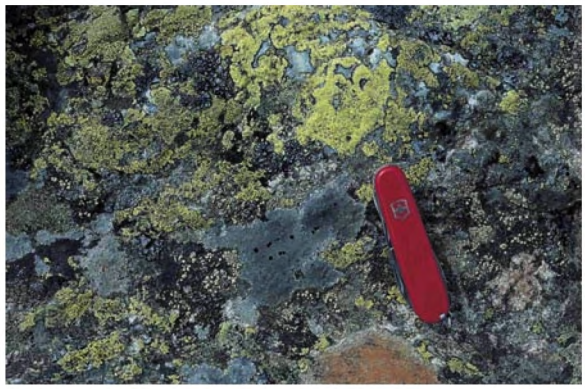
1.5

Water Deficiency (Drought)



Poikilohydric lichens are "world champions" in tolerating drought stress, which usually is accompanied by heat or cold and high light intensities. Despite the extremely harsh habitat, every square millimetre of this gneiss boulder in the Austrian Alps is occupied by more or less colourful crustose lichens. The community is dominated by the yellowish green *Rhizocarpon* cf. *geographicum*.
Photograph E. Beck.

Recommended Literature

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- Ingram J, Bartels O (1996) The molecular basis of dehydration tolerance in plants. Annu Rev Plant Physiol Plant Mol Biol 47:377–403
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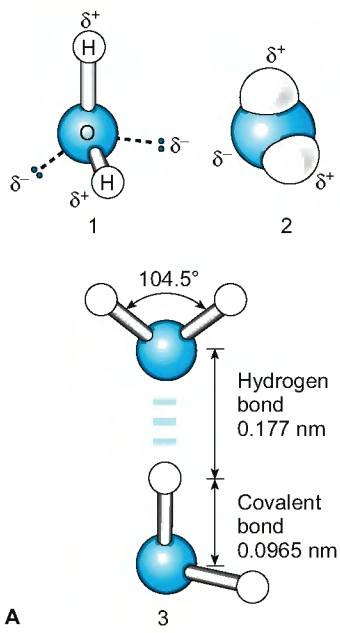
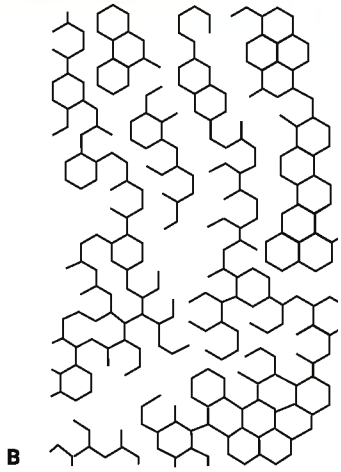
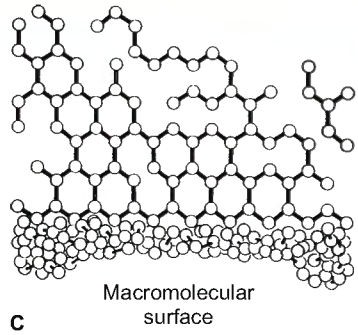
Life requires water in the liquid state and is, thus, based on the physicochemical properties of the **water molecule** causing the so-called **anomalies** (Box 1.5.1). These properties result from the dipole nature of the molecule $H^{\delta+}$–$O^{\delta-}$–$H^{\delta+}$; the dipoles produce H-bonds between the individual molecules and thus guarantee a high degree of cohesion with, at the same time, low viscosity. The results are variable or flickering (mobile) clusters or aggregates, which continuously exchange individual molecules. Despite the low molecular mass of the water molecule, water is, therefore, in the liquid rather than the gaseous state at temperatures between 0 and 100 °C and standard air pressure. Also based on the dipole nature of water is its adhesion to polar surfaces, for example, cell walls, and its capillary action based on the high surface tension. Adhesion, capillary action, together with cohesion and low viscosity, are decisive factors for the transport of water from roots to leaves (cohesion theory of water conductance). Another consequence of the dipole nature of the water molecule is its suitability as solvent for polar and polarisable compounds. In addition, water develops a structuring force in amphiphilic systems (hydrophilic–hydrophobic) giving rise to lipid micelles and contributing to the tertiary structures of proteins. These so-called hydrophobic interactions are based on the strength of the interaction between the hydrophilic domains of the molecules with each other and with the water clusters, as well as on the hydrophobic domains between one another. The term **hydrophobic in-**

Box 1.5.1 The physico-chemical properties (anomalies) of water







Due to their dipole nature, water molecules [Fig. A (1), (2)] associate via hydrogen bonds to a three-dimensional lattice (clusters; B, from Larcher 1994), which is in permanent molecular rearrangement. The physico-chemical anomalies of water can be explained as a consequence of this cluster formation.

Water forces amphiphilic molecules into particular associative structures, e.g. biomembranes (C, from Larcher 1994). These, in turn, interact with the water molecules, forming an ordered water layer which at some distance from the membrane surface again takes on the nature of flickering (mobile) clusters.

Compound	Molecular weight	Melting point (°C)	Boiling point (°C)
H_2O	$[18]_x$	0	100
H_2S	34	−86	−61

Further biologically important physical properties of water are listed below.

Specific heat capacity	1 cal g^{-1} = 4.2 J g^{-1}
Latent heat of crystallisation	333.6 J g^{-1}
Latent heat of evaporation	2441 J g^{-1}
Surface tension (at 15 °C)	73.5 g sec^{-1}

teraction is slightly misleading, as there are also interactions between the water molecules and the hydrophobic domains of the amphiphilic molecules, which are, however, weaker than the interactions of the hydrophobic domains with one another. Water is a very effective heat buffer for organisms, because of its relatively high heat of crystallisation (freezing avoidance) and very high heat of evaporation (transpiration cooling). As its radiation absorption is beyond the borders of the visible spectrum, water does not absorb visible light and thus does not interfere with photosynthesis or processes regulated by blue or red light.

Water Potential

The thermodynamic state of water is described by the water potential, ψ, which is, figuratively speaking, a measure of the energy required to remove water molecules from any water-containing system. As the (chemical) potential of pure water is taken as a reference system, and under the actual conditions is defined as zero, ψ is a measure of a difference in the chemical potentials. Commonly, the water potential of a system is expressed in the dimension of pressure and not of energy: If the chemical potential of water is related to the molar volume, the dimension

"pressure" results. Other details and the derivation of the definition of water potential are given in Chapter 2.2, Eq. (2.2.7) (see also Chap. 1.3).

The water potential of a cell is made up of several components:

$$(-)\psi = (-)\pi + (-)\tau + P,$$

where π is the **osmotic potential** (negative sign) of the equilibrated cellular solutions in a steady state [see Chap. 2.2, Eq. (2.2.6)], τ the **matrix potential** (negative sign) and P the **pressure potential** (cell wall and tissue pressure, positive sign). The pressure of the wall (plus tissue) is numerically equal to the **turgor pressure** (the pressure of the protoplast on the cell wall) and has the opposite, positive, sign in the water potential equation. In a hydrated cell, τ cannot be measured. It becomes apparent only after the cell has been desiccated to such an extent that only the water bound to cellular and subcellular structures remains. To remove that portion of cellular water requires either extreme low (negative) water potentials or extreme high pressures (see Chap. 1.3.6.4).

Intercellular and Intracellular Water Flow, Aquaporins

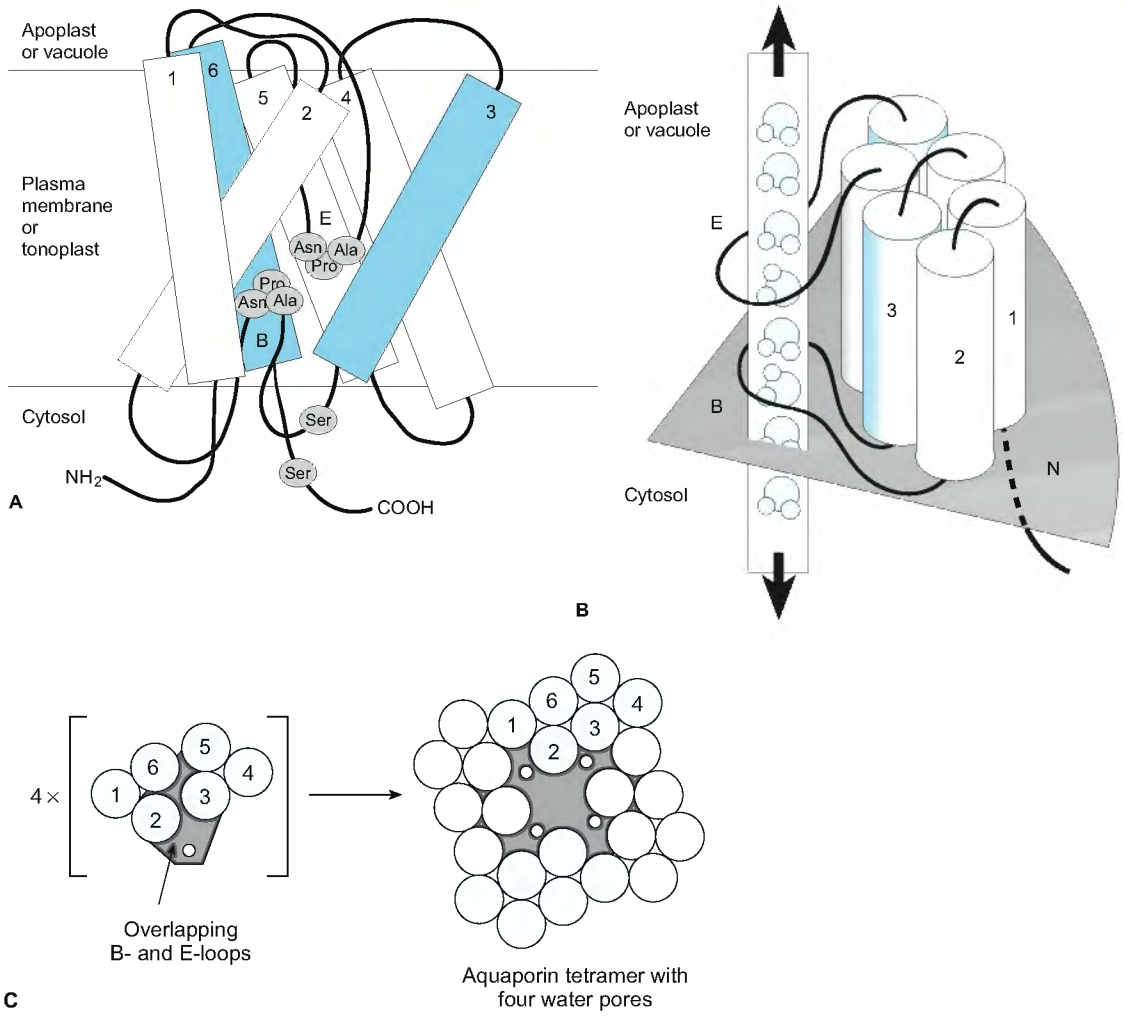
A water potential difference between two systems (e.g. two cells) corresponds to a voltage in an electrical circuit and causes the flux of water in the direction of the system with the more negative potential, provided that the pathway is conductive to water (**hydraulic conductivity**). The flux of water may be in the apoplast, i.e. through and along the cell walls (**apoplastic path**) or also through the protoplasts (**symplastic path**; see Chap. 2.2, Fig. 2.2.8). The importance of the symplastic path for water transport has been greatly underestimated, but the discovery of **aquaporins** (Box 1.5.2) has gained more attention. Aquaporins are specific proteins, with a molecular mass of 15–30 kDa, which form water channels through cellular membranes (Johannson et al. 2000). Usually, oligomers accumulate to complexes. The density of such aquaporin aggregates in a membrane determines its hydraulic conductivity, which is in the range 2×10^{-8} to 10^{-5} m s^{-1} MPa^{-1}. Water flow through these channels is characterised by the high permeability of the membrane ($P_f \approx 700$ µm s^{-1}) and a low activation energy of the transport ($E_a = 8$–12 kJ mol^{-1}). Additional to this pathway is the so-called **lipid pathway**, namely the diffusion of water molecules through the lipid bilayer of the biomembrane, which is characterised by a low permeability and a high activation energy ($P_f \approx 10$ µm s^{-1}; $E_a = 48$–64 kJ mol^{-1}). Water transport by these two pathways can be differentiated by the kinetic data, and by blocking the aquaporins with heavy metals, for example, Hg^{2+}, as most of the aquaporins contain cysteine in the helices spanning the membrane.

Usually, the permeability for water of the tonoplast is considerably higher than that of the plasma membrane. As the water relations of the cell are dominated by the vacuole, the high water permeability of the tonoplast guarantees fast equilibration of the intracellular water potentials upon changes in the cell's water status. In *Arabidopsis* aquaporin clusters, so-called **plasmalemmasomes** (Robinson et al. 1996) have been described. They are thought to establish a direct connection between the plasmalemma and the tonoplast. Other membrane transport proteins, e.g. ion channels, also contribute to water transport, as they form hydrated pores. Depending on the structure of these pores, each transported molecule or ion carries up to 25 water molecules over the membrane. The effectiveness of an aquaporin, however, is about 20 times higher.

1.5.1 Water Balance of Drought-Stressed Cells

Upon drought plants lose water to the atmosphere: if water uptake cannot keep pace with water loss, transpiration is mainly fed from the vacuoles. It flows via the plasmalemma into the apoplast from where it evaporates into the intercellular spaces. Because of the decrease in volume caused by water loss, the osmotic potential in the protoplast is increased (i.e. becomes numerically more negative). At the same time, the turgor pressure as well as the cell wall pressure decrease. At the so-called turgor-loss point the cell wall is completely relaxed and both pressures are zero. Consequently, the water potential of the cell is then equal to its osmotic potential. In this state plants show substantial wilting. With further loss of water, wilting increases, as the cell walls are not only relaxed, but in responding to the intracellular suction bend in-

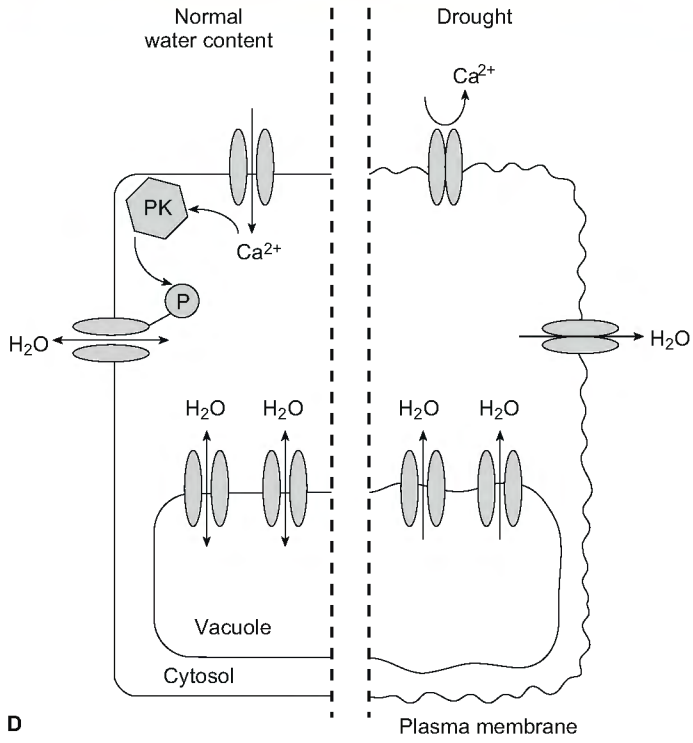
Box 1.5.2 Aquaporins



A shows the hourglass model of an aquaporin monomer. The labelled amino acids in the centre form the constriction of the hourglass, while the membrane-spanning helices, *1*, *2*, *3*, and their counterparts, *4*, *5*, *6*, form the "container". The loops *B* and *E* form the actual water pore (B). The two halves of the hourglass are homologous, but inverted in their orientation in the membrane: Helix 1 corresponds to helix 4, helix 2 to 5 and helix 3 to 6. Aqua-

wards giving rise to cell cytorrhysis (Chap. 1.3.6.1), if their rigidity is low enough to allow folding. As described for the case of freezing dehydration, this happens because the water-imbibed cell wall (Fig. 1.5.1) does not allow any air to penetrate. Suction develops and correspondingly a negative pressure potential (−1 to −2 MPa) increases the water potential of the cell. As a consequence, more water is retained in the cell than would correspond to the osmotic potential alone. Despite the increase in the water potential of the cells by a negative pressure potential, during prolonged drought, cells can lose so much of their water that dehydration damage arises, breaking down the bilayer structure of the membranes whereupon the osmotic system of the cell collapses.

Box 1.5.2 Aquaporins (continued)



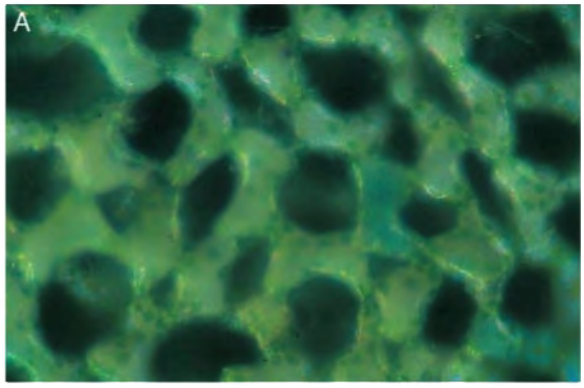
porins form complexes (C). A model with four monomers is shown (CHIP 28 from erythrocytes), which also represents the most common form of water pores in plants. The model applies to the aquaporins of the plasma membrane (PIPs) and of the tonoplast (TIPs). The efficiency of some aquaporins can be controlled by protein phosphorylation and dephosphorylation (D). The model shows the state under sufficient water supply (*left*) and under drought (*right*). An as yet unidentified osmoregulator holds the cytosolic Ca^{2+} concentration high and a protein kinase keeps the aquaporin phosphorylated, which keeps the pore open. Protein phosphorylation takes place at the serine residues labelled in A. Under drought stress the cytosolic Ca^{2+} concentration decreases, the aquaporin becomes dephosphorylated and the pore closes. In this way, water loss is reduced. It is presumed that selective regulation of the protein phosphorylation keeps the pores in the tonoplast open, so that the water potential of the cytosol is little affected by water loss from the cell (A and D after Kjellbom 1999; B after Schäffner 1998; C after Jung et al. 1994).

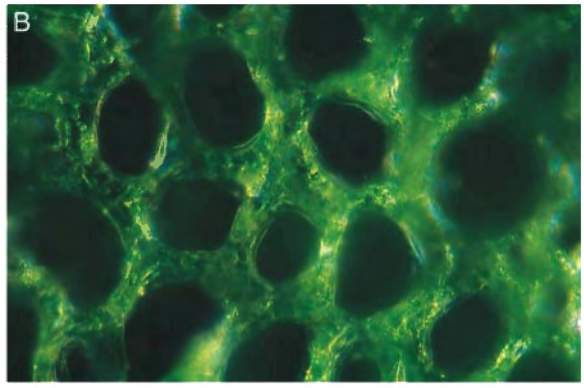
Water Storage

Water storage tissues, for example, of succulents, have special cellular water relations. Cell walls of succulents are very flexible (but not extendable) and thus convey a high water storage capacity (**hydraulic capacity**) to the cells. Upon water loss, the cell walls of these living tissues fold like a concertina, or the cells as a whole collapse cytorrhytically, and this reduces the cell volume. The flexibility (elasticity) of the cell wall, with which they react to pressure changes, must not be mistaken for expansion or contraction. It is a passive change in the shape of the cells depending on the **elasticity modulus** ε of the cell wall:

$$\varepsilon = \frac{dP}{dV/V} \text{ [MPa]}$$

where dP is the change in turgor and dV/V the relative change in volume.





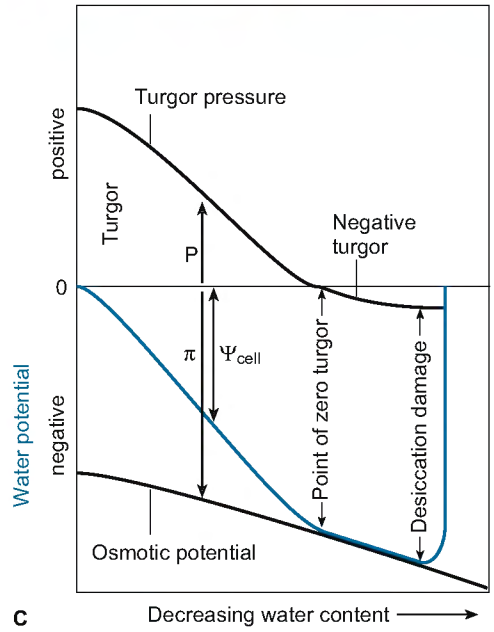


Fig. 1.5.1 Desiccation of plant cells upon exposure to air. A Spongy parenchyma of *Pachysandra terminalis* in turgid state. B The same tissue after 2 h exposure to air. The collapse of the cells, whose volume has shrunk by more than 50%, is clearly visible. C Water relations of a cell in the course of desiccation. After loss of turgor the protoplast remains attached to the cell wall, due to the matrix potential of the latter, and a negative wall pressure (tension) develops. A, B, photos by J.J. Zhu, C after Larcher (1994), modified

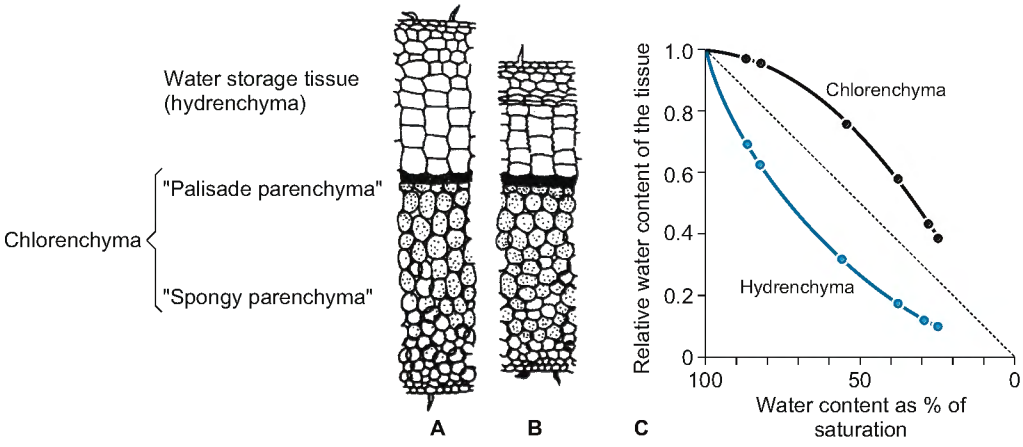


Fig. 1.5.2. Water storage in leaves of peperomia (*Peperomia trichocarpa*). A, B Cross sections of water-saturated leaves (A) and the leaves after drying (B). The hydrenchyma shrinks much more than the chlorenchyma. This phenomenon is shown quantitatively in C: at a total water content of 50% saturation, the portion of water in the hydrenchyma has dropped to 25%, whilst the water content of the chlorenchyma is still 75%. (After Larcher 1994)

For soft plant tissues, $\varepsilon \leq 5$ MPa, so larger changes in volume create only small changes in turgor (P). For leaves of sclerophyllous trees, ε is between 30 and 50 MPa, and they have only limited ability to change their shape upon water loss. Thus, softer tissues are able to shrink substantially, whilst harder tissues cannot. Water storage tissue (**hydrenchyma**) possesses cells with a particularly low elasticity module, i.e. they can easily take up and release lots of water. Leaves with marked water storage tissue, usually surrounded by the chlorenchyma (e.g. *Peperomia*), therefore change their cross section in accordance to their state of water. Because of the different elasticity modules, only the water storage tissue, but not the chlorenchyma, shrinks (Fig. 1.5.2).

Many water storage tissues consist of dead cells, for example, the *velamen radicum* of the air roots of orchids, where water uptake and storage depend mainly on capillary forces.

1.5.2 Cellular Reactions to Drought Stress

At the cell level, water deficit is not only a result of drought, but also of salt stress and of frost. The loss of up to 90% of the cell water is a necessary process during seed and fruit ripening and thus is a regular component of a plant's development. Plants have, therefore, developed biochemical mechanisms to cope with drought stress. Our understanding of the molecular processes triggered by drought and of the adaptation to water shortage is, however, still patchy. There is no doubt that each cell has more than one mechanism for reacting to drought; there is, so to say, a multiple safety net. However, not all changes in cell biology occurring upon drought stress are specific to that situation. Some strains are primarily targeted to other stressors, e.g. metabolic changes triggered by cold or pathogen attack can also be effective to cope with dehydration stress. In addition to specific stress-directed reactions, desiccation interferes with the normal housekeeping machinery of the cell. This holds in particular for **poikilohydric plants** (see Chap. 2.2.1.1) where photosynthesis is reduced in order to minimise the danger of photooxidation. In the resurrection plant *Craterostigma plantaginea* expression of genes of the photosynthetic machinery is attenuated, probably concomitantly with the induction of genes whose products – proteins and metabolites – increase drought resistance of cells directly or indirectly. A key role in coping with **dehydration stress** is attributed to the phytohormone **abscisic acid** (ABA; see Fig. 1.5.3 and Box 1.5.3), which triggers fast reactions as well as long-term adaptation. Complying with the above-mentioned complexity of stress-induced cellular reactions, ABA-independent reactions are also well known. Current knowledge shows cellular responses to drought to be no less complicated than the responses to heat stress.

1.5.2.1 Perception of Dehydration Stress

Water loss may cause the following changes:

- shrinkage of the protoplast,
- concentration of cellular solutions,
- decrease or loss of turgor,
- changes in the water potential gradient across membranes,
- in the worst cases, disintegration of biomembranes and denaturing of proteins.

Which of these phenomena play a role as cellular signals in plants has not yet been determined. Probably it is the change in the water potential or pressure gradient across membranes. **Osmosensors** are known from *E. coli* and yeast. Their functions were shown in complementation studies with mutants deficient in osmotic adaptation (Wurgler-Murphy and Saito 1997; see also Box 1.4.5). One of the transmembrane osmosensors of yeast, Sho1p, forms a protein of four closely packed membrane-spanning peptides with a peripheral C-terminal domain that is responsible for triggering signal transduction. If the osmotic gradient across the plasma membrane is increased, Sho1p activates a four-step MAP kinase cascade, leading finally to the production and accumulation of glycerol, which is an osmoprotectant of yeast. Successful complementation of the osmosensor-deficient mutant of yeast with a corresponding gene of *Arabidopsis* (*ATHK1*; Shinozaki and Yamaguchi-Shinozaki 1997) leads to the assumption that similar systems of drought perception are also effective in plants. Due to the multiplicity of stress responses of plants, it must be concluded that plant cells have several receptor systems which might render tissue specificity (Bonetta and McCourt 1998). Receptors for ABA should be differentiated from direct osmosensors, as

Box 1.5.3 Biosynthesis, metabolism and inactivation of ABA

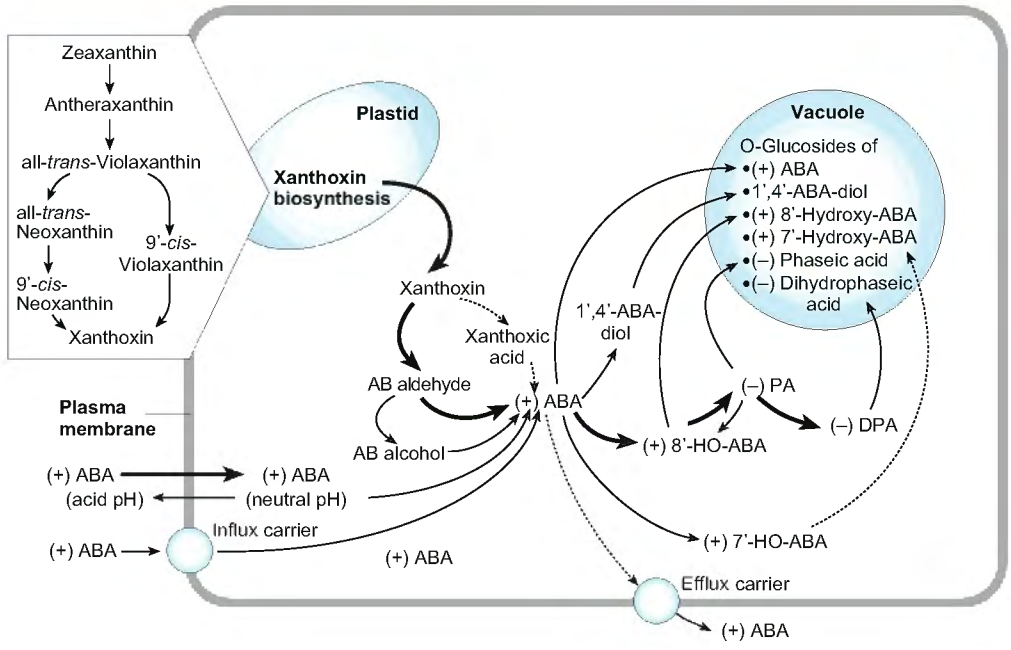


Fig. 1

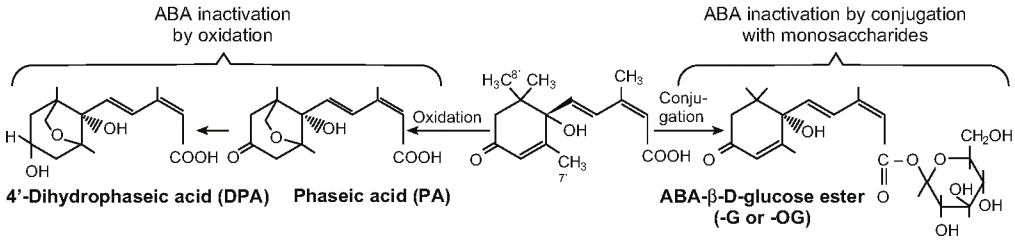


Fig. 2

As far as is currently known, ABA is a derivative of chloroplastic xanthophylls. These are not synthesised via the mevalonate pathway, but originate from the chloroplastic DOXP pathway (1-desoxy-D-xylulose-5-phosphate pathway; Lichtenthaler 1999). Key enzymes of ABA biosynthesis are zeaxanthin epoxidase (ZEP), encoded for by the gene *ABA2*, and 9-*cis*-epoxycarotenoid dioxygenase (NCE), encoded for by the gene *VP14*. NCE can cleave the xanthophylls: all-*trans*-violaxanthin, all-*trans*-neoxanthin, 9′-*cis*-neoxanthin (Cutler and Krochko 1999).

