acyltransferase (GPAT) from chilling-resistant plants (*Arabidopsis*) uses almost exclusively 18:1-ACP as substrate, whereas the enzyme from chilling-sensitive plants (e.g. cucurbits) can transfer fatty acids from both 18:1-ACP and from 16:0-ACP (see Box 1.3.6). When glycerol-3P-acyltransferase from cucurbits is overexpressed in tobacco (A), a transgenic plant is produced with a large proportion (76%) of saturated fatty acids in phosphatidylglycerol: If, in contrast, the enzyme from *Arabidopsis* (B) is used, the percentage of unsaturated fatty acids in this lipid increases. In *Arabidopsis* the wild type exhibits hardly any saturated fatty acids in phosphatidylglycerol, so that the proportion of unsaturated fatty acids cannot be increased any further; but the opposite experiment – to increase the proportion of saturated fatty acids – is feasible by means of gene technology. What influence does the altered proportion of unsaturated fatty acids have on sensitivity to low temperatures? Increasing the proportion of *cis*-unsaturated fatty acids increases chilling tolerance, both of photosynthesis and with regard to macroscopically visible damage after exposure to cold. Conversely, the resistance decreased when the proportion of saturated fatty acids was increased. (After Nishida and Murata 1996)

Box 1.3.6 Lipid biosynthesis in plants (After Frentzen and Heinz 1983, see also Ohlrogge and Browse 1995)



Desaturases are important for the development of chilling resistance in plants: acyl-ACP desaturase (3), acyl-lipid desaturases (13) [including prokaryotic acyl lipid desaturase (8)], glycerol-3P-acyltransferase (4) and possibly also the 1-acylglycerol-3-P-acyltransferase (5), which can only use fatty acids as substrates which are esterified or occur as thioesters (ACP).

have indeed resulted in increased cold resistance (Fig. 1.3.12).

1.3.5.2 Imbalances in Metabolism Caused by Cold

There have been extensive investigations on damage caused by imbalances of metabolism which has been slowed due to low temperatures. Such imbalances usually result primarily from the different Q_{10} values for the biophysical and biochemical processes involved.

Metabolic Imbalances

Diurnal fluctuations of enzyme activities and their metabolite pools of plants favouring warm conditions are particularly sensitive to cold. If such plants are kept in permanently weak light under otherwise normal surrounding conditions, an endogenous metabolic rhythm of so-called free **circadian** (i.e. periodicity of about 24 h) **oscillations** becomes evident. Two key cytosolic enzymes, **sucrose phosphate synthase** (SPS) and **nitrate reductase**, show this phenomenon. In correspondence with photosynthetic processes, their activities are higher during the light period and lower during the night, with a free periodicity of about 26 h. If the photosynthetic production of triosephosphate in the chloroplast and its further metabolism to sucrose in the cytosol are not synchronised, imbalances naturally result. One reason for this is that phosphate released in the cytosol during sucrose synthesis must be returned to the chloroplast from which it was withdrawn due to its having been bound to triose during the export of carbohydrate into the cytosol. In the example shown in Fig. 1.3.13 the free oscillations of SPS during a 12 h lowering of the temperature from 26 to 4 °C were disturbed to the extent that an exactly phase-reversed oscillation resulted upon a return to normal temperatures, in which minimal activity was exhibited during the light period and maximum activity during the dark period. A corresponding shift in rhythm also occurred with nitrase reductase. However, not all endogenous rhythms of warmth-loving plants are affected by chilling. For example, the endogenous oscillations of stomatal movement in avocado leaves maintained their original frequency even upon cooling, despite photosynthesis itself being strongly inhibited (Allen and Ort 2001).

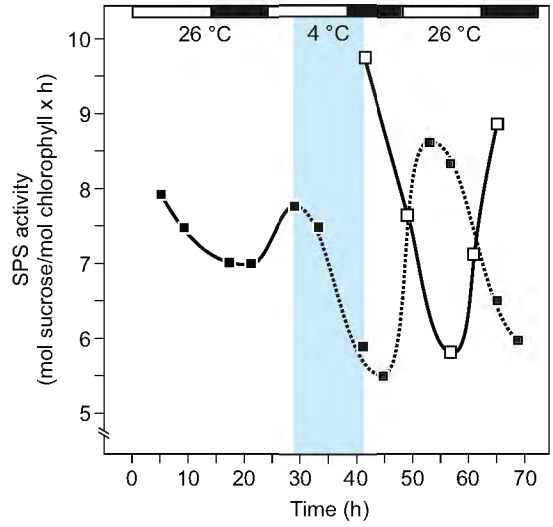


Fig. 1.3.13. Shift in endogenous metabolic rhythm caused by *chilling*. Tomato plants were grown at normal temperature (26 °C) in continuous weak light and the endogenous oscillation of SPS activity was observed (■). The oscillation period was about 26 h ("circadian"). The rhythm of the previously applied light–dark phases is shown in the *bar* at the top of the diagram. In a second experiment, plants having been treated in the same way were cooled to 4 °C between 29 and 41 h; this resulted in a reversal of the endogenous oscillation of SPS (□) because the rhythm was delayed. (Jones et al. 1998)

Chilling is also very important when it occurs together with high light intensity (Fig. 1.3.14). In such a stress combination the thylakoid system is usually overloaded, as the photosynthetic carbohydrate metabolism cannot keep up with the light reactions because of the low temperatures. As a consequence, photosystem II (PS II) in particular is inhibited, so-called **photoinhibition** due to light inactivation of the sensitive D1 protein (see Chap. 1.2.1.3). To regenerate complete photosynthetic capacity, the damaged D1 protein must be replaced. According to current knowledge, the five steps shown in Fig. 1.2.8 are required: removal of the inactivated D1 protein; synthesis of new pre-D1; insertion of pre-D1 into PS II; processing of the pre-D1; and reconstitution of the entire core of PS II.

The degree of unsaturation of the thylakoid lipids affects particularly the insertion and the processing of pre-D1. It is therefore assumed that the chilling tolerance of plants with a higher degree of unsaturation is based on a more efficient exchange of damaged for intact D1 proteins.

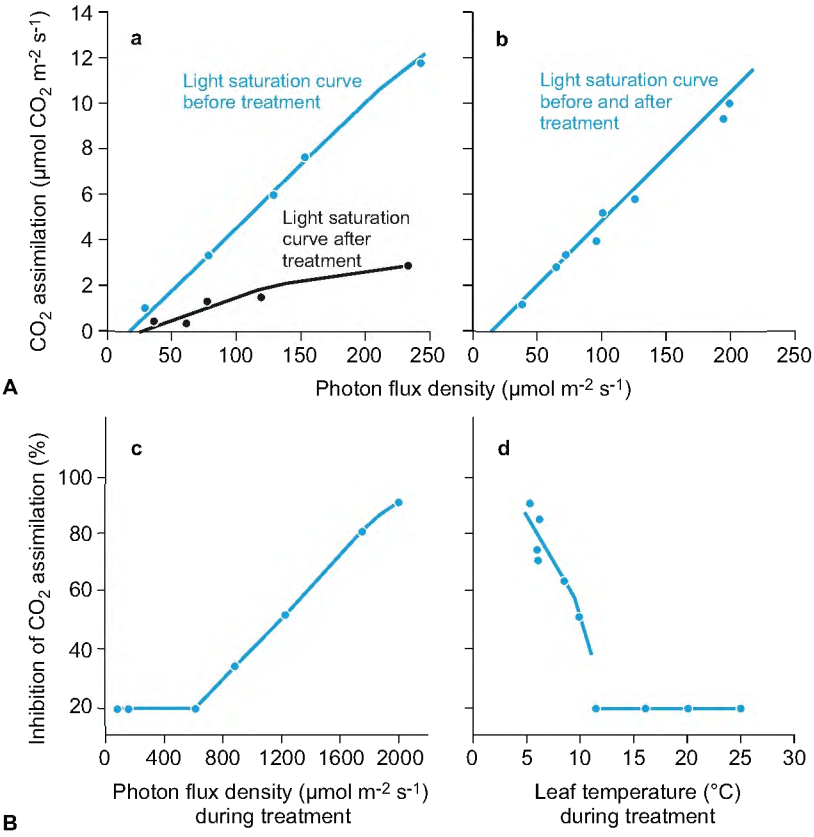


Fig. 1.3.14. The impact of a combination of stresses: The effect of chilling and light on photosynthesis. A Runner bean leaves (runner beans are tropical plants and thus chilling sensitive) were kept for 6 h at 6 °C under intense illumination (*a* 2000 µmol photons m^{-2} s^{-1}) and the light saturation curves for CO_2 assimilation were measured before and after the treatment. The stressed leaves showed a drastic inhibition of photosynthesis, even 1 h after treatment. When the leaves were exposed to only weak light (*b* 70 µmol photons m^{-2} s^{-1}) during the chilling, no inhibition was observed. However, light saturation curves were only measured up to about 250 µmol photons m^{-2} s^{-1}. B In this experiment, the bean leaves were exposed to different light intensities during a 3-h chilling period (5.5 °C). Above about 500 µmol photons m^{-2} s^{-1} photosynthesis begins to become inhibited (*c*). If the light intensity is held constant at 2000 µmol photons m^{-2} s^{-1} and the temperature is varied, the inhibition decreases as expected with rising temperature and does not occur at all at above about 10 °C (*d*). (After Powles et al. 1983) see also Powles 1984)

1.3.5.3 Metabolic Imbalances Often Lead to Oxidative Stress

It is nowadays thought that photosystems are mainly damaged by so-called oxidative stress. The stressors are termed **ROS** – reactive O_2 species. These not only consist of oxygen as such, but also include the products of the reactions of activated oxygen with metabolites and structural components of the cell, e.g. biomembranes (Box 1.3.7). In an undamaged cell living without particular stress, these ROS form at only very slow rates which present no problem for the endogenous detoxification and repair systems. Such reactive molecules, however, are produced in much larger amounts upon metabolic imbalances, under stress or in senescing cells, and can cause damage before they are detoxified. In the following, some situations and reactions are described in which ROS are formed:

Oxygen in the air possesses two unpaired electrons and thus occurs in the rather unreactive triplet state. Oxygen in this state is not able to react with molecules in the singlet state, but can react with others in the triplet state. An example is the reaction with chlorophyll, which has passed from the first singlet state into the first triplet state due to the loss of excitation energy. This chlorophyll is able to transfer its remaining energy to the O_2 molecule (exciton transfer), which is thus subjected to spin reversal and becomes extremely reactive **singlet oxygen** ($^1O_2$). $^1O_2$ has a pair of very labile electron

Box 1.3.7 Oxygen-activating reactions

1. Photodynamic reactions

$P^* + {}^3O_2 \rightarrow P + {}^1O_2$; singlet oxygen ${}^1O_2 + RH \rightarrow ROOH$; if RH is, for example, an unsaturated fatty acid, a fatty acid hydroperoxide is produced. ROOH can be reduced via electron donation (Fe^{2+}, Cu^{1+}) and so form alkoxyradicals:

$$ROOH + Fe^{2+} \rightarrow RO^{\bullet} + OH^- + Fe^{3+};$$
$$RO^{\bullet} + RH \rightarrow R^{\bullet} + ROH$$

this can be the start of a chain reaction

2. Reductive oxygen activation

In the presence of reducing agents with an ε value < -160 mV, oxygen can accept an elec-

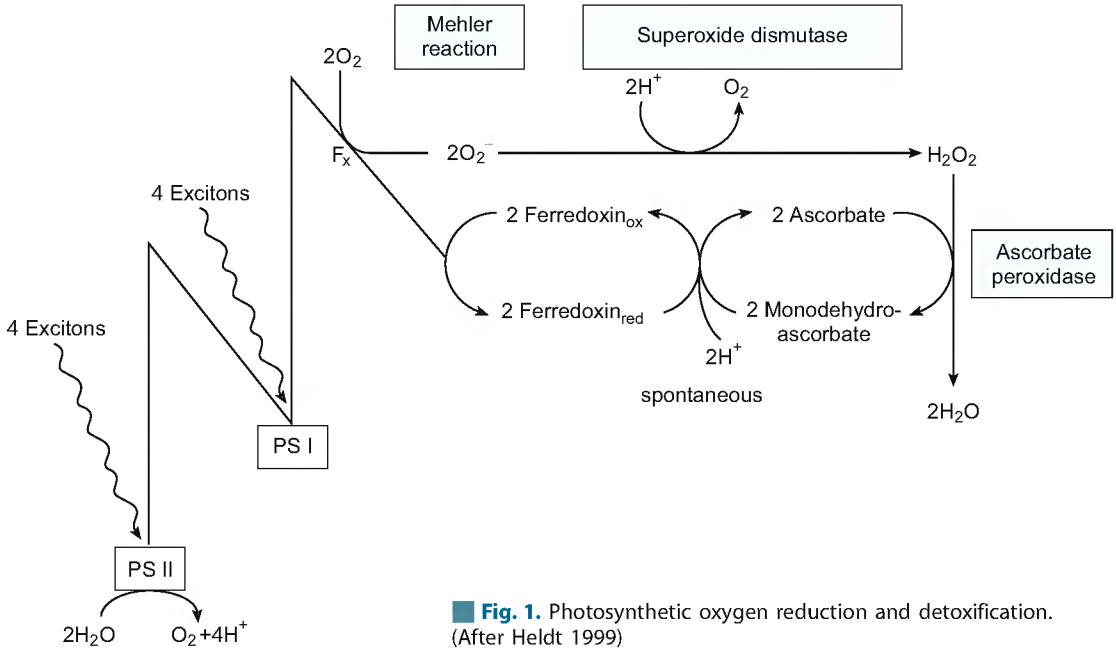


Fig. 1. Photosynthetic oxygen reduction and detoxification. (After Heldt 1999)

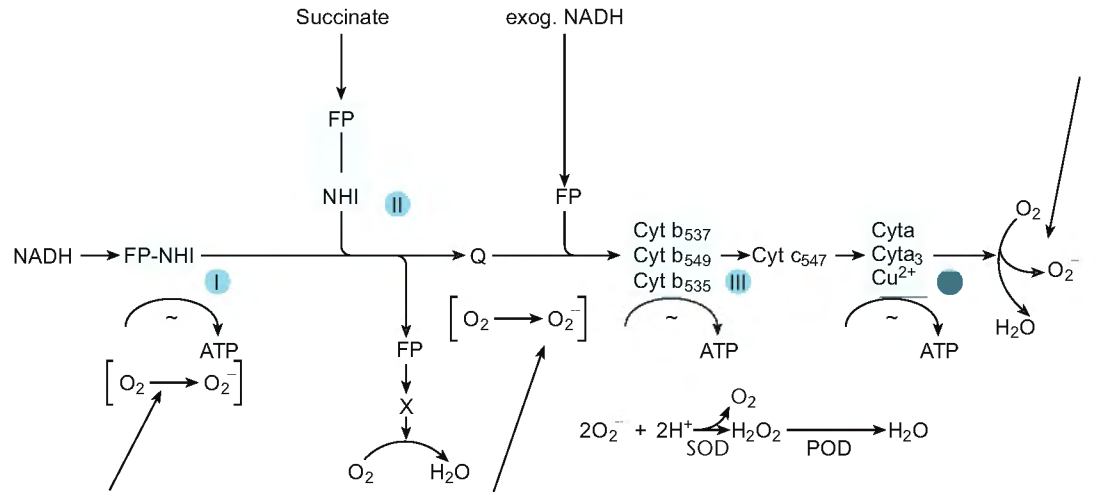


Fig. 2. Oxygen activation in the mitochondrial respiratory chain. *FP* Flavoprotein; *NHI* non-haeme-iron; *POD* cytochrome-c-peroxidase; *I, II, III, IV* protein complexes of the respiratory chain; *SOD* superoxide dismutase. (After Elstner 1996)

Box 1.3.7 (continued)

tron and become superoxide or an oxygen radical:

$$^3O_2 + e^- \rightarrow {}^3O_2^-$$

Superoxide can originate in photosynthesis (electron transfer to O_2 instead of to NADP), in the respiratory chain (from iron-sulfur proteins or from hydroquinone, during the stepwise reduction of oxygen by cytochrome a/a_3), as well as in reactions with monooxygenases.

Superoxide dissimilates spontaneously (10^4 times faster with SOD, however);

$$2O_2^- \rightarrow H_2O_2 + O_2$$

H_2O_2 can be reduced monovalently, e.g. by Fe^{2+}: **Haber-Weiss reaction, Fenton chemistry:**

$$H_2O_2 + Fe^{2+} \rightarrow OH^- + OH^{\cdot} + Fe^{3+}$$

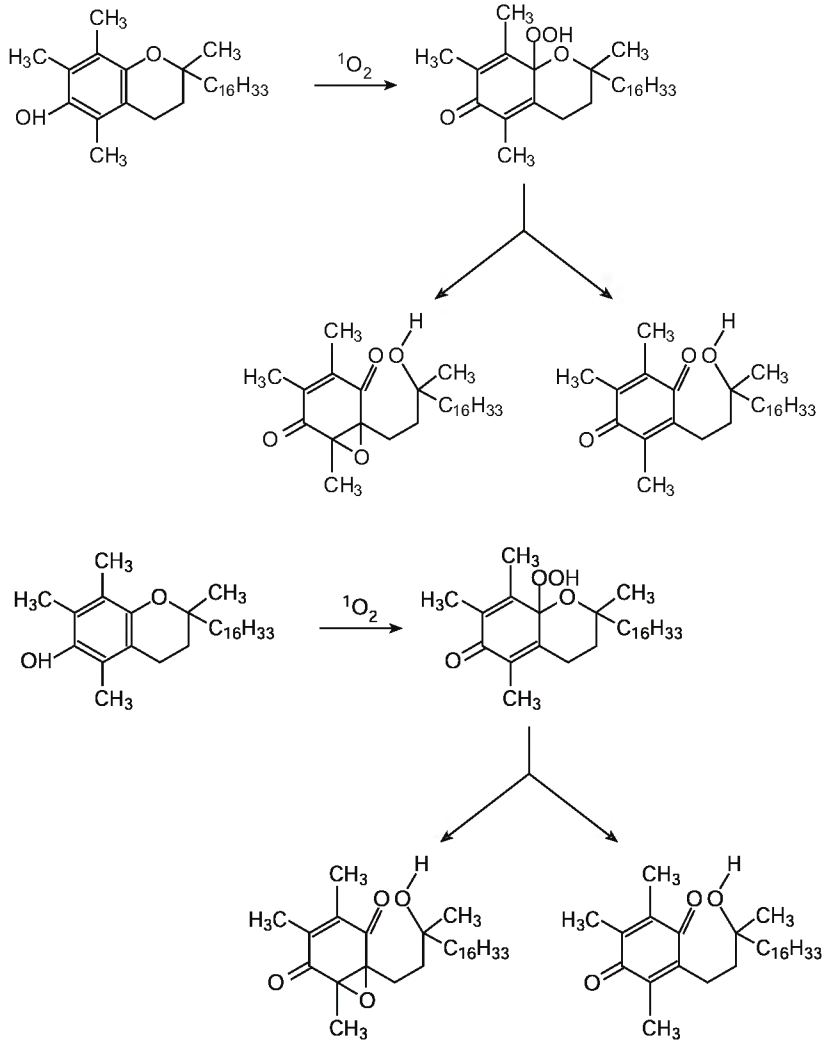


Fig. 3. Detoxification of singlet oxygen by tocopherol. (After Neely et al. 1988)

pairs which react with many organic substances, particularly readily with unsaturated compounds under formation of hydroperoxides.

Oxygen can also be activated by reduction. This happens, for example, in photosynthesis (see Box 1.3.7, Fig. 1) when no other electron acceptor is available for reduction by Fd, or when reduction is catalysed by the total herbicide methyl viologen (Paraquat). Oxygen can also be reduced by the respiratory chain (see Box 1.3.7, Fig. 2), via sulfur-iron proteins, via hydroquinone or via damaged endooxidases which have effected only partial electron transfer to O_2. The superoxide or oxygen radical anion is formed, which dismutates spontaneously to hydrogen peroxide and oxygen, a reaction which takes place 10,000-fold more rapidly in the presence of superoxide dismutase.

Hydrogen peroxide can be reduced monovalently [e.g. via the oxygen radical ion and iron-(III) ions], whereby the **Haber-Weiss reaction** gives rise to the even more reactive **OH radical**, which immediately causes oxidative damage in its immediate environment.

In the case of chilling, the rate of metabolic NADPH consumption (re-oxidation of NADPH to NADP) is less than the rate of NADP reduction due to the low temperature, i.e. NADPH accumulates and the NADP pool becomes "over-reduced". The photosynthetic electrons are then transferred by linear electron transport to oxygen (**Mehler reaction**) and superoxide is thus formed. This and ROS formed in this connection oxidise methionine and histidine residues of the D1 protein and thus cause its inactivation.

As shown in the above, ROS are not only formed at low temperatures, but also under the influence of other stressors, such as light. The establishment of a multifaceted **anti-oxidative system** is thus a general stress reaction, not a specific one. This anti-oxidative system consists of a number of enzymes and their substrates, the so-called radical scavengers.

The Anti-oxidative System Detoxifies Reactive Oxygen

Plants have recourse to several biochemical mechanisms to detoxify ROS (Box 1.3.7, Fig. 3):

1. Quenchers and Scavengers
   The group of quenchers and scavengers consists of small molecules which react with ROS in often non-enzymatic reactions and thus detoxify them. These are protective compounds localised in the membranes such as tocopherol and many carotenoids, but they also include water-soluble compounds such as ascorbic acid and phenol derivates – particularly flavonoids and coumarins. They react with $^1O_2$ as well as with the OH radical and thus intercept the most toxic ROS (thus the terms quencher, scavenger). The OH radical, which is very effective in its own vicinity, reacts particularly well with flavonoids. It is thus not surprising that the key enzymes of flavonoid biosynthesis, phenylalanine-ammonium lyase and chalcone synthase, are formed to a particularly great extent in frost-hardy plant organs during frost hardening or during stress by chilling coupled with high light intensities (see Chap. 1.2; Box 1.2.5). Flavonoids are also formed in response to infections and wounding, and are then called **phytoalexins**. They thus have a multiple role in stress defence.
2. Enzymatic ROS Detoxification
   ROS are enzymatically detoxified in the compartments in which they are most frequently formed, i.e. in the chloroplasts, mitochondria and peroxisomes, during the course of which water is usually produced. Corresponding enzymes have also been observed in the cytosol (Box 1.3.7, Fig. 3).

The water-soluble, detoxifying enzyme system consists of superoxide dismutase (SOD), a peroxidase – usually ascorbate peroxidase, (mono)-dehydroascorbate reductase and the NAD(P)H-dependent glutathione reductase, and is known as the **ascorbate-glutathione cycle** (Box 1.3.7, Fig. 3). The enzyme activities present in the individual compartments can be very high, so that the system works very efficiently. During frost hardening the individual enzymes of this detoxification pathway are additionally activated or more strongly expressed.

1.3.5.4 Repair Systems Mend Damage to Biomembranes, Nucleic Acids and Proteins

A number of repair mechanisms are known which remove peroxidised fatty acids and their resultant products (hydroxyoctadecadaic acids, so-called HODEs) from membranes (most membrane lipids remain quite generally for only a very short time in the membrane), detoxify

them and replace them with intact fatty acids. Such repair mechanisms also exist for damaged nucleic acids (see Box 1.2.4). Damaged proteins are repaired by means of the chaperone system or are degraded after marking with ubiquitin.

1.3.6

Frost

Stress caused by low temperatures is greatly increased in the case of frost due to freeze-desiccation. Secondary strains such as high light intensity play, of course, an additional important role at temperatures below 0 °C. Frost also occurs in tropical climates, namely in tropical high mountains. As the temperature in these regions only falls below the freezing point for a matter of hours, freezing avoidance mechanisms are very important. In contrast to chilling, frost is characterised by seasonal hardening (acquisition of frost resistance or frost tolerance) and dehardening. These processes involve many physiologically important aspects. Particularly topical are the many so-called cold-related proteins (CORs) which have been discovered recently. These constitute an important starting point for introducing frost hardening into cold-sensitive crop plants, in addition to the genetic engineering of biomembrane frost hardening described in Chapter 1.1.6. It should be mentioned in this regard that 70% of the annual harvest losses in the USA was caused by cold and drought in the 1980s, while insects, weeds and pathogens together were responsible for only about 10%. Late frosts caused damage of more than US$ 1 billion during 1981–1985 in southern Florida (Yelenosky 1985).

1.3.6.1

Freezing Temperatures and Biomembranes

The manner in which chilling affects membrane fluidity applies in principle to frost as well, but to a more severe extent. Measurements of fluidity by means of electron spin resonance or fluorescence polarisation show primarily the stiffening effect which a high protein/lipid ratio has on the fluidity of biomembranes at low temperatures. Particularly protein-rich biomembranes, such as those of the thylakoids, reduce the protein/lipid ratio during frost hardening. In addition, a desaturation of the fatty acids in the membrane lipids is often observed. More details in this regard will be given in Chapter 1.3.6.9.

It is important to realise that the freeze-desiccation of frost-hardened tissues is inevitably accompanied by a more or less pronounced shrinkage of the protoplast.

As the biomembrane cannot expand or shrink like a balloon would [the intrinsic elastic flexibility of a biomembrane should not exceed 2–3% (Wolfe and Steponkus 1983)], shrinking processes are often associated with a removal of lipids from the membrane and expansion processes with the insertion of lipids. During reversible freeze-desiccation, this material must be deposited in such a way as to be immediately available upon thawing (Fig. 1.3.15).

A quite similar problem occurs during plasmolysis. Steponkus et al. (1983) showed with isolated protoplasts from rye mesophyll cells that damage to frost-sensitive cells occurs during thawing, because the amount of material available for re-incorporation is no longer sufficient for the "areal growth" of the plasma membrane. The resulting lysis of the cells was called "expansion-induced lysis". Protoplasts of frost-hardy winter rye leaves did not show this phenomenon. They do not deposit the material removed from the membrane during contraction within the cell, but deposit it at the external side of the membrane where it is not accessible to lipases. The different behaviour of the lipids depends on the degree of their unsaturation: Frost-sensitive plasma membranes of protoplasts can be artificially "hardened" if they are treated with a surplus of unsaturated lipids (in the experiments phosphatidylcholine with one- or two-fold unsaturated fatty acids was used) and a partial exchange of the membrane lipids takes place. Even though protoplasts are an excellent in vitro system for the study of many processes, they can only provide answers to some partial aspects of frost hardening in biomembranes.

In considering the freezing of cellular water, it is very important to regard the involvement of the cell wall in stabilising the protoplast. The freezing of cell water has often been compared to plasmolysis – a very different process. During freezing, the cellular fluid must crystallise in the intercellular spaces, as otherwise biomembranes bordering the ice crystals would disintegrate, because the hydrophobic interactions of the lipids which stabilise the membranes require the presence of liquid water. This also means that no ice

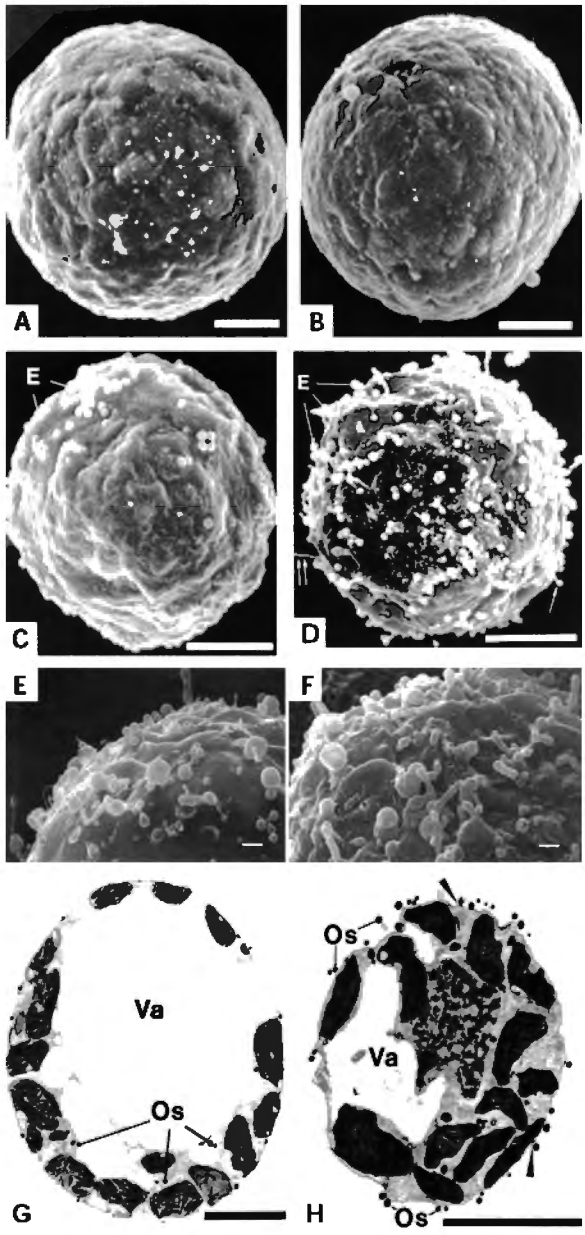
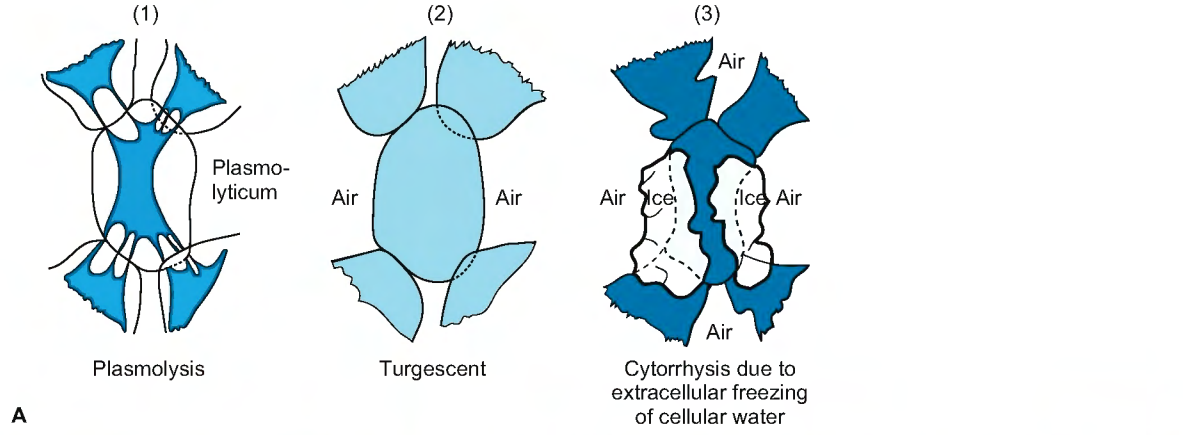


Fig. 1.3.15. Behaviour of isolated protoplasts from rye leaves in isotonic and hypertonic solutions. Protoplasts were isolated from leaves of frost-sensitive (A, B) and frost-hardened (C, D) rye plants and incubated in isotonic medium (A, C, G) and in a medium with a concentration double that of the isotonic medium (B, D, H). The outer surface of the non-hardened protoplasts remained almost smooth on shrinking (B), i.e. lipids excluded from the membrane were displaced to the interior of the protoplast. Protoplasts from frost-hardened cells deposited their excluded lipids in extrusions of various shapes (E) on the outside of the plasma membrane when they shrank, where they cannot be degraded by endogenous lipases (D). The transmission electron micrographs (G, H) also show the shrinking (compare the size of the vacuoles in G and H) and the lipid extrusions (*arrow*) which are strongly contrasted by osmium staining (*Os*). The *bar* corresponds in each case to 5 µm. E An enlarged section of a protoplast from frost-hardened leaves in hypertonic medium. F The surface of a protoplast from non-hardened leaves in hypertonic medium, after fusion of the protoplast with liposomes consisting of dilinoleoylphosphatidylcholine. Protoplasts which deposit lipids internally on shrinking burst upon swelling in isotonic medium, because the lipids are degraded by phospholipases. Lipids which are deposited externally can be reintegrated into the membrane upon swelling of the protoplast. The *bar* corresponds to 1 µm. (After Steponkus et al. 1983, 1988)

may be formed between the plasma membrane and the cell wall. This space, which in the case of plasmolysis is filled with the plasmolyticum, would have to fill with air during the extracellular freezing of cellular liquid if the protoplast were to withdraw from the cell wall. The air would have to enter through the pores of the cell wall. These pores are very small (about 4 nm diameter in the primary wall), and they are filled with water due to the high matrix potential of the cell wall. It would require a suction of more than 80 MPa to suck air through the cell wall pores (Zhu and Beck 1991). Such high suction has never been observed in living cells, however. The protoplast is therefore not normally able to detach itself from the cell wall during the exogenous freezing of the cellular liquid of the protoplast, and the entire cell must collapse upon the dehydration resulting from freezing (Fig. 1.3.16).

Cells which are not particularly rigid, e.g. those of the mesophyll, indeed wrinkle and fold under these conditions (**freezing cytorrhysis**). Since the cell wall itself cannot shrink, the plasma membrane must not reduce its surface significantly. The problem as to the extra- or intracellular deposition of membrane material de-



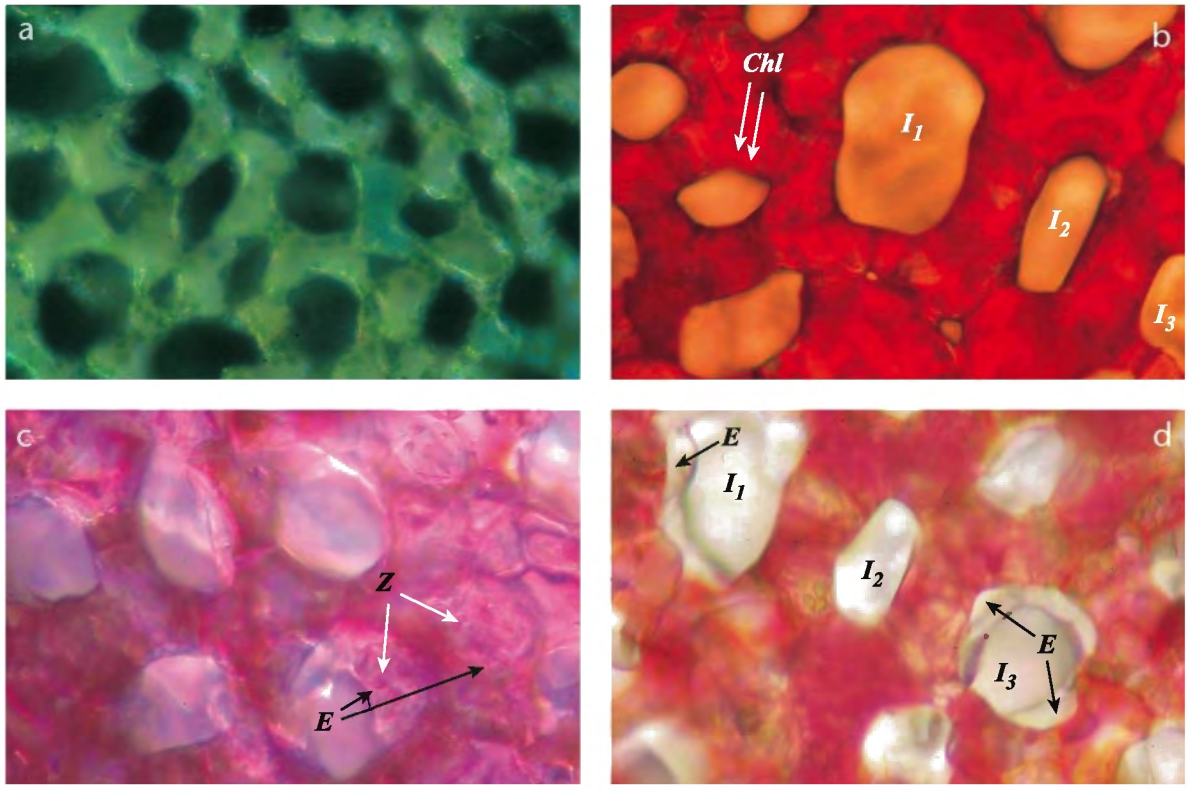


Fig. 1.3.16. A Plasmolysis and freezing of mesophyll cells from a leaf of *Pachysandra terminalis*. During plasmolysis (*1*), the plasmolyticum intrudes into the space between the cell wall and the protoplast, and the cell wall is relaxed. During freezing (*3*) the protoplast remains attached to the cell wall despite the export of cell fluids into the intercellular spaces, because the cell wall is air-tight in the swollen state. The cell wall dents or buckles, depending on the extent of the freeze-desiccation. Since crystallisation takes place extracellularly, no ice is formed between the cell wall and the protoplast. B: *a–d* Spongy parenchyma of a leaf of *Pachysandra terminalis*. *a* Surface view of the spongy parenchyma following removal of the lower epidermis; chloroplasts can be clearly seen in the cells. *b* Spongy parenchyma after taking up neutral red through the leaf stalk. *Chl* Chloroplasts. *d* The same section of the spongy parenchyma at −12 °C (I_1–I_3 indicate the same intercellular spaces). Deposition of colourless ice (*E*) can be clearly recognised within the intercellular spaces. *c* Spongy parenchyma was stained with neutral red and then killed before cooling to −12 °C. The ice crystals are not restricted to the intercellular spaces, and they contain drops of coloured cell sap (*Z*). Note also the change in the neutral red colour resulting from acidification as a consequence of killing the tissue. (Photos J.J. Zhu)

scribed above is thus of hardly any consequence in this regard. Freezing plasmolysis does not occur even in rigid cells which are tightly integrated into a tissue if the cells are to survive the freezing (see Chap. 1.3.6.3).

1.3.6.2 Intra- and Extracellular Formation of Ice

Amphiphilic lipids (lipids with a hydrophobic and a hydrophilic pole) only unite to form micelles and membrane surfaces if they are in a sufficiently hydrophilic medium, i.e. stabilised by a structured water film. If this structured water film is removed, the stabilising effect of the hydrophobic interactions is lost and the lipids aggregate in droplets: This is called the lipid hexagonal II phase (Fig. 1.3.17). An example of lipids in this hexagonal II phase is the lipid droplets (plastoglobuli) in senescing chloroplasts. Intracellular ice formation probably starts at biomembranes, because of the structured water film associated with them. This ice formation, however, would remove the membrane-stabilising component and consequently the membrane would disintegrate. Earlier research described this as the perforation of biomembranes by ice crystals, because it is the hexagonal, pointed forms of the ice crystals that form at temperatures above −100 °C which cause the dehydration. Extremely rapid cooling (cooling rates of > 1000 °C/min) down to the temperature of liquid nitrogen, so-called **vitrification** (which can only be carried out with very small sample volumes of a few mm^3), leads to solidification of the cell liquid into amorphous ice, which does not have the destructive effect just described. However, there is a danger of recrystallisation of the amorphous ice into hexagonal, damaging crystal forms upon the slow thawing of the samples. Vitrification is an artificial, special case which does not take place in nature because of the very rapid cooling rates required. However, it is important for the preservation of bacterial cultures, for example.

Whether the crystallisation takes place inside or outside the cell depends on several factors. One of these is the rate of cooling: The faster the tissue cools, the greater is the danger of intracellular ice formation (Fig. 1.3.18). This is not damaging in the case of vitrification, due to the very abrupt and sudden deep-freezing of the entire tissue. Another factor which determines where ice is deposited is the state of the plasma membrane: Frost-hardened membranes allow water (more exactly: the liquid of the cell) to exit into the apoplast more easily than do frost-sensitive membranes.

The mechanism of this efflux of water has not yet been explained. It is often assumed that ion pumps (especially H^+-ATPases) are inactivated in the cold, and that ions and other dissolved materials diffuse out of the cell into the water film surrounding the intercellular spaces and are followed by water. The liquid accumulating in the intercellular spaces now requires a trigger for crystallisation. Such a nucleation stimulus can originate from the xylem if the formation of ice first starts there (Gross et al. 1988) or if it rapidly spreads from a nearby nucleation centre. On the other hand, certain surface structures of the cell walls or of microorganisms which are found predominantly in the substomatal respiratory cavity can also trigger the nucleation.

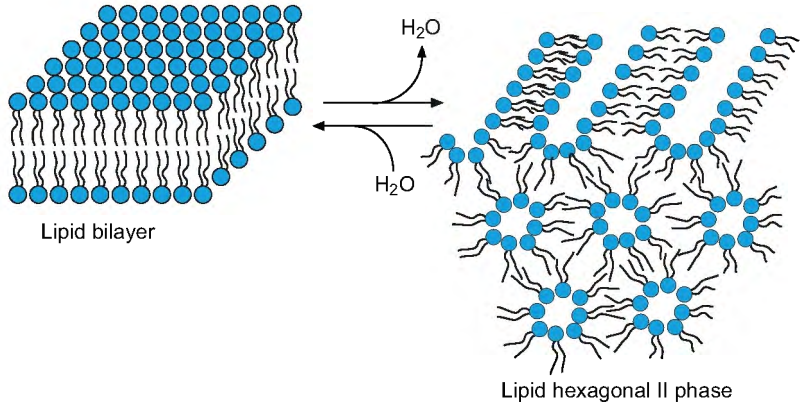


Fig. 1.3.17. Disintegration and reconstruction of a phospholipid bilayer by water removal (e.g. freeze-dehydration) and rehydration. Phospholipids form lipid drops or threads in the hexagonal II phase. (After Crowe et al. 1983)

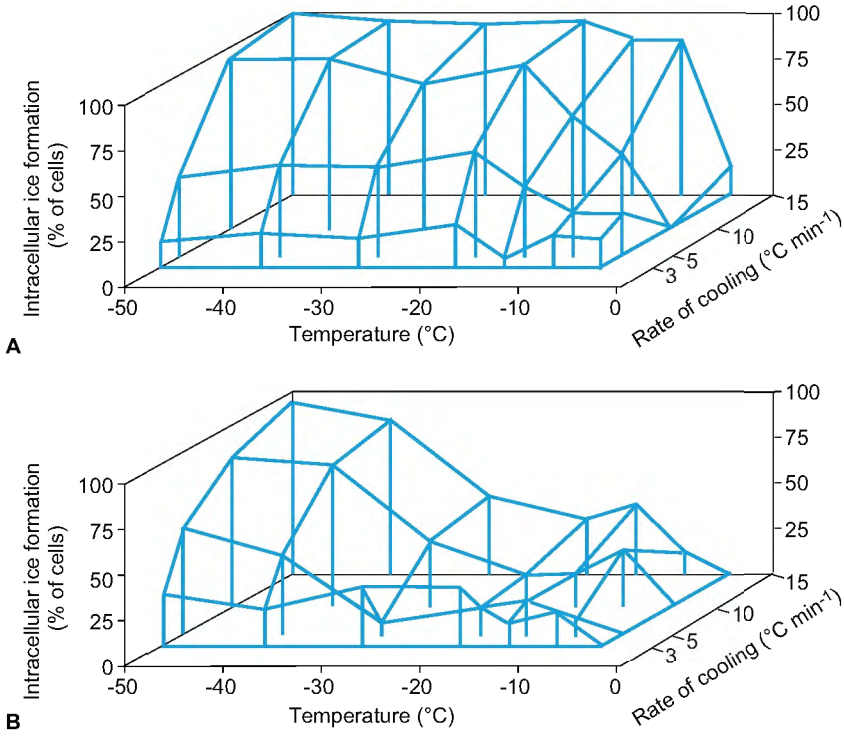


Fig. 1.3.18. Formation of intercellular ice *in* protoplasts of frost-sensitive (A) and frost-hardened (B) rye leaves as a function of the rate of cooling and the minimum temperature (*abscissa*). Protoplasts from frost-sensitive leaves do not permit the exclusion of cellular liquids during cooling, so that ice forms intracellularly. Consequently, the cells are lethally damaged. (After Dowgert and Steponkus 1983)

1.3.6.3 Heterogeneous Nucleation; the Example of INA Bacteria

The molecular details of the surface structures of plant cell walls which are active in nucleation, i.e. which trigger the formation of crystals at relatively low frost temperatures, are not yet known. However, it is known that such a phenomenon does exist. Examples are winter cereals and leaves of other winter-hardy plants, e.g. speedwell (*Veronica* spp.) and some species of box trees. Extracts of the internal surfaces (intercellular spaces) of these plants contain proteins which are formed during frost hardening. It is very probable that these proteins trigger the early nucleation, but this has not yet been shown definitely. The principle of nucleation initiation has been investigated thoroughly for a number of microorganisms which live on plant surfaces and trigger the crystallisation of water there (so-called heterogeneous nucleation). These microorganisms are called INA bacteria (*ice nucleation active bacteria*). The more effective their surface structures are in ordering the water clusters into a matrix-like arrangement, the more efficient they are as catalysts for crystallisation, and the lower is the degree of supercooling required for crystallisation (Fig. 1.3.19 and Table 1.3.6).

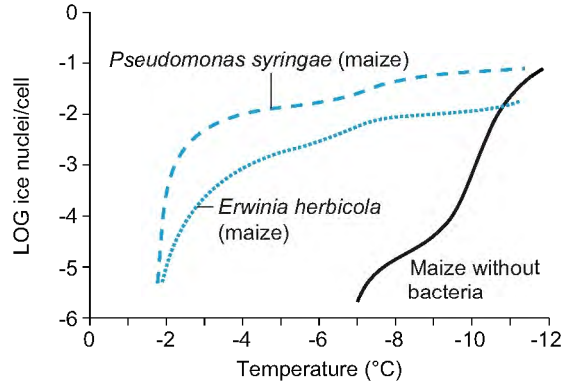


Fig. 1.3.19. The effect of INA bacteria on ice formation in maize leaves. Upon incubation with *Pseudomonas syringae* at –4 °C, 0.01 ice crystals per bacterial cell were determined, i.e. one ice crystal per 100 bacteria. (After Lindow 1982)

In these cases, the nucleation activity can be related at least in part to certain proteins, the so-called ice nucleating proteins (INPs). The amino acid sequence for one INP from *Pseudomonas syringae* is known: a particular octapeptide repeats itself 122 times in this polypeptide. Deletion mutants show that a 68-fold repetition of the motif is sufficient to trigger nucleation. If the periodicity of the octapeptide is changed, the nucleating activity is lost immediately

Table 1.3.6. Ice nucleation activity of bacterial cultures. Thirty drops (each 0.01 ml) of test material were placed on a controlled surface and the temperature was slowly lowered from room temperature to −25 °C. The temperature at which the first ice crystals formed (T_1) and the temperature at which 90% of the drops were frozen (T_{90}) were recorded. (Maki et al. 1974)

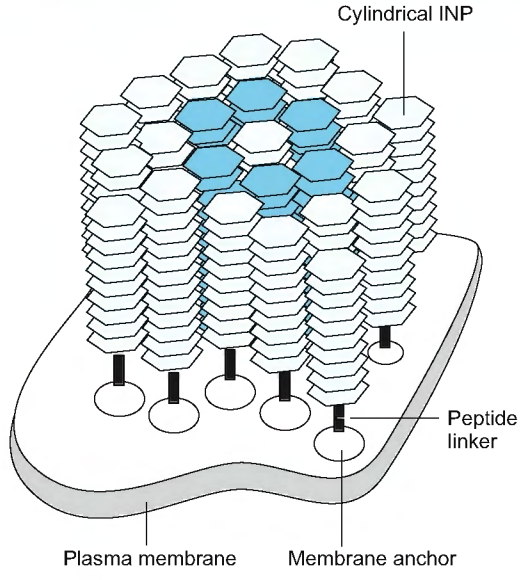
Bacterial species	(Ice) nucleation temperature (°C)	
	T_1	T_{90}
Pseudomonas syringae C9	−2.9	−3.5
Pseudomonas syringae	−3.2	−3.9
Pseudomonas aeruginosa	−7.5	−17.8
Staphylococcus epidermidis	−6.9	−19.5
Escherichia coli	−8.3	−17.1
Enterobacter aerogenes	−9.6	−17.0
Proteus mirabilis	−8.0	−19.4
Proteus vulgaris	−7.8	−17.0
Bacillus subtilis	−10.6	−18.0
Bacillus cereus	−6.9	−17.0
Pure culture medium	−9.2	−17.0

(Green and Warren 1985). No relationship between the periodicity and the dimension of the distance between the water molecules in the hexagonal ice crystal could be shown. Such proteins could force a *quasi* crystalline fixation of the water molecules which would minimise the supercooling required to "quieten" the water clusters (see Box 1.3.8).

INA bacteria often cause great economic damage when they settle on plants which are able to tolerate moderate cold, but not ice formation, such as maize, strawberries and citrus fruits. In the presence of *Pseudomonas syringae*, an ice crystal is formed per 100 bacteria already at −4 °C. The natural occurrence of such bacteria on plants varies with the season (Fig. 1.3.20), among other things.