Figure 1 shows the metabolism of (+)-ABA at the cellular level. The first steps of ABA biosynthesis, until cleavage of the 9′-epoxycarotenoids by the dioxygenase, occur in the plastids. The cleavage product xanthoxin after export from the plastid (by an unknown mechanism) is metabolised in the cytoplasm to (+)-ABA. The *bold arrows* show the main route and *fine arrows* the shunt via ABA-alcohol, whilst the pathway via xanthoxic acid is still disputed (*dotted line*). The fast import of ABA from the apoplasm into the cell is induced by the neutral pH of the cytoplasm (after Cowan 2000).

The main pathway of ABA degradation as shown in Fig. 2 starts with the hydroxylation of the 8′-carbon, followed by a cyclisation to phaseic acid which is converted by a reductase to dihydrophaseic acid. The three degradation products are physiologically inactive. Hydroxylation of the 7′-carbon is also known, but similar to the direct reduction of the ABA keto group to 1′,4′-ABA diol is quantitatively insignificant. The formation of conjugates of ABA and its derivatives, in particular with glucose, is physiologically relevant, as these compounds are water-soluble and are exported from the cytoplasm into the vacuole (after Taiz and Zeiger 2000).

ABA is already a stress signal at the cellular or subcellular level. The biochemical structure of ABA sensors has not yet been elucidated.

1.5.2.2 ABA-Mediated Stress Response

Mild drought stress leads to a ten-fold increase in the endogenous ABA concentration (Fig. 1.5.3). This applies to higher, as well as to lower, plants. Mutants lacking the ability to synthesise ABA are not viable, as they wilt even with the slightest drought stress. However, they may be kept alive by adding ABA[1]. The receptor for the dehydration signal leading to ABA synthesis and accumulation is unknown.

Even though the biochemistry of **ABA sensing** is not yet known, it is clear that there are ABA receptors on the outside of the cell membrane and inside the protoplast (Bray 1997). The receptors may be activated or inactivated by interaction with an effector protein. Prenylation (farnesylation) of that protein leads to its binding to the receptor and to its inactivation. In this way, the sensitivity of the cell for ABA may be adjusted (Bonetta and McCourt 1998). However, ABA is not the only signal molecule that is recognised by the ABA receptors. The derivative (+)-8′-methylene-ABA is more efficient, probably because this compound persists longer than ABA, i.e. is metabolised more slowly. The cyclic ADP-ribose (cADP-Rib) and nicotinic acid-adeninedinucleotide phosphate have been identified as **secondary intracellular messengers** of the ABA signal (Quatrano et al. 1997). They probably react to the ABA signal and affect intracellular calcium stores, triggering increase in free cytosolic Ca^{2+}. This acts as a further secondary messenger, starting one or more phosphorylation cascades which directly or indirectly lead to metabolic responses (strain reactions) to drought stress.

Processes induced by ABA are either fast reactions, as seen in stomatal closure, or slow reactions connected to the expression of ABA-responsive genes.

Reaction of Stomates to ABA

The ABA signal is detected at the outer face of the plasma membrane of guard cells, causing a fast decrease in the turgor and closure of the stomates (Schroeder et al. 2001).

The compartment that is effective in the control of ABA-triggered reactions is thus the apoplast of the guard cells. ABA, as a **signal originating in the roots**, reaches the leaves via the xylem stream and, by **ion trapping**, is quickly absorbed by the protoplast. Usually, the apoplast is more acidic than the cytosol, whose pH is around 7. Thus ABA, as an acid, is protonated to a higher extent in the apoplast (pH 5–5.5) than in the cytoplasm. The protonated, i.e. undissociated, acid easily diffuses through the plasma membrane into the cytosol where it is trapped after dissociation. The apoplastic concentration of ABA, which is that concentration perceived by the outer face of the plasma membrane, is, therefore, low. It has been recently shown that stressful situations, such as low water availability in the soil, lead to an alkalisation of the xylem sap by up to one pH unit (Wilkinson 1999). This decreases the efficacy of the cytosolic ion trap and shifts the pH-dependent equilibrium between the intra- and extracellular ABA concentrations in favour of the apoplast

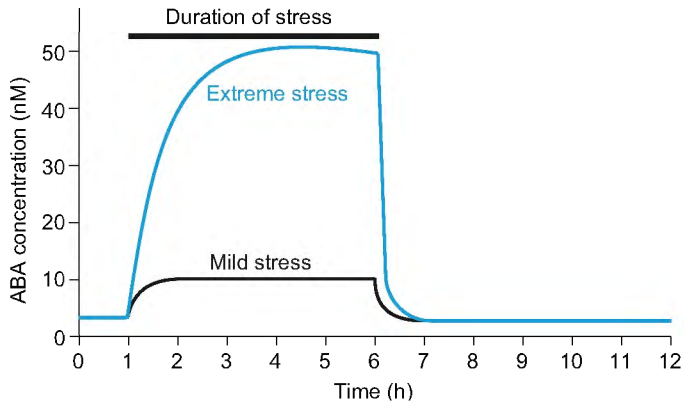
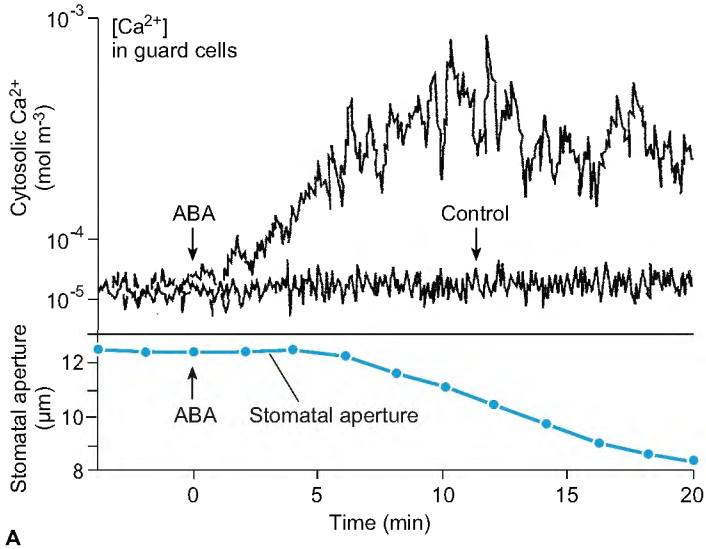
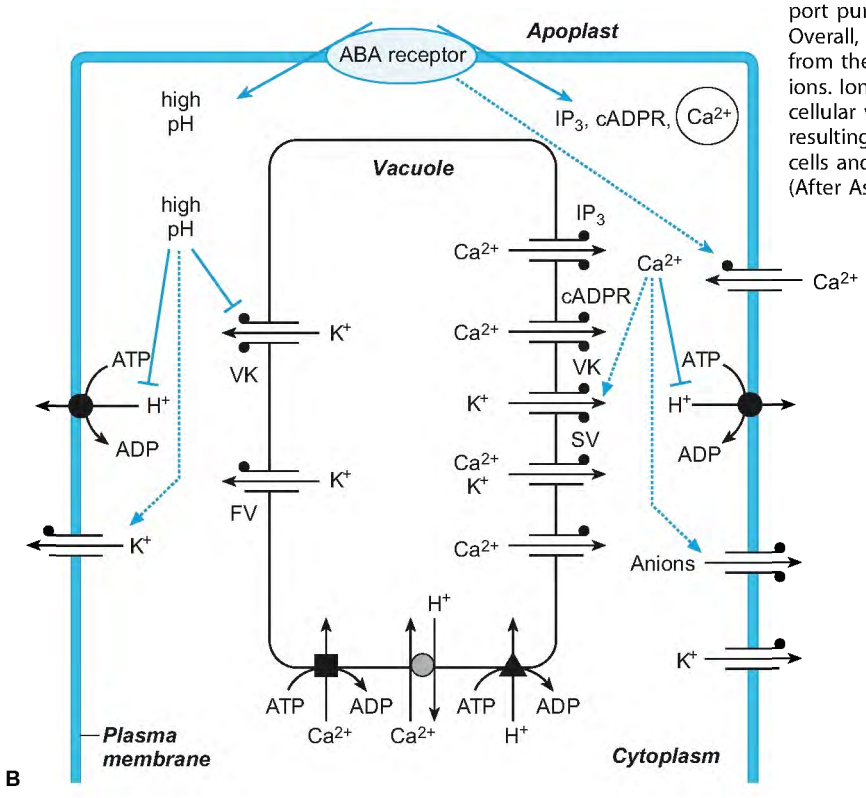


Fig. 1.5.3. Computer simulation of ABA export from the guard cell protoplasts into the surrounding apoplasts following alkalisation of the latter upon drought stress. The simulation of the distribution of ABA is based on the ion trap principle. Following rehydration, the original pH relations are restored and ABA diffuses back into the cytoplasm. The duration of the applied stress is shown by the *black bar* at the top of the figure. (Hartung 1996)

[1] Fungi also synthesise ABA, probably directly from mevalonic acid (Walton and Li 1995)



A



B

Transporters

Ligand-gated channels

Channels

Activation and/or stimulation

Inhibition

SV Slow vacuolar cation channel

VK Vacuolar K^+ channel

FV Fast vacuolar cation channel

Fig. 1.5.4. Regulation of stomatal closure by ABA. A Immediately after application of ABA to isolated epidermal strips the concentration of free Ca^{2+} in the guard cells increases. Only 5 min later the stomates begin to close (after Taiz and Zeiger 2000). B Simplified scheme of stoma closure caused by ABA. ABA affects the ion relations in the guard cells in many ways, but mainly by increasing the intracellular pH (*left part of figure*) and by directly and indirectly increasing the intracellular concentration of free calcium (*right part of figure*). Many ion channels are regulated by Ca^{2+} or metabolites (gated channels), and others open or close in response to the membrane potential and are thus dependent on the pH gradient across the plasma membrane. Some reactions are controlled by both pH and the concentration of free Ca^{2+} (e.g. proton export pumps of the plasma membrane). Overall, there is a gross net export of K^+ from the cell, followed by export of anions. Ion export leads to the shift of cellular water to the neighbouring cells, resulting in turgor loss of the guard cells and finally to closure of the stoma. (After Assman and Shimazaki 1999)

and, in turn, increases the strength of the ABA signal (Fig. 1.5.3).

Moreover, the ABA concentration in the xylem fluid increases under that kind of stress. Neither the structure of the ABA receptor nor the biochemistry of its reaction with ABA is known. Knowledge of the ABA signal cascade starts with an increase in the cytosolic calcium concentration by import of Ca^{2+} from the apoplast and by release from intracellular reservoirs. In addition, the cytosolic pH also increases. Because of this and the increased calcium level, proton-exporting pumps and potassium-importing channels are inactivated and potassium-exporting channels are activated. Ca^{2+} also activates anion channels (chloride or malate) in the plasma membrane. Flow of such anions from the vacuole into the cytoplasm has been shown, but the transport mechanism and the nature of the activator of the channels are still debated. For many of these channels, a guard cell-specific control is assumed. Thus, there is a direct and a calcium-dependent signal transduction pathway (Fig. 1.5.4) and both have the same effect, i.e. a concerted reaction of ion channels resulting in an export of potassium and anions (e.g. chloride and malate) from the guard cell protoplast. As a consequence of the decreasing osmotic potential of the guard cells and the corresponding increase in the water potential of the neighbouring cells, water moves from the guard cells to the neighbouring cells. Because of the concomitant decrease in the turgor in the guard cells, the stomata close. As mentioned above, the sensitivity of the plasma membrane towards ABA can change in the course of the day; this could be due to salts or other phytohormones transported in the xylem sap.

Besides the reaction to ABA, guard cells also react to other signals, e.g. CO_2 concentration or light (see also Chap. 2.2, Fig. 2.2.17). Increased concentrations of CO_2 reduce stomatal conductivity (Morison 1998) while light acts as a signal for the opening of stomata. It has been shown that it is mainly the blue light portion of the daylight that triggers ion influx and stoma opening (Zeiger and Zhu 1998).

Regulation of Gene Expression by ABA

The control of gene expression mediated by ABA aims at two different groups of gene products: **functional** and **regulatory proteins**. The latter may be components of regulation cascades leading to the synthesis of functional proteins (enzymes or protective proteins; Fig. 1.5.5).

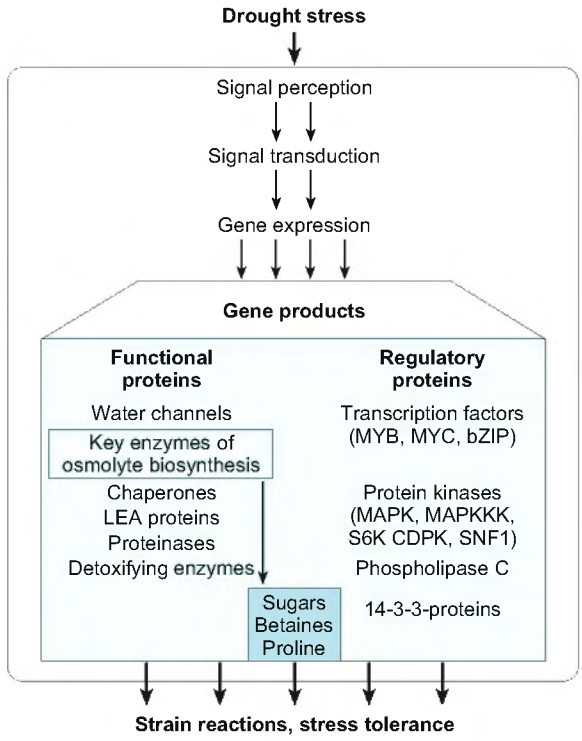


Fig. 1.5.5. The response of plant cells to stress caused by desiccation. (After Shinozaki and Yamaguchi-Shinozaki 1997)

The regulation of gene expression at the transcription level requires the interaction of *cis*-acting elements in the promoters with corresponding transcription factors, usually DNA-binding proteins. The *cis*-elements are fairly well known, but knowledge of transcription factors is still patchy. The **ABA responsive *cis*-element** is a highly conserved motif, the so-called ABRE (ABA-responsive element), which consists of the base sequence Pur**PyrACGTGG**PyrPur (Pur, Pyr: purine base, pyrimidine base). The central piece of this element is the sequence CACGT(G,C), the so-called **G-box**, which is typical of promoters of many stress- and light-responsive genes.

Changing of only two of the conserved bases in ABRE reduces the responsiveness of this *cis*-acting element enormously. The transcription factors binding to ABRE are the G-box-binding proteins, which belong to the so-called **basic leucine zipper** (so-called bZIP proteins), containing several basic amino acids after the zipper motif. How ABA activates the bZIP protein so that it binds to the ABRE is not yet known.

Natural genes usually contain several ABREs (Fig. 1.5.6 A). Thus, those promoters that contain a single ABRE react only weakly to ABA. They

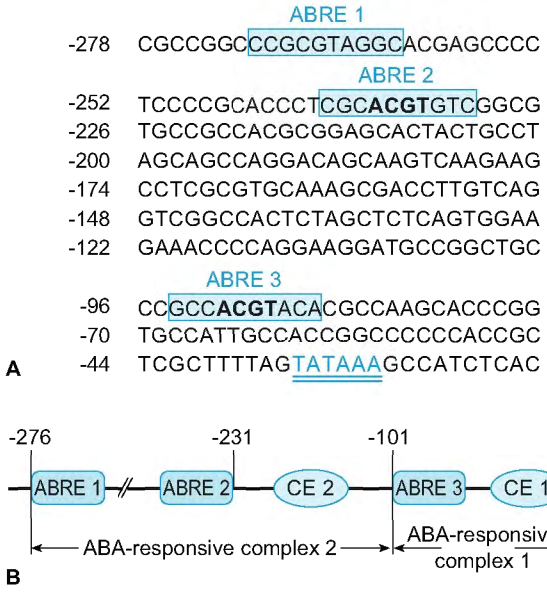


Fig. 1.5.6. The HVA22 promoter from barley (*Hordeum vulgare*) contains several ABA-responsive elements (ABREs) and further *cis*-active elements (CEs). A The complete HVA22 promoter with the ABREs 1–3 and the TATA box. B ABREs and CEs form a complex that determines signal specificity. (After Shen and Ho 1995)

require a second promoter element for strong reactions. The dehydration protective protein of barley is called the coupling element (CE3), with a base sequence similar to that of ABRE (PurACGCGTGTC) and can even be experimentally replaced by it, without affecting gene expression. Both elements bind the same ABA-responsive bZip-factor TRAB1 (Hobo et al. 1999). This factor also binds to other ***non-G-box*-ABREs**.

The specificity of the gene response to ABA requires further DNA-binding proteins. In the *rab17* gene of maize, nine promoter elements have been identified, which bind to such proteins. Five of these elements are ABREs, four contain other motifs. Six of these *cis*-elements induce the strong expression of the *rab 17* gene in the course of embryo maturation in the ripening seed, three are responsible for expression of the gene specifically in leaves (Busk et al. 1997). The principle of combining two or several promoter elements ("bipartite promoter"), for example, a general ABA-responsive element with one element mediating signal and site specificity, is widespread (Fig. 1.5.6 B; Shen and Ho 1995).

It is assumed that certain DNA-binding proteins, such as the VPI, represent a new class of nucleus-specific factors, so-called **DNA chaperones**, which modify the structure of DNA in a way which is not sequence-specific and thus enables the binding of other regulatory factors.

Not all ABA-responsive genes possess a G-box containing ABRE. They are activated by products of other genes, the synthesis of which is induced by ABA (Fig. 1.5.7). The gene *Rd22*, expressed in drought-stressed *Arabidopsis* plants, probably coding for a protective protein, reacts to ABA but does not possess an ABA-responsive element in its promoter. This promoter possesses motifs known to bind frequently occurring transcription factors, e.g. homologues of the human gene activators MYC and MYB (Hiroshi et al. 1997). The bZIP proteins mentioned above belong also to that group of transcription factors. In this case, they react with motifs similar to the G-box in the promoter. This mode of ABA-mediated induction of genes whose promoters do not contain an ABRE is apparent in drought but has been shown also for salt and cold stress and, of course, after application of ABA.

1.5.2.3 ABA-Independent Gene Activation by Drought

Besides gene expression mediated by ABA, there are several examples in which drought alone or together with salt or cold stress lead to activation of a promoter without participation of ABA. The best-investigated gene of this type is *Rd29A*, which is activated in all vegetative tissues of *Arabidopsis* upon desiccation and probably codes for a thiolprotease. Such ABA-independent genes contain also a consensus sequence in the promoter (TACC-GACAT), the so-called **dehydration responsive element (DRE)** or **C-repeat** (Yamaguchi-Shinozaki and Shinozaki 1994). It is assumed from the frequent occurrence of this motif that specific DNA-binding proteins (DRE-binding proteins) exist. It could be shown that the *Rd29A* promoter contains a DRE as well as an ABRE. However, the ABRE is only activated during prolonged drought stress. The DRE was also found in promoters of genes activated by cold, the so-called COR genes (cold response), but then was ultimately controlled by ABA via a further cascade.

Figure 1.5.7 presents an overview of the pathways leading to gene expression induced by drought.

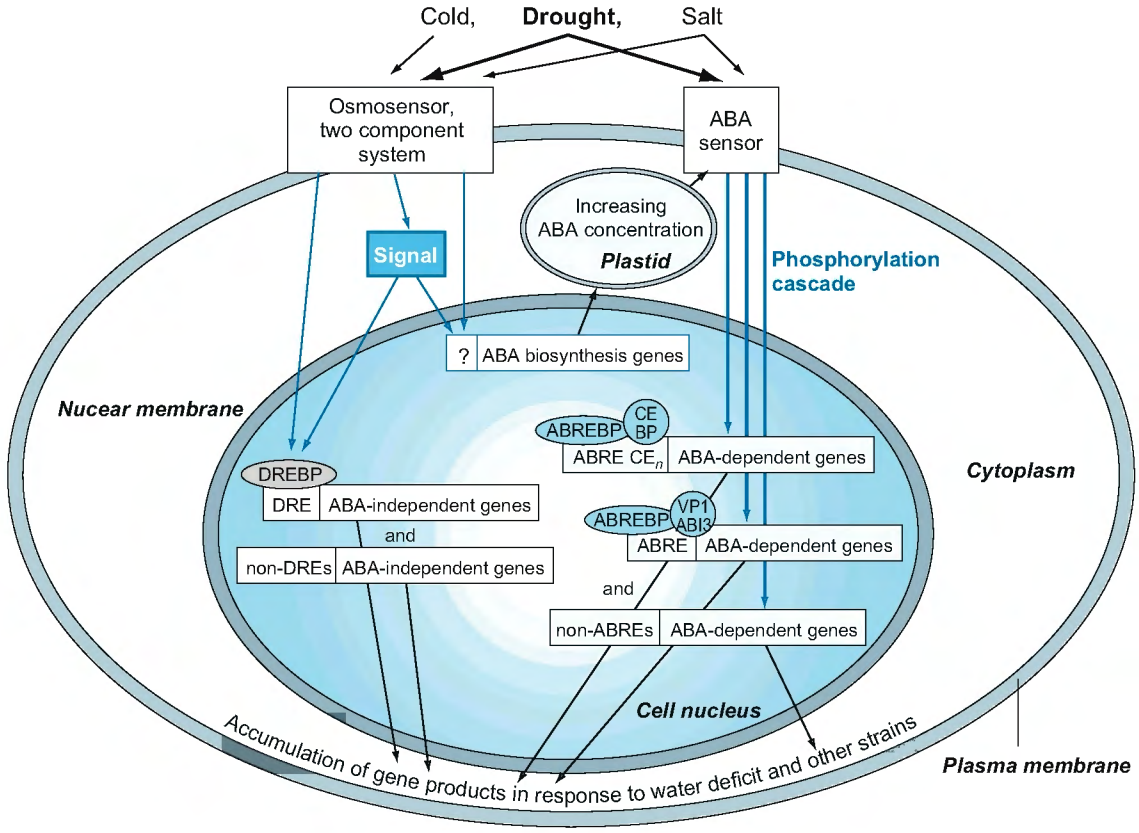


Fig. 1.5.7. Multiple pathways which lead to induction of gene expression upon stress by desiccation. *DRE* Dehydration responsive element; *DREBP* DRE-binding protein; *ABRE* abscisic acid binding element; *ABREBP* ABRE-binding protein; *CE* coupling element; *CEBP* protein binding to CE; *VP1/ABI3* sequentially akin proteins which regulate a MYB-related transcription factor. (After Bray 1997)

1.5.2.4 Signal Transduction

There is very little information on transduction cascades, from the various drought signals to the promoters of genes. It is well established that Ca^{2+} and inositol 1,4,5-trisphosphate, liberated from the membrane lipid phosphatidylinositol by phospholipase C, act as secondary messengers, giving rise to protein phosphorylation and dephosphorylation at the next lower level of the signal transduction cascade. In some cases, the participation of MAP kinases (mitogen-activated protein kinases) has been shown (Neill and Burnett 1999). It should also be noted that there are internal receptors for ABA in the cell (see Sect. 1.5.2.2) and that an interaction of various signals, a so-called signal cross talk with signals like gibberellins, requires further biochemical mechanisms (Shinozaki and Yamaguchi-Shinozaki 1997).

1.5.2.5 Function of Proteins Induced by Dehydration Stress

As mentioned above, water deficit induces proteins, some of which increase the stress tolerance of cells more or less directly (functional proteins), and others which are part of a signal transduction chain (regulatory proteins; see Fig. 1.5.5), leading finally to the synthesis of functional proteins.

Functional proteins are mainly aquaporins, or enzymes which catalyse the biosynthesis of **osmolytes** (compatible solutes, e.g. various carbohydrates, amino acids and betaines), also proteases for the degradation of damaged proteins (ubiquitin, thiolproteases) and ROS-detoxifying enzymes[2], such as catalase, SOD, ascorbate peroxi-

[2] During drought, stomata close (see Chap. 2.2, Fig. 2.2.19), resulting in CO_2 shortage if the light intensity is high. This leads to the formation of ROS and radicals.

dase, glutathione-S-transferase, etc. Overexpression of these genes or of gene constructs, in which the corresponding proteins are directed by specific signal peptides into sensitive cell compartments, leads to increased drought tolerance and to a stabilisation of metabolic processes, such as photosynthesis (this will be discussed in greater detail in Chap. 1.6.2, salinity stress).

A special group of protective proteins are **dehydrins, LEA** or **Rab proteins**, which occur in all plants (Campbell and Close 1997); apparently, they do not possess any enzymatic activity, but are very effective in protecting the cellular membranes and proteins with quaternary structures (Close 1996). Unfortunately, the nomenclature of these protective proteins is not yet conclusive[3].

These protective proteins occur regularly during desiccation of the embryo and the endosperm when seeds ripen. Discovered in 1981, they were therefore called **late embryogenesis abundant (LEA) proteins**. They belong to the group of drought-induced proteins, the promoters of which contain one or several ABREs, and thus can be directly induced by ABA. Therefore, they have also been termed **Rab (responsive to ABA) proteins**. Most of them occur not only during seed ripening, but form and accumulate also during dry periods and upon osmotic stress in all plant organs. Being of particular importance for poikilohydric plants, they are also known as **dehydrins**. According to their structural characteristics (consensus sequences) and the high proportion of certain, usually hydrophilic, amino acids, LEA proteins were classified into 18 families, representatives of four of which are widely distributed.

Most of the known LEA proteins have not yet been isolated as proteins but are only known as genes or as mRNAs, which become prominent during drought. They are all nuclear coded and occur mainly in the cytoplasm, but also in the nucleus. Most strikingly, they cannot be denatured by boiling or acid treatment, probably because they do not have a globular structure, but rather form **random coils** or **amphiphilic α-helices**. Such helices carry on one lateral side hydrophobic, on the other side, hydrophilic functional groups (Fig. 1.5.8). Random coil proteins contain significantly more water than helical proteins. The size of the LEA proteins, however, is very variable, ranging from 15 to almost 200 kDa.

[3] One of these proteins is also known under the name of osmotin (see Chap. 1.6.2.3).