INA bacteria are used in meteorology (after killing by γ-radiation) for the production of artificial snow and to induce cloud formation above dry areas. They have also been used for the protection of cereal seed against seed beetles: Bacterial powder eaten by the beetles reduced the

Box 1.3.8 Ice nucleators



Nucleation temperature (°C)	Estimated molecular mass (kDa)	Number of INPs required for nucleation
-12 to -13	150	1
-3	870	60
-2	19800	132
-1	83700	558

The schematic diagram shows an INP superstructure ("ice nucleator") on a bacterial membrane. It is assumed that the membrane lipid phosphatidylinositol is the anchor of the individual INPs in the membrane (after Hew and Yang 1992, see also Burke and Lindow 1990). The table shows the correlation between the size of an ice nucleator and the reduction in supercooling temperature. (After Hew and Yang 1992, see also Burke and Lindow 1990).

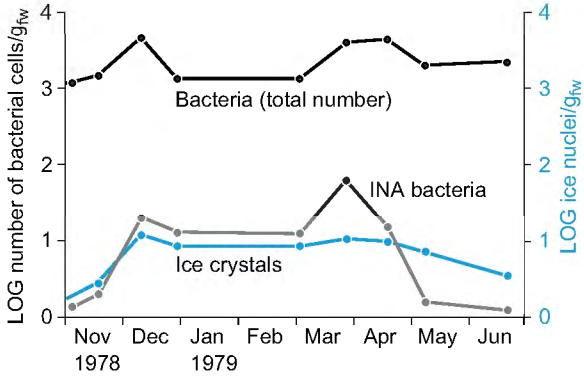


Fig. 1.3.20. Seasonal dependence of the number of all bacteria (total number) and INA bacteria, as well as the formation of ice nuclei at –9 °C, on avocado leaves. (After Lindow 1982)

Table 1.3.7. The effect of INA antagonists on the formation of ice nuclei upon frost

Bacterial culture	Damage Change in leaf colour after 1 week
Control (*Pseudomonas syringae*)	95%
Mutant A 510	12%
Mutant A 509	18%
Mutant A 507	27%
Mutant A 506	33%
Mutant A 508	51%

Pear leaves were inoculated with the respective bacterial culture 3 weeks prior to frost, to give the bacteria time to establish themselves. Frost damage was quantified by assessing necrosis. (After Lindow 1982)

ability of the insects to supercool to such an extent that they were killed by internal ice formation upon a frost shock of –10 °C, a temperature which is completely harmless to the seed (Fields 1993). In agriculture, however, attempts are being made to dilute out the INA bacterial infestation of crop plants by replacement with antagonists. These are obtained by plating out wash solutions from leaves and cooling them to –5 or –9 °C. Antagonists of the INA bacteria should be present wherever there is no ice formation. These organisms are mostly natural mutants of the original bacterial strains (Fig. 1.3.21 and Table 1.3.7).

Successful production of antagonists has also been achieved by altering the octapeptide periodicity of the INPs. However, the release of these transformed bacteria into the environment has not been permitted (Wilson and Lindow 1993), partially because of fears that they would interact with meteorological processes, particularly with cloud formation. Plants able to tolerate extracellular freezing appear to profit from an early triggering of nucleation, however, as this minimises the very unstable state of supercooling.

1.3.6.4 How Much Water Freezes in Frost-Hardy Tissues?

Liquid water can be distinguished from frozen water by nuclear magnetic resonance (NMR) spectroscopy. If the total water content of, for example, a leaf is known and the residual liquid water content is determined at different frost temperatures, so-called freezing curves are obtained which display the percentage of frozen water at each frost temperature (Fig. 1.3.22).

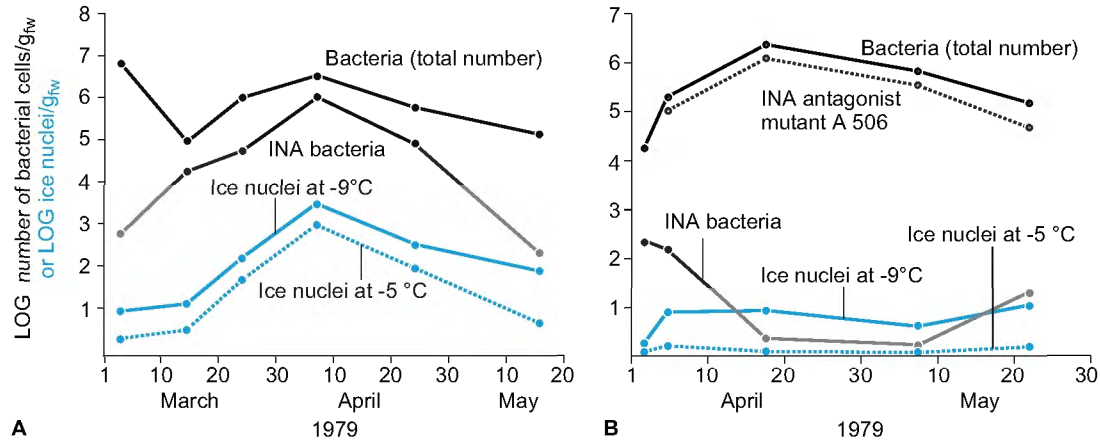


Fig. 1.3.21. Formation of ice crystals by INA bacteria on pear leaves (A) and displacement of the INA bacteria by mutants which are ineffective in nucleation (B). (After Lindow 1982)

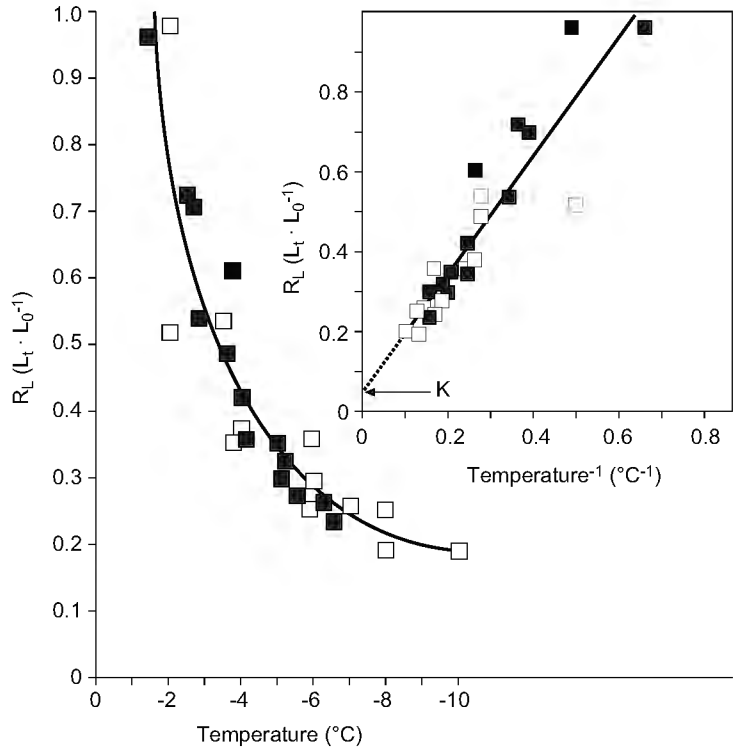


Fig. 1.3.22. Freezing curve of frost-hardened ivy (*Hedera helix*). The *curve* shows the proportion of liquid water (R_L) of the total water content (L_0) in dependence on the frost temperature (t). Ideally, a rectangular hyperbola is obtained which can be depicted as a simple linear reciprocal relationship (*insert*). The point at which the line cuts the ordinate (K=4.9%) indicates the proportion of the water which can theoretically not freeze. ■ R_L calculated from $P_{(t)}/V_{(t)}$ and $\Psi_{leaf(t)}$. □ R_L measured. (After Hansen and Beck 1988)

The lower the temperature, the more water is present in the form of ice, i.e. the water must be excreted from the cells into the apoplast. The curve can be mathematically transformed to obtain a straight line which cuts the ordinate at the so-called K-value. This specifies the proportion of the water which cannot freeze due to binding to macromolecules. This value is in the order of a few percent of the total water content.

The proportion of frozen tissue water also depends, of course, on the original concentration of the cellular liquid. It may be assumed in a first approximation that there is a physicochemical balance in a frozen leaf between the unfrozen, residual solution of the cells and the extracellular ice. This is because the vapour pressure of water is reduced over both ice and a solution, and the water potential is thus correspondingly larger (numerically more negative: see Box 1.3.9):

$$\Psi \text{ ice} = \Psi \text{ cell} = \Psi \text{ tissue} \approx \Psi \text{ leaf}$$

The water potential of a cell consists of the osmotic potential π, the matrix potential τ and the pressure potential ("wall pressure", P). Water potential, osmotic potential and matrix potential have negative signs, whereas the pressure potential, the wall (+ tissue) pressure, has a positive sign.

$$(-)\Psi \text{ tissue} = (-)\pi + (-)\tau + \text{P}$$

At moderate frost and only little freeze-desiccation, τ is still 0, but P already approaches 0. At the turgor-loss point (P=0) the following applies:

$$(-)\Psi \text{ tissue} = (-)\pi = (-)\Psi \text{ ice}$$

and dehydration stops. In this case, the degree of dehydration is determined solely by the concentration of the cellular liquid. There is a steady-state water potential between cell compartments; otherwise the intracellular water fluxes would change the volumes of the compartments. If this relation applies, and this is usually so in the case of mild frosts, an **ideal equilibrium freezing** takes place (Fig. 1.3.22).

If more water remains in the liquid state than would be expected upon ideal equilibrium freezing, a **non-ideal equilibrium freezing** occurs. This is usually observed at moderate frosts of below −8 °C, at which rates of water loss of about 90% frequently occur. In such a case $(-)\tau$ and/or P must contribute to the water potential of the frozen plant organ. During severe freeze-desiccation, the cell wall is strongly invaginated and thus develops a suction instead of the pressure which it usually exerts: P becomes negative

Box 1.3.9 Freezing of water and aqueous solutions

In liquid water, clusters of water molecules are in temperature-dependent motion (**Brownian molecular motion**). For crystallisation to take place, this movement must be minimised as far as possible. If factors which trigger crystallisation can be avoided, it is possible to cool pure water to −39 °C, whereupon it suddenly freezes. At this temperature larger clusters are formed as a consequence of the limited mobility of the water molecules, so-called critical embryos of about 190 H_2O molecules, which then initiate crystallisation. As the nucleation in this case originates from water itself, it is called **homogeneous nucleation**. The cooling energy expended for this crystallisation is released as heat of crystallisation. This spontaneous development of heat can be measured as a **freezing exotherm** and thus serves to monitor the freezing process (Fig. 1).

The melting process during thawing also requires energy, which is called the heat of fusion and can be recognised by a delay in warming during the course of the thawing process.

If nucleation is initiated in other ways, e.g. by seeding with ice crystals, supercooling does not need to take place to such a great degree. Depending on the type of initiation, crystallisation can already take place upon supercooling to only a few degrees. This is called **heterogeneous nucleation**, and is again dependent on the production of critical embryos, i.e. the reduction of Brownian molecular motion. Particular hydrophilic surface structures can force the water cluster into a type of lattice structure which facilitates crystallisation. An only small degree of supercooling is then required to initiate nucleation.

The vapour pressure (P) of water over ice is less than that over supercooled liquid water (see Table), and the water potential Ψ of ice is accordingly more strongly negative ("larger") than that of supercooled water at the same temperature. Put another way: More energy must be expended to remove a water molecule from crystalline ice than to remove it from liquid water.

$$P_{ice} < P_{supercooled\ water}$$

$$\Psi_{ice(T)} = \frac{R \times T(K)}{\overline{V}} \times \ln \frac{P_{ice(T)}}{P_{water(T)}}$$

$\overline{V}$ = mole volume of ice

$$\Psi_{ice} = \frac{RT}{V_W} \ln \frac{P_{ice}}{P_{water}}$$

The dependence shown by the black triangle is calculated from the following equation:

$$\Psi_{ice} = \frac{\Delta H_f}{V_W 273} t$$

The values of the dots showing the dependence were determined psychrometrically. For the theoretical basis of the equations, see Hansen and Beck (1988).

As the vapour pressure of water above ice (as well as above water) is directly dependent

Table. Partial pressure of water vapour (mbar) above ice and above supercooled water in relation to temperature

(°C)	0	1	2	3	4	5	6	7	8	9
Ice										
−30	0.38	0.34	0.31	0.27	0.25	0.22	0.20	0.18	0.16	0.14
−20	1.0	0.94	0.85	0.77	0.70	0.64	0.57	0.52	0.47	0.42
−10	2.6	2.4	2.2	2.0	1.8	1.7	1.5	1.4	1.2	1.1
0	6.1	5.6	5.2	4.8	4.4	4.0	3.7	3.4	3.1	2.8
Water										
−30	0.51	0.46	0.42	0.38	0.35	0.31	0.28	0.26	0.23	0.21
−20	1.3	1.2	1.1	1.0	0.88	0.81	0.74	0.67	0.61	0.51
−10	2.9	2.6	2.4	2.3	2.1	1.9	1.8	1.6	1.5	1.4
0	6.1	5.7	5.3	4.9	4.5	4.2	3.9	3.6	3.3	3.1

Box 1.3.9 (continued)

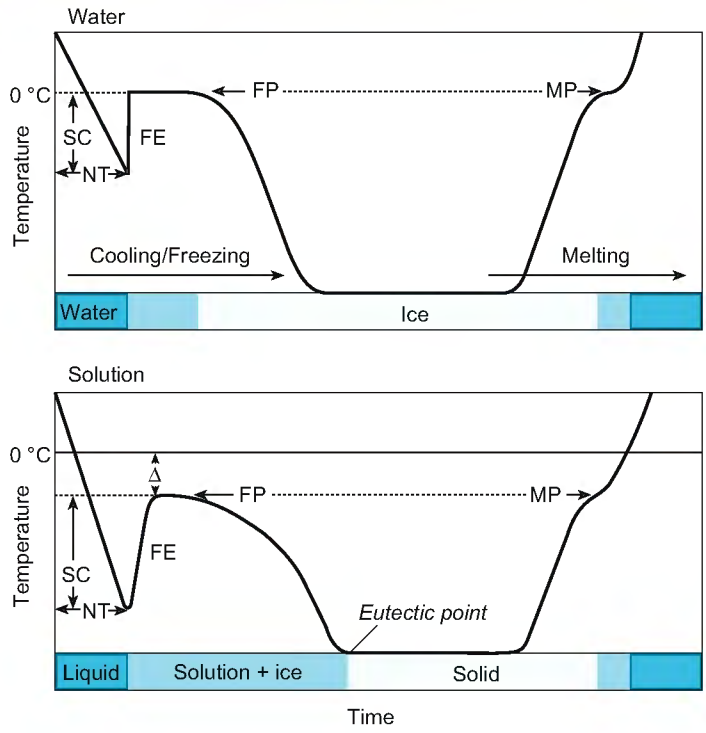


Fig. 1. Temporal sequence of freezing and melting of water and an aqueous solution. *FP* Freezing point; *FE* freezing exotherm; *MP* melting point; *SC* supercooling; *NT* nucleation temperature; *D* lowering of the freezing point of the solution. (After Sakai and Larcher 1987)

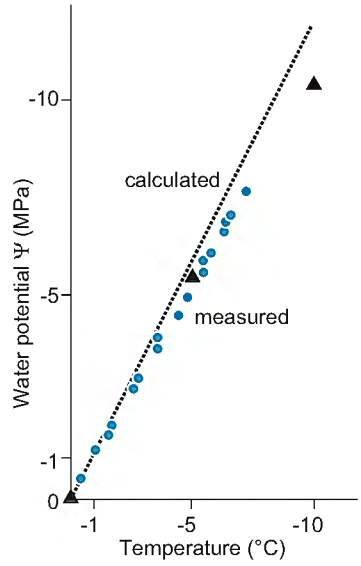


Fig. 2. Temperature dependence of the water potential of ice. The *dotted line* is calculated from the equation

on the temperature, the water potential of ice is also linearly dependent on the temperature (Fig. 2).

The vapour pressure of water over aqueous solutions is also lowered. The extent of the reduction depends on the concentration of the solution, more precisely on the mole fraction (M) of the water in the solution (**Raoult's law**):

$$P = P_0 \times M_{water}$$

$$M_{water} = \frac{mol_{solvent}}{mol_{solvent} + mol_{solute}}$$

[mole fraction]

In the same way, it follows that the free energy of the water in a solution depends on its mole fraction:

$$G_{water} = G_{0water} + RT \ln M_{water}$$

and

$$\Delta G = RT \ln M_{water}$$

It follows from this that the water potential of solutions Ψ_π, or following the modern nomenclature π, is more negative than that of pure water:

Box 1.3.9 (continued)

$$\pi = \frac{RT}{V_{water}} \ln(1 - M_{solute})$$

since M_{solute} is generally small, then

$$\ln(1 - M_{solute}) \approx -M_{solute};$$

substituting the equation for the mole fraction of the solute into the equation

$$M_{solute} = \frac{mol_{solute}}{mol_{solute} + mol_{water}},$$

gives

$$\pi \approx -\frac{n_s RT}{\overline{V}_{water}(n_s + n_{water})};$$

in a dilute solution

$$\overline{V}_{water}(n_s + n_{water}) \approx \overline{V}_{water} n_{water} = V_{water}$$

and

$$\frac{n_s}{V_{water}} = Cs \text{ [concentration]}$$

$$\pi = -RT \times C_s$$

It follows, furthermore, that the freezing point/melting point is lower than that of pure water and that it, too, depends on the mole fraction or concentration of the dissolved substances (s):

$$\Delta_{Tm} = 1.86 \times n_s$$

$n_s = mol_{solute}$ in 1 kg water (molality)

The molal constant for the lowering of the freezing point of water is 1.86 °C. The more concentrated the solution is, the greater is the lowering of the freezing point.*

In ideal solutions pure water freezes first, whereupon the remaining solution becomes concentrated. At a particular temperature, the **eutectic point**, the remaining solution then also freezes. In colloidal solutions such as are present in a cell, a small proportion of the water which is firmly bound to the macromolecules remains liquid even at very low temperatures, e.g. in liquid nitrogen. This proportion of the water can be determined by means of a diagram, in which the proportion of liquid water at various frost temperatures is plotted against the reciprocal of the temperature, and is given at the point at which the line crosses the ordinate (cf. Fig. 1.3.22). This residual water, in which enzymes are dissolved, is a problem in frozen food. At freezer temperatures these enzymes naturally function only very slowly, but hydrolytic enzymes in particular are quite stable and are usually not destroyed during freezing. Only few degradation products are to be reckoned with when frozen foods are used immediately after thawing. However, if the thawed material is later refrozen, the hydrolases (e.g. proteases) will have been provided with favourable conditions for their activity and the frozen products can suffer from a loss of quality.

* This applies strictly only to dilute, i.e. ideal solutions. In concentrated solutions, interactions occur between the dissolved particles, which result in an apparent reduction of the concentration. The factor a, by which the concentration appears to decrease, is termed the activity factor.

(negative turgor) and strengthens the osmotic potential. A matrix potential develops similarly during strong dehydration. All three potentials now act together:

$$(-)\Psi \text{ tissue} = (-)\pi + (-)\tau + (-)P = (-)\Psi \text{ ice}$$

This occurs even at only moderate frost in the ground-covering plant *Pachysandra terminalis* (Fig. 1.3.23).

Significantly less water freezes in this case than would be expected from the prevailing osmotic relations, and the effect of the negative pressure potential becomes particularly evident. However, the influence of the matrix potential may also become apparent during such strong dehydration. At the extent of the freeze-desiccation shown, 12% more liquid water (32% residual water compared with 20% at −7 °C) signifies considerably less strain on the cells.

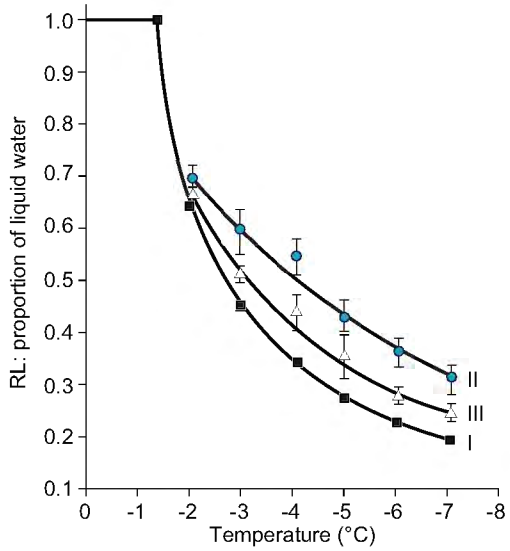


Fig. 1.3.23. Freezing curves for *Pachysandra terminalis*. *Pachysandra* leaves show non-ideal equilibrium freezing: the residual amount of unfrozen water measured in the tissue at various frost temperatures (*curve II*) is significantly greater than that calculated from the concentration of the cellular solutions (*curve I*). In this case, the cell wall as well as the cell sap must therefore develop a "negative pressure potential" corresponding to suction. This is the result of the close contact between the plasma membrane (protoplast) and cell wall. The share of the matrix potential in the suction can be determined by killing the tissue prior to the freeze-desiccation (*curve III*). The more severe the frost is, the greater is the proportion of the total potential due to the matrix potential. (After Zhu and Beck 1991)

Table 1.3.8. Reduction of the freezing point by antifreeze proteins as measured by thermal hysteresis

Organism	Tissue	Reduction in freezing point (°C)
Bony fish	Muscle	0.7–1.5
Insects	Digestive tract, muscle	3–6
Bacteria		0.3–0.35
Fungi	Fruiting body	0.3–0.35
Mosses	Whole plant	0.3–0.68
Horsetail or Dutch rush (*Equisetum hyemale*)	Rhizome	0.2
Ferns	Shoot	0.25
Winter wheat	Leaves	0.2
Carrot	Tuber	0.4
Carrot	Shoot	0.15
Poplar	Twig	0.22

1.3.6.5 Antifreeze Proteins

A further possibility to impede ice formation and thus to decrease cell desiccation is constituted by special proteins which are excreted from the protoplast into the apoplast and influence the formation of ice there. These have been termed antifreeze proteins (AFPs) or thermal hysteresis proteins (THPs). Antifreeze proteins have long been known in fish and insects (DeVries and Cheng 1992; Duman et al. 1992), where they apparently prevent or alleviate the freezing of body fluids. In these animals they do appear to significantly reduce the freezing point in a non-colligative (non-physicochemical) manner.

An effect of these proteins is illustrated in the test carried out to detect their presence: The growth of an ice crystal and the formation of new ice crystals in a very diluted AFP solution can be observed under the microscope during cooling. It is thereby observed that the temperature required for nucleation is significantly lower, and an existing ice crystal grows much more slowly during cooling than in pure water; the melting point, however, is not affected. This results in hysteresis during freezing and thawing, and is the reason why these proteins are also called THPs (Table 1.3.8).

Antifreeze proteins are a mixture of small to medium-sized proteins which can be extracted from the tissue of frost-hardy leaves, but not of frost-sensitive leaves (Urrutia et al. 1992). They are divided into several groups: Antifreeze proteins and antifreeze glycoproteins are both subdivided into further groups based on the tertiary structures of the two major types of protein. There are clear conceptions as to how these proteins function at the molecular level, although this is not yet true of plant AFPs.

The already classical antifreeze glycoproteins of cold-water fish are built around the repeating tripeptide -[Ala-Ala-Thr]-, where the threonine bears a sugar residue.

The AFP of the winter flounder is also characterised by such repetitive sequences, namely of an undecapeptide. The 11 amino acid units consist mainly of alanine with rhythmically interspaced polar amino acids (Thr, Asp and Asn). These are ordered in such a way that their polar residues are all on one side of the helix (Fig. 1.3.24), so that the protein has a hydrophilic and a hydrophobic side. The hydrophilic face of these proteins is thought to attach to the main growing surface of the ice crystal, and the hydrophobic side of the protein, which is now turned to the outside, makes it difficult for further water clusters to associate with the ice

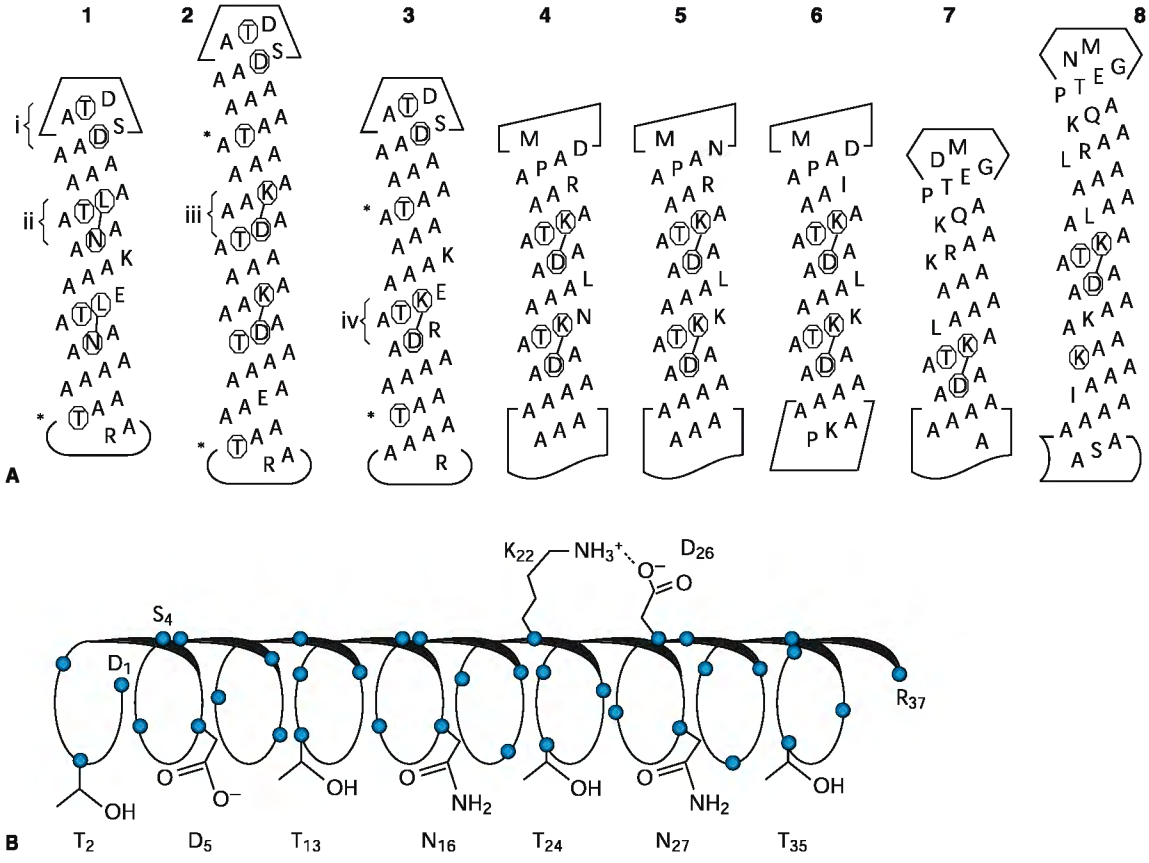


Fig. 1.3.24. Antifreeze proteins (AFPs) of fish. A Various alanine-rich α-helical AFPs. *1* Winter flounder; *2* yellow tail flounder; *3* plaice; *4–8* different AFPs from sculpin. The N- and C-terminal caps, which stabilise the α-helical structures, are shown by structure symbols. Ice- (water-) binding motifs (*i–iv*) are shown by *circles*. B Side view of the AFP B of the winter flounder, in which the unilateral orientation of the hydrophilic groups can be clearly recognised. K22 and D26 form a salt bridge (amino acids are specified in *single letter code*). (After Sicheri and Yang 1995)

crystal. The matrix for this attachment is set by the ice crystal, while the manner in which the attachment takes place is determined by the protein. Since α-helical polypeptides are dipoles, the AFP preferentially attaches to the prism surfaces of the ice crystal in an antiparallel manner. Due to the blocking of its normal growth surfaces, the ice crystal now grows less rapidly and extends mainly in the direction of the c-axis. This leads to the formation of small, needle-like crystals (Antikainen et al. 1996; Fig. 1.3.25).

It is quite evident that the effect of AFPs in delaying freezing depends on the concentration of the proteins. Since they must be bound to the surface of the ice to achieve their effect, they can be said to be used up. This also explains why AFPs do not provide a general protection against freezing. AFPs reduce the formation of ice in the organism, but they cannot suppress it completely. The partially frozen state of the tissue liquid arrived at in this manner is certainly more stable during supercooling than the completely liquid state, which tends to freeze abruptly. It is quite plausible that many small ice crystals which are inhibited in their growth and cloaked in proteins are less damaging to the organism than is a large crystal. The protein cloak of the ice crystal is possibly the deciding factor in frost protection by AFPs. A further effect of AFPs could be to prevent recrystallisation of ice concomitant with the formation of larger ice crystals during the thawing process (Carpenter and Hansen 1992). This is of particular importance with regard to ice crystals in the body fluids of cold-water fish and insects, where the formation of larger ice crystals would lead to circulatory damage. The combination of INPs and AFPs could constitute an effective method of regulating the formation of ice crystals in tissues (Griffith et al. 1993; Worland and Block

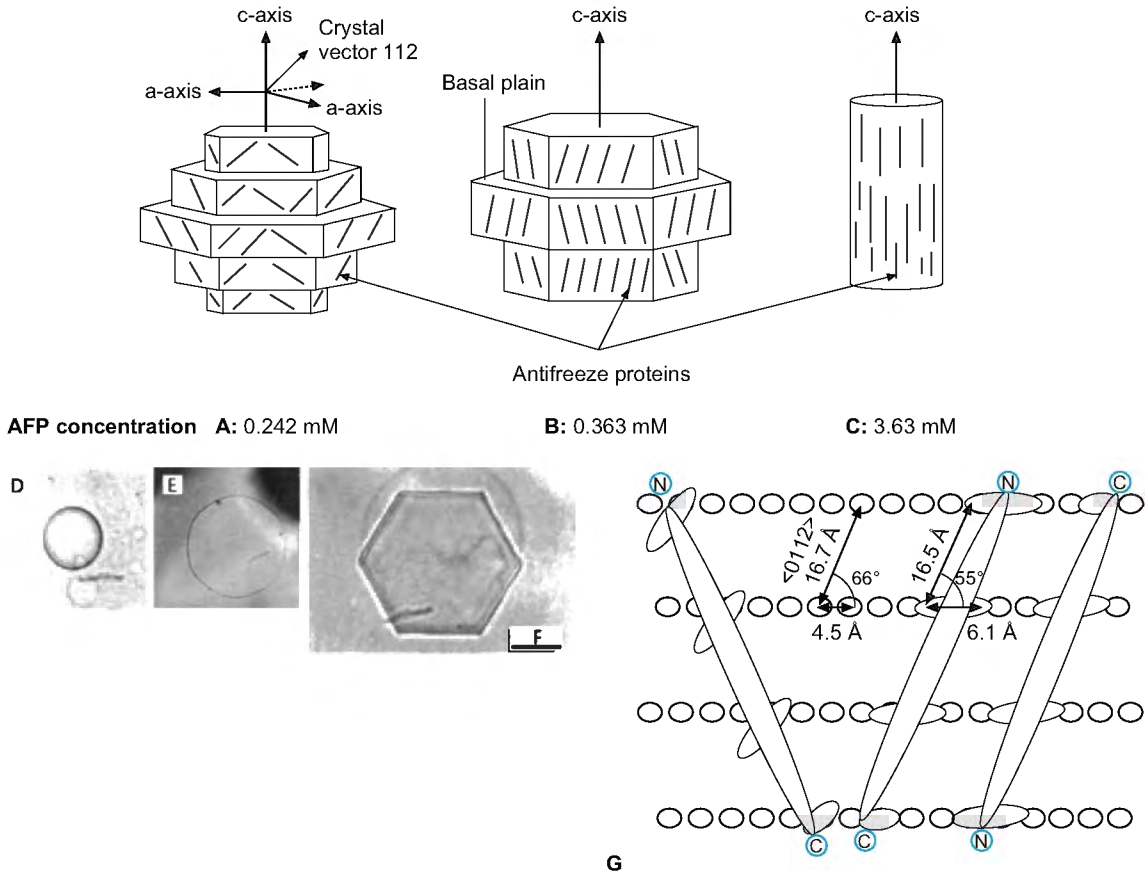


Fig. 1.3.25. The effect of antifreeze proteins (AFPs) on the formation of ice crystals. A–C Schematic drawings of crystals in the presence of increasing concentrations of winter flounder AFP. In the most dilute solution, there is not yet any interaction between the individual protein molecules, which orient themselves mainly according to the ice crystal vectors. In the more concentrated solutions, the protein molecules interact and bind in an antiparallel manner to the prism surfaces. E, F Micrographs of ice crystals which have formed in the presence of protein extracts from the apoplast of unhardened (E) and frost-hardened rye leaves (F). Length of the *bar*: 25 µm. For comparison an ice crystal in pure water is shown in D. G Molecular model of the binding of the winter flounder protein (*grey*) to an ice crystal (*circles* denote water molecules). The four ice-binding motifs correspond to the distance between the water molecules in the ice crystal. (A–C after Yang et al. 1988; D–F after Griffith et al. 1992; G after Sicheri and Yang 1995)

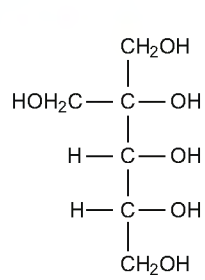
1999). Attempts to transform potato plants with the AFP of flounder genetically engineered to optimise codon usage yielded plants with significantly increased cold tolerance (Wallis et al. 1997).

1.3.6.6 Protection of Biomembranes and Freezing Tolerance

Soluble material accumulates in the residual liquid of the cell to an enormous extent during extracellular freezing. In addition to the danger of membrane disintegration as a consequence of water withdrawal (see above), severe stress is incurred by the concentration of ions at membrane surfaces. High ionic charge at the membrane surface changes the membrane potential, and can thus also lead to membrane disintegration or at least to the dissociation of peripheral proteins.

The Degree of Frost Hardiness Depends on the Extent and Quality of Membrane Protection

Relatively high concentrations of soluble, low molecular weight, so-called cryoprotective substances can be found in frost-tolerant plants [polyols, sucrose and its galactosides raffinose and stachyose, poorly degradable carbohydrates such as hammamelitol (Fig. 1.3.26), amino acids, polyamines and many more]. These compounds "dilute" the ionic charge at the membrane surface and, at the same time, stabilise the bilayer struc-



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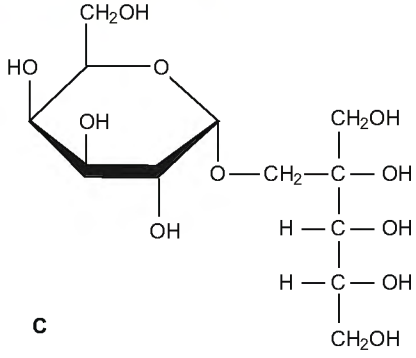

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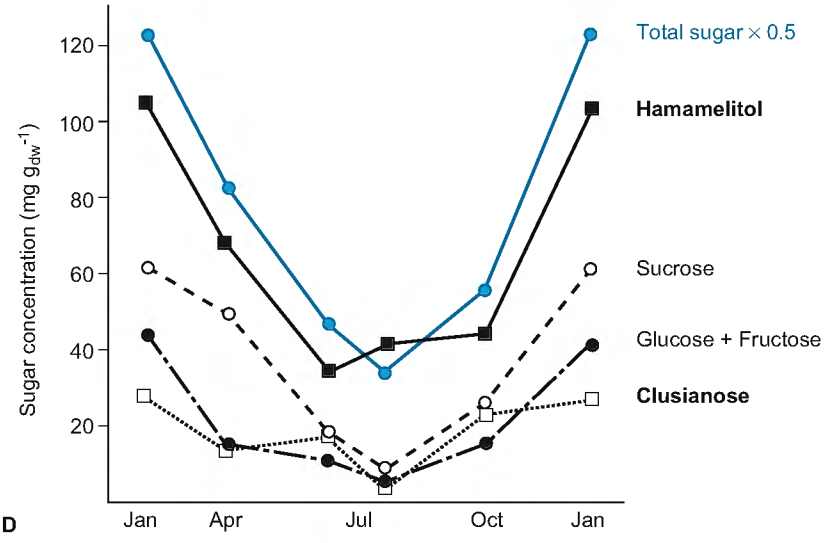


D

Fig. 1.3.26. Frost protection in the eastern Alpine *Primula clusiana* Tausch (A). Hamamelose = 2-hydroxymethyl-D-ribitol (B), and its galactoside clusianose (C), accumulate irreversibly in the overwintering leaves during the cold season (D). These old leaves die subsequent to the formation of new leaves in the following vegetation period without the branched chain sugar derivatives, which are present in high amounts, having been removed for further use. The accumulation of hamamelose can also be induced by cooling the plant in summer. (Photo E. Beck)

ture in being weakly polar (as is water). The main effect of cryoprotective substances is thus to protect membranes. Their effect in lowering the freezing point first comes into play during freeze-desiccation of the cells. For example, the sap expressed by pressure from frost-hardy spruce needles is only 1.5 osmolar, corresponding to a reduction of the freezing point by 2.8 K. This is physiologically insignificant, as a greater degree of supercooling is required to trigger crystallisation. The cell liquid becomes more concentrated during freeze-desiccation, however, and would be ten-fold more concentrated at a residual volume of 10%. The colligative (physicochemical) properties of the cryoprotective substances would then, of course, be of considerable importance. In the example of spruce needles, this would result in the lowering of the freezing point by 28.5 K, which would prevent the freezing of the residual liquid water.

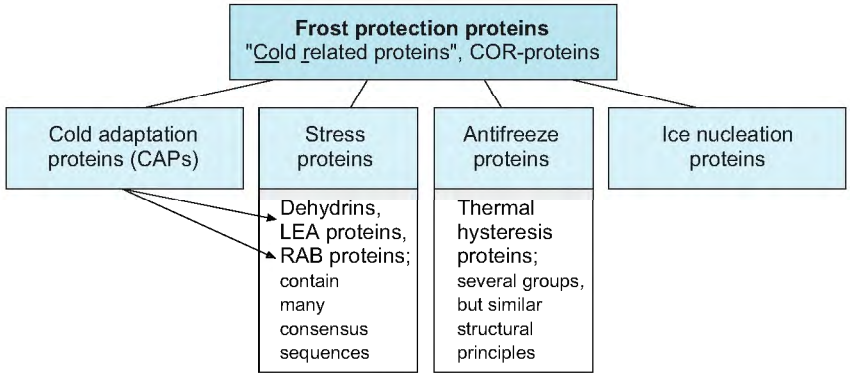


Fig. 1.3.27. The different types of frost protection proteins

Freezing Protection Proteins

So-called cold protection proteins (CORs) or cold adaptation proteins (CAPs), which can play an essential role in the development of frost hardiness, are of particular contemporary interest. Many such proteins and corresponding genes, which become expressed upon cold treatment, have been described since the late 1980s (Fig. 1.3.27). Among them are antifreeze proteins and isoforms of housekeeping enzymes with a lower temperature optimum such as, e.g., lactate dehydrogenase (in animals) or PEP carboxykinase (Sáez-Vásquez et al. 1995), as well as enzymes for the biosynthesis of cryoprotectants.

Membrane-protective proteins also belong to this group, proteins which interact with the surfaces of membranes and stabilise these during freeze-desiccation. They are similar to the so-called dehydrins, extremely hydrophilic protective proteins which are formed during drought stress or during regulated desiccation, e.g. during seed ripening. They usually have a molecular mass of between 15 and 50 kDa (see Chap. 1.5.2.5), but very large (~200 kDa) CORs have also been described recently. These proteins are rich in glycine, are boiling- and acid-stable and possess a high proportion of random-coil secondary structure. On a molar basis they are about 20,000–40,000 times more effective than sucrose in conserving membranes. These proteins are described in detail in Chapter 1.5 on drought stress. There are some indications that cold protection proteins can be membrane-specific; e.g. a 7-kDa protein has been found which specifically protects chloroplast thylakoids (Hincha and Schmitt 1992). Even though heat shock proteins have been linked with cold tolerance, the CORs and CAPs are not related to them in any way either in structure or function. Like antifreeze proteins, most CORs and CAPs are amphiphilic but, in contrast to the AFPs, they attach with their hydrophobic side to the structures they are protecting, e.g. membrane surfaces. Their hydrophilic side faces the cytosol. This attachment results in the displacement of lower molecular weight solutes such as ions from the membrane surface. As is the case with dehydrins, it is possible to induce the synthesis of CAPs by treatment with abscisic acid (ABA, see Chap. 1.5.2.3) in place of cold treatment. Treatment of citrus plants with ABA and other growth regulators was successful in conferring cold tolerance, but had a number of negative side effects such as disadvantageous biomass distribution and premature fruit shedding. Thus spraying with such chemicals cannot be recommended as a general method for artificially improving the frost-hardiness of threatened crops (Yelenosky et al. 1987).

1.3.6.7 Avoidance of Freezing

Supercooling

The avoidance of the formation of ice crystals at freezing temperatures leads to **supercooling**. The supercooled state of plant tissues can usually be maintained for only some hours, in which case it can, for example, help plants to cope with the nightly radiation frosts which occur regularly in high mountain regions of the tropics. Supercooling has been observed to persist at length only in tissues possessing very rigid cell walls. Leathery leaves, for example, can remain unfrozen at temperatures of −12 °C, and the buds of trees and the wood of certain species (e.g. of the North American *Cornus stolonifera*) are able to supercool at −30 to −50 °C for long periods (Ashworth 1993). Supercooling has the advan-

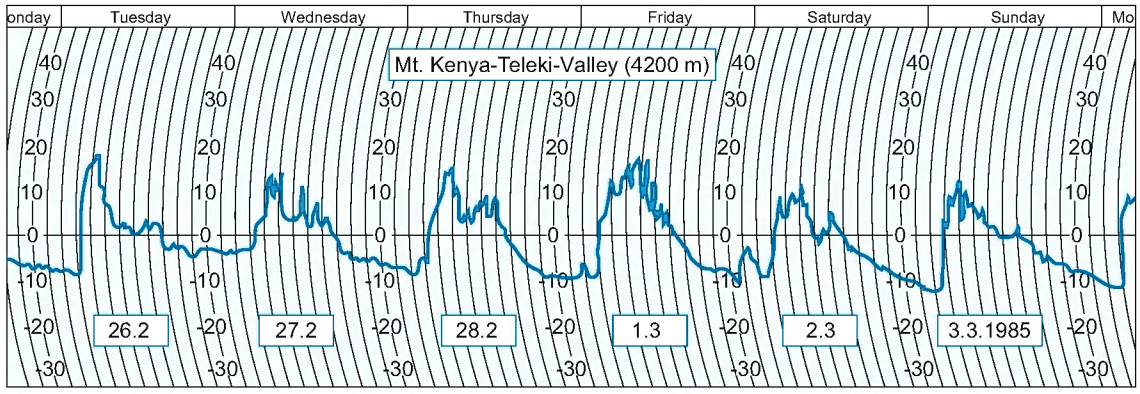


Fig. 1.3.28. Temperature courses (close to the ground) in tropical high mountains during the dry period: Hedberg (1964) characterized this recurrent-frost climate as summer every day, winter every night. Night frosts occur even during the rainy season, although the temperature then seldom drops to below −5 °C

tage over freezing tolerance in that the hydration of the cells is maintained and no dehydration stress occurs. Metabolic reactions are extremely slow at the low temperatures of supercooling to be sure, but during freeze-desiccation they cease completely. Supercooling also brings the risk of **intracellular ice formation** upon sudden nucleation, however (see Fig. 1.3.18), which then leads irrevocably to the death of the cell. This may be triggered by movement due to wind or animals in open spaces, for example. In general, those plants possessing nucleation barriers are not tolerant of freezing. Supercooling as a strategy may be regarded as a form of stress avoidance (avoidance of freezing).

Transient Avoidance of Freezing in Plants of Tropical High Mountain Regions

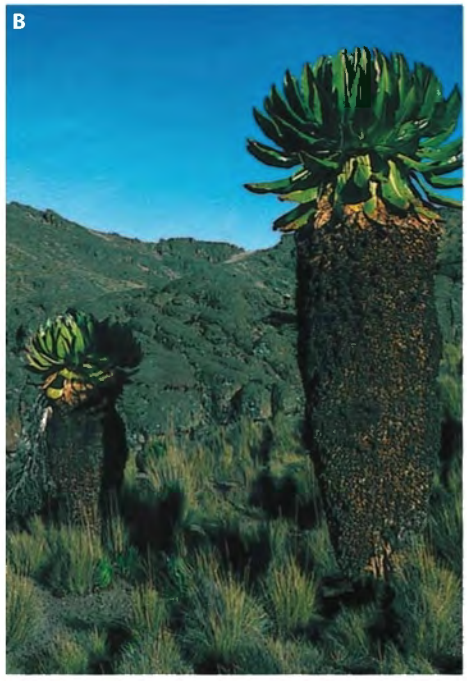
Typical of high mountain areas in the tropics is the so-called recurrent-frost climate: this is a special version of the daily fluctuation type of climate characteristic of the tropics, in which several hours of frost usually occur during the course of the night (Fig. 1.3.28).

Even though these frosts occur regularly, they do not last long and are only moderate (temperatures below −15 °C are very rare and occur at most in hollows), and the indigenous flowering plants have developed special facilities to provide temporary avoidance of freezing.

This is particularly evident in the tropical alpine giant rosette plants of the genera *Senecio*, *Lobelia* and *Espeletia*. These seemingly archaic plants (Fig. 1.3.29) bear a single large rosette of leaves of up to over 1 m in diameter and consisting of usually more than 100 large leaves at the end of the above- or below-ground stem. There are no marked quiescent periods during the course of the year and, therefore, young leaves are continuously formed and older leaves also die continuously. At least the first half of the development of the leaf takes place in a conical leaf bud in the centre of the leaf rosette, from which the leaves emerge after greening and carry out the rest of their longitudinal growth in the rosette. The principle means by which these plants avoid freezing are **supercooling** and **delayed cooling** (Beck 1994).

Supercooling has been shown above all for the South American *Espelitias*, the cell sap of which does not freeze even at −8 °C. Temperatures lower than this almost never occur in areas colonised by these plants, which are lethally damaged if ice formation is artificially induced. Delayed cooling can be achieved by insulation and via heat buffers. **Cloaking** of the above-ground stems of these plants confers permanent heat insulation through the dead, partly rotted leaves, which delay cooling but do not prevent the stem temperature from rising to 15–20 °C during the day under intense solar radiation (Fig. 1.3.29). A stem temperature in the range of between 0 and 20 °C is important, as a large share of the water required by the leaf rosette is supplied by the living pith tissue and does not stem from the weakly developed xylem with its extremely narrow vessel lumens. Water transport via living cells depends to a large degree on the temperature.