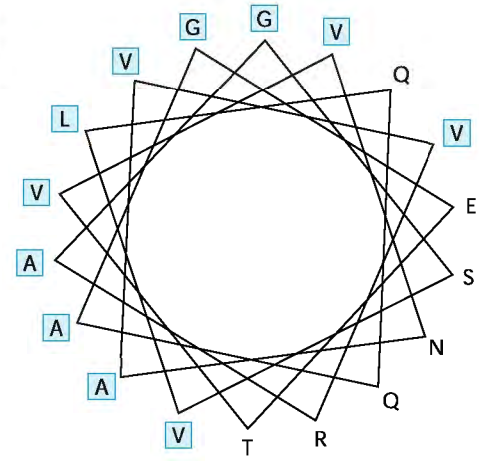
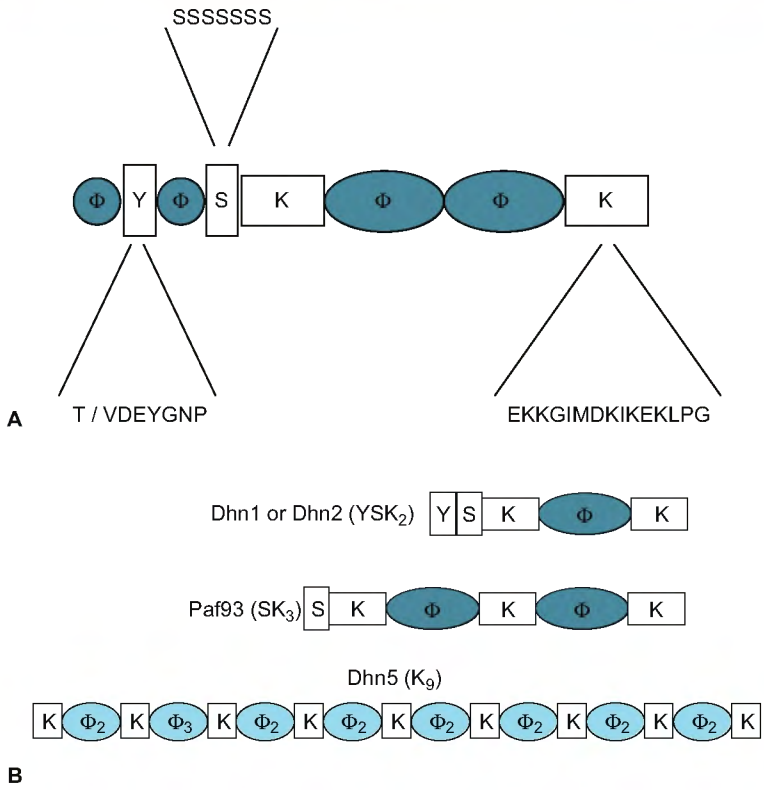


Fig. 1.5.8. Representation of a LEA protein as a helical wheel. Hydrophobic amino acid residues are highlighted by *boxes*, thus illustrating the amphiphilic character of the helix. Amino acids are represented by the *single letter code*. (After Curry and Walker-Simmons 1993)

Individual families of these proteins will not be discussed here in detail, only dehydrins as prominent representatives shall be described, as there are about 80 currently known types belonging to the LEA-D11 family. Of this family, a further 200 or so species are known from partial gene sequences or cDNAs. In some plant species, they are constitutively expressed, but always accumulate under dehydration, forming then between 0.1 and 5% of the total soluble protein or of the total mRNA. These proteins contain between 82 and 575 amino acids, and are modularly structured with many repeats. The 15 amino acids comprising **K(rich)segment** (EKKGIMDKIKEKLPG, the antigenic component of dehydrins) is highly conserved in cyanobacteria, lower and higher plants, and often occurs in several copies. There is also the **S(rich)segment** containing up to seven serine residues which can be phosphorylated. The **Y(rich)segment** (T/VDEYGNP) is commonly at the N-terminus of many dehydrins in one or several copies. These modules are interconnected by less conserved segments, the so-called ***Φ*-segments**. The amino acids cysteine and tryptophan are completely missing, which could explain the stability of these proteins against denaturing treatment and chemicals. The YSK_2 dehydrin from maize seeds is given as an example of the modular structure of dehydrins (Box 1.5.4).

Many dehydrins contain a bipartite nuclear localisation sequence, suggesting a protective role of these proteins in the cell nucleus. They

Box 1.5.4 Typical structure of dehydrins



The YSK_2-dehydrin from maize seeds shows the typical structure of a dehydrin with Y- and K-consensus sequences (A). The number of serine residues in the S-segment is variable. *Φ* indicates less conserved sequences, which may be repetitive in individual dehydrins (after Close 1997). B shows dehydrins from barley with different combinations of the conserved motifs: YSK_2, SK_3, and K_9 (after Campbell and Close 1997).

can accumulate in "protein bodies", but also can stabilise cellular membranes by preventing the disintegration of the bilayer into lipid droplets (hexagonal structure of lipids; see Fig. 1.3.16) upon attenuation of the stabilising water film. The hydrophobic stretches of the amphiphilic *α*-helices interact with the surface of the membranes, whilst the hydrophilic parts are oriented towards the cytoplasm (see Fig. 1.3.24). Preventing the formation of globular lipid structures, they have been called "reversible chaperones". Protection of membranes with dehydrins hinders the accumulation of low-molecular ions at the membrane surface upon withdrawal of cellular water which would result in a change of the membrane potential of that membrane. Dehydrins are very suitable for genetic transforma-

Table 1.5.1. Growth of rice plants, transformed with the *HVA1* gene from barley under drought stress. The plants were exposed to several cycles, each consisting of 5 days of drought and 2 days of recovery. Leaf growth was measured before, and 3 days after, the onset of drought. Leaf growth rates of the two youngest leaves were measured. The height of the plants was measured after four stress cycles and an additional 2 days of recovery (Xu et al. 1996)

Line	Leaf growth rate (%)	Height of plant (cm)	Root fresh weight (g)
Wild type	69	22±1.4	0.9±0.1
Line 30	90	29±1.1	1.4±0.1
Line 36	129	37±1.8	2.1±0.1
Line 41	113	33±1.8	2.3±0.3

tion in drought-sensitive plants. For example, the *HVA1* gene from barley (*Hordeum vulgare*) was successfully transferred to rice; transgenic rice plants were considerably more tolerant to drought and salt (Table 1.5.1; Xu et al. 1996).

1.5.2.6 Accumulation of Osmolytes (Compatible Solutes) During Drought

In addition to desiccation-induced synthesis and accumulation of dehydrins, protection of cells and tissue by low molecular (mass) solutes is also very important. Formation of enzymes which catalyse the synthesis of such protective substances (**compatible solutes**) is also directly or indirectly induced by ABA (see Fig. 1.5.5). The protective action of compatible solutes on stressed cells is attributed to two effects:

1. Increase in the osmotic potential counteracts the osmotic dehydration of the cells. Compatible solutes, therefore, are also called **osmolytes**. They can achieve considerable concentrations, often 5–10% of the dry weight. Upon extreme water loss, e.g. during the drying of poikilohydric plants, solutions of sugars such as sucrose adopt a glassy almost solid state, which effectively prevents the disintegration of biomembranes into lipid droplets (the so-called hexagonal-II phase, see Fig. 1.3.16). However, only solutions of such carbohydrates which do not readily crystallise can achieve this state.

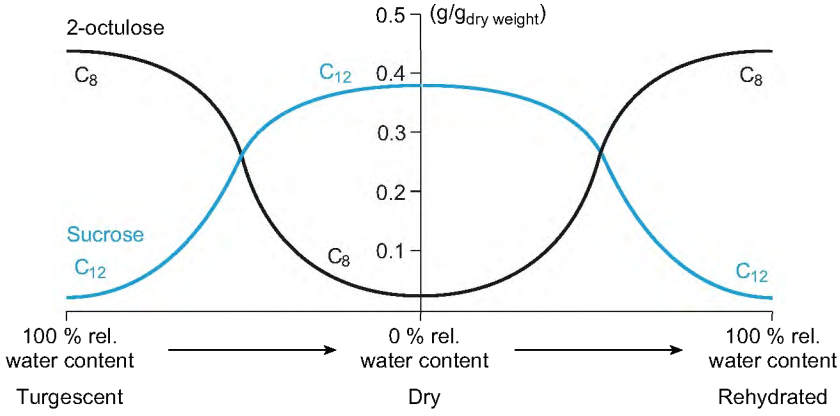


Fig. 1.5.9. Changes in the proportions of soluble carbohydrates in correspondence with the water relations of the resurrection plant *Craterostigma plantaginea*. In the fully hydrated state, leaves mainly contain the C_8 sugar octulose, which upon desiccation is almost quantitatively converted to sucrose, a compatible solute. This reaction is reversible. (After Bartels et al. 1993)

Table 1.5.2. Accumulation of metabolic products in organs of terrestrial plants under drought stress and their physiological effects

Type of compound	Compounds	Function upon drought	Cross-protection
Ions	Potassium	Osmotic balance, acquisition of macronutrients, sodium exclusion and export	Salt
Proteins	LEA/dehydrins, osmotin, SOD/catalases	Membrane and protein protection, pathogen-related proteins, detoxification of radicals	Frost, defense against pathogens
Amino acids	Proline, ectoin	Osmotic balance, membrane and protein protection	Frost
Sugars	Sucrose, fructans	Osmotic balance, membrane and protein protection, storage of carbohydrates	Frost
Polyols	Acyclic (e.g. mannitol), cyclic (e.g. pinitol)	Osmotic balance, membrane protection, storage of carbohydrates, radical scavengers	Frost, salt
Polyamines	Spermine, spermidine	Ion balance, protection of chromatin	Anaerobiosis
Quaternary amines	Glycinebetaine, β-alaninebetaine	Membrane protection	Frost, salt
Tertiary sulfonium compounds	Dimethyl sulfoniopropionate	Membrane protection	Frost, salt
Pigments and carotenoids	Carotenoids, anthocyanins and betalains	Protection against excess light and photoinhibition	

2. Because of their increased concentration and high water solubility, compatible solutes compete with other dissolved materials, particularly with ions of high charge density, displacing them from the surfaces of biomembranes and proteins. Owing to their hydrophilic characteristics, they are able to become a constituent part of the structured portion of the water film on the membrane surface. Carbohydrates which provide only relatively weak protection may become reversibly converted into more effective ones, such as sucrose during drought stress (Fig. 1.5.9).

Besides carbohydrates and their derivatives (sugar alcohols, cyclitols) in drought stress, quaternary ammonium bases (so-called betaines) and tertiary sulfonic acids (Fig. 1.5.10), amino acids (mostly proline, Box 1.5.5) and polyamines may accumulate as protective osmolytes (Fig. 1.5.11). In Table 1.5.2 such osmolytes are listed and characterised.

Besides dehydrins, enzymes which catalyse the biosynthesis of compatible solutes are also suitable targets for genetic transformation, in order to produce crop plants that are less sensitive to drought stress (Fig. 1.5.12).

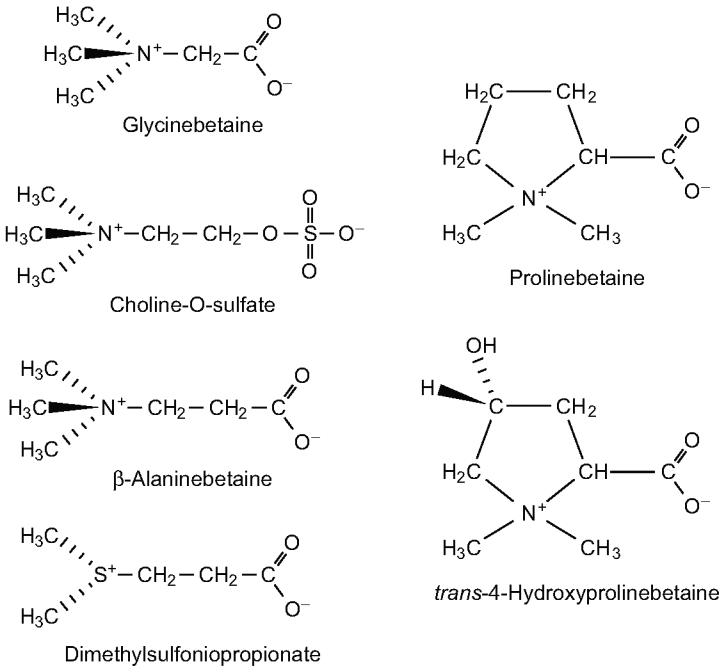


Fig. 1.5.10. Some quaternary amino compounds and tertiary sulfonium compounds which have been identified as compatible solutes in plants. The amphiphilic group $(CH_3)_3$–N^+...O^- renders these compounds their membrane-protective capacity. (Hanson 1993)

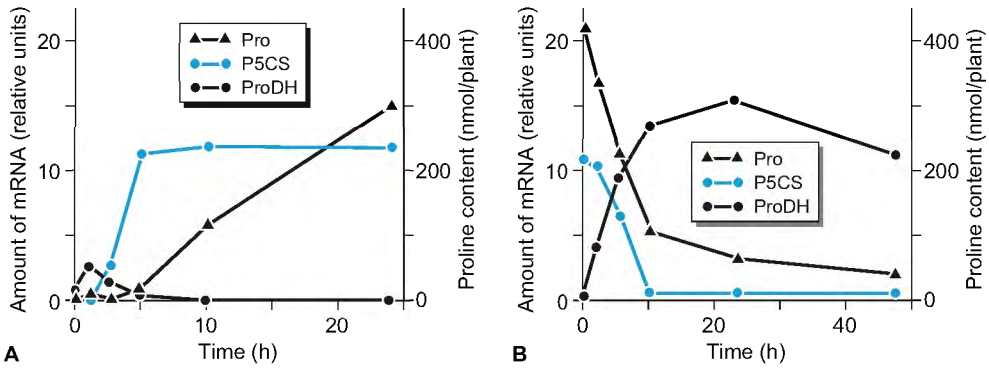
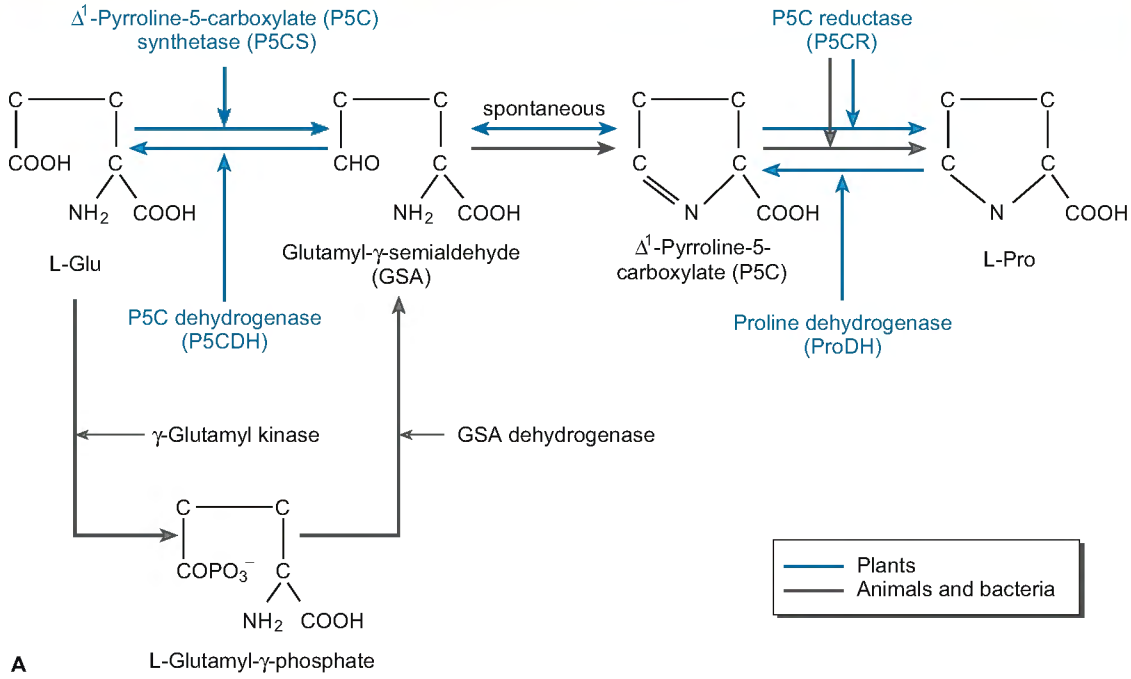
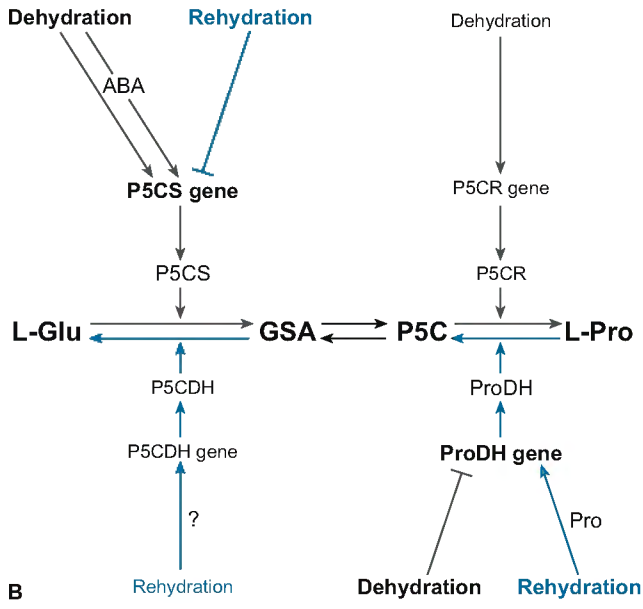


Fig. 1.5.11. Time course of accumulation and decrease in proline (Pro), the mRNAs for Δ^1-pyrroline-5-carboxylate synthase (P5CS), and for proline dehydrogenase (proline oxidase, ProDH) in *Arabidopsis* during the development of drought (A), and 10 h after rehydration (B). (After Yoshiba et al. 1997)

Box 1.5.5 Proline metabolism



A



B

A shows a comparison of biosynthesis and metabolism of L-proline in plants and in bacteria. In green plants, the main precursor of proline is L-glutamic acid. However, when subjected to osmotic stress and N limitation, ornithine can be the precursor. The initial enzyme in proline metabolism is proline dehydrogenase, often also called proline oxidase (after Yoshiba et al. 1997).

B shows the regulation of proline synthesis under drought stress and subsequent rehydration. In the unstressed plant, proline synthesis and degradation are in balance and the cellular proline concentration is very low. The rise in the proline level occurs by activation of the gene for Δ^1-pyrroline-5-carboxylate synthetase and concomitant inhibition of expression of the proline dehydrogenase (proline oxidase) gene. Activation of P5CS gene expression takes place dependently as well as independently of ABA. In the well-watered status, expression of this gene is extremely low. Upon rehydration proline dehydrogenase is activated by the high concentration of proline that accumulated during the period of drought. In contrast, the second gene involved in proline synthesis, the P5CR gene, is neither significantly influenced by drought nor by ABA (after Yoshiba et al. 1997).

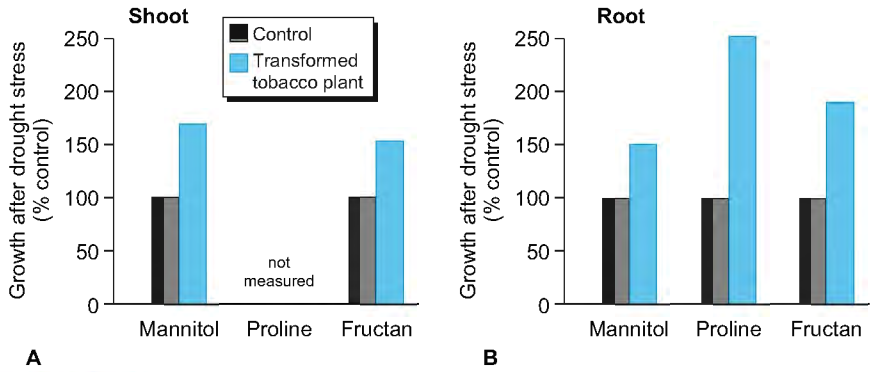


Fig. 1.5.12. Accumulation of osmolytes in genetically modified tobacco plants under drought stress. The gene for mannitol-1-phosphate dehydrogenase (from *E. coli*) or P5CS (from *Vigna aconitifolia*) or levan-sucrase (from *Bacillus subtilis*) was transferred into tobacco using *Agrobacterium tumefaciens*. All three metabolites accumulate in the transformed plants in comparison to the controls, and there was a significant increase in growth of the transgenic plants compared with the controls under drought, and in the case of P5CS also upon salt stress. (After Bray 1997)

1.5.3

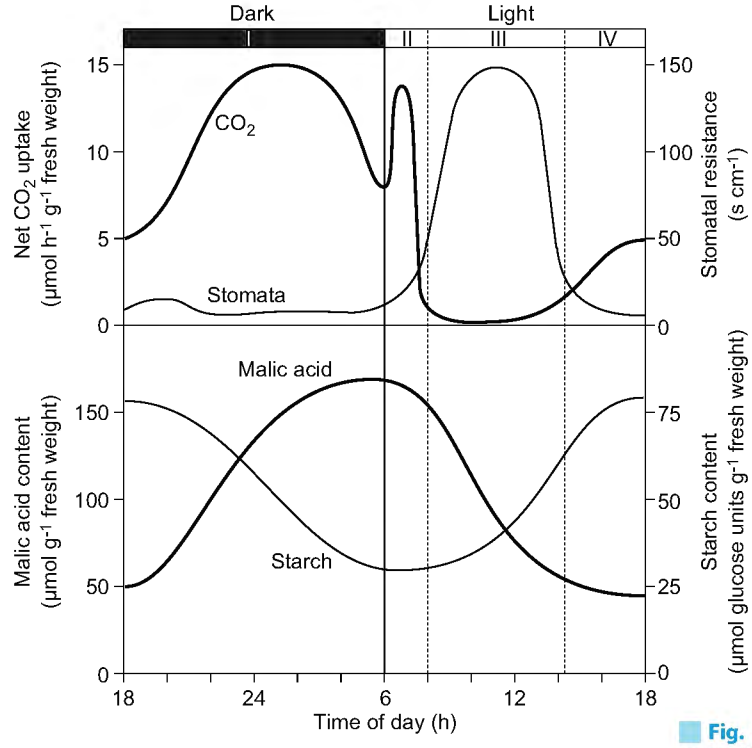
CAM (Crassulacean Acid Metabolism)

Recommended Literature

- Cushman JC, Bohnert HJ (1999) Crassulacean acid metabolism: molecular genetics. Annu Rev Plant Physiol Plant Mol Biol 50:305–332
- Griffith H (1989) Carbon dioxide concentrating mechanisms and the evolution of CAM in vascular epiphytes. In: Lüttge U (ed) Vascular plants as epiphytes. Evolution and ecophysiology. Ecological studies 76. Springer, Berlin Heidelberg New York
- Lüttge U (1993) The role of crassulacean acid metabolism (CAM) in the adaptation of plants to salinity. New Phytol 125:59–71

A particular type of metabolic response to drought is the so-called **crassulacean acid metabolism (CAM)**, which is also addressed in Chapter 2.4.1.2 (Fig. 2.4.3). Plants growing in dry and hot regions (where transpiration often greatly exceeds water loss) with this type of metabolism avoid "dying of thirst" and "dying of hunger" (due to limitation of photosynthetic CO_2 uptake by closed stomata) by opening their stomata only at night, when the humidity deficit of air due to low temperatures is fairly small. In this case, CO_2 is taken up in the dark and, catalysed by **PEP carboxylase**, bound to phosphenolpyruvate (PEP). The resulting oxaloacetic acid is converted into malic acid by **NAD-dependent malate dehydrogenase** in the cytosol. Malic acid is then stored in large amounts in the vacuoles (acidification up to pH 3), which can easily be tasted! During the day, malate is released from the vacuole (de-acidification) and converted, in the chloroplast, by the **NADP-dependent malic enzyme** into CO_2, reduction equivalents (NADPH) and pyruvate. CO_2 is assimilated in the reductive photosynthetic carbon cycle (Calvin cycle). Pyruvate may be metabolised in different ways: direct respiration (in the mitochondria) as well as phosphorylation to PEP by **pyruvate P_i dikinase** in the chloroplast are discussed. Phosphoenolpyruvate may leave the chloroplast via a specific **PEP translocator** and contribute to glycolytic or gluconeogenetic carbon flow. The product of photosynthesis, starch, is stored in the chloroplast and used for PEP formation during the night.

As CAM plants are able to fix only as much CO_2 by PEP carboxylation as PEP is supplied, those only relying on the CAM cycle would not be able to grow. In the extreme, CAM is thus a mechanism for survival but does not allow growth. With sporadic precipitation, and in the early morning hours and perhaps also in late afternoon, stomata usually open or are at least not fully closed, so that direct net photosynthesis and growth are possible. Gas exchange, content of malic acid and starch, as elements of photosynthesis, are shown for the course of a day in Fig. 1.5.13.



A

Phase	I	II	III	IV
Net CO_2 fixation	From the atmosphere	(From respiration)		
Malic acid accumulation	Vacuolar influx	Vacuolar efflux		
Storage glucan	Glycolysis	Gluconeogenesis		
PEPC	Active	Inactive		
PEPC kinase	Active	Inactive		
Rubisco	Inactive	Active		
ME/PEPCK	Inactive	Active		
Stomata	Open	Closed		
Photoperiod	Dark	Light		

B

Fig. 1.5.13. Typical gas-exchange curve (CO_2 uptake rate and stomatal resistance = stomatal conductance^{-1}) of a CAM plant. The changes in concentration of the typical metabolites starch and malate are also presented. Gas exchange shows the phases *I* nocturnal CO_2 fixation; *II* dawn with the start of CO_2 fixation by Rubisco; *III* assimilation of the internally released CO_2 by Rubisco with stomates closed; *IV* stomates begin to open after the internal CO_2 has been depleted and daytime temperatures are dropping. In B, the time courses of the different reactions are shown in different shades of *blue*. A from Lüttge et al. (1994); B from Cushman and Bohnert (1999)

1.5.3.1 Flexibility of CAM

CAM plants are superior to other photosynthetic types in their **water use efficiency (WUE)**, but their photosynthetic rates and growth rates are much lower (Table 1.5.3).

During long periods of drought, stomata remain continuously closed, even at night; fixation and assimilation of CO_2 are limited to carbon dioxide generated internally in the plant tissue by respiratory processes. This state is called **CAM idling** (Fig. 1.5.14). The photosynthetic assimilation of internal CO_2 provides some protection from photooxidation. However, the low internal CO_2 content, at the usually extremely high radiation, is not sufficient to allow a substantial flow of electrons and full relief of the photosystems. Consequently, radicals are formed and oxidative stress occurs. Therefore, CAM idling is of only limited use, as it can only be sustained for a relatively short time, and because even with closed stomata water is lost by cuticular transpiration.

Many bromeliads show diurnal CAM metabolism, even though their stomata are not – or are only partially – closed during the day when they perform "normal" C3 photosynthesis. This is called **CAM cycling**. An extreme example of CAM cycling occurs in the usually submerged

Table 1.5.3. Water use efficiency, photosynthesis and biomass production of C3, C4 and CAM plants. (After Lüttge et al. 1994)

Type of photosynthesis	C3	C4	CAM
Water use efficiency (g water/g C)	450–950	250–350	18–100 (nighttime); 150–600 (daytime)
Maximum rate of net photosynthesis (μmol CO_2 m^{-2} s^{-1})	9–25	25–50	0.6–8
Growth (g biomass m^{-2} day^{-1})	50–200	400–500	1.5–1.8

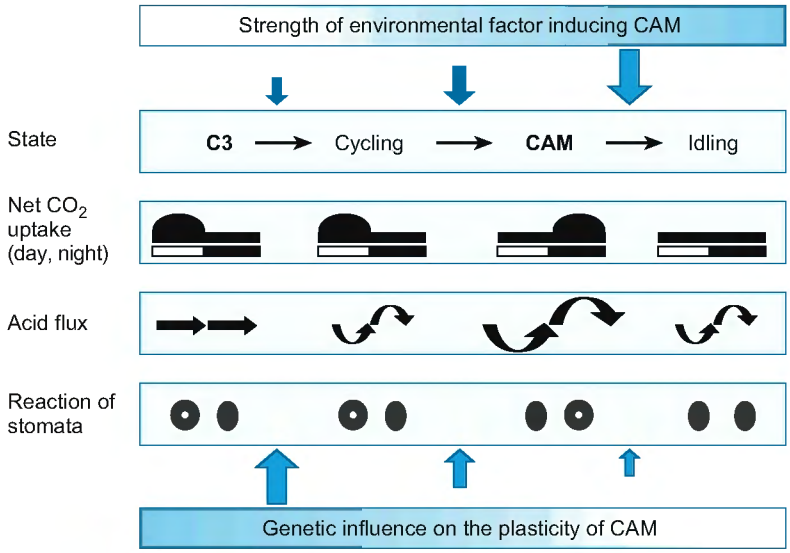


Fig. 1.5.14. Plasticity of CAM in facultative CAM plants. (After Cushman and Bohnert 1999)

water plant *Isoëtes* (*howellii*). The epidermis of this plant has, of course, no stomates and the plant does not experience water stress (Keely 1998); the reason for the CO_2 fixation during the night is assumed to be lack of CO_2 in the usually acidic waters where *Isoëtis* grows.