The so-called **night buds** of the giant rosettes provide thermal insulation in accordance with the prevailing temperature (Fig. 1.3.30): When the temperature falls at night the rosette leaves bend in towards the top and thus form a closed



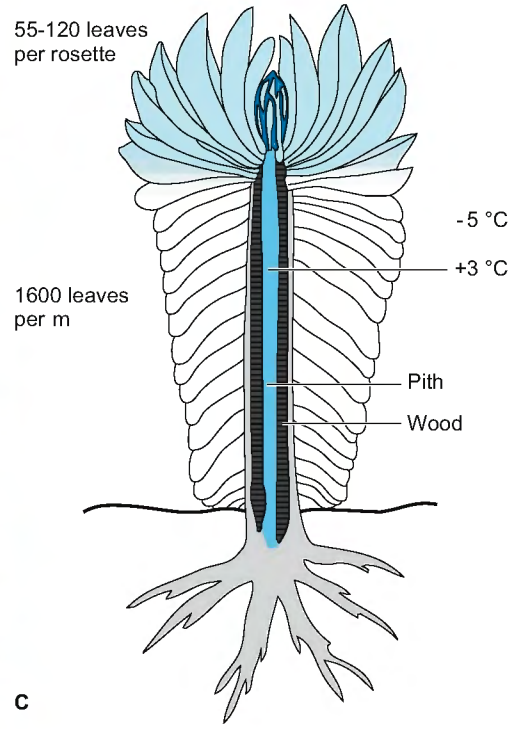


Fig. 1.3.29. Rosette plants of the genera *Senecio* (A–C) and *Espeletia* (D) from the alpine tropics. Neither the East African *Senecio keniodendron* (endemic to Mt. Kenya) nor *Espeletia corymbosa* from the Andes lose their dead leaves, but retain them as an insulating cloak around the stem. The effect of this insulating layer is indicated in the schematic diagram (C) which illustrates the inner structure of the plant. The massive pith serves as a water reservoir, as the xylem itself is only relatively weakly developed. The Espelitias are called "Freilejones" (fat monks) on account of the appearance of the cloak of dead leaves. (Photos E. Beck)

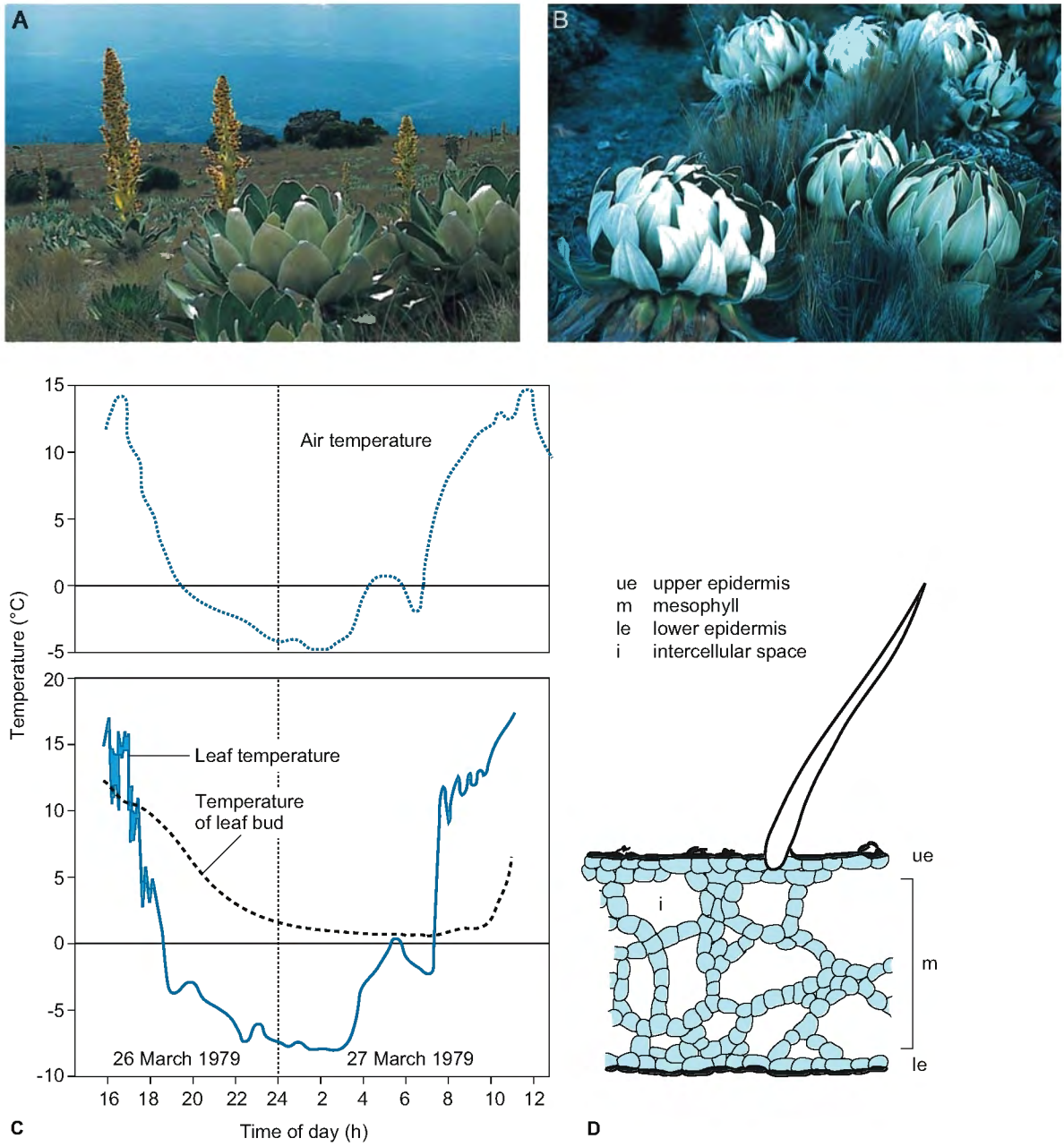


Fig. 1.3.30. Protection against freezing by adaptive insulation in *Senecio keniensis* (endemic to Mt. Kenya). Rosettes open during the day (A) and close when it becomes cold, forming a so-called night bud (B). The insulative effect (C) applies to only the young leaves in the interior of the rosette. While the outer leaves are frozen stiff, the inner ones remain at above the freezing point. The leaves of the giant rosette plants (D) frequently exhibit a "polystyrene foam" structure which increases the insulative effect (*Lobelia keniensis*). (Photos E. Beck)

shell around the still-growing buds and young leaves still in the bud cone (Fig. 1.3.30 B). This night bud consists of many layers of leaves and thus provides considerable insulation. Although the outer leaves are frozen stiff in the morning, the temperature inside does not fall below the freezing point (Fig. 1.3.30 C). Upon warming in the morning, the rosette opens within a few minutes and is then immediately ready for photosynthesis.

Water is a very effective heat buffer on account of its high specific heat and its even higher latent heat of crystallisation (Fig. 1.3.31). The inflorescence of *Lobelia telekii* contains several

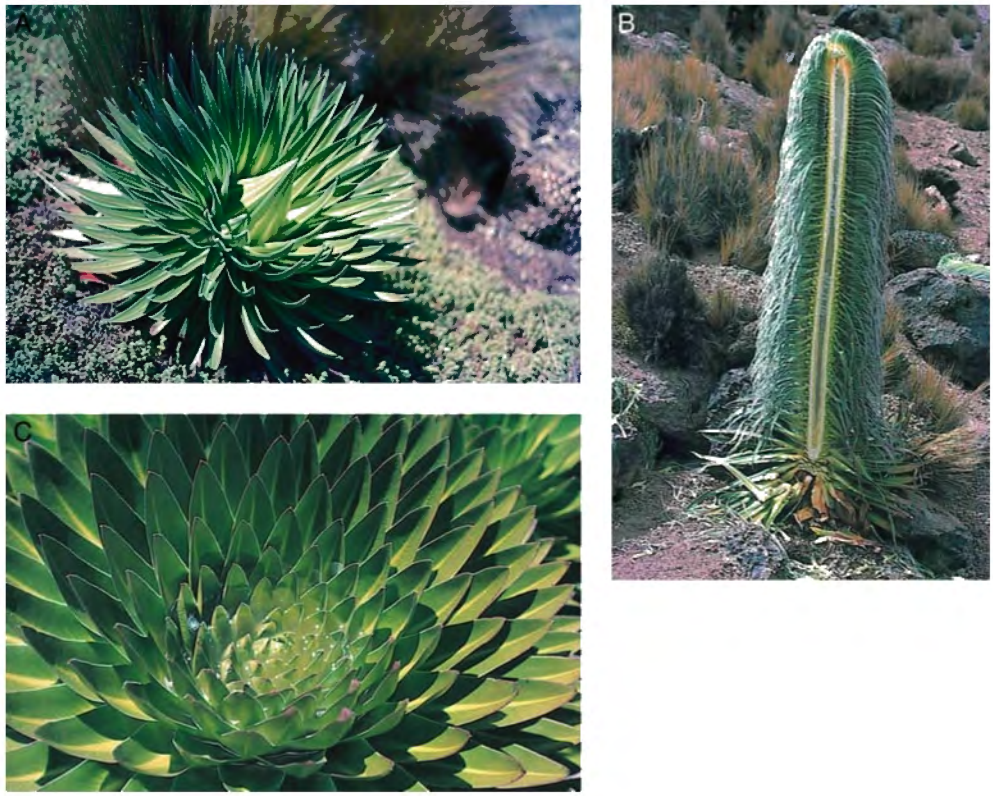


Fig. 1.3.31. Water or aqueous solution as a thermal buffer. A, B *Lobelia telekii* in the rosette stage and during flowering, respectively. The rosette clearly shows the structure of a "giant rosette". The young leaves develop in the centre of the rosette and, closely appressed, form a cone-shaped leaf bud. The infloresence is up to 3 m in height (B) and contains 3 l of watery pith fluid according to Kroog et al. (1979). From the specific heat of water (4.2 J/g×K) and the heat of its crystallisation (331 J/g), a thermal buffering capacity of 1005 kJ can be calculated. At –6 °C only 2% of the liquid in the pith was frozen, according to which only 1.8% of the total heat buffering capacity was used. C The numerous leaves (>150) of the rosette of *Lobelia deckenii* (Mt. Kilimanjaro) form cisterns in which rain as well as gutated liquids accumulate. A rosette contains more than 2 l of such liquid. This liquid is pushed up during the formation of the night buds and surrounds the leaf bud as an additional heat buffer. (Photos E. Beck)

litres of water in its pith cavity with an enormous heat capacity: At an exterior temperature of –6 °C only about 2% of this liquid was frozen and only 1.8% of the heat storage capacity was made use of. The cistern-like bases of the rosette leaves also collect litres of water, which is only partially frozen after cold nights and thus constitutes a considerable heat reservoir (Fig. 1.3.31C). Investigations have shown that all of the leaves of a giant rosette (except, seemingly, the leaves of the genus *Espeletia*), including those still in the bud, can tolerate freezing. What is, then, the value of the avoidance of freezing? Daily de- and rehydration processes evidently put a great strain on tissues: Outer rosette leaves that more or less freeze every night age relatively quickly. Tips of leaves which are exposed to the sunlight in their frozen state yellow after only a few days. It therefore appears to be advantageous for tissues to avoid freezing stress even when they are frost-hardy.

1.3.6.8 Seasonal Tolerance to Freezing

Phases of frost hardening and dehardening alternate with the seasons in native "lowland" plants. Signals for hardening or dehardening are the day length and temperature. In nature both factors work together; in experiments they also act individually. Short day length and a slow decrease in temperature to a low, but not yet damaging, value induce rapid hardening (~–1 K/day). The phytochrome system participates in the signalling that leads to the effect of day length: far-red light stimulates hardening, red light dehardening. Since resistance to freezing is

a process that affects the whole plant, hardening and dehardening are extremely complex processes which are currently being intensively studied at the level of cell biology. Some well-investigated individual processes, such as the formation of cryoprotective substances including proteins, have already been mentioned. A particularly important process is the adaptation of biomembranes to heat and cold. According to present knowledge, frost hardening is not possible during the main growth period because the cell wall is probably not able to cope with the strain of water loss during freeze-desiccation.

Molecular Biology of Frost Hardening

Even though *Arabidopsis thaliana* can develop at most only moderate frost resistance, research using this plant has yielded important insights into frost hardiness due to the complete sequencing of the *Arabidopsis* genome and the relative ease of carrying out mutant studies (Thomashow 1999). The constitutive "frost hardiness" of this plant has been shown to be genetically independent of its ability to harden to frost. Constitutive frost hardiness is understood as the degree of frost hardiness which can be measured with plants grown at room temperature. Constitutive as well as induced frost hardiness constitute a syndrome, i.e. they involve the concerted action of many genes (more than 100 genes have been reported: Xin and Browse 2000). The most important features which occasion frost hardiness have been treated in detail in earlier chapters. The existence of the syndrome "frost hardiness" or "frost hardening" suggested a search for "master genes" which regulate the frost-hardening process. Such master genes were found at the level of transcription factors and transcription activators (Fig. 1.3.32), e.g. the activators of the CBF group (CRT-Repeat Binding Factor) that bind to the promoter element AAGAC which occurs in many COR genes. CBF1 has also been described as DREB1B (Drought Regulated Element Binding factor 1B; see Chap. 1.5: stress due to drought), which again illustrates the phenomenon of cross-protection. The induction of some COR genes by abscisic acid (ABA) via the transcription activator "bZip" (alkaline leucine zipper) can be understood in the same context. Figure 1.3.32 shows how such master genes can activate several other genes at the transcription level, and how the regulatory effects of several master genes can overlap. The identification of a master gene begs the retrogressive question as to how this master gene is activated. An increase in cal-

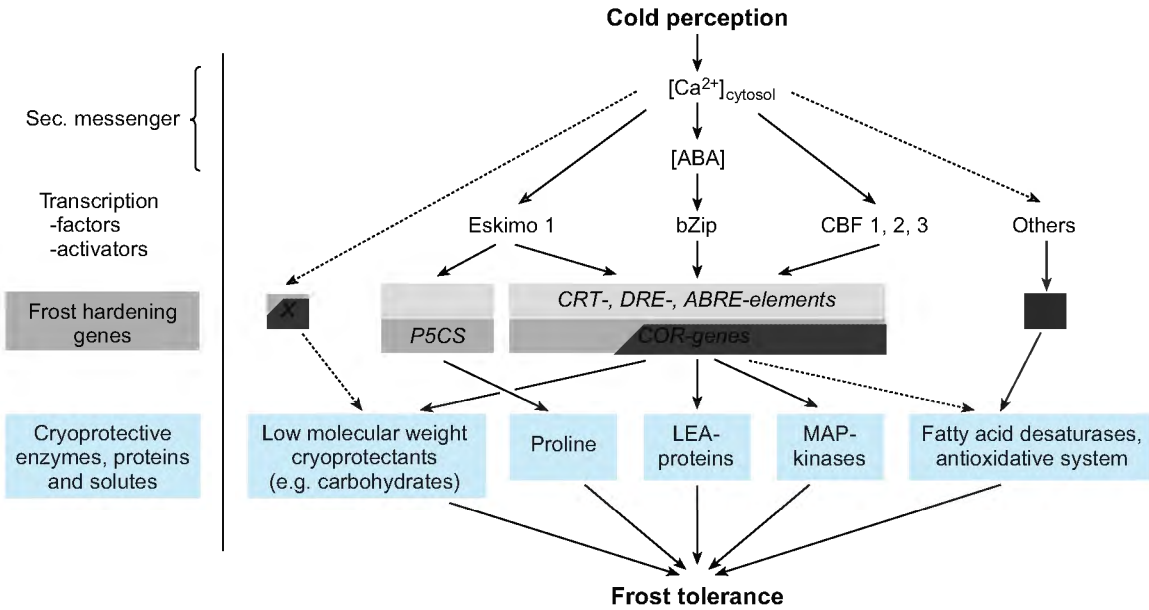


Fig. 1.3.32. Molecular biology of the induction of frost hardening. Overview of the established (*continuous arrows*) and presumed (*dotted arrows*) signal transduction pathways in *Arabidopsis thaliana* which lead from the perception of the cold signal (molecular basis unknown) to the development of frost hardiness (maximum degree of resistance is to −12 °C). *ABA* Abscisic acid; *bZIP* alkaline leucine zipper; *CBF* CRT-repeat binding factor; *DRE* drought-regulated element; *ABRE* ABA-responsive element; *P5CS* gene for Δ-1-pyrroline-5-carboxylate synthetase (cf. Box 1.5.5). (After Xin and Browse 2000)

cium concentration in the cytosol appears to be a decisive trigger in this regard (Tahtiharju et al. 1997). At any rate, such an increase is observed immediately after the onset of a frost hardening program and precedes gene activation. It is not yet known how the increase in the cytoplasmic calcium concentration is triggered. It is supposed, however, that the membrane stiffening due to cold is involved in the stress perception (Vigh et al. 1993; see also the section on stress perception in Chap. 1.1).

1.3.6.9
Frost Hardening Decreases Efficiency

The "strategy" of winter-green plants such as conifers for coping with frost is to promote the extracellular freezing of tissue water. Photosynthesis ceases when the formation of ice takes place due to the severe dehydration and low temperatures. About 30% of the chlorophyll-binding antenna proteins of the chloroplasts of Scots pine are degraded during the course of frost hardening (pines appear somewhat yellow-green in winter). This can be seen as a protection of the photosynthetic membranes against light stress during sharp frosts (Hansen 2000). It results in a considerable restriction of photosynthetic function in the non-frozen state, however. Experiments in this regard on the CO_2 assimilation of pine needles have shown an about 80% reduction in potential photosynthetic capacity (see Chap. 1.1.3, Fig. 1.1.7). This limitation of photosynthesis does not necessarily have negative consequences, however, as respiration is also considerably restricted at lower temperatures (Hansen and Beck 1994).

1.3.6.10
Secondary Stress: Frost Drought

Plants cannot take up water from frozen soil, and thus are in danger of suffering drought damage during permanent frost. Since the activity of roots depends on temperature, the uptake of water and nutrient salts deteriorates even at temperatures of a few degrees above the freezing point (Chap. 2.3). Plants of warmer regions already have similar difficulties at temperatures of about 5–10 °C: In various citrus species cold-induced drought damage is already evident at +5 °C.

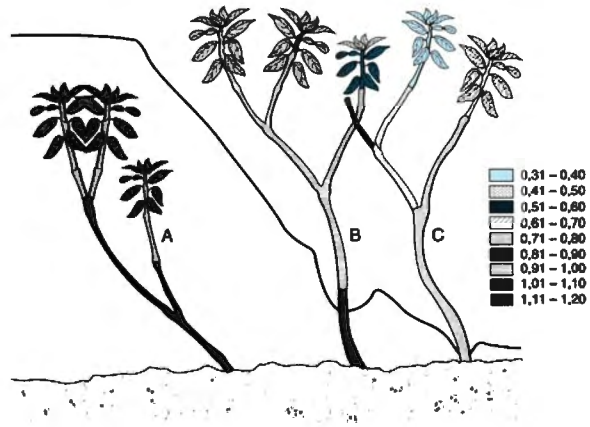


Fig. 1.3.33. Frost drought. Water contents (in g H_2O/g dry wt.) in leaves and shoots of the Alpine rose (*Rhododendron ferrugineum*) in winter. A Continually covered with snow; B only the stem basis covered by snow; C on frozen ground without any snow cover during the entire winter (measurement in March). (After Larcher 2003)

Frost drought occurs particularly at sites bearing little snow, when the soil is deeply frozen and the sun's rays induce transpiration while an effective water delivery from the frozen stem and soil is impossible (Fig. 1.3.33). This is accompanied by increased danger of embolism in the xylem and in the cells as well. During prolonged frost drought, the volume of the cells, e.g. in conifer needles, shrinks to a fraction of the volume in the turgescent state. According to Larcher (2001), the tree line is determined by the duration and the severity of soil frost and by the extent of snow cover. Snow cover provides protection against desiccation and extremely low temperatures during severe frosts.

Snow cover also causes considerable strain, however: The pressures incurred lead to snow and ice breakage, light incidence is reduced (under a 20-cm-thick snow cover the residual light is 1–15% of that in the open) and gas exchange is impeded. The situation under prepared ski runs is particularly critical: Reductions of biomass production by 20–30% and in extreme cases by up to 70% have been observed (Cernusca 1984).

Table 1.3.9. Plants and cold: an overview

Temperature range (°C)	Plant response	Survival strategy	Example	Description
+8 to 0	Sensitive	None; unable to adapt	African violet	"Chilling-sensitive"
+8 to 0	Tolerant	None; unable to adapt	Tomato, potato	"Chilling-tolerant"
0 to –12, possibly lower	Tolerant	Lipid desaturation, cryoprotective substances	Spruce, pine, ivy in summer state	Frost-tolerant, but not tolerant to freezing
0 to –70, possibly lower	Tolerant	Lipid saturation, cryoprotective substances, lipid enrichment	Spruce, pine, ivy in winter state	Tolerant to frost and freezing

1.3.7

Concluding Comments

The expositions having been made in the section on "cold" show that the stressor low temperatures gives rise to a whole battery of strain reactions. The rather ambiguous term "syndrome" is often used in the literature to designate the multilayered changes which are thereby brought about, e.g. the frost-hardening syndrome. Table 1.3.9 summarises the various aspects of the cold syndrome of plants.

Summary

1.

General

1. Every organism in nature is subject to limiting temperatures which define the temperature range in which active life is possible; these can in some cases significantly exceed or be lower than the limits of the temperature range of liquid water. Living beings (microorganisms) which have a temperature optimum of above 100 °C are able to exist only under high pressure, in the deep oceans. All living organisms evidence so-called cardinal points of temperature compatibility: Cold death, cold rigidity, the range of active life, heat rigidity and heat death. Living organisms which are adapted to low temperatures are said to be psychrophilic, those requiring high temperatures are thermophilic to extremely thermophilic, while most organisms are mesophilic. Not all parts of a plant have the same temperature compatibility; this also applies to the different stages of their development.
2. Plants are able to adapt to conditions other than their "normal" temperature conditions, within certain limits. This requires that the lipid composition of the biomembranes be modified, so that the membranes are able to maintain the liquid-viscous state at all temperatures which may occur. If the temperature conditions significantly approach the limits of active life, high or low molecular mass protective substances ("chemical chaperones") are additionally formed. Plants are also able to adapt in this way to large temperature fluctuations occurring during the course of a day.

2.

Heat

1. The typical strain triggered by heat is the production of heat shock proteins and the switching off of housekeeping genes. These reactions already start at only a few degrees above the optimal temperature of an organism; this means that plants live close to their heat stress limits. Heat avoidance mechanisms are, therefore, of great importance. A small leaf area, appropriate phyllotaxis, a strong indument and transpirational cooling all work against the overheating of leaves.
2. The heat shock response is one of the best-understood stress reactions at the molecular level. Proteins denatured by heat associate with heat shock proteins (HSPs, chaperones). Certain HSPs are normally present in association with heat shock transcription factors (HSFs). The HSFs are released and activated (trimer formation) as a result of the reaction with denatured proteins. The active HSF then reacts with corresponding palindromic sequences in the promoter region of the heat

shock genes, the so-called heat shock elements. This leads to the expression of these genes and to the production of HSPs. The HSPs "re-nature" heat-damaged proteins or mark them for proteolytic degradation. Their concentration then increases in the cell and they thus bind free HSFs again, which leads to the switching off of the production of further HSPs.

3. The way in which housekeeping genes are switched off is also at least basically understood at the molecular level. Heat prevents the association of the subunits of the spliceosome and thus the processing of mRNA. The unspliced mRNA of the housekeeping genes is stored in conjunction with HSPs in heat shock granules. Translation is also switched off by inactivation of an initiation factor. Heat shock proteins evidently do not require this initiation factor and their mRNA does not contain introns, so that it does not need to be spliced.

3. Cold

1. With respect to the stressor cold, a distinction is made between damage arising at temperatures above the freezing point (damage through chilling in subtropical and tropical plants) and damage occurring upon frost and the subsequent formation of ice in the plant. Ice formation results in freeze-desiccation of tissues and cells, and frost damage is often damage due to dehydration. The freezing of water within the cell (intracellular ice formation) is always lethal (the exception is the artificial "vitrification" employed to conserve organisms).
2. Cold damage is primarily damage to membranes. This applies to chilling as well as to frost. If the proportion of high melting point lipids or the protein/lipid ratio is too high, large membrane domains are immobilised and their functions are lost when the temperature is lowered. Membrane damage is also caused by severe freeze-desiccation and the resulting accumulation of charged solutes (e.g. salt ions) at membrane surfaces.
3. Tropical and subtropical crop plants usually cannot harden to cold, or only to a very small extent. Metabolic imbalances and disturbances of cellular metabolism occur due to the different temperature tolerances of various metabolic reactions, even at temperatures not yet low enough to account for membrane damage. The inactivation of ion pumps, particularly of those associated with the energy supply or the homeostasis of membrane potentials, is the major cause of the damage referred to as being due to membrane "leakiness".
4. Energisation of the photosynthetic apparatus when photosynthetic metabolism is disturbed leads to the formation of reactive oxygen species (ROS) which cause further damage, particularly through the formation of radicals in the biomembranes. This then exceeds the capacity of the plant's well-developed detoxication system to intercept the ROS and the reactive products which they subsequently form. The photoinhibition observed during the over-energisation of photosystem II, particularly in connection with chilling, is mainly triggered by ROS. ROS are not formed only in the context of photosynthesis, but also during dysfunction of the respiratory chain and in other reactions in which molecular oxygen participates. ROS detoxification systems occur, on the one hand, in membranes, e.g. as evidenced by tocopherol and xanthophylls in the photosynthetic membranes. In the cytosol or in the stroma of the chloroplasts, ascorbate and glutathione are the main compounds involved in the detoxification of ROS, but flavonoids also play a role.
5. Frost causes stress not only because of the low temperatures involved, but additionally due to (freeze-)desiccation. Therefore, the exact cause of the incurred damage cannot always be determined unequivocally. Freezing of tissue and cell water must always occur outside the cells in the intercellular spaces. Water must thus exit from the cells into the intercellular spaces prior to freezing. Cold inactivation of pumps in the plasma membrane could be a reason for this phenomenon. Ice formation in the intercellular spaces requires a nucleation trigger. Bacteria are often found on the epidermal surface of the plant, but also in the substomatal intercellular spaces, which act as nucleation triggers.
6. Bacteria which are active in nucleation, the so-called INA bacteria, possess "nucleators" consisting of aggregates of INPs (ice-nucleating proteins), whose molecular construction favours the fixation of water clusters into ice matrix-like structures and thus reduces the supercooling required for crystallisation. Nucleation-active proteins have also been ex-

tracted from the intercellular spaces of plant tissues, but their molecular effect has not yet been clarified. Ice formation, which takes place at relatively mild freezing temperatures, reduces the danger of the sudden intercellular freezing of cell water which takes place when supercooling has progressed too far. On the other hand, there are many plants that are frost-tolerant but not tolerant to freezing. Avoidance of nucleation is the only means of surviving frost for these plants (which include many crop plants).