Many CAM plants, particularly those from the families of Crassulaceae and Aizoaceae, are C3 plants with a facultative, i.e. inducible, CAM. Perennial facultative CAM plants are able to switch between the two modes of photosynthesis many times during their life: C3 photosynthesis – CAM – CAM idling – C3 photosynthesis, ...

Drought or high salinity, and in some cases also day length (short day in *Kalanchoë*, *Aloe* and *Opuntia*), can also induce CAM. In some species, dependence of CAM induction on the age of the individual plant has been described: This holds for agaves or cacti, whose saplings cannot produce vacuoles that are large enough for the storage of sufficient malic acid, as do the adult individuals (Fig. 1.5.15).

CAM induction need not occur in all photosynthesising organs of a plant. The succulent stem of *Frerea indica* (an asclepiad) performs

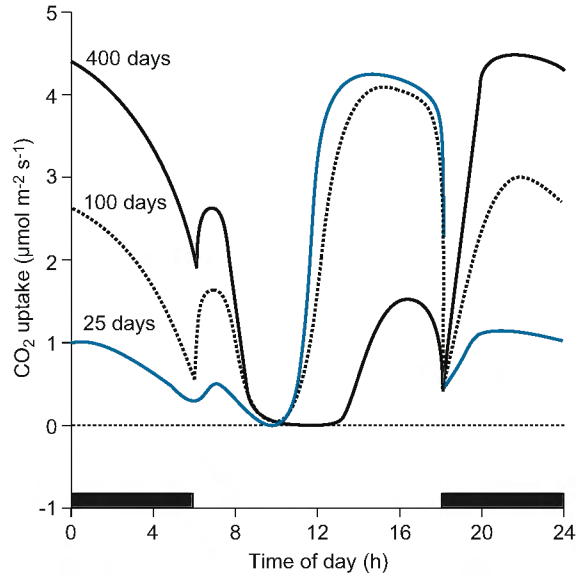


Fig. 1.5.15. Daily net CO_2 uptake by seedlings and plantlets of *Agave deserti* in its first 13 months. The seedlings were 25 days old and 14 mm in height; at 100 days their average height was 31 mm, and 400-day-old plantlets were 63 mm in height. The four phases of diurnal CO_2 uptake typical of CAM plants were only clearly visible after 1 year of growth, despite the fact that the young plants are capable of CO_2 fixation at night. (After Nobel 1988; Larcher 1994)

CAM, whilst the green leaves at least in rainy periods photosynthesise by the C3 mode; in tropical *Clusia* species, with opposite leaves at a node, one leaf may perform C3 photosynthesis, whilst the opposite leaf uses CAM (Lüttge 1987). This ecophysiological plasticity allows the C3-CAM intermediates optimal adaptation of their photosynthetic capacity to the prevailing conditions, making effective use of the humid season or of sporadic precipitation.

Whether a facultative CAM plant, or a part of the plant, follows C3 photosynthesis or CAM photosynthesis can be realised using the **δ^{13}C value**. Carbon dioxide in the air consists of 98.89% of the ^{12}C isotope and 1.11% of ^{13}C. The content of the radioactive isotope ^{14}C is, compared with those values, negligible (10^{-10}%). Rubisco consumes CO_2 as substrate and discriminates more strongly between ^{12}C and ^{13}C than does PEP carboxylase (PEPC), which uses HCO_3^- instead of CO_2. Carbon assimilated only by Rubisco thus contains less ^{13}C than that fixed first by PEPC. The **change of ^{13}C** in the biomass, therefore, allows the realisation of the mode of CO_2. The ratio of ^{13}C:^{12}C is determined by mass spectrometry and calculated with the following formula:

$$\delta^{13}\mathrm{C}[‰] = \left(\frac{^{13}\mathrm{C}/^{12}\mathrm{C}\text{ of sample}}{^{13}\mathrm{C}/^{12}\mathrm{C}\text{ of standard}} - 1\right) \times 1000$$

The standard is a defined limestone.

δ^{13}C values of C3 plants are around –28‰, those of C4 plants at –14‰, and for CAM plants with predominantly nocturnal CO_2 fixation between –10 and –20‰, and for daytime CO_2 fixation between –25 and –34‰.

1.5.3.2 CAM Causes Problems in Biochemical Regulation

Various instructive reaction schemes were developed to show the biochemistry of CAM (see Lüttge et al. 1994; Heldt 1997) and therefore need not be presented here.

The understanding of CAM as PEPC-mediated dark fixation of carbon dioxide, more exactly of HCO_3^-, the transient storage in the form of malic acid in the vacuole and the release by the malic enzyme for the final assimilation by Rubisco, assumes a complicated network of regulatory characteristics and processes.

Two of these metabolic processes will be discussed in more detail, namely the storage of malic acid in the vacuole and the competition for CO_2 between PEPC and Rubisco.

Storage of Malic Acid in Vacuoles

The tonoplast is the membrane that solves the problem of regulating transitory malic acid storage in the vacuole: During dark fixation of carbon dioxide, malic acid must be imported against the concentration gradient into the vacuole (and be retained there), but, during daytime, flow (probably even controlled) in the opposite direction must be enabled. The import of malic acid is well understood, but export still poses certain unsolved questions. A pH value of 7 (to perhaps 8) is assumed in the cytosol; at those values malic acid is dissociated as the pH value of both carboxyl groups is in the acidic range (C1: at pH 3.1; C4: at pH 5.1). Import of divalent malate ions into the vacuole would soon come to an end and can only be maintained if the negative charges are balanced by positive charges. This is achieved through the **proton-ATPase of the tonoplast (V-ATPase)**, which is supplied with energy from glycolysis together with a **pyrophosphate-dependent proton pump**. Uptake of the divalent malate ion into the vacuole takes place via **dicarboxylate channels** which transport malate as well as fumarate. Because of the acidification of the vacuole (to pH 3.5–3.0) malate becomes increasingly protonated and finally only malic acid is present. This is also important for osmotic reasons, because three osmotically active solutes ($2\times H^+$ and malate) unify. Little is known about the efflux of malic acid from the vacuole. The undissociated acid could permeate the tonoplast via the "lipid path" (see Sect. 1.5.1). Some results also suggest a carrier in the tonoplast which catalyses a malate/$2H^+$ symport. However, why this carrier is not active during dark fixation has not yet been elucidated. It is interesting that a decrease in temperature during the night is an absolute requirement for acidification: During warm nights, there is no accumulation of malic acid in vacuoles (Lüttge et al. 1996).

The CAM-PEP Carboxylase as Pacemaker Enzyme

PEPC is a cytosolic enzyme which binds bicarbonate to PEP in an irreversible reaction:

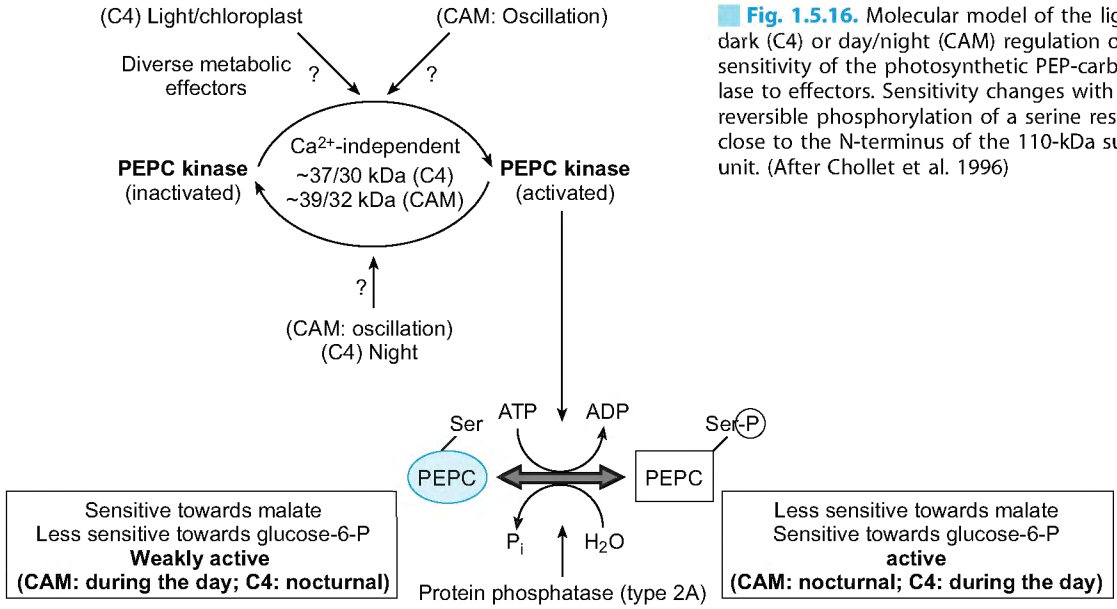


Fig. 1.5.16. Molecular model of the light/dark (C4) or day/night (CAM) regulation of the sensitivity of the photosynthetic PEP-carboxylase to effectors. Sensitivity changes with the reversible phosphorylation of a serine residue close to the N-terminus of the 110-kDa subunit. (After Chollet et al. 1996)

$$PEP + HCO_3^- + Mg^{2+} \rightarrow \text{oxaloacetate} + P_i + Mg^{2+}$$

In the reaction, the energy-rich phosphate group of PEP is transferred to the bicarbonate ion forming carboxyphosphate as an intermediate.

The final product of the reaction, oxaloacetate, is an unstable β-keto acid and is reduced by NAD malate dehydrogenase to the stable malate. The NADH originates from glycolysis.

Since PEPC does not react with CO_2 but with HCO_3^-, the carboxylation of PEP is insensitive to O_2, in contrast to the carboxylation of RuBP by Rubisco.

PEPC is an extensively analysed plant enzyme because of its function as the pacemaker enzyme of CAM. Differences in the reported kinetic parameters (Michaelis constant, v_{max}) are not because of the different sources of the enzyme but are due, most of all, to differences in the isolated protein; often a protein was isolated which had been partially truncated by proteolysis. Furthermore, the enzyme also reversibly forms dimers or tetramers, which originally was regarded as allosteric regulation of the mechanism. The activity of the enzyme is allosterically influenced by effectors. Glucose-6-phosphate and triosephosphate stimulate activity, whilst malate and aspartate have a strong inhibitory effect, particularly at a slightly alkaline (cytosolic) pH. The regulation of the CAM-PEPC has been shown to take place via the reversible phosphorylation of a serine residue of the 110-kDa subunit (e.g. Ser 8 for millet, Ser 15 for maize) near the N-terminus. Phosphorylation at this single site renders the enzyme insensitive to malate; even in the presence of this allosteric inhibitor it remains in the active form, in particular as it now reacts more sensitively to the positive effectors mentioned above. The phosphorylation of CAM-PEPC occurs through a strongly regulated **protein kinase**, dephosphorylation (and inactivation) by a **protein phosphatase** of the type 2A. This phosphatase does not show any fluctuations in activity, in contrast to the kinase. The activity of the protein kinase is controlled diurnally, which, in contrast to the C4-PEPC, does not react to a change from dark to light (Fig. 1.5.16).

The question why CAM-PEPC does not compete during the day for CO_2 released by malic enzyme (in the chloroplast) can be answered by the consideration that the dephosphorylated enzyme is inhibited by malate. In addition to the post-translational regulation of activity by phosphorylation and dephosphorylation, there is another regulation through gene expression, which was observed during the induction of CAM in C3/CAM intermediates. The genome of *Mesembryanthemum crystallinum*, for example, contains two genes for PEPC, *Ppc1* and *Ppc2*. Regulation of expression is induced by drought, ABA and high salinity, where certain transcription factors recognise AT-rich regions in the promoter.

The problems of transitory malic acid storage in the vacuole and the diurnal oscillations of CAM-PEPC activity have been discussed here in more detail, but CAM also shows other peculiarities which are only partly understood, e.g. the question of what happens to the pyruvate formed during the release of CO_2 in the chloroplast. Some scientists assume it is used in regeneration of PEP catalysed by pyruvate P_i dikinase, analogous to the C4 pathway of photosynthesis, whilst others believe that respiration in the mitochondria is more probable. A further unsolved question is the opening of the stomates during the dark period, as stomata usually close in the dark (blue light is the effective component of the white light for the opening of stomata, see Fig. 2.2.15).

1.5.4

Anatomical and Morphological Adaptation to Drought

Besides physiological adaptations to drought, there are several anatomical and morphological responses of plants to this stressor: enlargement of root systems, improvement of hydraulic conductance and water transport systems, reduction of transpiring surfaces, increase in the stomatal density, production of a hairy tomentum, apoptosis of assimilation organs (shedding of leaves at the beginning of dry periods), and many more (see Chap. 2.2). All these changes are, of course, based on physiological processes; these and their regulatory mechanisms are still, to a large extent, poorly understood.

Summary

1. Life requires liquid water and is thus based on its particular physico-chemical properties which result from the dipole nature of the water molecule (the so-called anomalies of water).
2. The water potential, ψ, is a measure of the thermodynamic state of water in any system and is given in the dimension of pressure. The components are the osmotic potential of the cellular liquids, π, the water potential of the solid cell components (e.g. the cell wall), the so-called matrix potential, τ, and the pressure potential, i.e. the pressure of the cell wall on the protoplast which is numerically equal to the turgor pressure, i.e. the pressure of the protoplast on the cell wall.

$$(-)\psi = (-)\pi + (-)\tau + P$$

3. A water potential gradient between two systems (e.g. plant–air or plant–soil) causes water flow from the system with the lower (numerically less negative) water potential to that with the higher (numerically more negative) water potential. This is the reason for water loss from plants to the atmosphere. As a consequence of large water loss the protoplasts of the plant cells shrink concomitantly with a decrease of the wall pressure (which completely disappears at the turgor loss point or may even convert to a suction) and the plant wilts. During prolonged drought, so much water may be lost by transpiration that cellular membranes disintegrate, as can happen upon the loss of water by freezing dehydration.
4. The capacity of the "lipid path", i.e. the permeation of water through the lipid bilayer of the biomembrane, is not sufficiently high for a fast equilibration of water potential gradients in a cell. In these membranes, special integral proteins, so-called aquaporins, give rise to the high permeability of most biomembranes for water. The highest hydraulic conductivity of cellular membranes is usually with the tonoplast.
5. Desiccation is a natural phenomenon in the life cycle of a plant, for example, during seed ripening. Therefore, plants must have the capability to react in a manifold way to this stress. One reaction sequence is triggered by the phytohormone abscisic acid (ABA), but at least one other is ABA-independent. In these reaction cascades, transcription factors are synthesised, which upon interaction with other promoters switch on several genes: these genes may code for further regulatory factors or for proteins which contribute either directly (e.g. protective proteins) or indirectly (compatible solutes) to the maintenance of cell viability. The desiccation sensor or osmosensor of plants is not yet known; from yeast and bacteria such proteins are known however.
6. ABA synthesis is not via the mevalonate pathway, as previously assumed, but results from

degradation of the xanthophyll zeaxanthin. Biosynthesis of zeaxanthin, in turn, is synthesised in the recently discovered DOXP pathway (1-deoxy-D-xylulose-5-phosphate pathway).

7. Receptors for ABA are on the outer surface of the plasma membrane, as well as in the interior of the cell. Though their molecular structure is not yet known, modes of changing their effectiveness have been elucidated. In addition, details are known about secondary intracellular messengers (e.g. cyclic ADP ribose). Calcium, inositol-trisphosphate, protein kinases and protein phosphatases further take part in signal transduction. Reactions induced by ABA are either fast reactions such as the regulation of the stomates or slow reactions resulting from the expression of ABA-sensitive genes.
8. In stomatal movement, the apoplastic ABA signal is perceived by a receptor in the plasma membrane and the increase in the intracellular concentration of free calcium is one of the early steps in signal transduction. This activates anion channels, through which anions (malate, chloride) leave the cell, changing the membrane potential and triggering K^+ efflux. More than 90% of the ions exported from cells must first be released from the vacuole into the cytosol. The corresponding channels are also regulated by cytosolic free Ca^{2+}. Because of ion export from the guard cells and the concomitant release of water, turgor declines and the stomates close. In addition to their reaction to ABA, guard cells also react to other signals, e.g. blue light or the internal CO_2 concentration.
9. Cellular reactions to drought result from ABA-induced as well as from ABA-independent gene expression. The promoters of ABA-responsive genes contain the ABRE motif. This motif, in turn, contains the so-called G-box as "core motif", that is typical of promoters of many stress- and light-responsive genes. In order to bind the corresponding transcription factor, the promoter requires either several ABREs and/or binding enhancing elements. Further elements are required for organ-specific expression. Such functionally diversified promoters are called "bipartite promoters".
10. ABA-independent reactions to drought result from gene expression triggered by desiccation, high salinity or even cold. Promoters of such genes contain the so-called DRE element, to which specific transcription factors bind. There are also promoters which possess both elements, the DRE and the ABRE motif. As far as is known, the latter becomes effective only during prolonged drought or cold. Under these conditions the promoter changes to an ABA-responsive one.
11. The final gene products are proteins, which immediately or by their products improve the stress tolerance of cells: aquaporins, proteases, ROS-detoxifying enzymes, and enzymes which catalyse the synthesis of compatible solutes. A special group of protective proteins are the LEA or Rab proteins, which are attributed to the family of dehydrins. They are not catalytically active, but are very efficient in protecting membranes and protein complexes. Dehydrins are usually medium-sized proteins which either form amphiphilic helices or only random coils. Usually, they have a modular (segmented) structure and are resistant to boiling and denaturing by acids as they do not contain cysteine or tryptophan. In some cases, detailed understanding of the protective effect of these proteins has been achieved. Dehydrins and compatible solutes are the major components of membrane stabilisation in poikilohydric plants.
12. Dehydrins have proven useful for the transformation of drought-sensitive plants (crop plants). The same applies to enzymes which catalyse the synthesis of compatible solutes.
13. CAM is a special physiological adaptation of plants in hot and dry regions. Their stomata remain closed during the hot day and open only at night. CO_2 originating from bicarbonate is bound to PEP by phosphoenolpyruvate carboxylase (PEPC). The resulting chemically unstable oxaloacetate is stabilized by reduction to L-malate (malate dehydrogenase). Malic acid accumulates in the vacuole as an ATP-dependent proton pump shuffles protons into that organelle. During daytime, malic acid is released from the vacuole and after entering the chloroplast is decarboxylated by malic enzyme. The CO_2 is fixed by Rubisco and the pyruvate formed from the original PEP is either phosphorylated with pyruvate-P-dikinase to PEP or broken down in respiratory metabolism. Loading and unloading malic acid into and from the vacuole create particular biochemical problems

which are partly solved by using different pathways. Another problem is the reversible inhibition of PEPC during daytime to allow the CO_2 to be assimilated by Rubisco: A CAM plant-specific PEPC has been demonstrated which in its dephosphorylated form (daytime) is inhibited by malate, whereas the activity of the phosphorylated enzyme (nighttime) is not affected.

14. CAM plants have a much higher water use efficiency (WUE) than plants photosynthesising by the C3 or the C4 mechanism, but their growth is considerably slower than that of other homoiohydric plants.
15. Many CAM plants are able to switch from CAM to C3 photosynthesis if the environmental conditions are favourable (facultative CAM plants). Such change may be seasonal or diurnal, e.g. C3 photosynthesis may be performed for a short time in the early morning or late in the afternoon, when the stomata are not yet fully closed as for the rest of the day. During prolonged periods of drought, stomata remain closed even at night and photosynthesis is restricted to internally produced CO_2. Under these conditions growth is not possible (CAM idling). Crassulacean acid metabolism with completely or partly open stomates during the daytime is called "CAM cycling".
16. From the stable carbon isotope ratio (the so-called δ^{13} value), it is possible to detect whether a plant has photosynthesised by the C3, C4 or CAM mode: C3: –28‰, C4: –14‰, CAM: –10 to –20‰.

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1.6

Salt Stress (Osmotic Stress)

Salt lakes are almost uninhabitable for plants because of the enormous osmotic potential of the substrate, which is often also very alkaline due to the high soda ($NaCO_3$) content. Nevertheless, plant life can be found in such habitats. The white expanses in the picture of Lake Magadi in southern Kenya are not snow, but salt incrustations. The banks of sediment in the lake are overgrown with thick layers of algae. The shoreline also supports, in part, a vegetation of halotolerant bushes. Photo E. Beck

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That stress occurs when certain environmental factors exceed or are below the useful or tolerable range of intensities becomes particularly obvious with nutrients. The concentrations at which nutrients occur or exert their effect often differ greatly from element to element and from plant to plant (see also Chap. 2.3). This is particularly apparent for ions which are not obligate nutrients, such as sodium. This ion – in contrast to the case with animal metabolism – plays no essential role in the metabolism of plants, but it often occurs at high concentrations as sodium chloride where plants grow. Salts found in nature (mainly NaCl and $CaSO_4$) may occur at concentrations which are either toxic, tolerated or even required as an osmoticum, and plants are accordingly referred to as **glycophytes** (non-salt plants) or **halophytes** (salt plants). In the following, stress caused by high **NaCl concentrations** will be the main topic of discussion.

The electrical conductivity (EC_e) of salts in liquid solutions is used for the standardisation of salt contents and is expressed in [Siemens (S)/m] or [mS/cm]:

$$1\,\mathrm{S} = \frac{1}{\mathrm{Ohm}}\,[\mathrm{Mho}]$$

1 S/m corresponds to –0.36 MPa and the dimension Mho is the reciprocal of Ohm, the measure of electrical resistance.

The conductivity of seawater corresponds to 4.4 S/m; that of water used for irrigation must be lower than 0.2 S/m. Soils with a conductivity of >0.4 S/m are termed saline soils. The salt concentration of seawater (3%: 480 mM Na^+, 50 mM Mg^{2+} and 560 mM Cl^-) corresponds to

Table 1.6.1. Salt tolerance of important agricultural plants grown under a range of high salt concentrations. EC_e is a measure of salinity and is the electrical conductivity of the water extracted from the saturated soil solution. (After Marschner 1986)

Plant type	Maximum tolerance (EC_e in mMho/cm)	Reduction of harvest per EC_e unit above the tolerance value (%)
Barley	8.0	5.0
Sugar beet	7.0	5.9
Wheat	6.0	7.1
Soybean	5.0	20.0
Tomato	2.5	9.9
Maize	1.7	12.9
Green bean	1.0	19.0

Table 1.6.2. Yields of salt-tolerant barley plants from the gene and seed banks of the USA upon cultivation on Californian sand dunes and irrigation with seawater supplemented with phosphate and nitrogen fertilisers. (After Lüttge et al. 1994)

Barley variety	Yield (t/ha)
Standard variety on saline soil (average)	0.83
Var. XXI, 1st experiment	1.08
Var. XXI, 2nd experiment	1.50
World average on non-saline soil	2.00

an osmotic potential of –2.7 MPa. Table 1.6.1 shows the salt tolerance of various crop plants.

Irrigation water in dry areas contains between 0.1 and 1 kg mineral salts per cubic metre. While this is significantly below the damage threshold (–0.15 MPa), it can still lead to soil salinity and to the loss of useful agricultural land in a few years due to the intense evaporation.

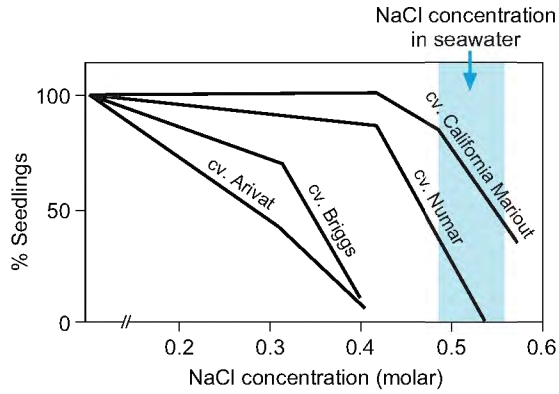


Fig. 1.6.1 Salt tolerance of various barley varieties, measured by the development of young plants on salt-containing substrates. (After Lüttge et al. 1994)

It follows from the theory that life arose in the sea that salt tolerance must have been an original characteristic of living systems; sensitivity to salt should then be seen as a loss of this original characteristic. "Regaining" salt tolerance of important crop plants is accordingly one of the most important aims of plant breeding. An example is the improvement of salt tolerance in barley (Fig. 1.6.1 and Table 1.6.2), which has per se the highest salt tolerance of all cereals (see Table 1.6.1).

1.6.1

Physiological Effects of Salt Stress (NaCl)

- Barkla BJ, Pantoja O (1996) Physiology of ion transport across the tonoplast of higher plants. Annu Rev Plant Physiol Plant Mol Biol 47:159–184

1.6.1.1

Effects on the Ion Metabolism of the Cell ("Primary Effects")

Sodium and chloride ions are regarded as being biologically aggressive osmolytes on account of their small ionic diameters and high surface charge densities and their consequent strong tendency to attract water. High concentrations of these ions in the apoplast accordingly lead to imbalances in water and ion relations. **Stress** caused by **salinity** is thus both **dehydration stress** and **ionic stress**. The latter rapidly leads to destruction of biomembranes, because ion imbalances result not only in altered concentration relations, but also in changes of membrane potential. Homeostatic ion concentrations, i.e. cytosolic concentrations maintained at equilibrium in glycophytes not subject to salinity stress, are in the ranges of 100–200 mM K^+, 1–10 mM Na^+ and Cl^- and 0.1–0.2 mM Ca^{2+} (Fig. 1.6.2; Niu et al. 1995).

These ion concentrations are kept constant in plant cells in the main by electrophoretic flows, i.e. passive flows which are coupled to H^+-ATPases and H^+-pyrophosphatases in the plasma membrane and the tonoplast (in contrast to animal and yeast cells, plant cells do not possess Na^+/K^+-ATPase pumps). In addition, import and

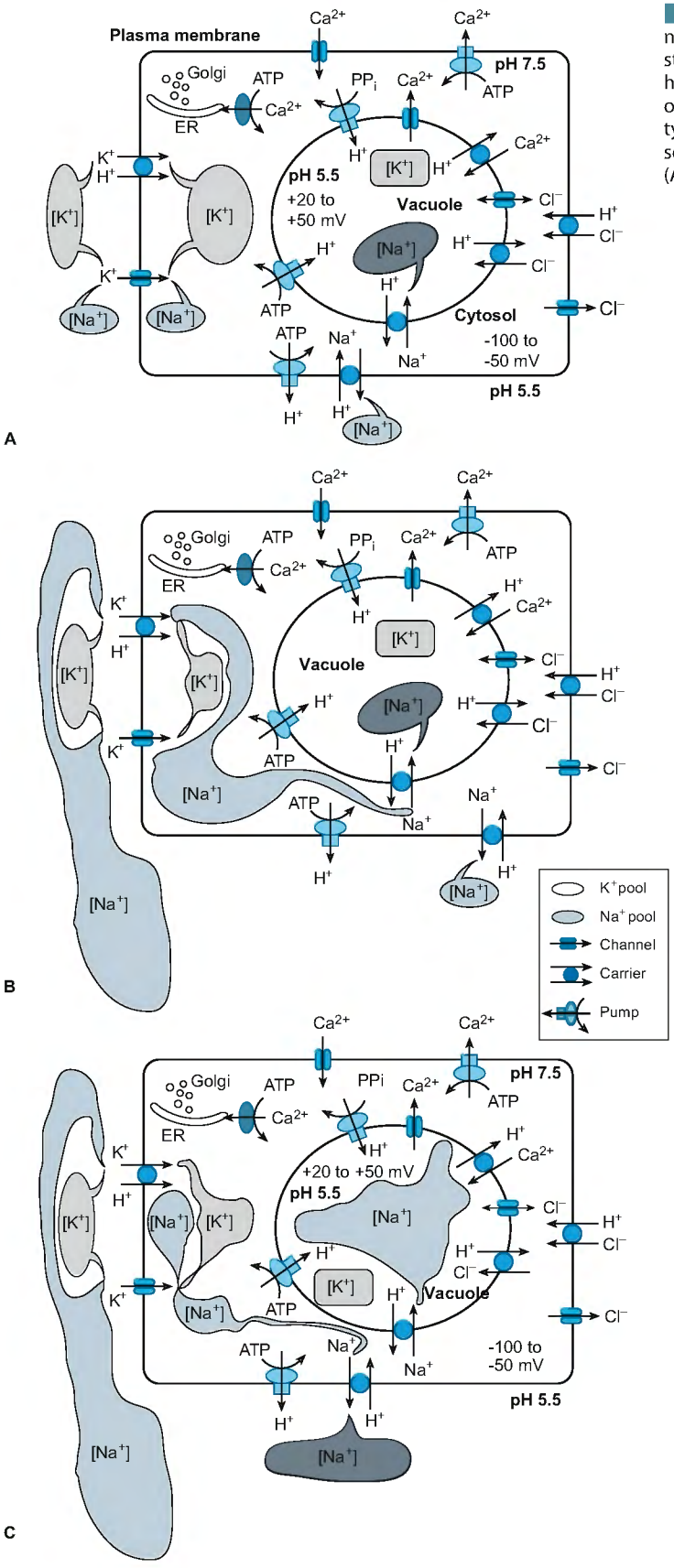


Fig. 1.6.2. Ion relations in plant cells in the non-stressed (A) and strongly salt-stressed (B) states, as well as subsequent to adaptation to high salt concentrations (C). The schematic overview incorporates all hitherto described types of transport systems that have been associated with ion homeostasis under salt stress. (After Niu et al. 1995)

Table 1.6.3. Transport systems potentially involved in ion homeostasis in the plasma membrane and the tonoplast. (After Hasegawa et al. 2000b)

Substrate	Subcellular localisation	Direction	Source of energy or counter ion
	Pumps		
H^+	Plasma membrane	Apoplast	ATP
H^+	Tonoplast	Vacuole	ATP
H^+	Tonoplast	Vacuole	PPi
Ca^{2+}	Plasma membrane	Apoplast	ATP
Ca^{2+}	Tonoplast	Vacuole	ATP
	Channels		
K^+ (Na^{2+})	Plasma membrane	Cytosol	Membrane potential
K^+ (Na^{2+})	Plasma membrane	Cytosol	H^+ (symport)
K^+	Plasma membrane	Cytosol	H^+, Na^+ (symport)
Ca^{2+}	Plasma membrane	Cytosol	Membrane potential
Ca^{2+}	Tonoplast	Cytosol	Membrane potential
Cl^- (anion)	Plasma membrane	Apoplast	Membrane potential
Cl^- (anion)	Tonoplast	Vacuole	Membrane potential
	Carriers		
Na^+	Plasma membrane	Apoplast	H^+ (antiport)
Na^+	Tonoplast	Vacuole	H^+ (antiport)
Ca^{2+}	Tonoplast	Vacuole	H^+ (antiport)
Cl^- (anion)	Plasma membrane	Cytosol	H^+ (symport)
Cl^- (anion)	Tonoplast	Vacuole	H^+ (antiport)

export channels for K^+ and anions which are regulated by the membrane potential (so-called gated channels) have been described, as well as a Ca^{2+}-ATPase for Ca^{2+}. The activity of these pumps and channels results in a membrane potential of 0.2 V at the plasmalemma (the cytosolic side is negatively charged) and a tonoplast potential of between 0 and +20 mV, with the cytosolic side again being negatively charged. When the volumes of the participating apoplast, cytosol and vacuole compartments are taken into account, the membrane potentials give rise to pH gradients, whereby the normal pH value for the cytosol is always 7 and that of the apoplast and the vacuole is 1–2 units more acidic. An overview of the transport systems at present known to participate in the ion homeostasis of higher plants is given in Table 1.6.3.

High salinity places this homeostasis under considerable stress, because particularly Na^+, but also Ca^{2+}, enter passively into the cell along the concentration gradient and form large pools there. The accumulation of positive charges in the cytosol breaks down the natural barrier of the membrane potential for Cl^- and, consequently, leads to a massive influx of this anion through the anion channels.

In particular the potassium relations of the cell are threatened by high sodium concentrations. On the one hand, K^+ and Na^+ compete for the not particularly selective, but very efficient, K^+ uptake systems. These are channels and K^+/Na^+-symporters: low affinity cation transporters, or LCTs. At high apoplastic Na^+ concentrations the potassium uptake by the cell is accordingly strongly reduced, and the cytosolic potassium pool shrinks (Fig. 1.6.2 B).

On the other hand, the flooding of the cytosol with Na^+ results in increased activity of proton pumps, especially of the plasma membrane ATPases but also of the tonoplastic Na^+/H^+ antiport system, and thus in an increase in ATP consumption.

The situation becomes further aggravated in that increased proton transport also results in changes in the intracellular pH relations. External application of NaCl at seawater concentrations results in an alkalisation of the cytosol of barley roots of between 0.5 and 1 pH unit. This alkalisation detrimentally affects the activity of various cytosolic enzymes, particularly of those of catabolic energy metabolism (Katsuhara et al. 1997).

Uptake of Na^+ into the vacuole, which effects salt removal from the cytosol, requires a Na^+/H^+ antiporter (sodium-H^+ exchange) which has been found in barley roots and beet in addition to the above-mentioned H^+-transporting system. This antiporter couples the increased proton charge of the vacuole with sodium uptake. The synthesis of this transport system can be induced by salinity stress (Fig. 1.6.2 C). The so-

dium and chloride concentrations in the vacuoles of salt-stressed tobacco cell cultures were eight-fold greater than those in the cytosol (Binzel et al. 1988). A consequence of this is a further alkalisation – now of the vacuole – due to the proton-sodium antiport.

Flooding of the cell with Na^+ leads finally to an increased uptake of calcium by the cell and release of Ca^{2+} from cellular compartments, and thus to an increase in the size of the cytosolic Ca^{2+} pool. Since the cytosolic pool of free Ca^{2+} has signal function, the augmentation of this pool triggers regulatory processes in the cell, which can in the main be interpreted as constituting repair or adaptation reactions. The increase in the cytosolic Ca^{2+} concentration could take place due to water potential-induced calcium uptake similar to that occurring in the stomata (see also Chap. 1.5.2.4), or upon the opening of Ca^{2+} channels in the ER or tonoplast in connection with the inositol-triphosphate system.

1.6.1.2 Secondary Damage Caused by Severe Salt Stress

Salt stress, which makes excessive demands on the cell's ability to maintain homeostasis and thus leads to increased salt concentrations in the cell, results in functional disturbances. On the one hand, processes requiring balanced water relations, such as cell elongation, react particularly sensitively. Since a great deal of energy is consumed by the strain reactions and a considerable proportion of produced photosynthate is used in the production of compatible osmolytes, **cell division growth** is also detrimentally affected due to lack of building blocks and energy.

In salt-sensitive plants, e.g. maize and beans, even relatively low internal salt concentrations result in considerable reductions in growth (see Table 1.6.1). Indeed, the constitutive types of glycophytes and halophytes introduced at the beginning of this chapter can be relatively easily identified and even further subdivided on the basis of the sensitivity of growth to salt. Most of our highly efficient crop plants are unfortunately glycophytes which exhibit significantly reduced yields at even only relatively low salt concentrations.

In addition to growth processes, **photosynthesis**, particularly that of C3 plants, is detrimentally affected by elevated intracellular salt contents. Even though enzyme activities may be influenced by salt, the salt concentrations which build up in the chloroplast are usually too low to result in significant inhibition of the Calvin cycle. On the other hand, photosynthetic electron transport is impaired at even relatively low salt concentrations, whereby the nature of the detrimental effect has not yet been clarified. It is known that enhanced ROS formation takes place in the chloroplasts of plants under salt stress upon illumination (see Chap. 1.3.5). These ROS lead to damage of the photosystems, to chlorophyll degradation and, finally, to necrotic death of cells and tissues. Typical **salinity damage** symptoms become apparent (see Fig. 2.3.7 G), above all necroses at the edges of leaves and in the youngest generation of needles of conifer needles. It is interesting that the inhibition of photosynthetic CO_2 assimilation is not primarily due to an effect of salt on the opening of the stomata. The stomata remain functional even at high salinity. It must thereby be borne in mind that the damage having been caused results from NaCl taken up by the roots, in contrast to the direct effects of salt in salt sprays and splashings (e.g. at road edges) which damage old and young tissues in the same manner.

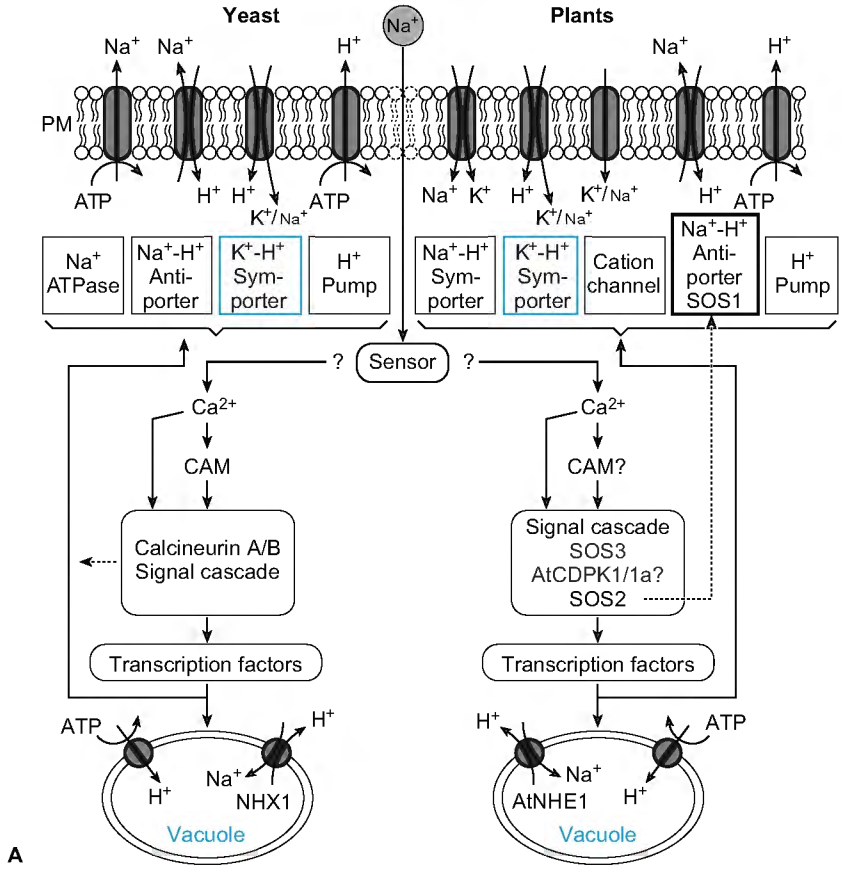
1.6.2 The Adaptive Response of the Plant Cell to Salt Stress

An osmotic stressor such as salt causes strain in plant cells, which can be interpreted as adaptation to the new osmotic conditions. Adaptation means new synthesis of proteins to alleviate the stress. In the case of osmotic stress, this alleviation comprises:

- reactions to regain ion homeostasis,
- reactions to adapt to the osmotic potential,
- synthesis of specifically acting, protective proteins.

1.6.2.1 Intracellular Signals

The adaptation commences with the perception of the osmotic stress. This has already been discussed for *Dunaliella* (see Chap. 1.1.4). Since lit-



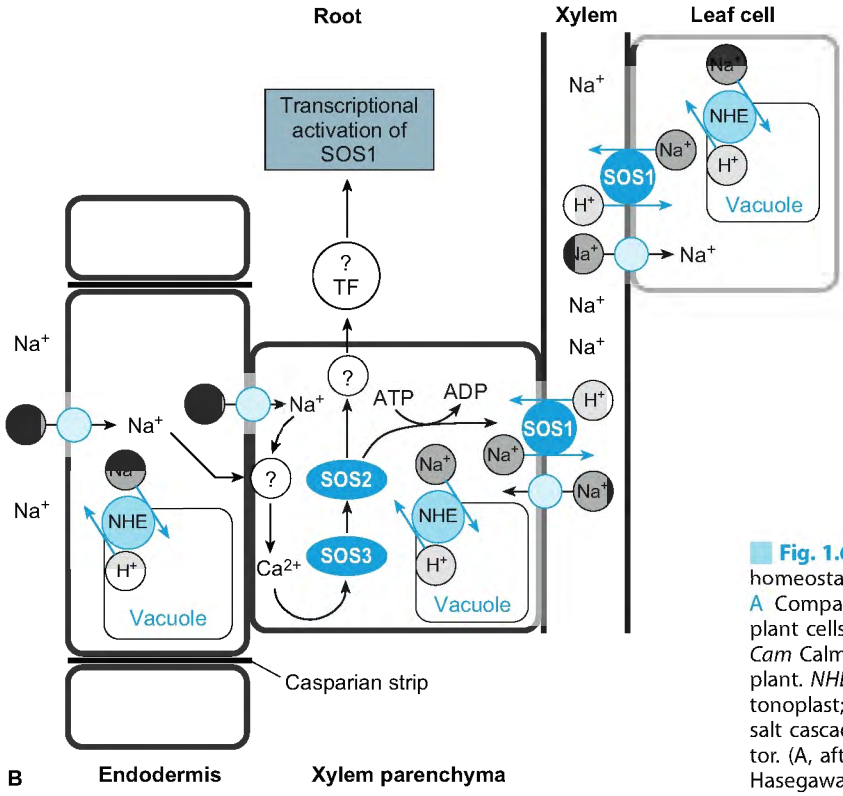


Fig. 1.6.3. Model of the regulation of ion homeostasis by Ca^+ under salt stress. A Comparison of the response of yeast and plant cells to salt stress modulated by Ca^+. *Cam* Calmodulin. B The SOS system in a plant. *NHE* Sodium-proton antiporter in the tonoplast; *SOS* protein of the overly sensitive salt cascade (see text); *TF* transcription factor. (A, after Bressan et al. 1998; B, after Hasegawa et al. 2000b)

tle is known about osmosensors, particularly as they apply to salt stress, the next step – the genesis of the signal – will now be considered. Genes and proteins have recently been identified which point to a biochemical connection between the osmotic adaptation of the plant and an increase in the cytosolic Ca^{2+} level. Yeast has served in this regard as a model, for which it has been known for some time that there is an interaction between Ca^{2+}, the subunit B of the calcium-binding protein calcineurin and salt tolerance. In this organism the protein phosphatase calcineurin activates on the one hand transcription factors, which lead to increased expression of a plasma membrane Na^+-ATPase and a tonoplastic Na^+/H^+ antiporter. On the other hand, active calcineurin modulates a not very selective K^+/H^+ symporter in such a way that it becomes both more effective and more strongly selective for K^+ (Fig. 1.6.3 A). A gene family has been correspondingly discovered in *Arabidopsis*, a mutation in which makes plants particularly sensitive to Na^+ and also to Li^+ ("salt overly sensitive": SOS). The functions of three SOS proteins have now been elucidated: SOS1 is a Na^+-H^+ antiporter in the plasma membrane, SOS2 is a serine-threonine protein kinase which activates SOS1, and SOS3 is a calcium-binding protein with an EF-hand domain typical of such proteins, which – after being activated – itself activates SOS2 (Fig. 1.6.3 B; Hasegawa et al. 2000). In addition to the direct activation of SOS1, the SOS cascade also positively influences the activation of transcription factors and leads to increased expression of the antiporter.

Corresponding to the ways in which plant cells respond to drought, there are several parallel calcium-dependent regulatory systems which apply to NaCl stress as well. These are only understood in part, however, e.g. a protein kinase (calcium-dependent protein kinase, CDPK) involved in the expression of a LEA protein or non-stress-specific MAP kinases. It is known that certain phytohormones also act as signals.

A strongly elevated abscisic acid (ABA) level has often been determined in salt-stressed plants (Fig. 1.6.4). An enhanced formation of ethylene has also been observed, whereas the cytokinin content usually decreases.

Since salinity has an ionic as well as a dehydration component (due to the binding of water to ions) and ABA plays an important role as a phytohormonal signal during drought (see Fig. 1.5.8), it cannot be excluded that the increased ABA level is – according to the type of plant and the conditions – related to the dehydration syndrome rather than to salt toxicity (Fig. 1.6.5).

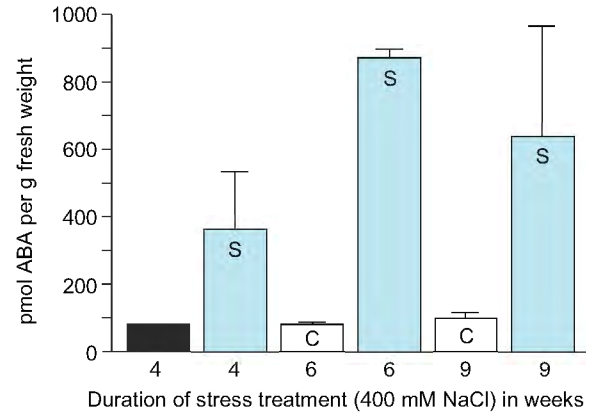


Fig. 1.6.4. Reaction of the endogenous ABA levels in leaves of *Mesembryanthemum crystallinum* to salt stress. The concentrations of the osmolytes proline, pinitol and ononitol, as well as the amounts of the CAM form of PEP carboxylase and the protective protein osmotin, increased along with the level of ABA. *C* Control (without salt); *S* 400 mM NaCl in the nutrient solution. (After Thomas and Bohnert 1993)

Nevertheless, a physiological relation between an increase in the ABA level and adaptation to salt stress does exist, as shown in an experiment in which the autonomous osmotic adaptation of tobacco cultures to salt was compared with an accelerated adaptation resulting from exogenous ABA supply.

As is the case with drought stress, numerous specific proteins can be shown to be expressed upon salt stress (see Chap. 1.5.2.2); the induction paths can be ABA-dependent or ABA-independent (Jin et al. 2000). However, it is again not clear with regard to these strain reactions whether the induction is due to drought or to ion effects.

In addition to the ABA level, the level of jasmonates in the plant responds to salinity. However, the behaviour of this group of hormones is very different to that of ABA. While the concentration of ABA increases only transiently upon salt shock (transferring plants from a salt-free medium to one with an elevated NaCl content) and then falls off again (Fig. 1.6.6), the concurrent increase in jasmonate is maintained over a longer period. Jasmonate accumulates about 20-fold upon longer-term salinity stress, whereas the ABA level increases only about 4-fold. Each of the hormones, if applied externally, induces

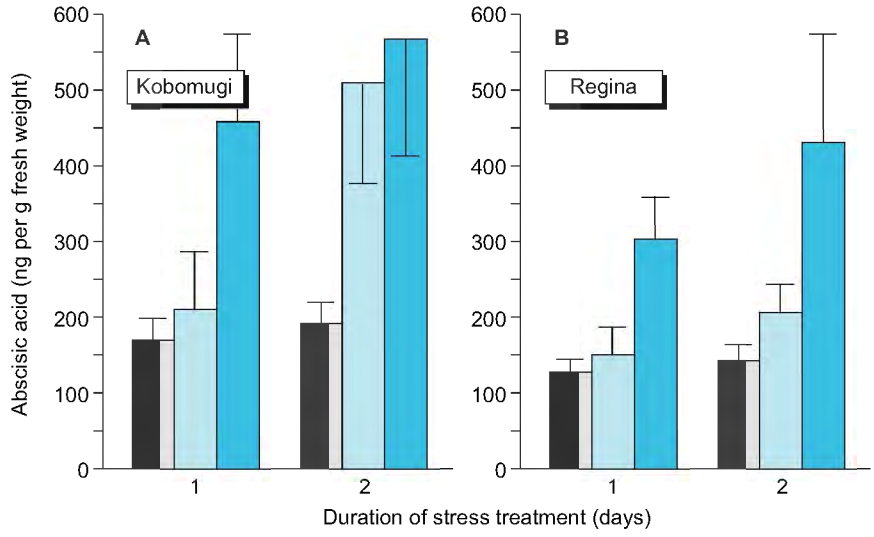


Fig. 1.6.5. The reaction of the ABA level in the leaves of two wheat varieties subjected to salt stress and to combined salt and drought stress. Control (*grey*), salt stress (200 mM, *light blue*), salt and drought (200 mM salt and 21% polyethyleneglycol, *blue*). (After Nagy and Galiba 1995)

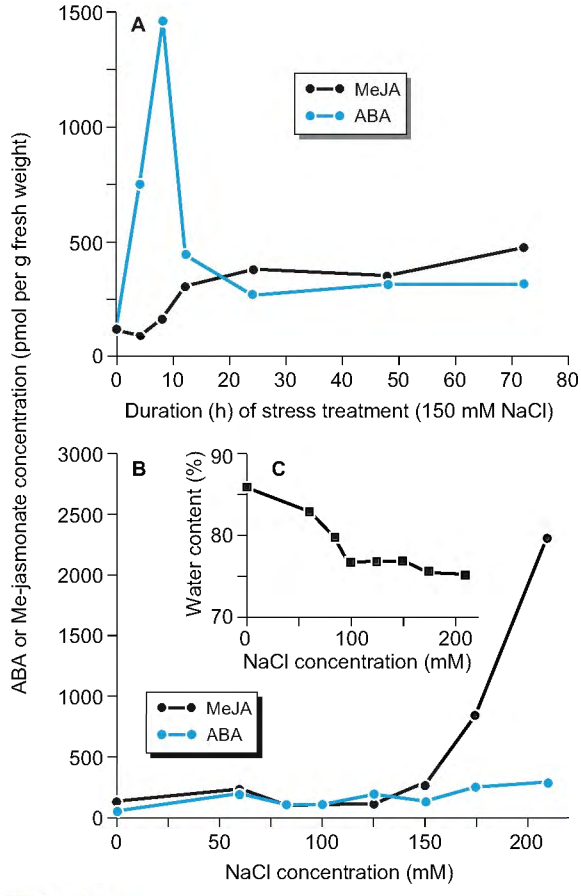


Fig. 1.6.6. Endogenous concentrations of ABA and methyl jasmonate in roots of rice plants subjected to different forms of salt stress. A "Salt shock": Transfer of seedlings from a salt-free medium to a salt-containing medium (150 mM). B ABA and methyl jasmonate contents of rice seedlings after 2 days of treatment with different salt concentrations. C Water content of the shoots after 2 days of exposure to salt stress of different intensities. (After Moons et al. 1997)

the expression of specific genes with only limited overlap. Together they tend to act antagonistically, however, with regard to gene expression (Moons et al. 1997).

1.6.2.2 Adaptation Reactions to Restore Ion Homeostasis

Glycophytes and halophytes basically make use of the same mechanisms to deal with salinity stress. Halophytes are able to adapt faster and to tolerate extreme salinity, whereas glycophytes adapt stepwise to develop tolerance to a moderate degree of salinity. In both constitutive types salt tolerance requires the expression of certain, similar genes. However, the transition from extremely salt-sensitive plants to extremely salt-tolerant plants is not clear cut, and differentiation between the two types is difficult because of the plant's ability to adapt.

Considering the many examples of salt-stressed glycophytes and halophytes having been investigated, it becomes clear that adaptation serves to regain ion homeostasis and the original membrane potentials and pH values. The better the plant is able to achieve this, the better it becomes tolerant of osmotic stress. However, regaining ion homeostasis does not signify a "return" to the original ion concentrations. Rather, the plant attempts to achieve homeostasis with the entire ionic load, including the NaCl that has been taken up (see Fig. 1.6.2 C). Depletion of the aggressive NaCl ions in the cyto-

plasm during adaptation requires considerable energy, which cannot be supplied with the normal provision of the cellular transport systems. Therefore, more of those proteins are synthesised which contribute either directly or indirectly to the removal of NaCl from the cytoplasm. Both the apoplast and the vacuole can be compartments for the "final" deposition.

Relief of the cytosol by removal of the Na^+ ions from the cytoplasm and their transport into the vacuole and the apoplast is effected mainly by means of Na^+/H^+ antiporters, both in the plasmalemma (e.g. in *Atriplex nummularia*; Hassidim et al. 1990; see also Chap. 1.6.2.1) and in the tonoplast (NHE, Fig. 1.6.3 B), e.g. in *Beta vulgaris* (Barkla and Blumwald 1991). These antiporters are induced by elevated cytosolic Na^+ (NaCl) concentrations and require high proton concentrations in the external medium and in the vacuole, i.e. effective H^+ pumps. These pumps are synthesised to a greater extent upon salt stress. If ion homeostasis is established, the increased expression of, e.g., the plasma membrane H^+-ATPase decreases once more. Adapted cells no longer exhibit enhanced pump activity. Figure 1.6.2 C shows the result of salt adaptation with regard to the distribution of ions. The Na^+ concentration in the cytosol has returned to the original value (Fig. 1.6.2 A), while that in the vacuole has increased many-fold. The original membrane potential and pH value have nevertheless been reestablished. Over-expression of a vacuolar Na^+/H^+ antiporter gene in *Arabidopsis* increased salt tolerance considerably (Apse et al. 1999). The K^+ status is the weak point in the cellular ion budget subsequent to an adaptation process, irrespective of ion homeostasis. The reduced growth of halophytes and glycophytes under salt stress is possibly related to the problematical provision of the cell with K^+ (see Box 1.6.1).

The effectiveness of salt elimination into the apoplast is supported, in many halophytes, by salt glands (Fig. 1.6.7), which lead to the deposition of NaCl onto the surface of the leaf, where it then crystallises. This applies particularly to some mangrove species, but also to desert plants (e.g. the genus *Reaumuria* and other Chenopodiaceae).

Other halophytes can enhance the sequestration of salt in the vacuole by developing large salt-storing mesophyll cells (**salt succulence**, Fig. 1.6.8 A) or by loading large **bladder hairs** with NaCl (Fig. 1.6.8 B). Both these excretion mechanisms are also termed **recretion**. Leaves which have accumulated high concentrations of salt are shed.

1.6.2.3 Adaptation of Osmotic Potential

Plants must possess a higher osmotic potential than their medium if they are to take up water and nutrients from a saline milieu, i.e. they must increase their own osmotic potential. It is thus inevitable that changes in their internal concentration relations must take place, something which necessitates further adaptive action. In addition to maintaining ion homeostasis, in-

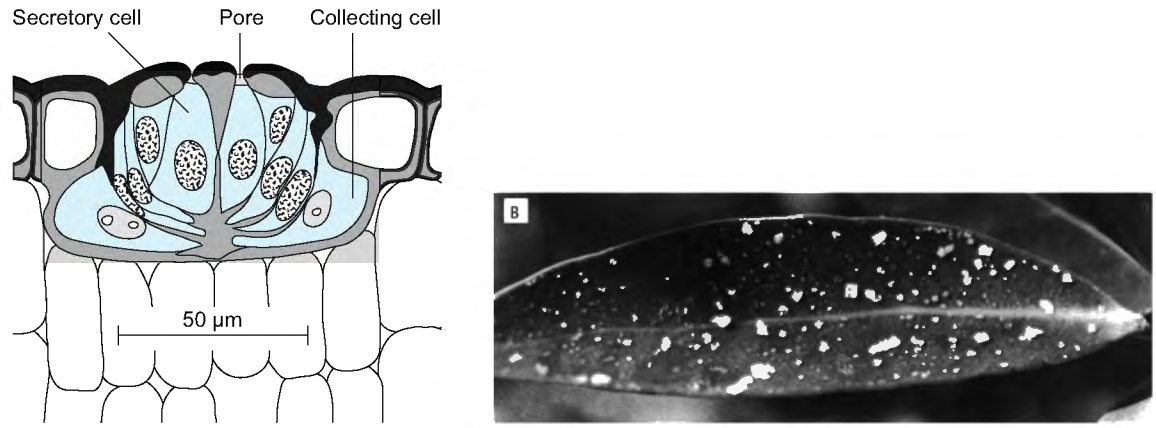


Fig. 1.6.7. Salt elimination ("recretion") via the apoplast. A Salt gland in the leaf of sea lavender (*Limonium vulgare*). B Leaf of the mangrove plant *Avicennia germinans* (*Verbenaceae*) showing salt crystals excreted via the salt glands. (Lüttge et al. 1994)

Box 1.6.1 Ion relations of salt-sensitive and salt-tolerant glycophytes under salt stress (after Speer and Kaiser 1991)

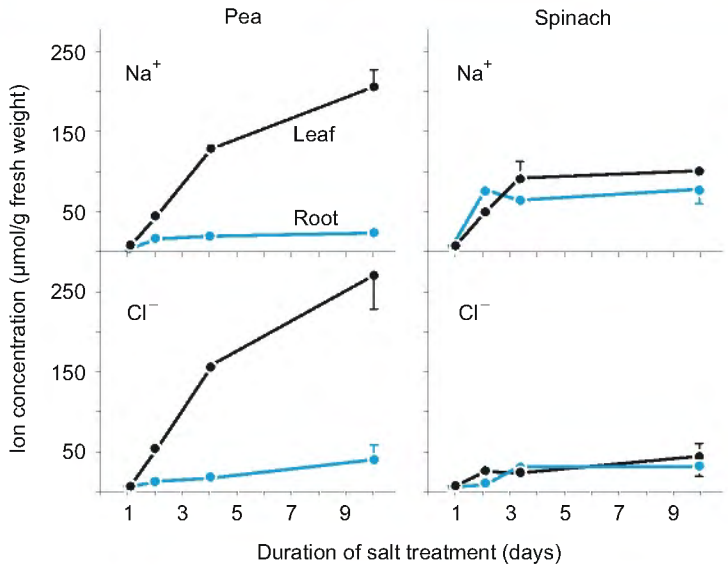


Figure. Salt content of the leaves and roots of pea and spinach plants during the course of growth on nutrient solution containing 100 mM salt for 10 days

Seedlings of pea (salt-sensitive) and spinach (salt-resistant, Chenopodiace) were transferred to nutrient solution and exposed to 100 mM NaCl. The ion concentrations of the roots and leaves (see Fig.), as well as the intercellular distribution of the ions, were monitored (see Table).