7. The desiccation of cells stemming from the freezing of cellular water is enormous even upon moderate frost. As a rule, more than 80% of tissue water is already frozen at temperatures of about −10 °C. Freezing ceases when the water potential of the (extracellular) ice, which depends on the (freezing) temperature, equals the water potential of the cell. This is called "equilibrium freezing", whereby ideal and non-ideal equilibrium freezing are distinguished. In ideal equilibrium freezing, the water potential of the freeze-desiccated cell is determined solely by its osmotic potential. In non-ideal equilibrium freezing, a negative wall pressure (suction) which arises during the desiccation of the cell augments the water potential of the cell, so that more water remains in the liquid state than would be expected from the osmotic potential. The plasma membrane must always remain in contact with the cell wall in an intact cell during freeze-desiccation, as the matrix potential of the cell wall prevents the penetration of air from the intercellular spaces. Shrinkage of the cell volume caused by freeze-desiccation therefore leads to (reversible) deformation of the whole cell and even to wrinkling of the cell wall (freezing cytorrhysis).
8. Extreme freeze-desiccation leads to the breakdown of biomembranes, as the ordering of hydrophobic interactions between membrane lipids requires the presence of liquid water. Membrane breakdown is further stimulated by high concentrations of charged solutes, which can drastically alter the membrane potential.
9. The degree of frost hardening depends not only on the composition of the lipids (and the protein/lipid ratio) of the biomembranes, but also on the effectiveness of so-called cryoprotectants. These are low molecular weight solutes such as many carbohydrates, which take over the membrane-stabilising role of water molecules during freeze-desiccation, and at the same time prevent the accumulation of ions at the membrane surfaces. In addition to low molecular weight cryoprotectants, frost-hardy plants also synthesise proteins which protect membranes and other proteins. These are, for example, proteins from the family of the dehydrins, which associate protectively with membranes during dehydration. The formation of such proteins is a good example of cross-protection, i.e. of a multiple protective system, the formation of which is triggered by one particular stressor but then also hardens the plant against other stressors (e.g. frost, drought and high salt concentrations). The synthesis of such proteins may also be induced by the plant hormone abscisic acid.
10. The phenomenon of cross-protection suggests the existence of a molecular "master switch". Indeed, in addition to the presence of plant hormones, there appears to be a higher hierarchical level of genes, the products of which activate a whole series of further genes. The products of these lower-level genes contribute to stress management either directly or indirectly (e.g. via the synthesis of low molecular weight osmolytes – here cryoprotective solutes). Such a system is called a "regulon". Finding such master-switch genes would be of particular interest with regard to increasing the stress tolerance of plants by genetic transformation. It seems more feasible at present, however, to transform plants by introducing several genes from the lower hierarchical level into a crop plant (Holmberg and Bülow 1998). Candidate genes for transforming plants with a master switch would be, for example, those encoding transcription activators such as DREB 1A, B, C and others (see Chap. 1.6: "proline"). These genes are high up in the cascade hierarchy for drought tolerance (Sarhan and Danyluk 1998).
11. So-called antifreeze proteins are well known in the animal kingdom, and have recently also been found in plants. They impede crystallisation of water and hinder the growth of ice crystals, thus delaying freezing, but not thawing. They are, therefore, also called THPs (thermal hysteresis proteins). The molecular structures and mechanisms of action of some of these proteins are known. They attach to ice crystals and turn their hydrophobic side towards the aqueous medium, thus making it difficult

for other water molecules to associate with the ice. The formation of many, small ice crystals instead of fewer large ones is the consequence.

12. Perennial plants from moderate and higher latitudes exhibit the phenomenon of seasonal frost hardening and dehardening. Frost hardening is accompanied at the molecular level by desaturation of membrane lipids, reduction of the protein/lipid ratios in biomembranes, and the synthesis of cryoprotective and ROS-detoxifying compounds and possibly also of proteins which promote or hinder ice nucleation. Plants have only a low photosynthetic capacity in the frost-hardened state. This is predominantly due to the degradation of the photosystems in the thylakoid membranes, but it also significantly reduces the danger of over-energisation in the frozen state. Arctic and tropical high mountain plants are permanently frost-hardy. The latter have developed various mechanisms to avoid freezing in accordance with the daily-frost climate, such as insulation and heat storage which can be of a permanent (cloaking of the stem) or transient (night buds) nature. Such mechanisms only work, however, because the nightly frost periods last, at most, for only a few hours before the tropical sun raises the temperature above the freezing point again.
13. Frost drought comprises a stress situation for the entire plant, for which there is no avoidance response except leaf shedding and also no specific tolerance. Frost drought results from the transpiration of above-ground plant parts when water cannot be taken up from the frozen soil. Frost-hardened plant organs can survive this situation only as long as the mechanisms for damage avoidance are not overtaxed.

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1.4

Oxygen Deficiency (Anaerobiosis and Hypoxia)

Nelumbo nucifera, the lotus flower, is a typical swamp plant in the monsoon climate of northern Australia. The rhizomes grow in the oxygen-deficient mud and are supplied with oxygen via an aerenchyma. The flow of air in the aerenchyma is driven by thermo-osmosis. Because of crocodiles, researchers in this area should exercise caution. Kapalgam Northern Territories. Photo E.-D. Schulze

Recommended Literature

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- Chang C, Bleeker AB (2004) Ethylene Biology. More than a Gas. Plant Physiol 136:2895–2899 (and subsequent articles in this issue)
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Common soils consist of **four components**: soil particles, water, air and organisms including plant roots (Fig. 1.4.1). Freely draining soils can only retain water in pores with diameters smaller than 10–60 µm. Even at water saturation up to the field capacity (see Chap. 2.2) the air-filled pore volume is 10–30% of the total soil volume. However, in partially or permanently waterlogged soils, there are almost no air-filled pores, as the air dissolves in the water.

Gas exchange in well-aerated soils is mainly through diffusion in the continuum of the air-filled pores, but is accelerated by a number of active processes in the soil and thus becomes a relatively fast process. For example, if oxygen is consumed by the respiratory activity of microorganisms and plant roots, oxygen from the atmosphere flows quickly into the soil following the concentration gradient. As a result, the partial pressure of O_2 in the soil air, at least in pore-rich soils, remains in the range of 15–20%. Similarly, CO_2 that accumulates in the soil pores quickly leaks out from the soil. The situation is completely different when gas exchange is via the water-filled pores of waterlogged soils. **Fick's first law of diffusion** describes the amount of gas diffusing per unit of time, i.e. the net gas flux, as depending on the diffusion coefficient D, on the size of the exchange area, and on the concentration gradient. At the same temperature, the diffusion coefficient of oxygen in water is about 10,000 times (exactly 11,300 times) smaller than in air. Oxygen has also a very low solubility in water (0.03 ml O_2 l^{-1} H_2O). Thus, gas exchange in waterlogged soils is very slow and oxygen becomes one of the limiting factors for growth and the development of plants. Oxygen supply to roots is enhanced by a temperature gradient as well as by water flow in the soil. For roots, the critical oxygen concentration is generally 5–10%, although some plants are able to grow at lower O_2 concentrations, e.g. cotton grass (*Eriophorum angustifolium*) roots grow at 2–4% O_2 (Armstrong and Gaynard 1976). The apparent tolerance of these plants is easily explained by the large intracellular channels extending from the

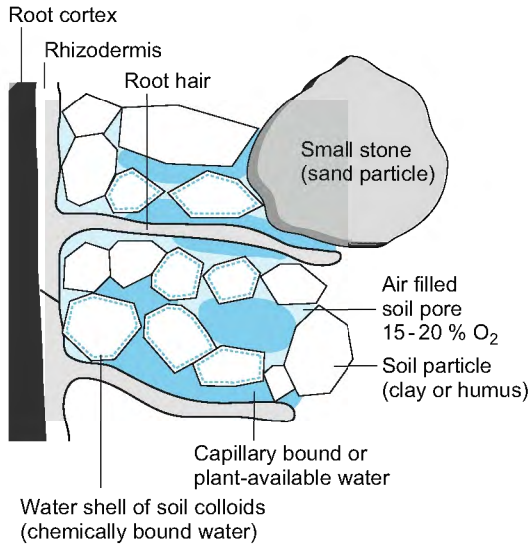


Fig. 1.4.1. Four-component system: Root/soil organism, soil particle, soil water (solution), soil air

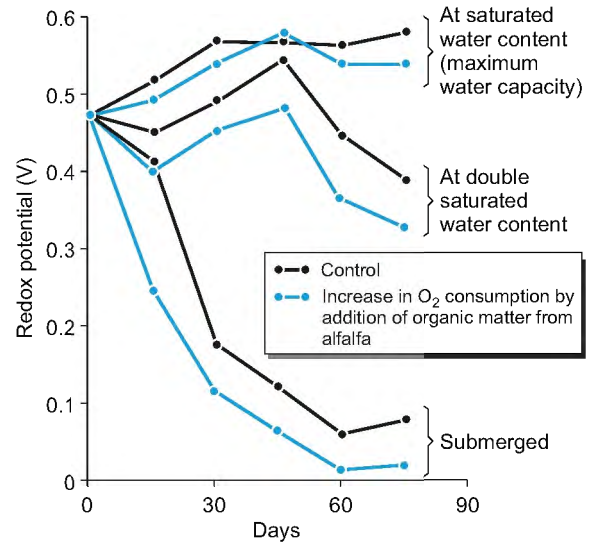


Fig. 1.4.2. Development of redox potential of a loamy clay soil as influenced by the water content and the amount of organic matter. (After Amberger 1988)

shoot and leaves into the roots maintaining a sufficiently high oxygen concentration in the root tissue. A similar **aeration tissue** (termed **aerenchyma**) is found in deep-water rice, the roots of which are able to grow at 0.8% oxygen (Armstrong and Webb 1985). In such cases, a corky exodermis is often produced, forming a gas-tight outer cell layer of the root that renders the escape of gases from the interior of the root into the soil very difficult. If such a diffusion barrier tissue is missing, oxygen leaks out of the aerenchyma to the surrounding soil, where the heavy metal ions in the immediate proximity of roots are oxidised forming "rusty spots and root channels" in pseudogley and are, thus, "detoxified" for soil organisms.

Regarding the relationship between oxygen concentration and metabolism, a situation, where biochemical reactions are not limited by partial oxygen pressure, is called **normoxia**. If mitochondrial ATP synthesis is affected, but not completely inhibited by low O_2, it operates under **hypoxia**. In the absence of oxygen (**anoxia**), oxidative phosphorylation in the mitochondria is negligible, compared with ATP synthesis by glycolysis and fermentation.

Long-term waterlogged soils have a negative redox potential because of the low oxygen partial pressure (see Box 1.7.1), i.e. they exhibit reducing properties. Oxygen entering such soils (e.g. through root or earth worm channels) is readily consumed by soil organisms.

The redox potential of soils decreases dramatically already after a few days of flooding (Fig. 1.4.2) and microaerophilic and anaerobic microorganisms start to grow. They mainly live on the organic matter of the soil as energy source, but require ions as electron acceptors that can be reduced. If nitrate is used as electron acceptor, giving rise to nitrite, N_2O and finally N_2 (denitrification), the process is termed nitrate respiration and, accordingly, in sulfate respiration sulfide is formed from SO_4^{2-} (see Chap. 3.3.3). Similarly, three-valent iron and four-valent manganese can be reduced to two-valent ions. In addition, CO_2 may be used as electron acceptor, resulting in the production of methane. Table 1.4.1 shows the sequence of redox reactions occurring in the soil when the redox potential decreases. Such reactions often consume protons, i.e. result in an alkalinisation of the soil.

However, reduced heavy metal ions are toxic. Thus, the growth of roots is not only inhibited by the lack of oxygen, but also by toxic ions in the vicinity of roots (Fig. 1.4.3). This applies particularly to the very sensitive symbiosis of plant roots with mycorrhizal fungi: Plants growing on waterlogged soils are very sensitive to pathogens and rarely form mycorrhizae; therefore, their capability of nutrient acquisition and growth is usually very limited (Table 1.4.2).

Two-thirds of the earth's land mass is flooded, at least occasionally (e.g. the monsoon regions of Southeast Asia or the areas at the lower

Table 1.4.1. Sequence of soil-bound redox reactions. The redox potential provides important information about the reactions in the soil, as these reactions take place in the sequence listed [i.e. sulfate is not reduced if iron(III) ions are still present]. (After Marschner 1986)

Redox reaction		Redox potential E (mV) at pH 7
Start of nitrate reduction (denitrification)	$NO_3^- \rightarrow NO_2^-$	450–550
Start of manganese reduction	$MnO_2 + 4H^+ + 2e^- \rightarrow Mn^{2+} + 2H_2O$	350–450
Absence of free oxygen	$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$	350
Absence of nitrate	$(\rightarrow N_2O \rightarrow N_2)$	250
Start of Fe^{2+} formation	$Fe(OH)_3 + 3H^+ + 1e^- \rightarrow Fe^{2+} + 3H_2O$	150
Fe^{3+} completely consumed		120
Start of sulfate reduction	$SO_4^{2-} + 10H^+ + 8e^- \rightarrow H_2S + 4H_2O$	–50
Sulfate completely consumed		–180
Methane formation	$CO_2 + 8H^+ + 8e^- \rightarrow CH_4 + 2H_2O$	<–180

Table 1.4.2. Effect of flooding on biomass production, and on nutrient content of leaves, of two flooding-sensitive and one flooding-tolerant representatives of the genus *Rumex*. (After Laan et al. 1989 a, b)

Species/conditions for growth	Dry weight of shoot (g)	Nutrient content of leaves (mol per g dry weight)				
		Nitrogen	Phosphorus	Sodium	Calcium	Magnesium
R. thyrsiflorus						
Dry site	15.2±1.4	1878±45	143±10	1238±37	323±11	435±22
Flooded	7.2±1.4	1006±64	44±2	355±15	190±2	218±3
R. crispus						
Dry site	13.6±1.4	1372±54	90±5	857±39	532±27	311±7
Flooded	12.7±2.5	702±35	58±10	347±23	315±13	160±8
R. maritimus						
Dry site	24.8±2.6	1018±91	59±2	478±11	615±19	401±4
Flooded	25.4±3.9	1052±52	59±2	272±10	761±40	398±11

Data are average values of five identical experiments ±SD

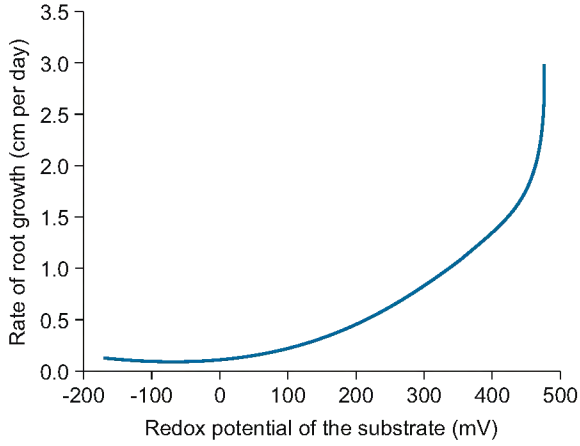


Fig. 1.4.3. Dependence of root growth of the grass *Spartina patens* on the redox potential of the soil. (After DeLaune et al. 1993)

reaches of the large Siberian rivers). Many higher plants, therefore, have developed mechanisms to survive hypoxia, the major stress factor. The frequency of flooding, and the tolerance of certain species, can be extrapolated from the zonation of the riparian vegetation.

Physiologically, **primary** and **secondary hypoxia** or anaerobiosis should be differentiated. In primary hypoxia, germination of a plant already takes place in an oxygen-deficient environment, which does not change during the whole lifetime of the plant. This applies, for example, to obligate marsh plants (Chap. 1.4.1.1). Secondary hypoxia occurs when plants normally growing in well-aerated soils are temporarily flooded.

1.4.1 Energy Metabolism of Plants Under Oxygen Deficiency

The daily oxygen demands of soils during the growth period of plants are in the range of 10–20 l/m^2, depending on the density of the roots and the activity of soil microbes. The minimum

Box 1.4.1 Energy metabolism of heterotrophic organs in normoxia and hypoxia

Energy charge of cells is usually determined by the degree of phosphorylation of the adenylate system (adenylate energy charge, AEC, often called EC).

The following formula is applied:

$$(A)EC = \frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]}$$

By definition, maximum EC equals 1. Since ADP possesses only one energy-rich phosphate bond, its concentration has to be multiplied by the factor 0.5. A cell supplied with sufficient oxygen has an AEC between 0.8 and 0.95. Under anaerobic conditions the energy charge may drop to 0.2 (see Fig. 1.4.4). Acute ATP deficiency leads to the breakdown of compartmentation as the energy-driven ion pumps are no longer sufficiently energised.

oxygen partial pressure in the soil for the growth of flooding-sensitive plants is at 2–3% (about 5 kPa). Inhibition of growth under hypoxic conditions is a multifactorial phenomenon, which is basically caused by the very low efficiency of the energy metabolism. During inhibition of mitochondrial respiration, many heterotrophic organisms and plant tissues are able to switch to fermentative metabolism. This type of metabolism, however, requires increased throughput of energy carriers such as glucose. Under these conditions, reserve material is quickly consumed and poisonous or stressful end products, such as ethanol or lactic acid, accumulate. Despite stimulation of glycolysis and fermentation by the so-called **Pasteur effect**, the **energy charge** of cells remains low so that during extended periods of hypoxia, or even short-term anaerobiosis, values of below 0.5 result (see Box 1.4.1). These values are too low for anabolic metabolism, i.e. for growth. Inhibition of phloem transport and phloem unloading is another consequence of the low-energy charge of the plant tissues. Thus cells are depleted not only because of the faster turnover of storage material, but also because of the shortage of stock. **Helophytes** (swamp plants) avoid the problems caused by hypoxia by the development of an **aerenchyma**.

1.4.1.1 Primary Hypoxia: Germination Under Hypoxic Conditions

Dry cereal seeds contain carbohydrates, mainly starch; to germinate they require catabolising enzymes: α- and β-amylase, amylopectin-debranching enzymes and α-glucosidases (maltase, diastase). Neither wheat nor barley seeds are able to germinate under anaerobic conditions, but rice can (Fig. 1.4.4). In the rice grain, starch debranching enzymes and α-glucosidases are present as inactive precursors, which are activated during germination, even without oxygen. Upon germination in the absence of oxygen, α- and β-amylases are synthesised de novo (Guglielminetti et al. 1995). This happens during the first 2 days of germination when the soluble carbohydrates already present serve as the energy source. After this, starch-catabolising enzymes become active and starch is hydrolytically degraded and the degradation products become available for further metabolism, predominantly as glucose-6-P and fructose-6-P.

1.4.1.2 Changes in Metabolism Caused by Hypoxia

Energy Metabolism Under Hypoxia and Anoxia

In addition to the energy deficit caused by hypoxia and anaerobiosis, toxic metabolic products accumulate, e.g. ethanol (see Box 1.4.1). Higher concentrations of this poisonous compound destroy the selective permeability of membranes and prevent formation of proton gradients and in turn the gain of energy. On the other hand, ethanol easily permeates through biomembranes and cell walls and thus only rarely reaches damaging concentrations of 50–100 nM in the cell. Acetaldehyde, the biochemical precursor of ethanol, is much more poisonous than ethanol, but is usually immediately reduced. It only accumulates when alcohol dehydrogenase is inhibited or

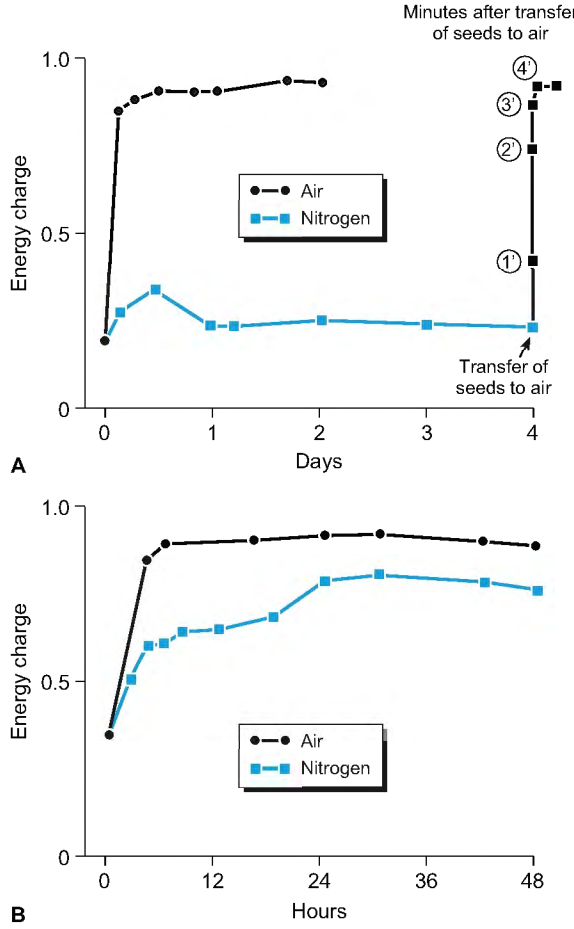


Fig. 1.4.4. The energy charge of lettuce seeds (A) or rice grains (B) during germination in air or under nitrogen. (After Pradet et al. 1985)

switched off by mutation or genetic modification. Acidification of the cytosol by lactic acid is another metabolically detrimental effect of hypoxia. When oxygen deficiency sets in, **lactate dehydrogenase** (LDH) is the first enzyme to be activated and the pH in the cytosol decreases as lactic acid forms and accumulates. As the pH optimum of LDH is in the neutral range, it inhibits itself upon acidification of the cytosol. **Pyruvate decarboxylase** is less susceptible to acidity and therefore takes over, producing acetaldehyde and, together with alcohol dehydrogenase, ethanol.

Biochemical Adaptation to Oxygen Deficiency

Besides anatomical-morphological strain reactions to the shortage of oxygen (as explained later), biochemical adaptation can take place: In addition to lactate, rice seedlings and sweet flag (*Acorus calamus*) rhizomes mainly produce basic amino acids [asparagine, arginine, γ-amino butyric acid (GABA)]. Synthesis of GABA from glutamate releases CO_2 and consumes protons, and is a reliable marker for metabolism under hypoxia:

$$^{-}OOC - CH_2 - CH_2 - CHNH_2 - COO^{-} + H^{+} \rightarrow {}^{-}OOC - CH_2 - CH_2 - CH_2NH_2 + CO_2$$

1.4.1.3 "Anaerobic Polypeptides"

Fine root systems and root meristems are particularly sensitive to oxygen deficiency. In species not tolerant to flooding, those parts of the root system used for water and ion uptake die off at oxygen partial pressures below 0.5–5 kPa and the plant becomes drought stressed, even if standing in water. Drought stress is indicated by an increased production of ABA that is distributed via the xylem sap, and leads to stomatal closure. As a consequence, the rate of photosynthesis decreases as well as growth and the plants finally become stunted while their leaves show strong **epinasty** (downward bending of the leaves and petioles because of increased growth of the upper side). Such phenomena are often observed in indoor plants which are watered too much, where hypoxia in the water-saturated soil leads to death of the root system and withering of the shoot.

In flooding-tolerant plants hypoxia triggers a change in gene expression. Synthesis of normal housekeeping proteins (see Chap. 1.3.4.2) attenuates and synthesis of about 20 or more so-called **anaerobic polypeptides (ANPs)** or **anaerobic stress proteins (ASPs)** is induced (Fig. 1.4.5). These are predominantly fermentative enzymes: **Alcohol dehydrogenase**, pyruvate decarboxylase, lactate dehydrogenase, glyceraldehydephosphate dehydrogenase, aldolase, glucose-6-P-isomerase, alanine aminotransferase and enolase have been identified. The promoters of the ANPs contain a consensus sequence, the so-called **anaerobic response element** (ARE; Christopher and Good 1996).