In both plants the salt concentration in the roots remains distinctly lower than the salt concentration externally applied, whereas the leaves of the salt-sensitive pea plant accumulate salt to a concentration 2.5-fold higher than that applied. In the relatively salt-resistant spinach, Na^+ accumulates in the roots and leaves up to the applied concentration, while Cl^-, as is the case with pea shoots, accumulates to only 50% of the concentration present in the medium.

It can be assumed that the ion concentrations in the cytosol are similar to those in the chloroplast, mitochondria and other organelles. Together with data from the figure, the following picture of adaptation becomes apparent:

The total ion load in the cytosol has remained the same or has even been reduced somewhat (spinach), while that in the vacuole has increased slightly. In contrast, the ion concentration has increased two- to four-fold in the apoplast, where it constitutes the least physiological inconvenience. However, the concentration relations of the individual ions have clearly been altered: The proportion of NaCl has increased drastically – with the exception of the cytoplasm – mainly to the detriment of K^+ and NO_3^-. This of course induces nutritional problems. These changes are considerably greater for pea than for spinach.

creasing the osmotic potential is the most immediate requirement for adaptation.

Halophytes make use of the available salt for this purpose (Fig. 1.6.9 A). The salt must be retained within the cell, and thus not eliminated. Figure 1.6.9 B shows that most of the salt is compartmented in the vacuole[1]. Only one fifth of the Na^+ concentration and one quarter of the Cl^- concentration present in the vacuole is found in the cytoplasm and its other organelles. Since, however, osmotic equilibrium must prevail within the symplast (protoplast), the cytosol must

[1] Of course, the capacity of the vacuole to store NaCl is limited. Long-term utilisation of the full capacity leads to rapid senescence of the tissue and to shedding of the leaves.

Box 1.6.1 (continued)

Table. Intracellular ion distribution and budgets in pea and spinach leaves grown without salt and after 10 days of adaptation to treatment with 100 mM NaCl. Only the most important ions are shown

Ion	Control/salt (C/S)	Pea [ion concentration (mM) in mesophyll]			Spinach [ion concentration (mM) in mesophyll]		
		Vacuole	Cytosol	Apoplast	Vacuole	Cytosol	Apoplast
K^+	C	104±23	53±5	13±4	260±31	147±25	10±4
	S	42±12	29±17	14±4	172±33	78±18	18±4
Na^+	C	3±1	19±4	1.5±1	0.5±0.1	26±16	1±1
	S	114±7	60±19	50±16	109±15	43±15	6±1
Mg^{2+}	C	12±3	6±2	4±2	37±6	17±6	2±1
	S	6.5±1	4±3	2±1	29±5	15±7	3±1
Cl^-	C	4±1	5±3	6±2	1±1	6±5	4±1
	S	245±34	60±35	89±67	50±5	9±4	14±6
NO_3^-	C	33±15	6±2	2.1±1.1	29±13	10±6	5±1
	S	0	1±1	0	19±8	4±1	6.6±5
$\Sigma_{cations}$	C	131	84	22.5	334.5	207	15
	S	169	97	68	339	151	30
Σ_{anions}	C	289	55	20	73	88	13
	S	292	77	96	106	53	26

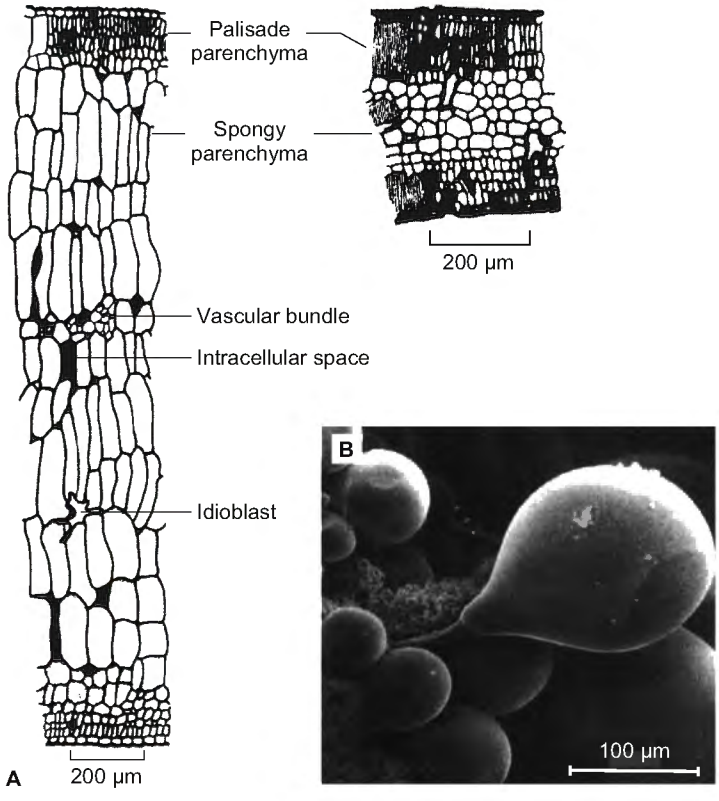


Fig. 1.6.8. Sequestration of salt in the vacuole. A Salt succulence in mangrove plants: development of salt-storage tissue from the spongy mesophyll of the leaves. *Right* Young leaf with still small, isodiametrical spongy parenchyma cells. *Left* Cross section of a fully grown leaf with large, elongated spongy parenchyma cells, in the vacuoles of which salt is deposited. The stone cell-like idioblast serves to stiffen the tissue. B "Bladder hairs" of the halophyte *Atriplex hymenelytra* (Chenopodiaceae). The salt is sequestered in the large vacuoles of the numerous bladder hairs and is thereby removed from the mesophyll. (A, after Larcher 1994; B, from Lüttge et al. 1994)

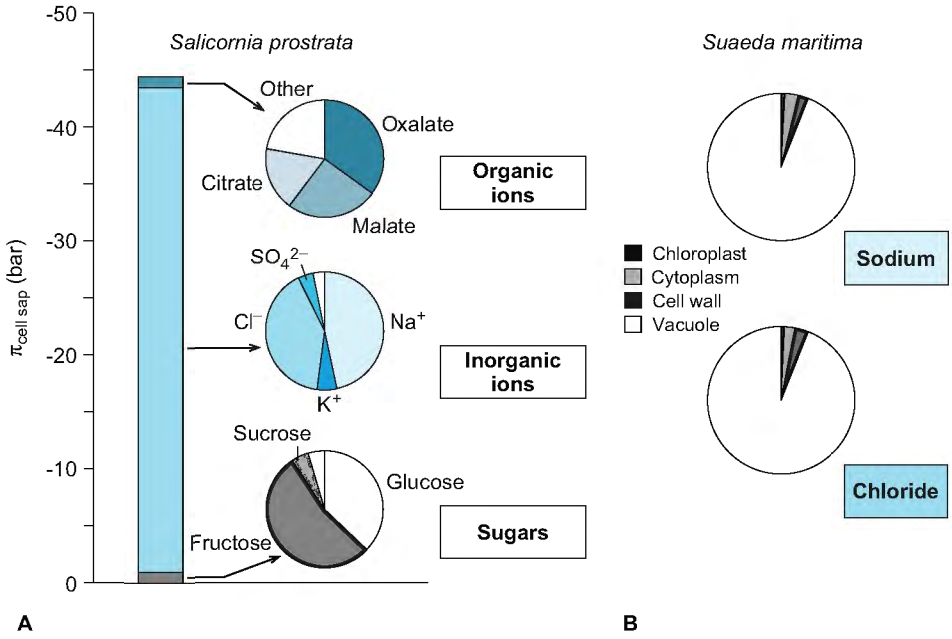


Fig. 1.6.9. A Osmotic potential of the cell sap of leaves of the halophyte *Salicornia prostrata* (Chenopodiaceae) with the components specified in the segments of the pie charts. B Intracellular distribution of the salt ions present in the leaves of the halophyte *Suaeda maritima* (Chenopodiaceae). (Larcher 1994)

have the same osmolarity as the vacuole. This is achieved in that the cell enriches the cytoplasm with compatible organic osmolytes. The adjustment of the osmotic potential of the cytoplasm by means of low molecular weight organic compounds is not as costly as it might appear at first sight. The volume of this compartment (with all its constituent organelles) is usually less than 10% of the volume of the vacuole, so that the required osmolarity is effected with a relatively small amount of solutes. The chemical nature of the organic osmolytes is discussed in the following section.

Compatible Solutes, Osmolytes

Salt adaptation is the classical field of osmolyte research, which has already been briefly considered in relation to dehydration stress (Chap. 1.5.2.2) and will be discussed in more detail here.

Stress osmolytes have two coupled functions:

1. Increasing the osmotic potential ("osmolyte") and thus the retention of water in the cytoplasm;
2. Multiple protective functions:
   - to maintain the integrity of membranes and proteins (dilution and displacement of aggressive ions from the surfaces),
   - to scavenge ROS, which are in general formed to an increased extent under stress, including salt stress.

Several groups of low molecular weight organic compounds, which belong to the carbohydrates and amino compounds, fulfil these functions (see Chap. 1.5.2.6, Fig. 1.5.11). In the following, the various osmolytes are discussed individually:

1. glycerol
2. polyols
3. proline
4. QACs and TSCs

1. Glycerol

The simplest osmolyte is **glycerol**. The green alga *Dunaliella*, which does not have a cell wall (see Chap. 1.1.4), adjusts to salt stress in the cytoplasm by rapid synthesis of glycerol. In this instance it is a predominantly osmotic phenomenon, as a cell without a cell wall is, of course, particularly endangered by changes in the osmotic potential of the medium. *Dunaliella* also responds with glycerol synthesis if the osmotic stress is artificially generated by polyols (Zelazny et al. 1995).

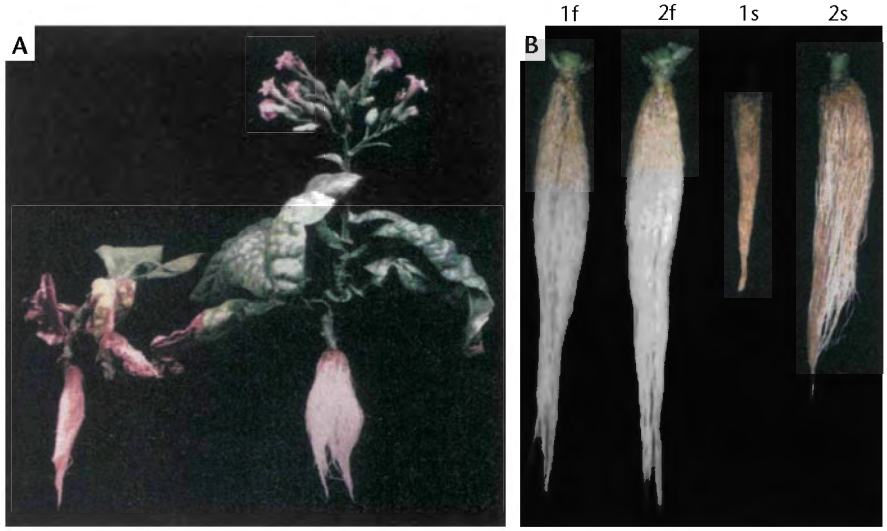


Fig. 1.6.10. Alleviation of salinity stress by constitutive expression of mannitol-1-phosphate dehydrogenase in transformed tobacco. The transformed plants synthesise and accumulate the osmolyte mannitol. A Control plants (*left*) and transformed plants (*right*) after 30 days of growth in nutrient solution containing 250 mM NaCl. B Roots of control (*1*) and transformed (*2*) plants in salt-free (*f*) and salt-containing (*s*) nutrient solution after 30 days of growth. (Tarczynski et al. 1993)

2. Polyols

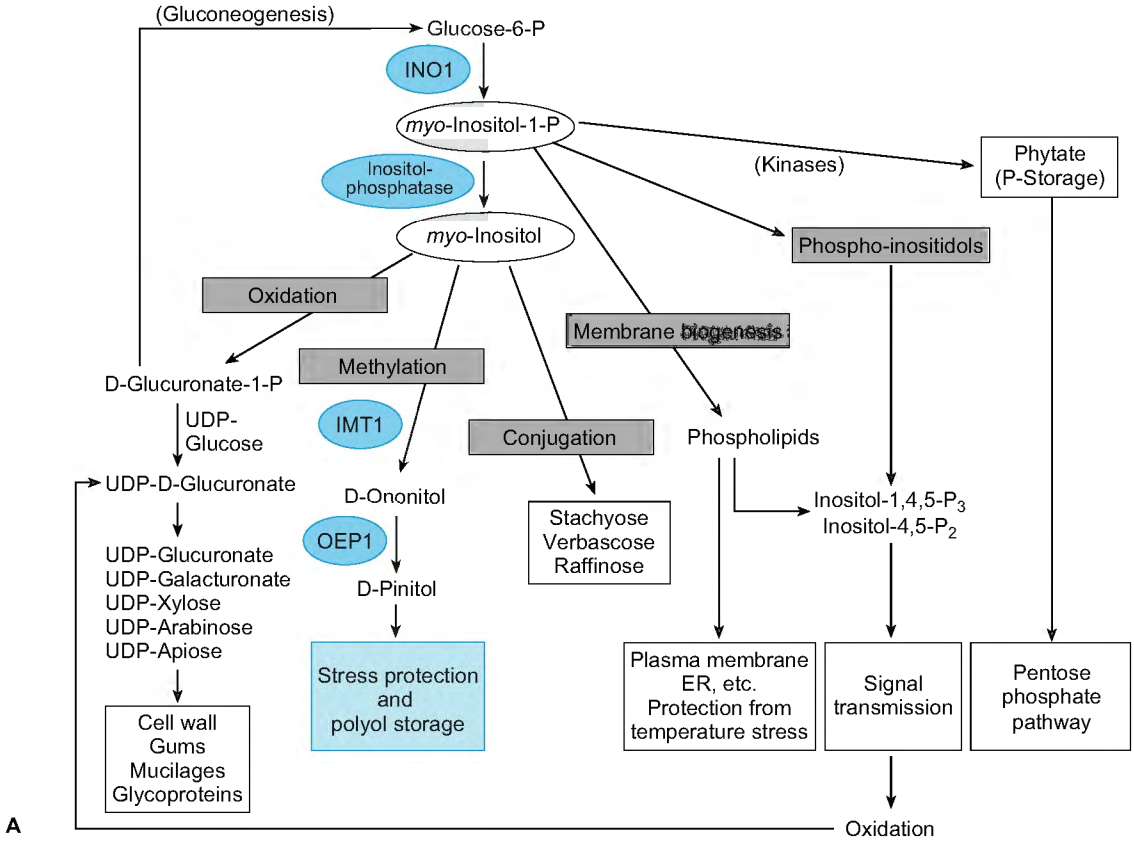
The second important subgroup of carbohydrates is composed of **polyols** (sugar derivatives). Their numerous hydroxyl groups bestow these compounds with hydrophilic properties, i.e. properties which closely approximate those of water clusters. Many algae of brackish water synthesise polyols rapidly and effectively as reduced derivates of monosaccharides in response to osmotic stress; they possess the corresponding dehydrogenases.

The **manna** of the Bible could well have been a polyol produced by desert plants (Crum 1993). Since the synthesis of these sugar alcohols requires only a single enzyme – a dehydrogenase which reduces the corresponding sugar – the sugar alcohols can easily be made use of to artificially enhance salt tolerance. Figure 1.6.10 shows the effect of the constitutive expression of mannitol in tobacco plants transformed with mannose dehydrogenase under the control of the S35 promotor. The mannitol accumulates, because wild-type tobacco plants do not produce the polyol and cannot further metabolise it when it is synthesised. Despite the carbohydrate expenditure required to produce this osmolyte, the transgenic tobacco plant does not grow notably more poorly than does the wild type in the absence of salt stress, but it does grow considerably better when stressed with salt.

Open-chain polyols occur in many plants, but they are more frequently found in glycophytes (e.g. in many Rosaceae) than in halophytes.

In addition to their osmotic effect, glycerol and open-chain sugar alcohols also provide protection against ROS when they accumulate (see Chap. 1.3.5). The reduction of monosaccharides (glyceraldehyde-3-phosphate and mannose- or fructose-6-phosphate) to their corresponding sugar alcohols requires reduction equivalents, i.e. electrons, which could also be transferred to oxygen under appropriate circumstances and would then contribute to the formation of ROS. Glycerol is a far more favourable osmolyte than a hexose in terms of the carbohydrate-to-hydrogen consumption rate. Transgenic organisms producing glycerol as an osmolyte thus show better growth than those making use of equiosmolar concentrations of higher sugar alcohols (Shen et al. 1999).

Cyclic sugar alcohols, so-called **inositols** or **cyclitols**, are very often accumulated as osmolytes in halophytes. They have the cell biological advantage over open-chain polyols in turning over slowly, i.e. they are withheld from rapid metabolism under deficiency situations (e.g. when photosynthesis is severely restricted because of stomatal closure) and are thus retained to function osmotically. Their biosynthesis originates from glucose-6-phosphate, and the great



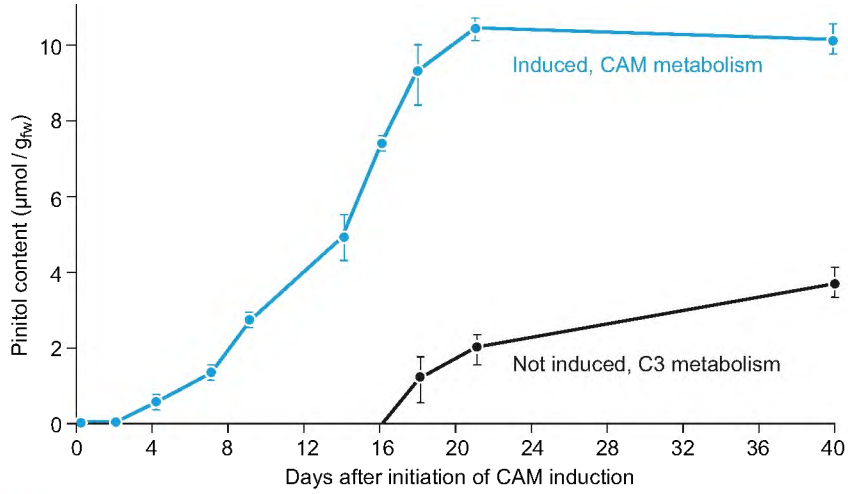


Fig. 1.6.11. A The physiological functions of *myo*-inositol and its derivates in plant cells (after Bohnert et al. 1995). B Pinitol accumulation during the induction of CAM in *Mesembryanthemum crystallinum* by irrigation with 400 mM NaCl solution. On day 21 the share of D-pinitol of the soluble carbohydrate fraction was 71% in the salt-treated plants and only 5% in the control plants. The experiment shows that a slight amount of pinitol synthesis also takes place in plants not subjected to salinity stress. (After Paul and Cockburn 1989)

Table 1.6.4. Contents of osmolytes in leaves of 6-week-old transgenic tobacco plants after 2 weeks of drought. The plants had been transformed with the gene for inositol methyltransferase from *Mesembryanthemum crystallinum*. (After Sheveleva et al. 1997)

Osmolyte	Leaf 11		Leaf 7	
	Day (μmol/g FW)	Night (% of value during the day)	Day (μmol/g FW)	Night (% of value during the day)
Proline	4.2	39	1.6	17
Glucose	7.0	7	5.7	25
Fructose	7.6	5	15.7	27
Sucrose	8.4	19	6.8	12
K^+	352	79	236	72
Na^+	24	113	11.1	96
Cl^-	200	91	129	88
myo-Inositol	6.4	137	3.8	88
Ononitol	34.6	89	25.8	77

variety of these cyclitols is synthesised by means of relatively few biochemical reactions (Box 1.6.2). As is shown in Fig. 1.6.11, inositol metabolism is linked with many cellular functions. The metabolically centrally placed *myo*-inositol is not accumulated as an osmolyte, but its metabolically inactive methyl derivatives are. Well known in this regard are mangroves, which accumulate above all bornesitol, pinitol and quebrachitol in their leaves (Popp and Smirnoff 1995).

In the facultative CAM plant *Mesembryanthemum crystallinum*, the gene for inositol methyltransferase is activated primarily by salinity and cold. Salinity additionally induces the transition from C3 to CAM metabolism. In this process the expression of inositol methyltransferase is induced in the leaves, and the synthesis of the key enzyme for inositol biosynthesis – inositol-1-phosphate synthase – is intensified coordinately (Nelson et al. 1998). D-Ononitol and D-pinitol thus accumulate in *Mesembryanthemum* during salt stress (and drought) (Fig. 1.6.11 B). The synthesis of the latter compound requires a further epimerase (OEP1), which transforms D-ononitol into D-pinitol (Box 1.6.2). At the same time, the osmolyte proline accumulates ten-fold. The induction of CAM and the accumulation of proline are both independent of ABA.

The induction of CAM and the production of osmolytes are not, however, obligately coupled in *Mesembryanthemum*: In young plants, which do not yet possess sufficiently large cells (vacuoles to store malic acid), CAM cannot be induced, or only to a small extent. Full CAM induction is only possible in plants older than 5 weeks. Younger plants are, however, able to activate specific protective measures under salt stress (polyol accumulation, regulation of ion homeostasis, facilitated water uptake).

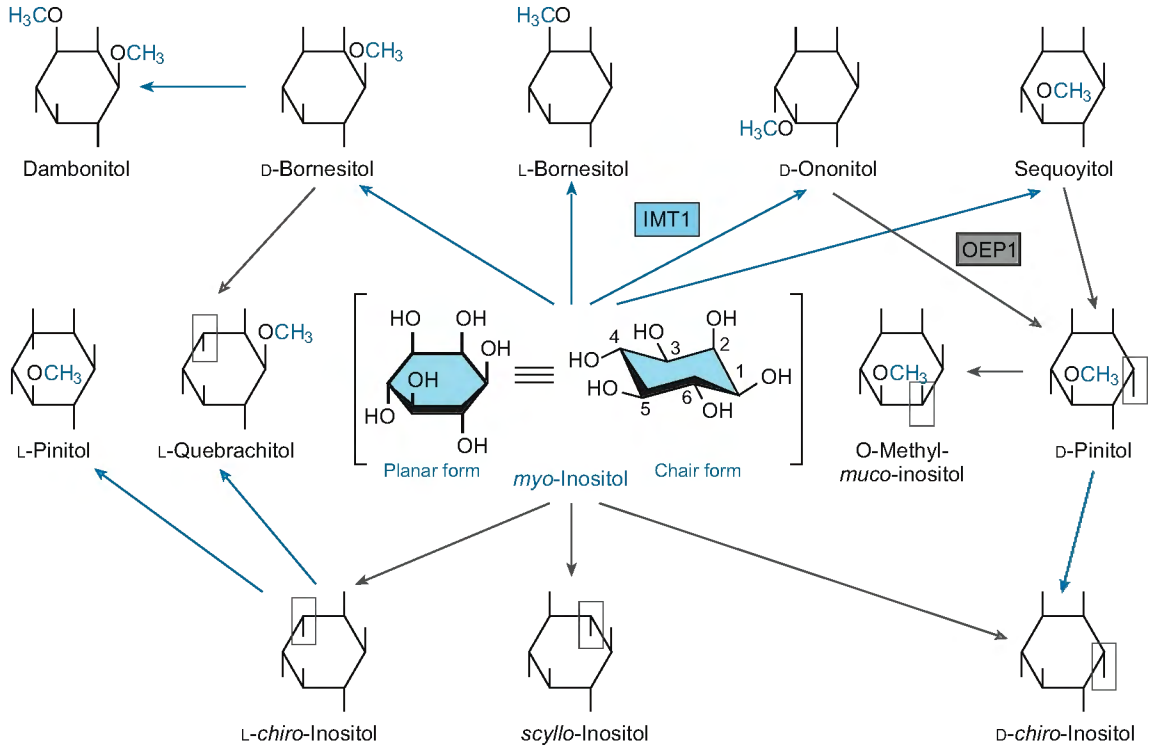
Considering the many ways in which cyclitols are incorporated into cell metabolism, it is difficult to ascribe a particular protective function to them. Whereas concentrations of metabolically active sugars (and also of proline) vary markedly between day and night, the concentrations of the cyclitols (and of the ionic osmolytes) remains largely constant during the day-night cycle (Table 1.6.4). This demonstrates, on the one hand, the sluggish metabolism of the methylated cyclitols, but this is also understandable in a cell biology context, as the osmotic stress is not subjected to day–night cycles either. The cyclitols are most probably also ROS scavengers (Smirnoff and Cumbes 1989).

3. Proline

In addition to methylated cyclitols, the amino acid L-proline (Box 1.6.3) accumulates during salt stress in a similarly ABA-independent manner. Proline is regarded as a general stress metabolite and accumulates at up to enormous concentrations in a great variety of stress situations, particularly also in relation to water deficiency. Proline is produced by microorganisms and green plants in different ways (see Box 1.5.5). *E. coli* synthesises proline from glutamate via glutamate-γ-semialdehyde and Δ^1-pyrroline-5-carboxylate. In eukaryotes, proline may also be formed from ornithine by δ-transamination. Upon osmotic stress, however, the activity of Δ^1-pyrroline-5-carboxylate synthetase (P5CS) increases, so that the

Box 1.6.2 Metabolism of *myo*-inositol

myo-Inositol is shown with its natural isomers; the formation of further inositols by methylation is indicated by *blue arrows* (methyltransferases, e.g. inositol methyltransferase 1, IMT1) and that by epimerisation is shown by *grey arrows* (e.g. ononitol epimerase 1, OEP1). The *grey boxes* show the site of epimerisation. (After Kindl 1994)



prokaryote pathway then dominates in all organisms (Delauney et al. 1993).

The endogenous proline level in tobacco (a glycophyte)[2] was drastically increased by the over-expression of Δ^1-pyrroline-5-carboxylate synthetase (P5CS; Fig. 1.6.12). Both the wild type and plants transformed with the plasmid pBI121 lacking the P5CS gene served as controls in these experiments. As shown in Fig. 1.6.12 A, B, proline synthesis was controlled by the availability of both water and – obviously – nitrogen. The proline level rose distinctly in the P5CS transformants upon water stress; however, the observed concentration of 6 mg Pro/g FW (~0.6%) was still far below that usually exhibited by salt plants. The osmotic potential thus did not differ from that of the wild-type. The osmotic potential of the wild-type and of the control transformant increased under salt stress (Fig. 1.6.12 A), but not that of the plant transformed with P5CS. This is because the P5CS transformant has a distinctly better developed root system[3]. In this case the stress-alleviating effect (Fig. 1.6.12 C–F) could indeed be traced to the accumulation of an osmolyte, namely proline, while in most wild plants other osmolytes accumulate in addition to proline (see Box 1.6.3).

4. QACs and TSCs

The last group of low molecular mass osmolytes to be discussed are the quaternary ammonium compounds (QACs) and tertiary sulfonium com-

[2] The fact that transgenic tobacco is used in so many experiments is not due to molecular geneticists being smokers, but is based on the ease with which tobacco can be genetically transformed!

[3] The reason for the better root growth is not known.