The trigger and signal chain leading to the induction of ANPs are still unknown. The formation of ANPs is suppressed above O_2 concentrations of 2–5%. In anoxia-tolerant plants mainly ethanol is formed due to the kind of ANPs which promote glycolysis and fermenta-

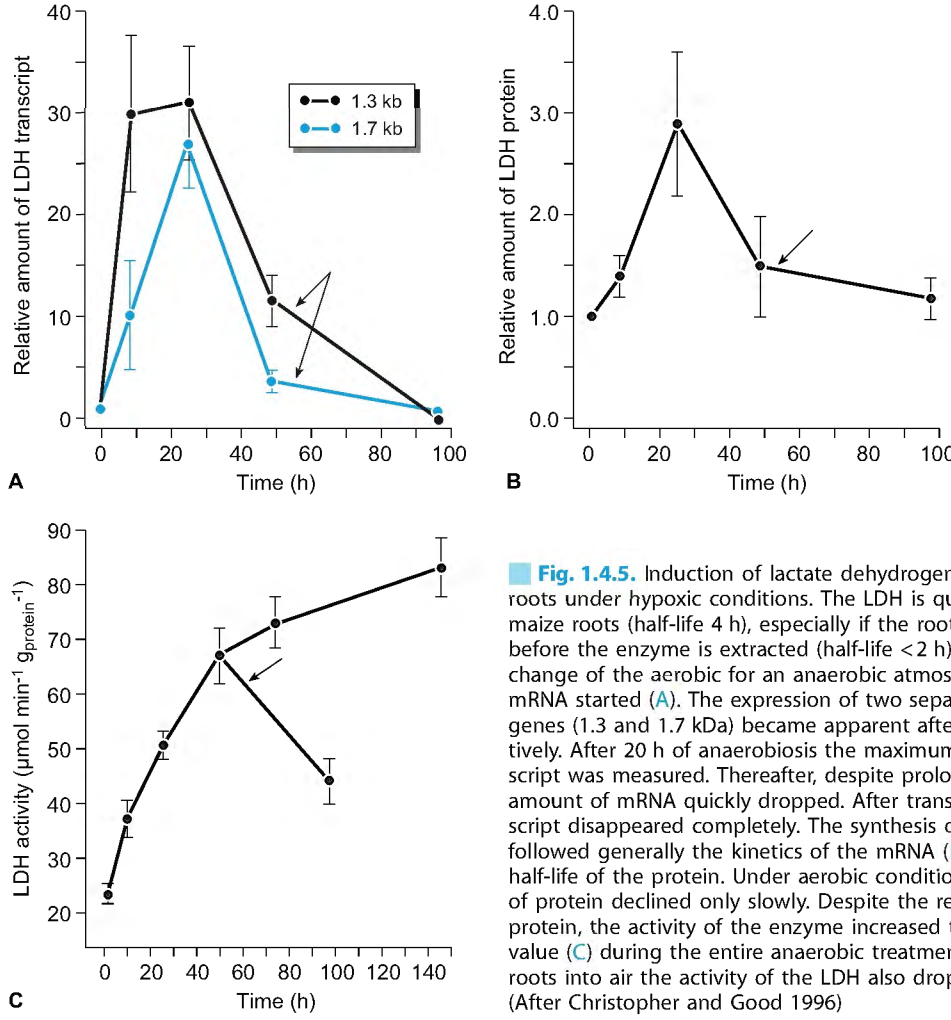


Fig. 1.4.5. Induction of lactate dehydrogenase (LDH)-ANP in maize roots under hypoxic conditions. The LDH is quickly broken down in maize roots (half-life 4 h), especially if the roots are kept in nitrogen before the enzyme is extracted (half-life <2 h). Immediately after the change of the aerobic for an anaerobic atmosphere, synthesis of LDH mRNA started (A). The expression of two separately regulated LDH genes (1.3 and 1.7 kDa) became apparent after 8 and 24 h, respectively. After 20 h of anaerobiosis the maximum amount of LDH transcript was measured. Thereafter, despite prolonged anaerobiosis, the amount of mRNA quickly dropped. After transfer into air the transcript disappeared completely. The synthesis of the LDH-ANP protein followed generally the kinetics of the mRNA (B), due to the short half-life of the protein. Under aerobic conditions (*arrow*) the amount of protein declined only slowly. Despite the reduction of the LDH protein, the activity of the enzyme increased to 3.5 times its original value (C) during the entire anaerobic treatment. Upon transfer of the roots into air the activity of the LDH also dropped drastically (*arrow*). (After Christopher and Good 1996)

tion and the energy charge of the tissue is kept high, thus maintaining gross metabolic activities.

Longer phases of anoxia (e.g. during winter) may be bridged in rhizomes and roots by periods of suspended metabolism, called **anaerobic retreat**. In spring, elongation growth starts again, but how this happens is still unknown.

Differences between anoxia-tolerant and anoxia-sensitive plants are gradual: There are neither absolutely tolerant nor absolutely intolerant species. Membrane lipids appear to play a crucial role: It appears to be important how fast membrane lipids are damaged and can subsequently be replaced. In intolerant species membrane lipids are damaged readily, while those of the tolerant species are more persistent (Fig. 1.4.6). Oxygen-dependent desaturation of lipids appears to be inhibited. In anoxia-sensitive plants, a higher proportion of saturated membrane lipids was found, in contrast to tolerant species where a higher degree of desaturation can be maintained for a longer period. Membranes of mitochondria and of the endoplasmic reticulum are particularly endangered. In pea seeds, mitochondria are destroyed after only 3–4 days of anoxia, whereas biomembranes of tolerant species remain functional for a long period.

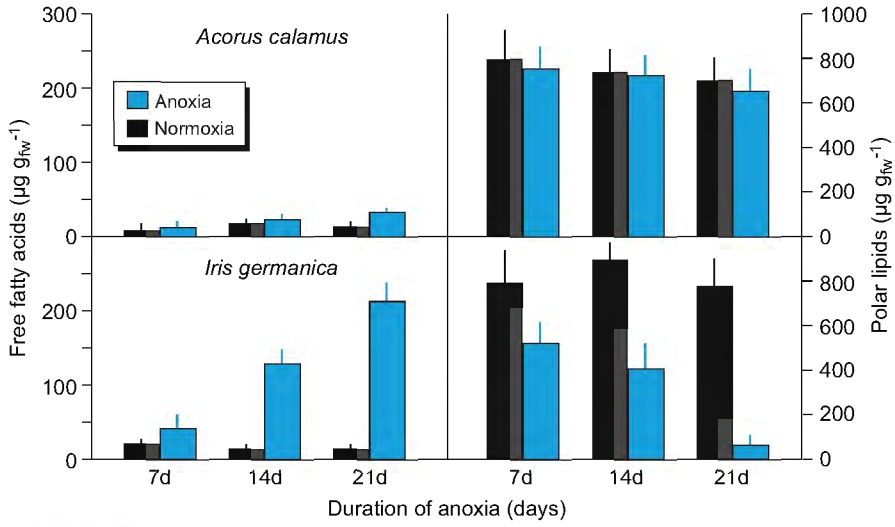


Fig. 1.4.6. Lipid degradation in rhizome tissue of iris which is sensitive to anoxia, and sweet flag (*Acorus*) which is insensitive. (After Jackson and Black 1993)

1.4.2

Anatomical-Morphological Changes Caused by Hypoxia

1.4.2.1 Elongation of Internodes

As with other stresses, there are also obvious avoidance reactions induced by hypoxia and anaerobiosis. Submerged plants usually produce strikingly long internodes. By such elongation growth, the apical parts of the shoots quickly reach the surface of the water and thus escape the hypoxic environment. This reaction has been particularly well studied in deep-water rice, where the submerged shoots elongate up to 25 cm/day.

A marked increase in the intercellular ethylene concentration is regarded as the phytohormonal signal which stimulates elongation growth. However, the real initial signal is the low oxygen partial pressure in the submerged internode, leading to increased expression of the ACC-oxidase gene (ACC: aminocyclopropane carboxylic acid) and finally to increased ethylene synthesis (Box 1.4.2). The ACC produced by the hypoxic roots is transported by the xylem stream into the better aerated shoot since the ACC-oxidase requires molecular oxygen as substrate. The increased ethylene partial pressure triggers the decrease in the endogenous ABA level and thus in turn increases the effectiveness of the elongation hormone gibberellic acid. Thus, at least four phytohormones participate in the elongation growth of shoots and petioles triggered by hypoxia: ethylene, ABA, auxin and gibberellin.

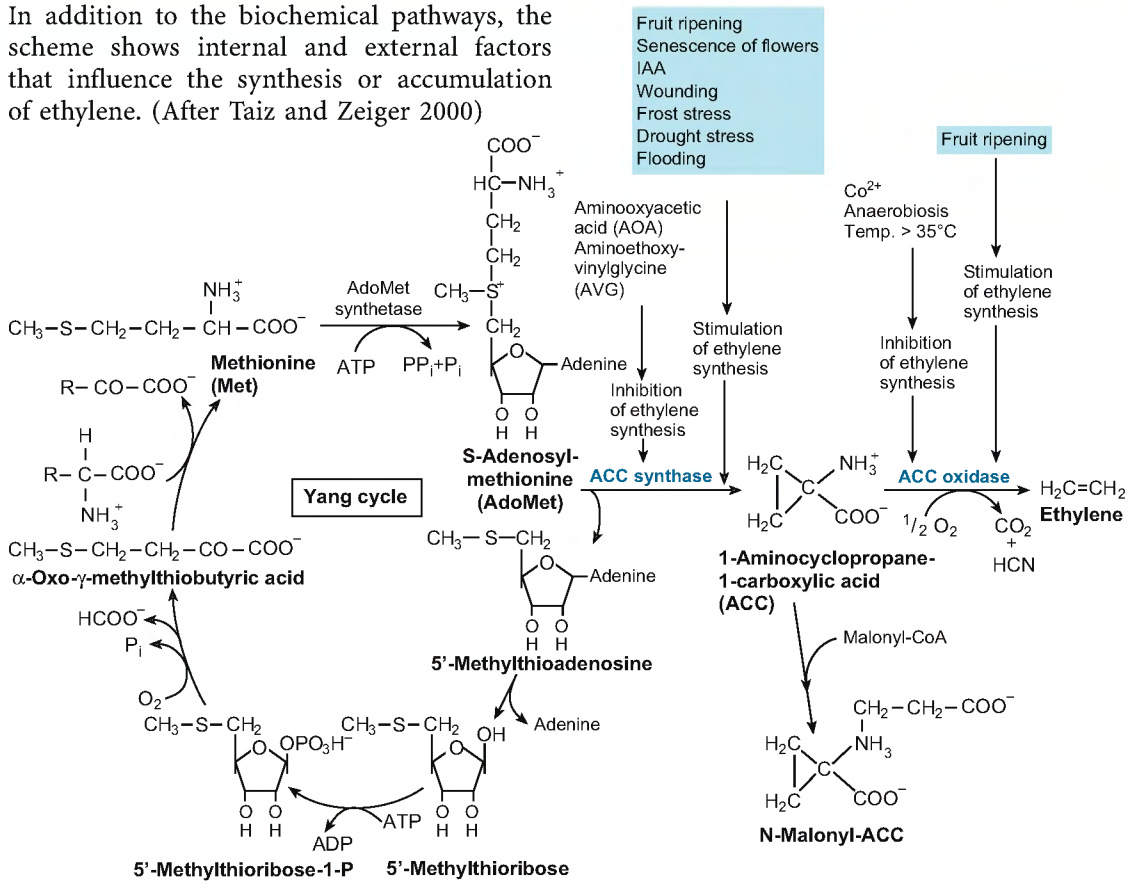
Expansins are cell wall proteins with a molecular mass of 24.5 kDa (Box 1.4.3). In the shoots of deep-water rice, two such proteins occur in the intercalary meristem and in the adjacent extension zone (but not in the differentiation zone) mainly around the vascular bundles and in the tissue lining the inner epidermis, i.e. towards the pith channel (Cho and Kende 1997a, b).

Submerged internodes accumulate considerably more expansins than those in air, and substantially expand their elongation zone. The molecular mechanism of expansins is still not well understood; it is assumed that they loosen the hydrogen bridges between the hemicellulose [(1 → 3),(1 → 4)-β-D-glucans)] and the paracrystalline segments of the cellulose microfibrils and thus increase the extensibility of the cell wall. Although this effect suggests a catalytic mechanism, expansins do not show any hydrolase activity.

Auxin also plays a role in shoot expansion: It stimulates acidification of the cell wall, probably by activating the ATP-dependent proton pumps in the plasma membrane. The effect of expansins can be mimicked by acidification of the cell wall. When rice grains were germinated under aerobic and anaerobic conditions, respectively, a

Box 1.4.2 Ethylene biosynthesis from S-adenosylmethionine (AdoMet) and formation of the precursor

In addition to the biochemical pathways, the scheme shows internal and external factors that influence the synthesis or accumulation of ethylene. (After Taiz and Zeiger 2000)

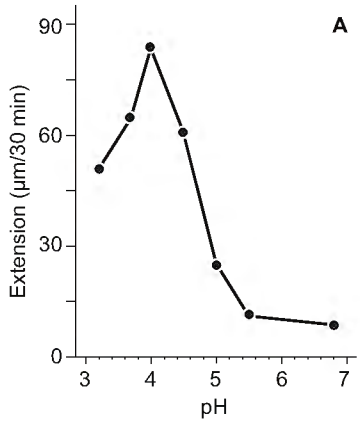


fast breakdown of auxin took place in air, but not under anaerobiosis (Mapelli and Bertani 1993; Mekhedov and Kende 1996). Persistence of auxin contributes to a prolonged elongation growth of the shoot.

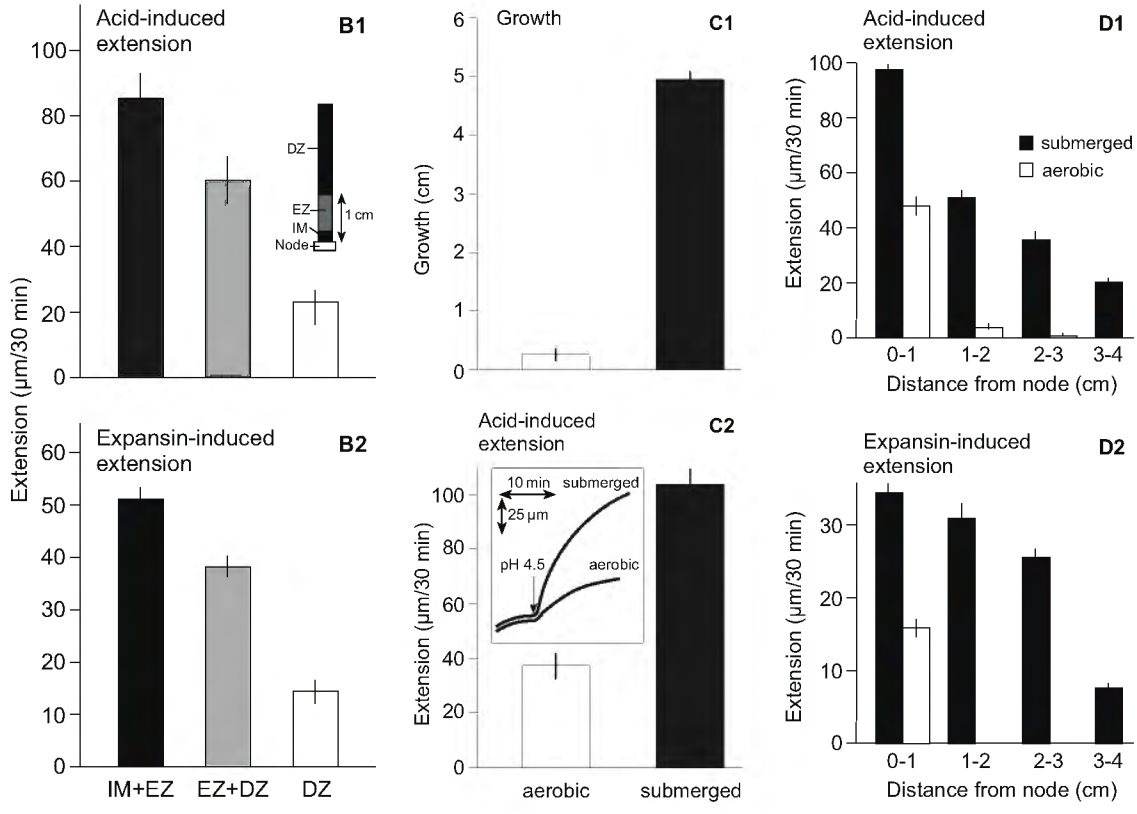
1.4.2.2 Formation of Aerenchyma

Under hypoxic conditions ethylene formation is increased, and it accumulates in and around roots and submerged shoots because of its low solubility in water. Concentrations of 0.1–0.5 ppm are sufficient to induce formation of intercellular space-rich tissues by **programmed cell death (PCD)** or **apoptosis**. PCD does not take place in differentiated older cells, rather an aerenchyma is initiated already at the end of the elongation zone of the organ. Formation of aerenchyma is lytic, e.g. in maize, *Luronium*, and *Nymphoides*, but usually schizogenous in petioles, e.g. of *Caltha*, *Rumex*, or *Filipendula*. Formation of aerenchyma is not restricted to helophytes and submerged plants; even terrestrial plants, such as maize and sunflower, may develop aerenchyma in roots and the basal part of the shoot. In many helophytes (such as rice and arrowhead), the formation of aerenchyma is genetically fixed (constitutive), and the induction of PCD is not dependent on oxygen deficiency or ethylene accumulation. Aerenchyma formation caused by hypoxia commonly results in irregular air spaces, whereas those constitutively developed show regular patterns of air channels. Aerenchyma allows air circulation in tissues, additionally supported by pressure ventilation (misleadingly often termed "thermo-osmosis",

Box 1.4.3 Expansin(s) and internode extension in deep-water rice



A shows the dependence of the extensibility of isolated cell walls on the pH. Cell wall preparations were incubated in media of different pH and subsequently subjected to tension; the stretching rate is shown. This technology was also used in the experiments presented in B–D. In B, the extensibility of different sections of the internode upon acid-induced growth (B1) and after addition of a protein extracted from cell walls ("expansin" preparation) (B2) is shown. *IM* Intercalary meristem; *EZ* elongation zone; *DZ* differentiation zone. C shows the effect of submergence on the elongation growth of the internode (C1) and the acid-induced extension of shoots which have grown aerobically and submerged (C2); the *insert* shows the plot of the recordings. D shows the influence of submergence on the length of the elongation zone. Shoots grown submerged have a considerably longer elongation zone than those grown in air. (After Cho and Kende 1997a, b).



Box 1.4.4 Thermo-osmosis

Table. Position of thermo-osmotically active layers and their average pore diameter. (After Grosse 1997)

Plant species	Tissue	Pore diameter (nm)
Nuphar lutea (yellow water lily)	Young leaf	700–1200
	Old leaf	>15000
Nelumbo nucifera (lotus)	(Air) leaf	30
Nymphoides peltatum (fringed water lily)	Young leaf	29
Alnus glutinosa (alder)	Cork cambium	14–113
	Cambium	>>100

Convectional air flow in intercellular spaces improves the gas exchange of plant tissues. This **thermo-osmosis**, in combination with aerenchyma formation, is often put forward as a mechanism to improve gas flow in tissues of helophytes, mangroves and plants of river basins. Thermo-osmosis includes several phenomena which are not dependent on biological activity but are of a purely physical nature. Easiest to understand is the efflux of gas from the tissue caused by evaporation of water from those cell surfaces bordering intercellular spaces; the more water evaporates, the greater is the partial pressure of the intercellular water vapour, but the volume of that gas efflux is very small. Gas fluxes arising from differences in temperature, e.g. between the leaves floating on the water surface and the rhizomes in the anoxic bottom of the pond, are more effective, as is also gas exchange caused by the differences in molecular mass of the gases involved. Such gas fluxes are called thermo-osmosis, as they require gas-filled pores or cavities, the dimensions of which are smaller than the mean free path of the gas molecules in Brownian motion (ca. 100 nm at atmospheric pressure and 20 °C). Therefore, not only plant tissues, but also ceramic filters or other porous materials with pore diameters of similar size to the intercellular spaces of plant material are used as **thermo-osmotic active layers** in studies (Table).

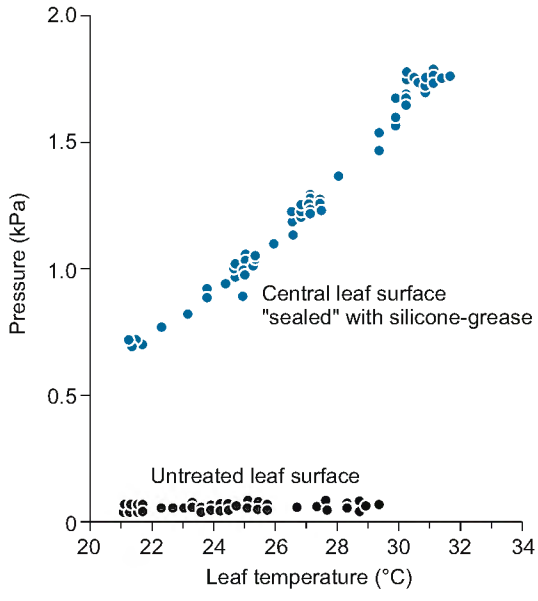


Fig. 1. The relationship between thermo-osmotically created pressure ("lacunar pressure") and temperature in a petiole of a young leaf of *Nelumbo nucifera*. An untreated leaf with open stomata was compared with a leaf in which the central portion of the surface (where the lacuna joins the petiole) was sealed with silicone. (After Dacey 1987)

Box 1.4.4 (continued)

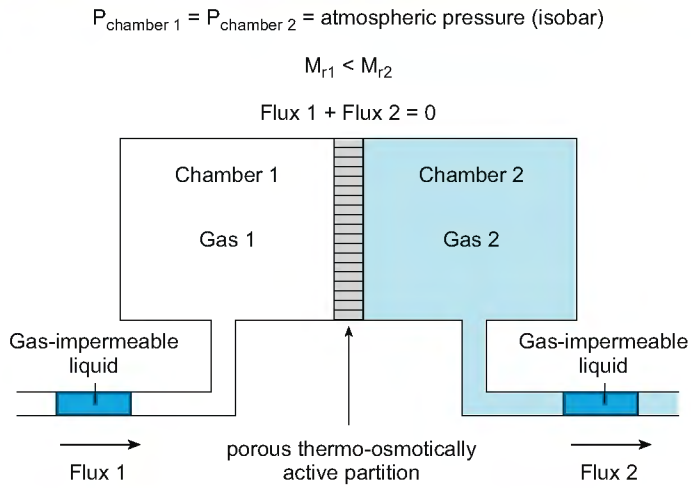


Fig. 2. Model of the isothermic formation of a thermo-osmotic gas flow in a porous layer. Gas flux originates from a concentration gradient of the gas on both surfaces of the porous layer. This gradient originates from the resistance to gas permeation of the thermo-osmotic layer whose pores are smaller than the mean free path length of the moving gas molecules. Gas 1, due to its smaller molecular mass, permeates through the layer easier than gas 2; therefore, there is a net gas flow from chamber 1 to chamber 2. The flow can be measured with the shift of liquid in the tubes. (After Schiwinsky et al. 1996)

Gas fluxes by thermo-osmosis are explained on the basis of two principles: A pressure difference because of a temperature gradient (when diffusion is limited, Fig. 1) and at the same pressure and temperature if the gases have different molecular masses (according to Graham's law, the relation of counter-fluxes of two gases through a thermo-osmotically active surface is inversely proportional to the square root of their molecular masses). Pressure always builds up if the mean free path of the gas molecules is longer than the available actual space and the gas molecules collide with the walls of the pores (Fig. 2). The major gas fluxes in a plant are those of nitrogen and oxygen; however, exchange of CO_2 and O_2, the molecular masses of which are substantially different (42 vs. 32), is physiologically more important.