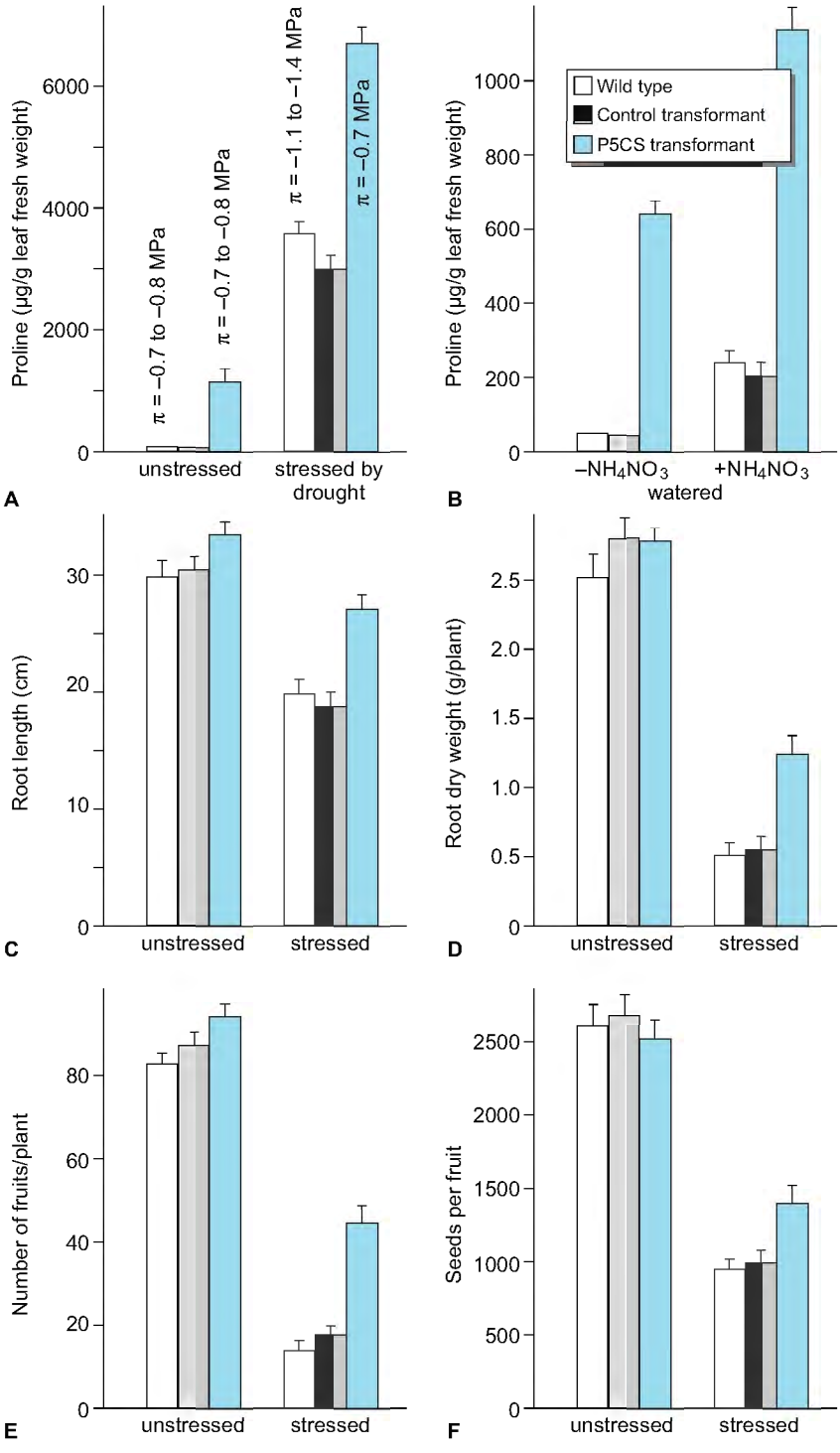


Fig. 1.6.12. Proline as an osmolyte. Tobacco plants were transformed by over-expression of Δ^1-pyrroline-5-carboxylate synthetase and thereby made less sensitive to osmotic stress. A Increase in the proline level in the leaves. B Dependence of the over-production of proline on the nitrogen supply to the tobacco plants. C–F Morphometric data for the wild-type and the transformants upon cultivation without stress and under salinity stress (500 mM NaCl). (After Kavi Kishor et al. 1995)

Box 1.6.3 How does proline function, and why is this particular amino acid suitable for stress amelioration?

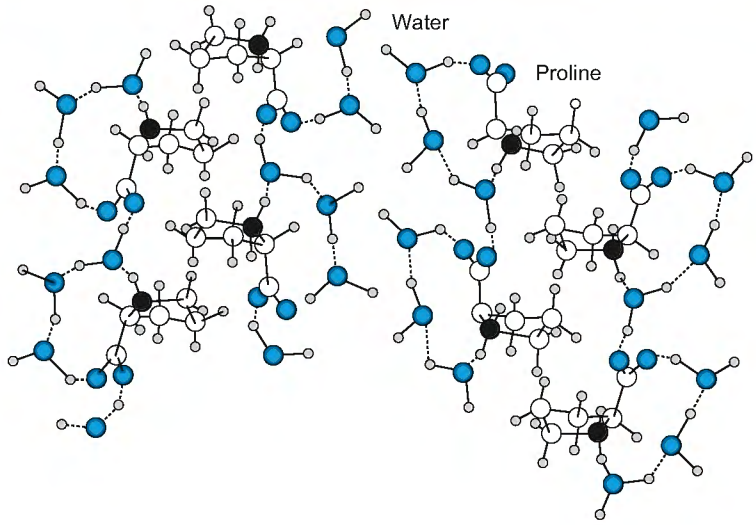


Fig. 1. Molecular model of an aqueous solution of proline. Both the water and the proline molecules form clusters. (After Schobert and Tschesche 1978)

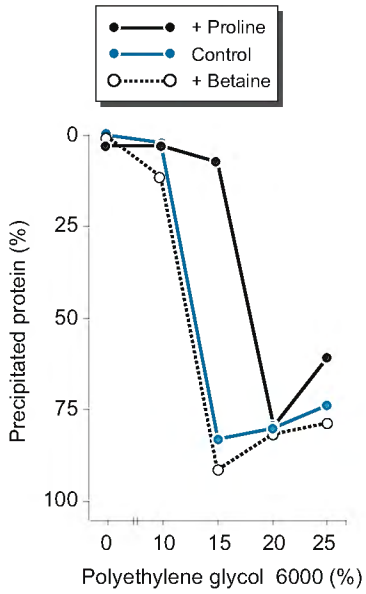


Fig. 2. Reduced precipitability of proteins due to water removal by polyethylene glycol in the presence of proline. (After Schobert and Tschesche 1978)

Proline (Pro) is a special amino acid which, due to its chemical structure, shows unusual properties in aqueous solution. It has the highest solubility of all the proteinogenic amino acids, but at higher concentrations it forms clusters which aggregate via hydrogen bonds between the hydrophilic side chains (Fig. 1). These clusters are strengthened by hydrophobic interactions between the rings. The amphiphilic Pro-multimers attach themselves readily to poorly soluble proteins, with the hydrophobic side to the protein surface and the hydrophilic side facing outwards, thus increasing the hydrophilic surface of the proteins. Pro accordingly works against the unfolding and denaturation of proteins which result from water removal, e.g. during salting-out by ammonium sulfate or precipitation with ethanol or polyethylene glycol (Fig. 2). It is interesting that another group of osmolytes, the betaines, does not work at all in this manner. It appears that there is something akin to a specificity for osmolytes, as if the individual classes of osmolytes have particular target structures and target molecules in the cell. This would also explain the parallel appearance of several different osmolytes in a single organism. Unfortunately, our knowledge in this regard is still extremely incomplete. Rice seedlings produce a large number of compatible solutes under salt stress, including many carbohydrates. It was thereby observed that Pro does not, e.g., protect against secondary oxidative stress (measured as chlorophyll bleaching), whereas trehalose effectively reduces this stress, as does also, e.g., mannitol and perhaps also cyclitols. Pro has no protective effect in these plants, at least not at low concentrations.

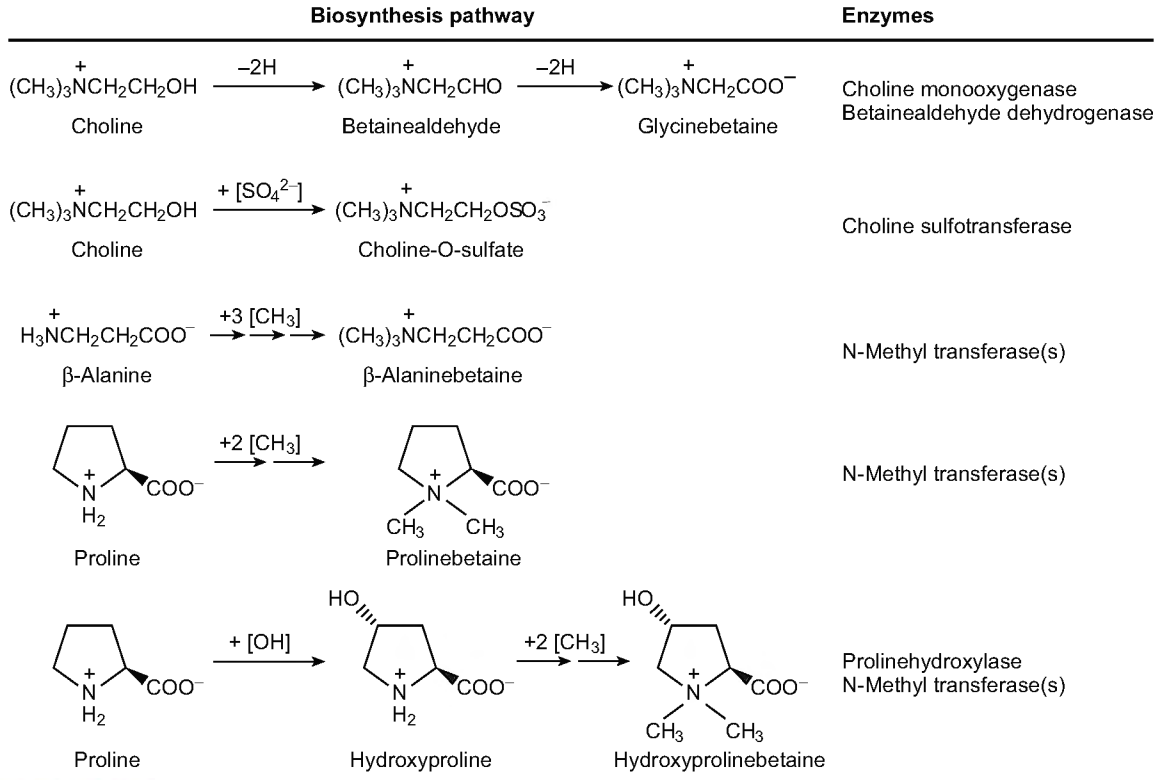


Fig. 1.6.13. Biosynthesis and structures of the zwitterionic QACs in higher plants. (After Hanson et al. 1994)

pounds (TSCs). These compounds are regularly found at high concentrations (>5 µmol/g dry weight; Rhodes and Hanson 1993) in halotolerant representatives of the Chenopodiaceae, the Poaceae and the Asteraceae, as well as in some halophilic genera of the Convolvulaceae and the Plumbaginaceae. They occur in heavy metal-tolerant species, too, and are by no means restricted to plants alone; bacteria, cyanobacteria and animals are also capable of producing these substances. A selection of these osmolytes has already been briefly referred to (see Chap. 1.5.2.6, Fig. 1.5.11).

The biosynthetic pathways leading to these compounds are now known (Fig. 1.6.13), and the corresponding genes have largely been cloned. **Glycine betaine**, the simplest representative of this family of substances, is not formed – as one might assume – by the methylation of glycine, but rather by the oxidation of choline by means of a ferredoxin-dependent monooxygenase. *β*-Alanine betaine, proline betaine and hydroxyproline betaine are, however, indeed formed by the transfer of a methyl group from *S*-adenosyl methionine. These osmolytes are zwitterionic, i.e. they have a cationic as well as an anionic centre.

It has been shown in the example of the salt-tolerant Plumbaginaceae that individual plants can synthesise and accumulate various species of these zwitterionic osmolytes. All accumulate **choline sulfate** and – with the exception of the mangrove *Aegialitis* – at least one betaine, some species even several. Since the mangrove plant *Aegialitis* actively secretes salt, it is probably less dependent on osmolytes and therefore produces only choline sulfate. For most representatives of the family, the stress consists of salinity and drought, and is sometimes compounded by oxygen deficiency due to the presence of salt crusts. Although there is no direct relationship between stressor combinations resulting from particular habitats and preferentially accumulated osmolytes, there appears to be a vague coincidence of choline sulfate and its derivative glycine betaine with drought stress, and of alanine betaine with salt stress (Hanson et al. 1994).

In addition to its osmotic function, choline sulfate undoubtedly also plays a role in the disposal of sulfate, which is important at marine sites. This is because sulfate cannot be excreted via the epidermal salt glands.

Table 1.6.5 A. Composition of the sap of maize leaves of the glycine betaine-producing line *Bet1/Bet1* and the *bet1/bet1* line lacking glycine betaine. The plants were grown on a defined soil mixture and watered with an inorganic nutrient solution. The salt treatment took place 3 weeks after sowing by means of a gradual adaptation from 42.5 to 127.5 mM NaCl during the course of 4 weeks. (After Saneoka et al. 1995)

Conditions of growth	Genotype	Organic solutes (mM)			Ions (mM)					
		Betaine	Amino acids	Sugars	K^+	Na^+	Ca^{2+}	Mg^{2+}	Cl^-	NO_3^-
NaCl-free	*bet1/bet1*	0.2	17.0	156.9	97	0.2	11.2	13.5	48.3	54.7
	Bet1/Bet1	3.2	21.0	149.5	102	0.2	11.4	14.0	43.9	46.8
With NaCl	*bet1/bet1*	0.2	22.4	88.1	112	13.3	13.0	13.0	194	32.7
	Bet1/Bet1	10.9	23.4	110.9	120	13.6	14.4	14.4	231	24.0

Table 1.6.5 B. Growth rates of the lines *bet1/bet1* and *Bet1/Bet1* under salt-free and saline conditions. Plants were grown as specified in Table 1.6.5 A; the salt treatment commenced 30 days after sowing. (After Saneoka et al. 1995)

Growth conditions	Genotype	Leaf growth rate (cm^2/day) after salt application			
		Days 5–10	Days 10–15	Days 15–20	Days 20–25
NaCl-free	*bet1/bet1*	289	268	233	175
	Bet1/Bet1	260	260	224	184
With NaCl	*bet1/bet1*	207	168	82	84
	Bet1/Bet1	218	190	121	121

Since glycine betaine has long been thought to be an efficient osmolyte, efforts have been made to develop lines of crop plants which produce large amounts of this compound upon salt stress by classical breeding. This has been achieved with maize: The sister lines *Bet1/Bet1* are glycine betaine producers, the homozygotic *bet1/bet1* are glycine betaine-deficient.

Table 1.6.5 A shows the enhanced glycine betaine accumulation of the *Bet1/Bet1* maize, particularly in relation to salt stress; the contents of Na^+ and particularly Cl^- are also distinctly higher on salt. The decrease in the concentration of carbohydrates in the expressed sap of the leaves (which is much higher than that of glycine betaine) correlates with the increase in the glycine betaine concentration. Presumably some of the carbohydrate carbon was used for the synthesis of the physiologically superior osmolyte glycine betaine.

Salt inhibits growth, but in this regard *Bet1/Bet1* is seen to suffer considerably less (i.e. it exhibits better leaf growth) than does *bet1/bet1* under salt stress (Table 1.6.5 B). The less impaired growth correlates with an at least partial maintenance of cell turgor under salt stress (0.06 MPa in *bet1/bet1* and 0.2 MPa in *Bet1/Bet1*; Saneoka et al. 1995).

Glycine betaine accumulates particularly in young tissues, where it competes with NaCl and somewhat reduces the content of the salt. There is, however, no indication that glycine betaine is transported within the plant. It evidently is stored at the site at which it is produced from choline (Nakamura et al. 1996).

Because glycine betaine ameliorates stress and plants are evidently unable to metabolise this compound and thus accumulate it, attempts have long since been made to transform crop plants lacking glycine betaine with the key gene encoding choline monooxygenase (CMO), which is required for glycine betaine synthesis. This is difficult in that this enzyme contains a Rieske-[2Fe-2S]-centre. Since glycine betaine synthesis takes place in the chloroplast, it must also be taken into consideration that the recombinant protein (CMO) would have to be transported into the chloroplasts. Such a transformation has recently been successful with tobacco (Nuccio et al. 1998). Accumulation of glycine betaine was, however, only weakly pronounced, because tobacco produces choline – the starting compound for glycine betaine synthesis – only in very small amounts (McNeil et al. 1999).

What Is the Special Protective Effect of QACs?

Transformation of the cyanobacterium *Synechococcus* with an *E. coli* glycine betaine synthesis cassette consisting of choline monooxygenase, betaine aldehyde dehydrogenase, a choline

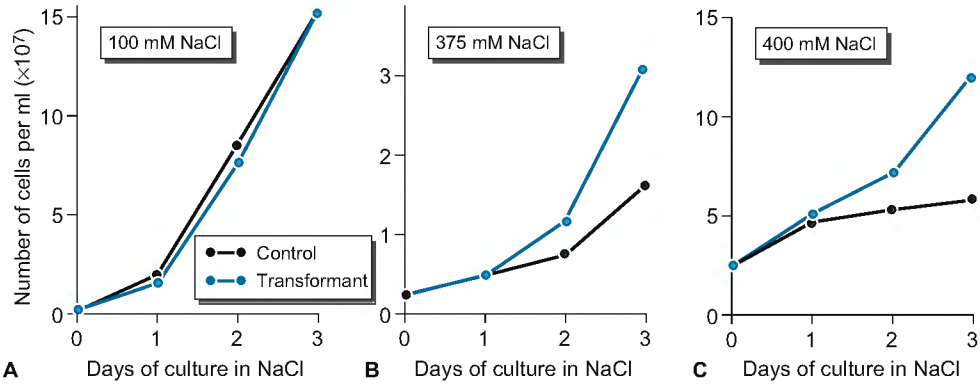


Fig. 1.6.14. Growth of wild-type and transformed *Synechococcus* cells under salinity stress. The cells were grown in choline-containing medium which was supplemented with NaCl at the indicated concentrations after 2 days (time 0 in the graph). The transformants contained the glycine betaine cassette. The glycine betaine concentration in the cells stressed with the higher NaCl concentrations was about 15 times higher than in the unstressed cells. (After Nomura et al. 1995)

Table 1.6.6. Effect of salinity on the photosynthetic electron transport of glycine betaine-accumulating *Synechococcus* cells (transformants) and non-accumulating *Synechococcus* (control) cells. *$DADH_2$* reduced 2,3,5,6-tetramethylphenylenediamine; *MV* methyl viologen; *PBQ* phenyl-1,4-benzoquinone. The reaction of photosystem I consumes (reduces) O_2 and that of photosystem II produces O_2. (After Nomura et al. 1995)

Reaction	O_2 production or uptake (μmol/mg Chl×h)			
	Synechococcus control cells		*Synechococcus* transformants	
	–NaCl	+200 mM NaCl	–NaCl	+200 mM NaCl
$H_2O \rightarrow CO_2$	57	40	52	39
PS I ($DADH_2 \rightarrow MV$)	–152	–96	–217	–177
PS II ($H_2O \rightarrow PBQ$)	71	60	78	77

transport protein and a regulatory protein led to glycine betaine accumulation in the transformants, in particular under high salt stress. These cells grew significantly better under salt stress than did control cells, although their growth was distinctly slower than at low salt stress (Fig. 1.6.14).

The reason for the improved growth is that the photosynthetic electron transport is less impaired by salt in the glycine betaine-containing cells (Table 1.6.6). Glycine betaine is synthesised in the chloroplast and performs its protective function principally in this organelle, where it appears to stabilise mainly the protein complexes of the photosynthetic membrane (Papageorgiou and Murata 1995). In this it differs markedly from the osmolyte glycerol, which has no stabilising effect on the protein complexes. Betaines are so-called **chaotropic compounds**, i.e. zwitterions, which counteract effects caused by ionic forces. They develop their maximum protective effect specifically at those sites where complexes or subunits of proteins threaten to dissociate as a result of salt stress.

DMSP (3-Dimethyl Sulfoniopropionate)

Less is known about TSCs than about QACs. The best-known osmolyte of this group is dimethyl sulfoniopropionate (DMPS), which has been found mainly in algae, but it has also been recently found in certain grasses and Compositae[4]. The starting material for the synthesis of DMSP is the amino acid methionine, which is metabolised to DMSP in marine algae and terrestrial plants according to different pathways. Since the starting compound and the end product are the same in each case, the pathways differ in principle only in the sequence of the individual steps involved (removal of the amino group, addition of a methyl group, decarboxyla-

[4] DMSP is the biogenetic precursor of the atmospheric trace gas dimethyl sulfide, which is released above all by algae.

tion). The various groups of land plants do synthesise DMSP in different ways, however, whereby they all require betaine aldehyde dehydrogenase for the last enzymatic step.

1.6.2.4 Induction of Protective Proteins by Salt Stress

The previous sections have made it clear that salt stress triggers numerous cellular reactions which are all interrelated and complement each other synergistically.

These reactions also include the synthesis of more or less specific protective proteins (Fig. 1.6.15). These may be, on the one hand, osmolyte-producing enzymes or, on the other, proteins from the LEA group (see Chap. 1.5.2.5) which ameliorate the dehydration stress triggered by high salt impact. In addition to these rather specifically acting proteins, another group of polypeptides is formed and accumulated during salinity stress whose function in relation to the stressor salt is not evident. This group includes both proteins which are also often formed during pathogen attacks and proteins of the cell wall and extracellular matrix.

The great variety of proteins which are synthesised and in some cases also strongly accumulated due to the effect of salt makes it difficult to assign the proteins to particular stress responses. The fact that LEA proteins are typical dehydration protection proteins (see Chap. 1.5.2.5) of course does not mean that they specifically ameliorate only the dehydration stress component of salinity.

One explanation for the occurrence of proteins which are not specific for salt stress in organisms subjected to salinity stems from the signal compounds leading to the strain resulting from salinity (see Chap. 1.6.2.1). Salt stress leads to an increase in the levels of ABA and jasmonate in the cell. ABA mediates the linkage of the responses of the cell to salt stress and drought. This stress response is carried over into the responses to stress caused by wounding or pathogen attack via the jasmonates (see Chaps. 1.10.1 and 1.10.2). However, a NaCl-dependent gene expression has been observed which can be assigned to neither ABA, nor jasmonate, nor the ethylene which is occasionally detected in connection with salt stress. This corresponds to the circumstances during drought stress which were described earlier (see Chap. 1.5.2.3).

Osmotins

Osmotins are proteins which accumulate in cells under salt stress and may constitute, e.g. in tobacco cell cultures, up to 12% of the total protein content. To the extent to which their expression is induced via ABA, they can also be synthesised and accumulated under drought conditions (Singh et al. 1989). If their expression is regulated via the jasmonate signal, they are also found upon pathogen attack. Although they were discovered during investigation of the cellular reaction to salt stress and were accordingly termed osmotins, these proteins are not specific for salt stress. Nowadays, they are rather classified as pathogen-related proteins (see Chap. 1.10.2) and referred to as osmotin-like proteins (OLPs), which are products of a multigene family.

Osmotins are relatively small proteins (M_r between 24 and 50 kDa) which are conspicuous in terms of their alkaline isoelectric point (Table 1.6.7). They have a net positive charge at the pH values of the cell, i.e. they are cations. As such, they are probably able to interact efficiently with most anionic membrane proteins (Kononowicz et al. 1993). They possess a large number of disulfide bridges, but no free SH groups (Zhu et al. 1995).

Several osmotins have been found in each of the plants that have been subjected to detailed study; they differ not so much in their molecular mass as in their isoelectric points and other protein characteristics. For tobacco, the best-studied species, a pre-protein has been discovered in addition to the mature protein, from which it could be concluded that mature osmo-

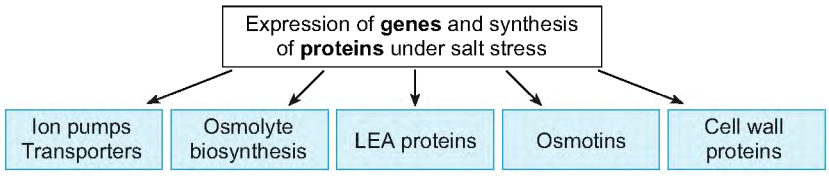


Fig. 1.6.15. The various groups of proteins induced by salinity stress

Table 1.6.7. Characterisation of osmotin and some related proteins ("OLPS"). a: known from the cDNA; b: probably targeted to the vacuole. (From various publications, above all Singh et al. 1989)

Type/constitutive type/ protein designation	Molecular mass (M_r) kDa	Isoelectric point	Expression	Characteristics
Atripex nummularia/halophyte				
pA8	23.8	8.3	Constitutive and ABA-controlled	a
pA9	23.8	6.9	NaCl-inducible, ABA-independent	a
Tobacco/glycophyte				
Pre-osmotin	28.5		ABA-, jasmonate-, and NaCl-inducible	Signal peptide present, soluble, protease-sensitive, probably ubiquitous in the cell
Osmotin I	26.0	7.8		
Osmotin II		8.2		Vacuolar inclusion bodies, protease resistant
Potato (*S. comersonii*)/glycophyte				
pA13	26.7	6.7	ABA-, cold-, NaCl-, wounding-, and infection-inducible	a, b
pA35	26.7	5.7	ABA-, cold-, NaCl-, wounding-, and infection-inducible	a, b
pA81	27.5	8.0	ABA-, cold-, NaCl-, wounding-, and infection-, inducible	a, b
Tomato/glycophyte				
Osmotin 24	24.0		Constitutive, ABA-controlled	
Osmotin 26	26.0		NaCl-inducible	
Thaumatococcus daniellii/glycophyte				
Thaumatin I and II	22.1	12.0		Weight for weight 3000 times sweeter than sucrose
Maize/glycophyte				
α-Amylase/trypsin inhibitor	22.1		Inducible by infection and wounding	

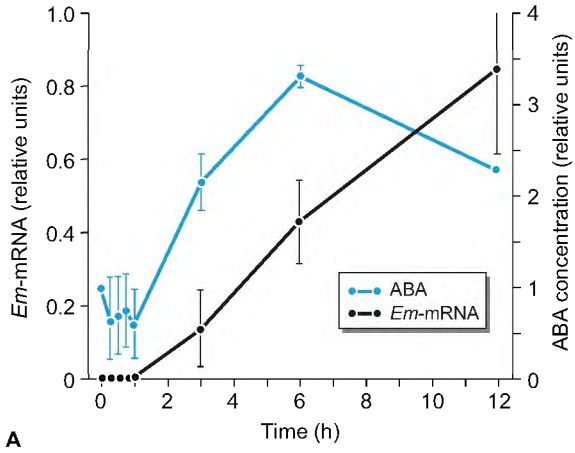
tins occur in specific compartments. However, the sequence which is considered to represent a signal peptide gives no indication as to the compartment to which the protein is targeted.

In tobacco cells, osmotin is found in a soluble form (osmotin I) and an insoluble form (osmotin II), the latter in the form of inclusion bodies in the vacuole. Because both forms react with the same antibody, it is presumed that osmotin II is an aggregation product of osmotin I (Singh et al. 1989).

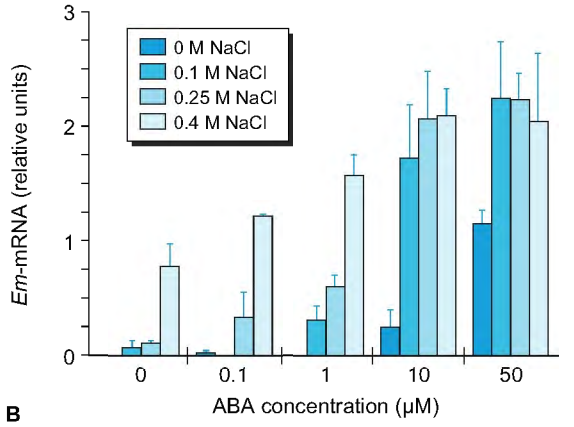
It is striking that there is a high degree of homology between the amino acid sequences of the osmotins and of proteins involved in defence against plant pathogens (the so-called PR proteins: see Chap. 1.10.12) or of proteins that can be induced by wounding (e.g. the inhibitor of animal α-amylase and of the trypsin of maize and other cereals). Although these proteins do not interact with antibodies against osmotins, much speaks for including them in the family of the osmotins (therefore the term OLPs, see above). It is particularly noteworthy that they are similar to **thaumatin**, a related protein from the East African plant *Thaumatococcus daniellii* (Marantacae) which tastes 100,000 times sweeter than sucrose on a molar basis. This protein has an extremely alkaline isoelectric point (IEP) on account of its particularly high content of basic amino acids. The secondary structure of thaumatin is known (de Vos et al. 1985), and it is thought that osmotins have a similar hydrophobic barrel structure of antiparallel pleated sheets with projecting hydrophilic loops. An interaction with biomembranes might be initiated via these loops (see below). At any rate, however, no other osmotins have any taste.