The upper part of a lotus leaf consists of a very dense palisade parenchyma whose intercellular spaces are sufficiently narrow to create a thermo-osmotic pressure, whereas the diameters of the intercellular channels in the petiole and rhizome are much too wide to create such a pressure. It is assumed that the gas pressure produced especially in the young leaves pushes air predominantly into the rhizomes from which it is released into the atmosphere via the lacunae of the old leaves. Gas flow occurs also within an individual leaf from the margin to the centre where the intercellular lacunae are located. Depending on the pressure in the interior of the aerenchyma, this leaks out of the leaf or enters the petioles and subsequently the rhizomes. Likewise, respiratory CO_2 produced in the rhizomes can escape from the plant via the lacunar systems of the rhizomes and the leaves.

The extent of gas fluxes may be considerable. In leaves of *Nuphar luteum* the thermo-osmotically produced pressure of 0.2 kPa (see Fig. 1) creates a gas flow of 50 ml min^{-1} through the lacunar system of the petioles (Dacey 1981; see also Armstrong 1990 and Kohl et al. 1996).

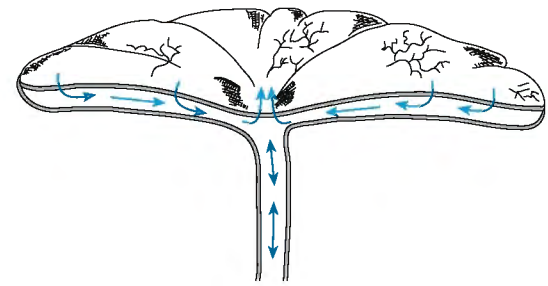


Fig. 3. Thermo-osmotic gas fluxes in an illuminated leaf of *Nelumbo nucifera*. Thermo-osmosis leads to a pressure resulting in an air flow towards the centre of the leaf. Much of that air exits from the leaf in its adaxial centre where the lacunae enter the petiole. Part of the gas can also flow into the rhizomes, following the pressure gradient. Conversely, air from the rhizome may also enter the atmosphere via the lacuna system of the petiole. (After Dacey 1987)

Box 1.4.5 The ethylene receptor

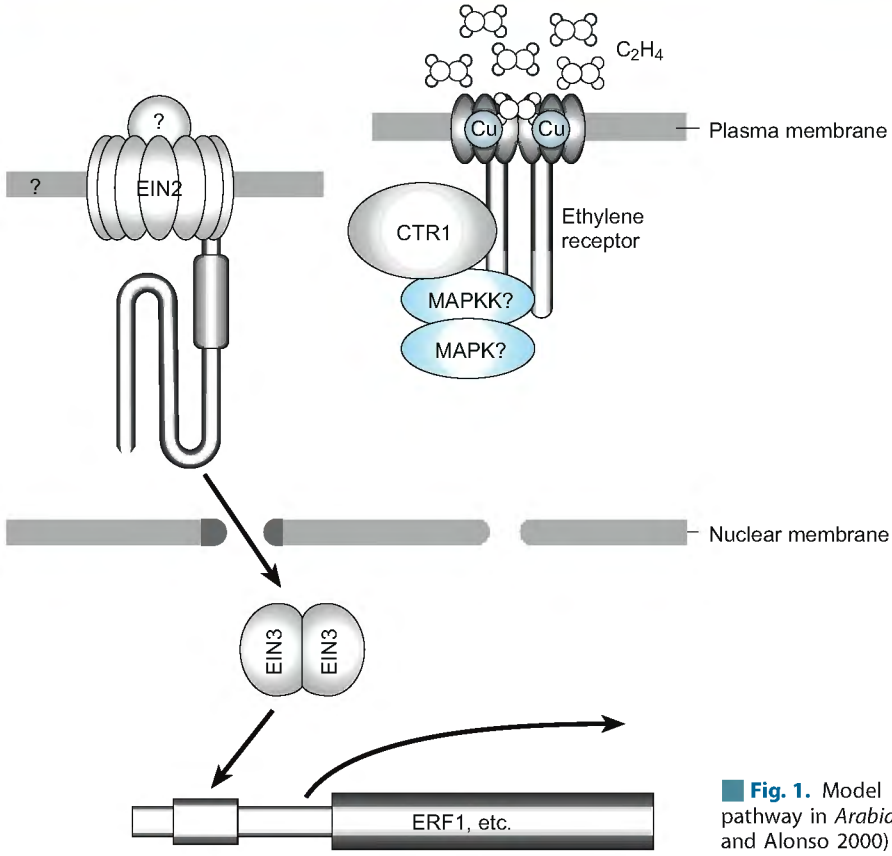


Fig. 1. Model of the ethylene signal pathway in *Arabidopsis*. (After Hirayama and Alonso 2000)

The ethylene receptors of *Arabidopsis thaliana* (Fig. 1) are relatively well understood. Ethylene induces in dark-grown *Arabidopsis* seedlings the so-called triple response consisting of an increased apical hook of the cotyledons, thickening of the hypocotyl instead of extension growth, and loss of gravitropical sensitivity. With the triple response mutants differing in reaction to ethylene may be found. Such mutants are divided into three groups: ethylene-insensitive mutants (*etr*, *ein*), constitutive ethylene-response mutants (*eto*, *ctr*) and tissue-specific ethylene-response mutants (*his*, *eir*). There are also "response to antagonist" (*ran*) mutants showing the ethylene response in the presence of the ethylene antagonist *trans*-cyclooctene. It has been shown, with the help of the *ran* mutants, that the ethylene receptor requires copper ions for the binding of ethylene. Probably they change their coordination number upon reaction with ethylene.

The structure of the ethylene receptor ETR1 is known. The active receptor is a homodimer that is probably located in the plasma membrane. ETR1 (as ETR2 and EIN4) belongs to the group of two-component histidine kinases (phospho-relay signal transducers, Fig. 2). These consist of a sensor (histidine kinase) and a response regulator. The histidine kinase contains an N-terminal signal input domain and a C-terminal kinase domain with a conserved histidine residue. The response regulator contains an N-terminal receiver domain with a conserved aspartate residue and a C-terminal signal output domain. The figure shows the well-analysed osmolarity sensor of *E. coli* as a simple example of the two-component histidine-kinase receptors. In this case, high osmolarity leads to auto-phosphorylation of the histidine residue of the sensor and to a transfer of phosphate to the aspartate residue of the receiver domain OmpR. The phosphorylation state of the (*E.*

Box 1.4.5 (continued)

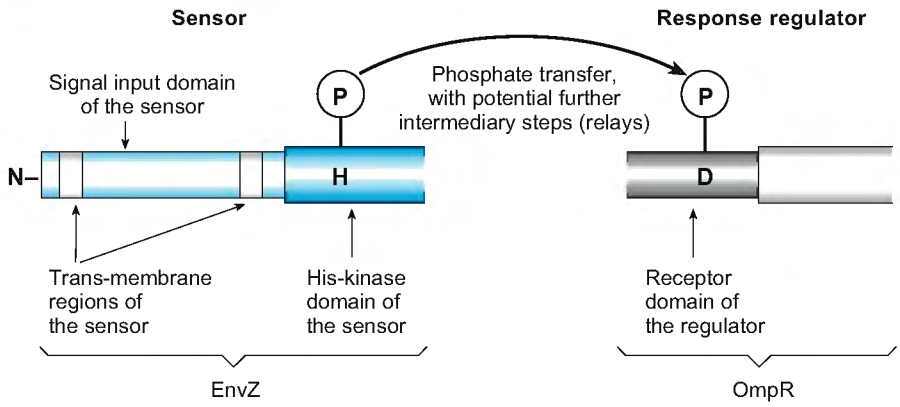


Fig. 2. Model of a two-component signal receptor. (After Urao et al. 2000)

coli) signal output domain changes its DNA-binding activity and thereby gene expression. In ETRI, for the internal transfer of the phosphate residue, a relay amino acid residue is positioned between the sensor and the regulatory domain and the phosphorylated regulatory domain does not react directly with the DNA in the nucleus, but with another protein, the CTR1 (constitutive triple response). CTR1 could react directly or indirectly via a cascade of MAP-kinases with another cell internal sensor (e.g. for divalent cations), the EIN2 protein, which is also membrane-bound. EIN2 would activate effector proteins (e.g. EIN3), which would then bind, after passing the nuclear membrane, to the promoter region of the ethylene response genes. Understanding the transduction of the ethylene signal is difficult because of the fact that the CRT must be inactivated for the ethylene response to occur. This means that binding of ethylene to the receptor inactivates the receptor and, in turn also CTR, so the inhibition of EIN is released and the signal chain becomes activated. For PCD triggered by ethylene, individual steps are not yet fully clear.

see Box 1.4.4). If light and CO_2 are available, submerged plants are able to produce oxygen photosynthetically, thus counteracting hypoxia.

Primary roots of terrestrial plants usually cannot tolerate hypoxia and die. Hypoxia-resistant plants (e.g. maize, ash, willow, *Forsythia*, *Rumex palustris*) are able within a few days to produce adventitious roots with a well-developed aerenchyma from basal shoot parts or the lower nodes (Fig. 1.4.7). These roots do not penetrate as deeply into the soil as the primary root system into a well-aerated substrate.

The individual cell layers of young adventitious roots are differently supplied with oxygen. The exodermis is the only cell layer which is usually oxygen-free; cell walls of this layer are often suberinised, thus preventing diffusion of oxygen from the interior of the root to the external medium. Formation of aerenchyma not only guarantees the aeration of tissues, but also reduces the number of oxygen-consuming cells in that tissue.

Lytic aerenchyma formation (i.e. from disintegration of cells; Drew et al. 2000) occurs selectively in the cortex of adventitious roots, starting in those parts of the tissue that are least supplied with oxygen. Lysis of cells often requires not more than 24 h. Ethylene induces this process more or less independently of normoxia or hypoxia, but hypoxia stimulates ethylene synthesis in root tips. Increased concentrations of CO_2 and ethylene itself also stimulate ethylene synthesis. Inhibition of ethylene synthesis, on the other hand, suppresses formation of aerenchyma. The genus *Rumex* comprises hypoxia-sensitive (*R. acesota*, *acetosella*) as well as hypoxia-tolerant species (*R. palustris*). In the flooding-tolerant *R. palustris* ethylene production is relatively low and almost the same under aerobic and anaerobic conditions. The internal ethyl-

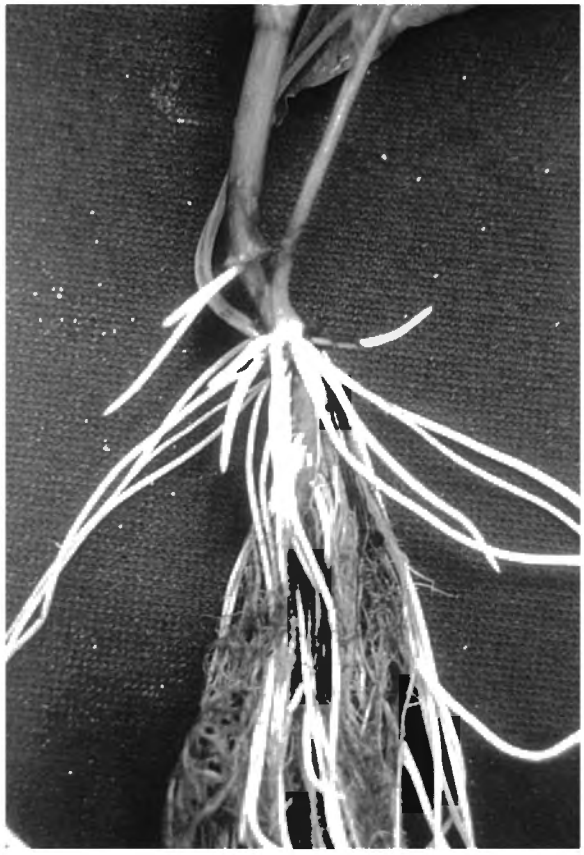


Fig. 1.4.7. The formation of adventitious roots in the flooding-tolerant *Rumex palustris* upon flooding of the root bed. The newly formed roots appear white as a consequence of the air-filled spaces in the aerenchyma and are thus clearly distinguished from roots grown under aerobic conditions which senesce under prolonged hypoxia. (After Laan et al. 1989a,b, 1991)

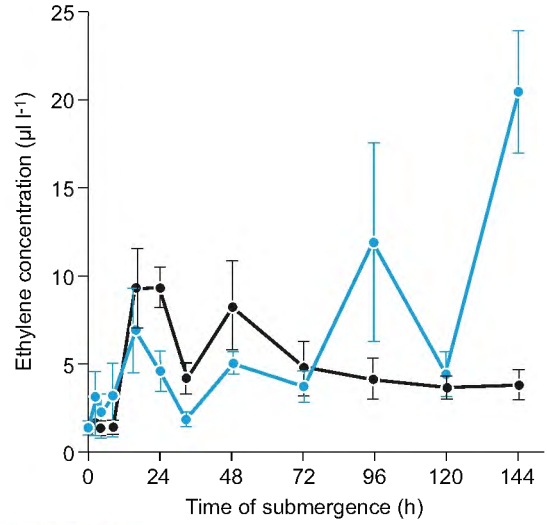


Fig. 1.4.8. The internal ethylene concentration in the tissues of two *Rumex* species of different flooding tolerance during extended waterlogging; *Rumex palustris* (•) is flooding-tolerant while *Rumex acetosella* is (•) flooding-sensitive. Decrease in the internal ethylene concentration results from inhibition of ethylene synthesis during the daily light period. (After Banga et al. 1996)

ene concentration even slightly decreases upon long-term submergence. In the flooding-intolerant sorrel, however, internal ethylene concentration increases with the duration of submergence (Fig. 1.4.8). A constant, low concentration of ethylene increases elongation growth, while a long-term increasing ethylene concentration, as in *R. acetosella*, causes premature senescence of the whole organ.

In addition to the above-mentioned elongation of internodes or senescence of individual cells (PCD) and whole organs (senescence), ethylene also induces ripening of fruits. Therefore, research has since long focused on the transduction of the signal emitted from ethylene. The first element of such a signal chain is the receptor and the ethylene receptor was the first receptor of a phytohormone to be elucidated; it belongs to the group of so-called two-component sensors (Box 1.4.5).

By combining various experimental setups and by using specific biochemical effectors, further insight could be gained into the mechanism of aerenchyma formation by PCD in the cortical tissue of maize roots (Fig. 1.4.9).

It has been shown that ethylene as a signal induces the protein kinase signal transduction pathway via a G-protein, Ca^{2+} and inositol-P, which leads to synthesis of lytic enzymes, e.g. cellulase and hemicellulase (Saab and Sachs 1996). Calcium activates endonucleases, so that the cell death is caused by a controlled breakdown of nucleic acid (by an endonuclease, activated by caspases) and not through breakdown of cell membranes, as after cell damage. Formation of aerenchyma is promoted by further effectors: For example, mechanical resistance of a heavy soil stimulates ethylene synthesis by the growing root and scarcity of minerals increases the sensitivity of the plant tissue to ethylene. Figure 1.4.10 shows a model of the biochemical processes that take place in the formation of aerenchyma.

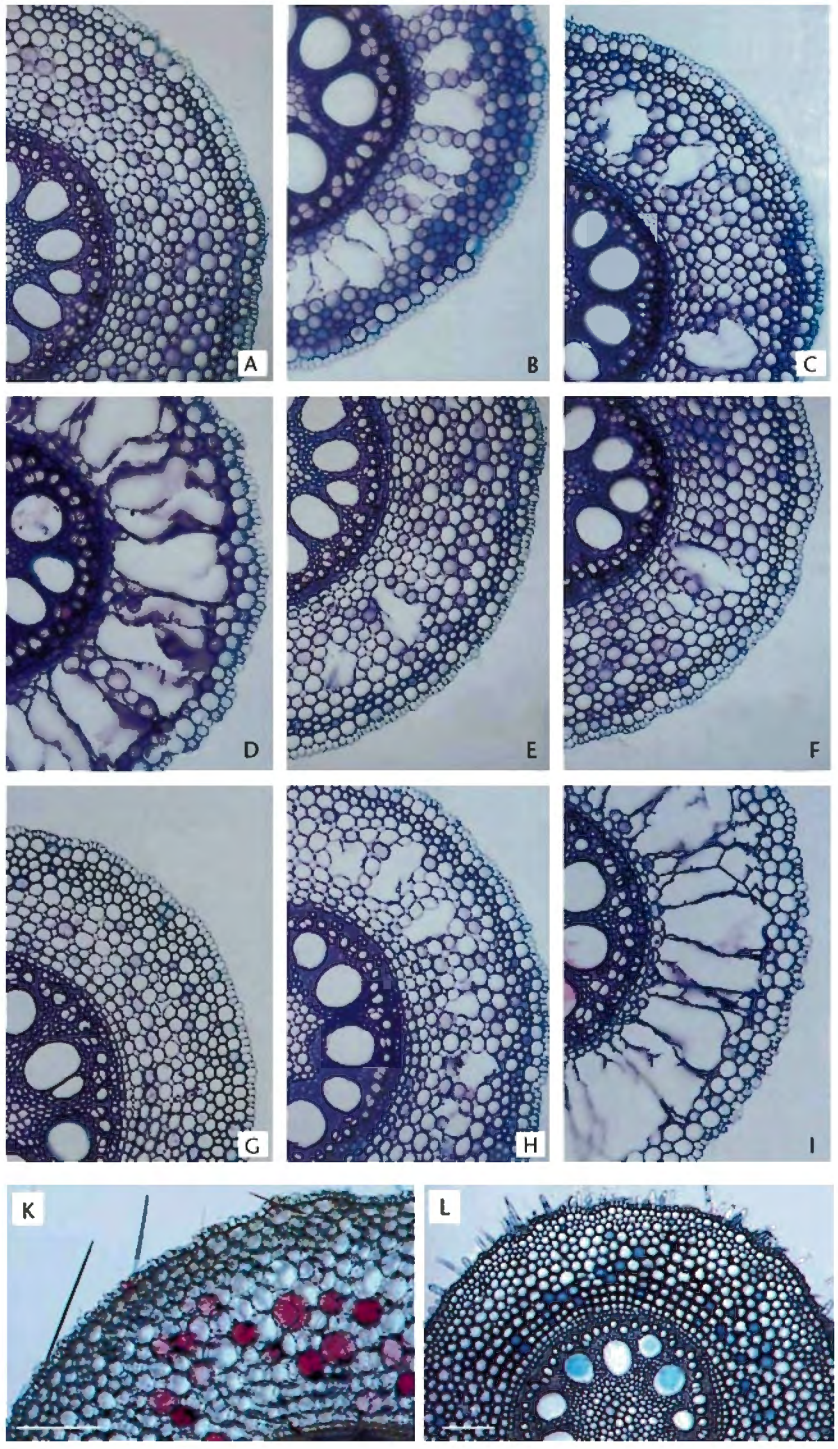


Fig. 1.4.9. Analysis of aerenchyma formation in a maize root. Ocadaic acid blocks protein phosphatases; GTP-γ-S activates G proteins; K252a inhibits protein kinases; neomycin inhibits inositol phosphate metabolism; EGTA complexes Ca^{2+}; disintegrating cells are coloured purple by neutral red and Evans blue accumulates in dying cells. (After He et al. 1996; Drew et al. 2000). A Normoxic; B hypoxic; C normoxic + ocadaic acid; D hypoxic + ocadaic acid; E hypoxic + neomycin; F hypoxic + K252a; G hypoxic + EGTA; H normoxic + GTP-γ-S; I hypoxic + GTP-γ-S; K hypoxic + neutral red; L hypoxic + Evans blue

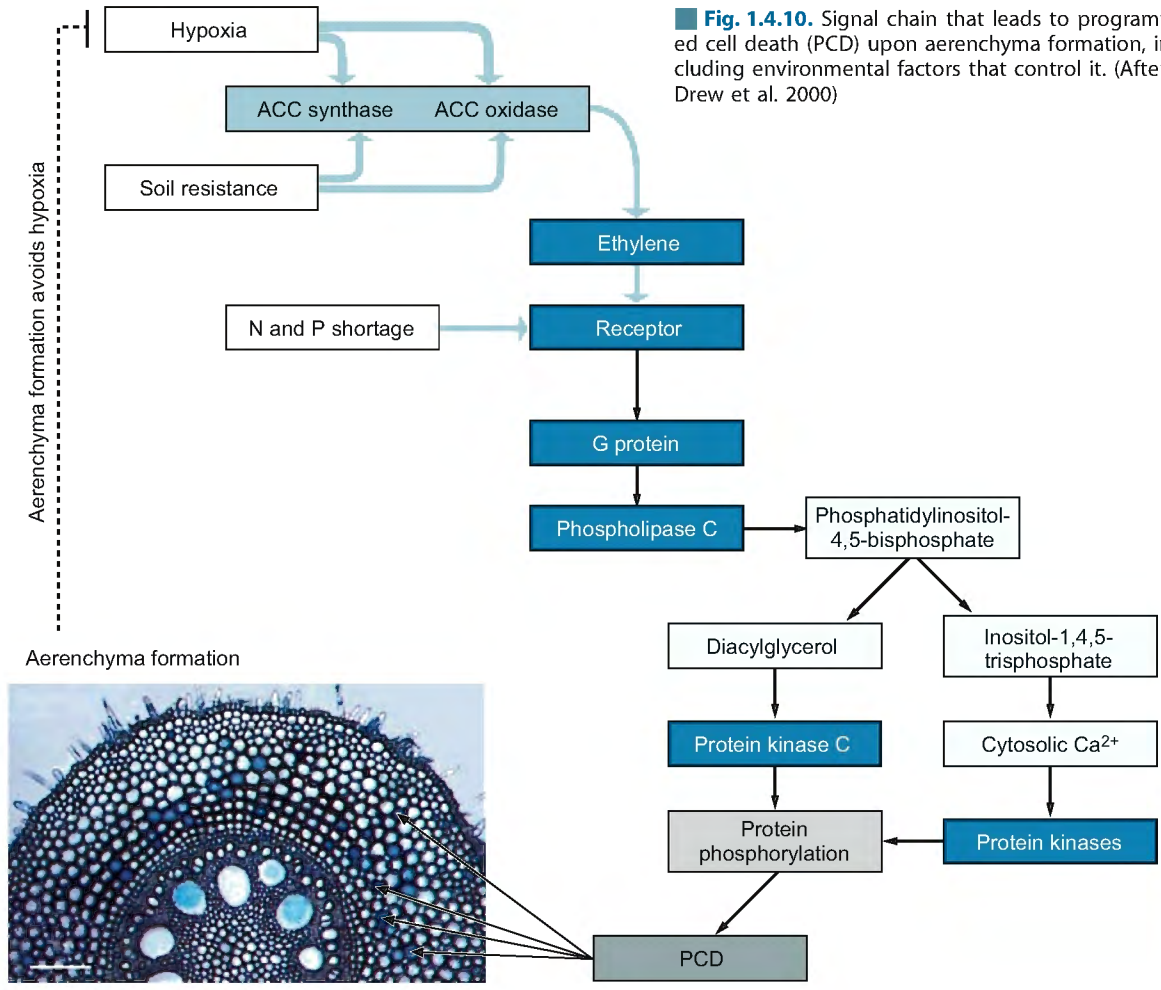


Fig. 1.4.10. Signal chain that leads to programmed cell death (PCD) upon aerenchyma formation, including environmental factors that control it. (After Drew et al. 2000)

1.4.3

Post-anoxic Stress

Tissues tolerating hypoxic stress are often damaged by subsequent aeration. This post-anoxic stress usually results from ROS (see Chap. 1.3.5.3). Cells in which metabolism is adapted to hypoxic or anaerobic conditions have a relatively negative redox potential, i.e. high electron pressure from a high NADH/NAD^+ ratio. In the presence of O_2 this leads to oxygen reduction and to the formation of ROS. During the hypoxic phase, activities of enzymes detoxifying ROS are decreased and the pools of scavenger metabolites are reduced so that the tissue is not capable of coping with increased oxidative stress. However, some plants are known to tolerate post-anoxic stress as, e.g., the yellow iris, where the enzyme superoxide dismutase is part of the ANP.

Summary

1. Lack of oxygen occurs after extended periods of soil flooding because the diffusion of oxygen in water is about 10,000 times slower than in air. The critical oxygen content required for growth of roots is about 5–10%. Helophytes (swamp-inhabiting plants) and submerged plants are able to live in partly hypoxic environments by supplying their roots and rhizomes with oxygen through an intercellular aeration system (aerenchyma). Gas flow in the interior of the plant is substantially accelerated by thermo-osmosis.
2. Normoxia, hypoxia and anoxia describe the conditions of sufficient, insufficient and deficient oxygen supply to organisms. Hypoxia and anoxia produce negative redox potentials of the soils, under which conditions oxidised heavy metal ions become reduced and in this form are poisonous. As a consequence of bio-