Expression of Osmotins

An increase in endogenous ABA concentration or an application of ABA stimulates adaptation to salinity and promotes the expression of osmotins. In most cases, however, it becomes apparent that osmotin synthesis is not exclusively, but only partly under the control of ABA, and that salt and ABA contribute synergistically to



A



B

Fig. 1.6.16. A Kinetics of ABA accumulation and Em transcript enrichment after treatment of rice suspension culture cells with 400 mM NaCl. The ABA content at time 0 was 0.79±0.27 nmol/g dry weight. B Interaction between externally applied ABA and NaCl in the induction of Em expression in suspension culture cells of rice. The cell cultures were simultaneously inoculated with ABA and NaCl and harvested after 24 h. Em is one of the LEA proteins of rice. (After Bostock and Quatrano 1992)

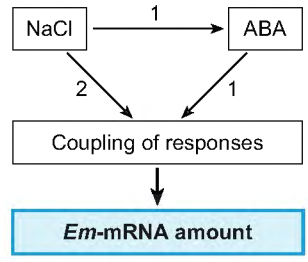


Fig. 1.6.17. Model of the interaction between NaCl and ABA in the induction of Em expression in suspension culture cells of rice. Salt can effect induction of the LEA gene Em via ABA (route 1) or directly, i.e. independently of ABA (route 2). Both routes converge on a common intermediate where they interact and lead to enhanced expression of the mRNA. (Bostock and Quatrano 1992)

the expression of osmotin mRNA (Grillo et al. 1995).

Salt is therefore required for the maximum expression of osmotin genes, and the accumulation of osmotins upon salt stress, too, requires a high osmotic potential. Osmotin synthesis evidently does not take place only under the transcriptional control of ABA and salt, but is also under the translational or post-translational control of both or one of these factors (La Rosa et al. 1992). The result is an increase in sensitivity towards ABA due to NaCl (see also Fig. 1.6.16).

Gene fusion products of the osmotin promoter and a reporter gene (GUS) have been used to study the spatial and temporal expression patterns of osmotins in the plant as well as the sensitivity of the promoter to the various types of stress. The region of the promotor which is responsive to ABA, ethylene, viral infections, salt, drought, UV, wounding, and fungal infections is quite large (~1100 base pairs); the minimum region required for the ABA effect is considerably smaller (the so-called fragment A of base pairs −248 to −108; Liu et al. 1995). The expression of the osmotin gene can be clearly separated from the accumulation of the protein itself. The latter can only be detected upon salt stress or fungal infection.

Corresponding to its stimulation by ABA, the osmotin gene is expressed in all situations in which drought results in an elevated ABA level: During the ripening of pollen grains, during the drying of seed pods, during the senescence of flowers, and in senescing leaves. An accumulation of osmotin does not take place in these cases, however. Osmotin does accumulate though during salt stress, particularly in the elongation zone of the root, in old leaves, in the xylem parenchyma of the shoot, and in the epidermis (Kononowicz et al. 1993).

Salt induces the expression of other protective proteins in addition to osmotins; best known are the **LEA proteins** and how these work. The expression of these proteins is partly dependent on ABA (see Chap. 1.5.2.2), but exhibits – as is the case with osmotin – at least one additional inducer, namely salt (see Figs. 1.6.16 and 1.6.17).

Induction by salt may be mediated via the "stress hormone" jasmonate in certain cases. Jasmonates induce the expression of many proteins with protective effects, amongst them os-

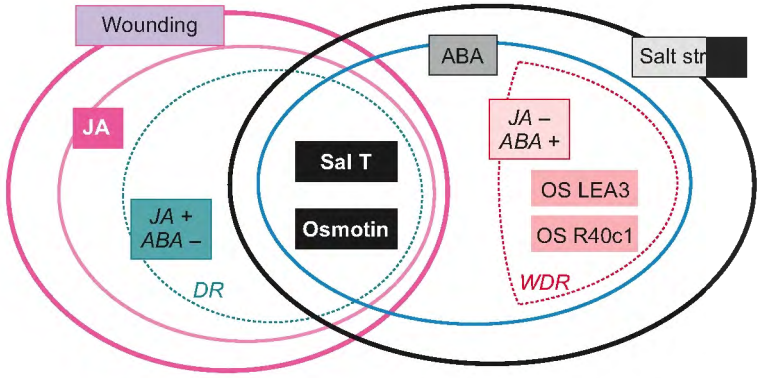


Fig. 1.6.18. Model of the effects of jasmonate and ABA on the expression of genes which are induced by salt stress. *ABA, JA* Gene expression influenced by ABA or JA or both; *JA+/ABA–* and *JA–/ABA+* antagonistic effects of JA and ABA. *Overlap* Synergistic effects; *WDR* water deficit response; *DR* defence response. *Sal T*, osmotin, *OS LEA3* and *OS R40c1*: genes. (After Moons et al. 1997)

motins and PR proteins. The expression of some of these (osmotins) is induced by both jasmonate and ABA. In this case, various stressors each probably preferentially trigger one of the two signal pathways to a particular extent. Special attention is thereby paid to the interaction of salt stress and stress due to infection or wounding, because it has become apparent that the response to one of these stressors causes a **"systemically induced resistance"** (immunity) to the other stressors. The next section will explain what this cross protection is due to.

The Function of Osmotins

In contrast to the LEA proteins (see Chap. 1.5.2.5), the function of osmotins in salt stress is unclear. If the conception of an unspecific function as a consequence of the induction pathway is not acceptable (it would be uneconomical in light of the sometimes enormous accumulation of osmotins), the cell biological role of the osmotins must be deduced from their relationship to PR proteins.

Transformation of potato plants with the osmotin gene from tobacco under the control of the strong (CaMV)35S promoter leads to osmotin accumulation and increased resistance to *Phytophthora infestans* (potato blight).

Osmotin inhibits the growth and spore germination of a number of pathogenic fungi such as *Phytophthora*, *Botrytis* and *Helminthosporium* in a manner which is nevertheless species-specific. It depolarises the cell membrane potential of the fungal hyphae by eliminating the pH gradient. This could be brought about by a two-fold mechanism: Thaumatin (see above) exhibits a tertiary structure fixed by eight disulfide bridges, of which the N-terminal domain has similarities to receptor-binding proteins. This highly conserved domain, which is also present in osmotin, could bind to certain regions of the fungal membrane and integrate its hydrophobic "barrel" of antiparallel β-pleated sheets into this membrane. This "barrel" would then produce a pore (a hole) in the membrane.

PR proteins and chitinases bind preferentially to actin and thus accumulate at the sites at which fungal hyphae penetrate into the cell, where the cytoskeleton is also amassed. The defence enzymes and the poration mechanism put the intruding fungal hyphae under particularly strong attack.

Can this situation be connected with osmotic stress? Pathogen attack destroys the host cell directly or during the course of the hypersensitive reaction (see Chap. 1.10.2), and the enzymes and substrates released damage the neighbouring cells, beginning with the plasma membrane. The breakdown of the selective permeability of this membrane leads to osmotic stress, whereby the toxins of the pathogen intensify the effect. This damage can be recognised macroscopically by the wilting of the infested plant parts and organs.

Fungal attack and osmotic stress are thus causally linked. Both induce not only the expression of the *OSM* gene, but also the accumulation of osmotin. Osmotic stress exerts itself mainly via ABA, pathogenic stress via jasmonate.

Osmotin and LEA proteins, as well as PR proteins, are expressed and accumulated via both pathways, and probably also by means of a third, ABA- and jasmonate-independent route (Fig. 1.6.18). Since osmotins are metabolically very inert, as indicated by the vacuolar inclusion bodies, it is understandable that the component of salt resistance contributed by osmotin is maintained for a long time after a salinity stress. Similarly, pathogen resistance (latent) is also maintained because of the supply of osmotin in the cell. Osmotins thus represent a very interest-

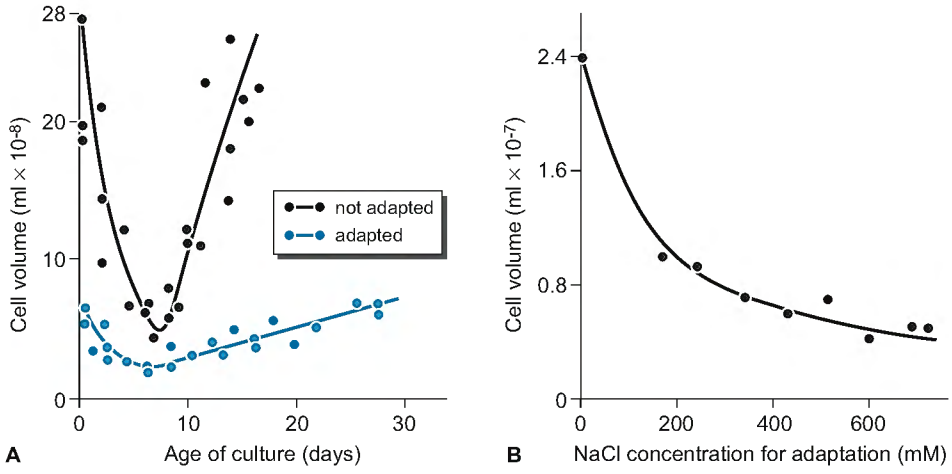


Fig. 1.6.19. Cell growth under salinity stress. A Cell volume during the course of a cell culture from the inoculum until into the stationary phase. The adapted tobacco cells grew at 428 mM NaCl in the medium. B Dependence of the cell size in the stationary phase on the salt content of the medium. (After Iraki et al. 1989a)

Table 1.6.8. Excretion of cell wall material from tobacco suspension culture cells in the stationary phase. The medium of the non-adapted cells contained no NaCl, that of the NaCl-adapted cells contained 428 mM salt. Drought stress was achieved with 30% polyethylene glycol. Uronic acid is a measure of pectin. (After Iraki et al. 1989b)

Cell type	(mg/25 ml cell culture)			
	Extracellular material (total)	Carbohydrate (total)	Uronic acid	Protein
Not adapted	26.0	18.4	7.2	0.4
Adapted to NaCl	28.0	24.1	1.1	2.8
Adapted to PEG	1.0	0.8	0.1	0.1

ing possibility for the molecular genetic transfer of pathogen and salt resistance on account of their longevity.

The Effects of Salt Stress on Cell Wall Proteins

Salt stress also leads to reactions in the cell wall. The structural proteins in this compartment are proline- and hydroxyproline-rich proteins (so-called extensins), and glycine- and arabinogalactan-rich proteins which are fixed in the cell wall by cross-linkage with one another or with polysaccharides (Showalter 1993). Since salinity-stressed cells are considerably smaller than non-stressed cells (Fig. 1.6.19), it was thought that changes in cell wall proteins could also take place under these conditions. Cells growing under osmotic stress often have considerably higher turgor than cells without salinity stress. Although their cell walls have the same thickness as those of non-stressed cells, they appear to have considerably less tensile strength (Iraki et al. 1989a). This was explained in terms of the walls of the cells adapted to salt containing much less cellulose and extensin than do those of the non-adapted cells. On the other hand, suspension culture cells adapted to salt excrete significantly more and above all more protein-rich cell wall material into the medium than do non-stressed cells (Table 1.6.8). These data show that salt stress inhibits cell wall metabolism, especially the dynamics of its polysaccharide metabolism (Iraki et al. 1989b).

The synthesis of cell wall-specific proteins is also influenced by stressors other than salt. Certain extensins are formed in greater amounts upon wounding or fungal infection. They become interconnected with one another or with hemicelluloses and pectin ("formation of papillae") under the influence of H_2O_2, an elicitor or high concentrations of GSH, and are thus insolubly deposited within the cell wall.

Salt stress evidently leads to increased synthesis of cell wall material, but not to its cross-linking, for which the so-called oxidative burst is required (Lamb and Dixon 1997). The polymers so formed are thus increasingly excreted into

the medium, especially since cell wall synthesis is inhibited (see above). Another protein secreted into the medium upon salt stress has been identified as chitinase, which is deposited in small pools in both the cell wall and the vacuole in non-stressed cells. Such chitinases are synthesised in increased amounts and secreted during a pathogen attack. The connection between osmotic stress and pathogenic stress can also be seen here.

1.6.3 Avoidance of Salt Stress

Plants growing in salty surroundings (e.g. along sea coasts) often exhibit the phenomenon of salt exclusion, which is interesting in the context of avoiding salt stress. In mangroves, e.g. *Rhizophoria* or *Sonneratia*, the solution in the xylem vessels is very dilute, constituting 0–1% NaCl compared with 3% salt in the environmental medium. Here, the principle of selective Na^+ exclusion (no or only very low affinity of the cation transporter for Na^+) appears to be very effectively realised. Ion barriers may occur in the rhizodermis or also further into the root. If such barriers do not exist, salt is taken up by the plant and distributed via the xylem stream. Both Na^+ and Cl^- are phloem-mobile and are thus usually transported from the leaves back to the shoot and then deposited in the parenchyma there (this happens when the older leaves are not the final site of deposition and are shed after being loaded with salt, as, e.g., in cereals; Colmer et al. 1995). A salt gradient is then formed from the base of the stem to the tip, and from the older to the younger leaves. The meristems, which are not yet connected to the vascular system and in which transport (including that of salts) takes place from cell to cell, are spared the burden of the salt load.

To conclude the chapter on stressor salt, the various aspects that have been discussed are summarised in Fig. 1.6.20.

Summary

1. In contrast to the case with animals, salt (NaCl) plays no or only a subordinate role in the metabolism of plants. Only true salt plants require salt to flourish optimally. A distinction is made between plants which are

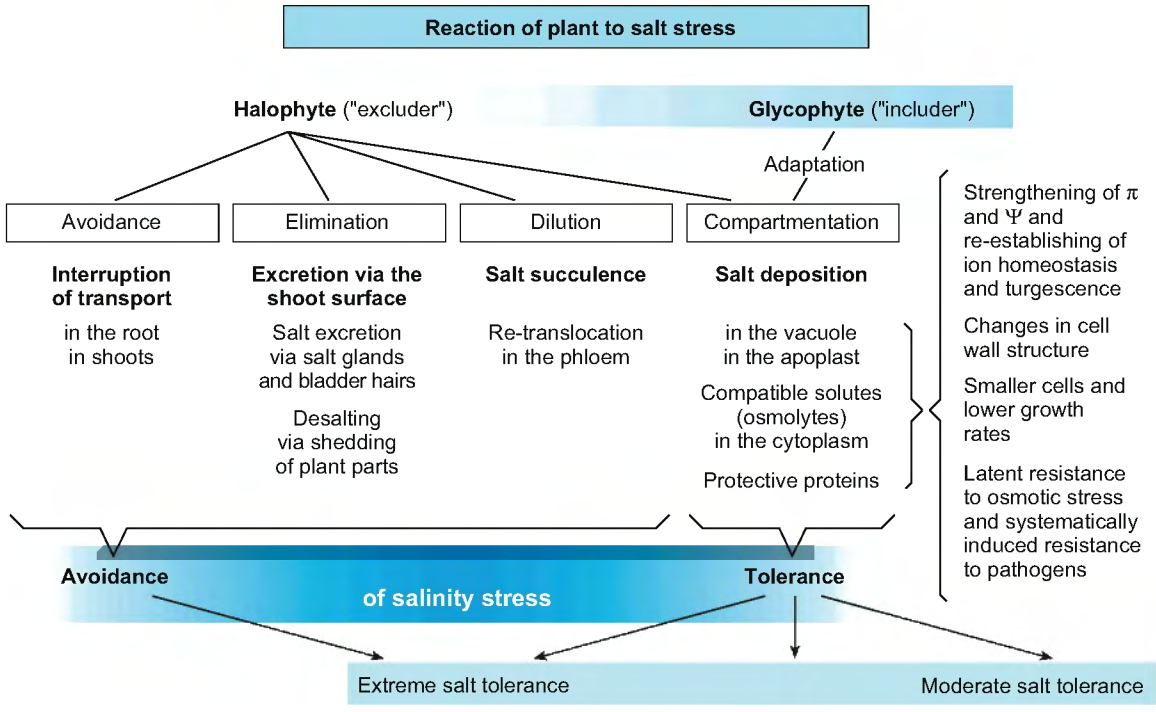


Fig. 1.6.20. Summary of the chapter on salt stress

not tolerant of salt (glycophytes) and those which are (halophytes).

2. Because of the small diameters of Na^+ and Cl^- ions and the consequent high charge density at their surfaces, both of these components of salt are considered to be aggressive osmolytes. In addition to the so-called salinity stress resulting directly from these ionic properties, the high charge densities of the salt ions also result in the binding and effective withdrawal of water (dehydration stress).
3. The most important membrane potentials in a functional plant cell are those of the plasmalemma (about 200 mV) and the tonoplast (up to 20 mV). The cytosolic side of the membranes is always the electronegative side. These potentials derive from the activity of ion pumps and channels, and result from the intracellular pools of cations and anions which the pumps and channels maintain in equilibrium. These equilibria (ion homeostasis) are dynamic, and their maintenance is aided by the action of proton-ATPases. This ensures that the pH values of the vacuole (about 5), of the cytoplasm (about 7) and of the apoplast (about 5) remain fairly constant. Non-stressed plant cells contain only very small cytoplasmic and vacuolar pools of Na^+ and Cl^-.
4. Salt stress leads to an influx of Na^+ and Cl^- and to augmentation of cytoplasmic Ca^{2+} pools. Na^+ and K^+ thereby compete for transport systems, with the result that not only the intracellular Na^+ pools increase in size, but also that the K^+ pool sizes shrink. The attempt by the cell to re-establish the original membrane potentials by increased pumping activity (particularly that of the H^+ pumps) results in the pH of the cytosol being shifted by up to 1 pH unit, which leads to changes in enzyme activities. The elevated calcium level triggers regulatory processes (activation of genes), which may be interpreted as adaptation to high salinity.
5. Growth by cell division and elongation is particularly affected, as are photosynthetic electron transport (with secondary damage due to ROS) and cell water relations. In particular young tissues often become necrotic.
6. Adaptive responses of plant cells to salinity stress include measures to re-establish ion homeostasis, to adjust the osmotic potential and to synthesise protective proteins.
7. How osmotic stress is perceived is still poorly understood (see Chap. 1.1.4). A signal cascade which reacts to the elevated Ca^{2+} level (SOS system) activates a Na^+-H^+ antiporter in the plasma membrane. Another signal chain, which is also calcium-dependent, leads to synthesis of LEA proteins and of protein kinases. Further signals result from augmentation of the intracellular concentrations of ABA and jasmonate, which induces the expression of numerous proteins.
8. Among the proteins which are newly synthesised or are synthesised in increasing amounts are those which serve to re-establish an ion homeostasis which exhibits changes in some of the pool sizes while maintaining the original membrane potentials and pH gradients, i.e. Na^+-H^+ antiporters in both the plasma membrane and the tonoplast membrane. Since these antiporters also require high proton concentrations, the synthesis of H^+-ATPases is also intensified. The salt tolerance of *Arabidopsis* was considerably increased by over-expression of one of the vacuolar Na^+-H^+ antiporters.
9. Halophytes are able to eliminate salt from the cytosol by excreting it from the apoplast onto the leaf surface or by forming large vacuoles in the mesophyll ("salt succulence") or in "bladder hairs". These excretion mechanisms are termed "recretion".
10. In addition to re-establishing ion homeostasis, increasing the osmotic potential of the cell is an important part of adaptation to salt stress. Halophytes are able to use salt as an osmoticum in the vacuole, whereas glycophytes, which do not (and cannot) produce such high osmotic potentials, usually augment their osmotic potential by means of organic osmolytes. Halophytes, too, must produce such compatible solutes to osmotically stabilise the cytosol, as they, too, must maintain a low salt concentration in this compartment. In addition to their osmotic function, organic osmolytes stabilise and protect biomembranes and additionally also often act as scavengers for ROS.
11. The most important osmolyte groups with respect to salinity are open-chain and cyclic polyols (sugar alcohols and cyclitols), which are only slowly metabolised and therefore guarantee long-term protection. The slow metabolism of the cyclitols is due to methylation (of *myo*-inositol). Sugar alcohols and

cyclitols are well suited for the transformation of crop plants to increase salt tolerance. Other important osmolytes are the amino acid proline, quaternary ammonium compounds (QACs, e.g. betaine) and tertiary sulfonic acid compounds (TSCs, e.g. 3-dimethyl sulfoniopropionate). They protect above all protein complexes against dissociation caused by salt.

12. In addition to LEA proteins and the proteins which catalyse the biosynthesis of osmolytes, protective proteins are synthesised and accumulated upon salinity stress. It is doubtful as to whether the functions they perform are really specific for salt stress. Amongst these protective proteins are osmotins and osmotin-like proteins, which are now regarded as belonging rather to the pathogen-related proteins, since their synthesis is also induced by pathogens. Osmotins have a strikingly alkaline isoelectric point; they are thus positively charged at the pH values of the cell and can accordingly readily interact with negatively charged membrane proteins. It is assumed, on the basis of their molecular structure, that they penetrate into the plasma membrane of the pathogen and form pores there, which make it easier for the host to combat the pathogen. Their function in relation to salinity stress is less clear. Osmotins, as well as the LEA proteins, are induced by ABA, jasmonate and ethylene and by the stressors which effect the accumulation of these phytohormones. They thus constitute a component of a system that cross-protects against all sorts of possible stressors. Because they are also deposited in vacuolar inclusion bodies, they are thought to provide long-term protection.
13. In addition to the production of the above-mentioned proteins, salt stress also influences cell wall metabolism. The cell walls of salinity-stressed cells contain less cellulose and fewer cell wall proteins (extensins). Salt stress evidently impairs the cross-linking of the extensins to one another and to the carbohydrates of the cell wall.
14. Avoidance of salinity stress: Plants of saline environments, e.g. mangroves, have developed a very effective mechanism for selective cation uptake. They evidently possess potassium transporters with no or only very little affinity for sodium, and are thus able to exclude Na^+ from their tissues. Other halophytes with a less selective K^+ uptake system make use of the fact that Na^+ and Cl^- are phloem-mobile ions which are able to circulate within the plant. They accumulate salt at the base of the stem, while the growing parts of the plant are kept largely salt free.

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1.7

Heavy Metals

Lower plants, algae, and especially fungi, are superior to higher plants in coping with heavy metals. In general metals are deposited on surfaces. A particular type of crust formation is the so-called desert varnish that is formed by biological activity. Rock inhabiting algae and fungi solubilise iron and manganese ions from the rocks and deposit them on the surface. The advantage of the crustaceous life form on rocks (mostly dolomite) is a higher CO_2 concentration, and a prolonged water availability after dewfall: Rocks absorb dew by capillary action. This moisture is sufficient for net CO_2 assimilation by endolithic lichens. Cliff drawings, close to Avdat, Negev, showing a rider on an ostrich hunt; ostriches were extinct in the Negev a long time ago.

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Heavy metals are metallic elements with a density ≥ 5 g/cm^3, namely the elements Ag, As, Au, Bi, Cd, Co, Cu, Cr, Fe, Hg, Mn, Mo, Ni, No, Pb, Pt, Sb, Sn, Ti, Tl, U, V, Zn, and Zr. In addition to their high specific weight, most of them can occur in more than one coordination number, i.e. they can become oxidised or reduced. They often occur as metallic components of enzymes which transfer electrons, for example:

- Fe in heme (e.g. in cytochromes) and in S-Fe proteins (e.g. in aconitase),
- Mn in photosynthetic water oxidase, in many dehydrogenases and in superoxide dismutase (SOD),
- Cu in cytochrome oxidase, in plastocyanin, in catalases and in other oxidases,
- Zn is often a component of dehydrogenases, certain species of SOD, of carboanhydrase and of nucleic acid binding proteins (zinc finger),
- Mo occurs in enzymes of N metabolism (e.g. in nitrogenase and nitrate reductase),
- Co in cobalamine (coenzyme B_{12} which is a derivative of vitamin B_{12}).

This listing is, of course, not exhaustive; nevertheless, it shows the requirement of plants for (some) heavy metals. Related to multiplicity of valences is also the tendency of heavy metals to form chelates, which are important for uptake by plants but also for sequestration in the plant.

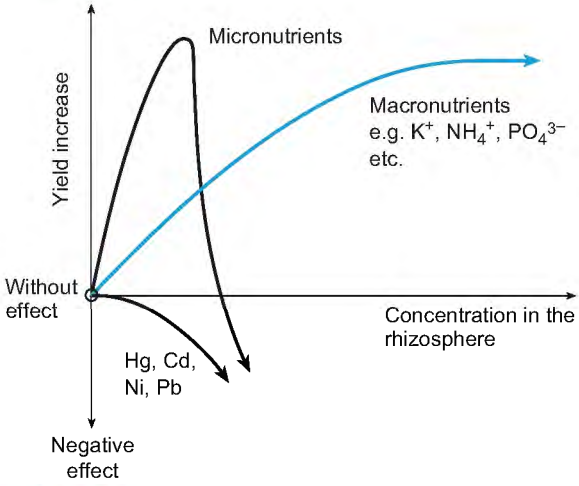


Fig. 1.7.1. Influence of ion availability in the rhizosphere on yield. (After Wallnöfer and Engelhardt 1984)

Some heavy metals are **micronutrients** (Fe, Mn, Zn, Cu, Ni, Mo, Co), others are potent **cell toxins**: Hg, Pb, Cd, Cr and As. However, also micronutrients may become toxic if they accumulate in the organelles of plant cells to higher concentrations. For heavy metals the concentration ranges of deficiency, optimal supply and toxicity are very close together (Fig. 1.7.1 and Table 1.7.1).

While the thresholds of symptoms of heavy metal deficiency are more or less independent of the plant species and within a relatively narrow concentration range, toxicity of heavy metals shows a broad concentration range (Table 1.7.1): Many plants are able to diminish uptake, detoxify or sequester (the so-called **hyperaccumulators**) heavy metal ions and thus avoid metabolic problems. Certain plants growing on soils with a surplus of heavy metals are called **metallophytes**. For example, *Silene vulgaris* is able to tolerate cadmium, cobalt, copper, lead, manganese, nickel and zinc in the substrate, *Festuca ovina, Agrostis tenuis* and *Minuartia verna* have ecotypes with similar metal resistance as *Silene vulgaris*, *Viola calaminaria* grows on soils rich in zinc and the fern *Pteris vittata* accumulates arsenic.

1.7.1
Availability of Heavy Metals

The heavy metal content of soils is usually sufficient for the nutrition of plants, it rather may be too high due to human activities. The total content of heavy metals of a soil is not crucial for the plant (e.g. Fe ca. 10,000 to 50,000 mg/kg, Mn up to 3000 mg/kg, Zn up to 300 mg/kg, Cu up to ca. 50 mg/kg), rather the availability of heavy metal ions is the determining factor. Availability, however, is a question of the solubility of heavy metals as ions or complexes, of the pH and the redox potential of the soil, and of the activity of plant roots and their association with mycorrhizal fungi. Unfavourable constellations of these factors may, at a similar total content, cause heavy metal deficiency or toxicity.

1.7.2
Heavy Metal Deficiency – Example Iron

1.7.2.1
Uptake of Heavy Metals

The effects of heavy metal deficiency on plants will be discussed for iron as an example. Except for poorly aerated mineral soils (gleys and pseudogleys), iron usually occurs in its oxidised, i.e. trivalent, form: goethite (α-FeOOH), haematite (α-Fe_2O_3) and as a lattice element in many minerals. Ferric oxides become insoluble in an alkaline environment and thus **iron deficiency** occurs in alkaline, well-aerated soils. In plants iron deficiency causes **chlorosis** particularly in the intercostal fields of leaves. This phenomenon intensifies at a high supply of phosphate and HCO_3^-, as insoluble ferric phosphates, hydroxides and (bi)carbonates are formed.

The plant root only takes up the ferrous ion. In the soil, however, the ferric ion is usually

Table 1.7.1. Assessment of the microelement concentrations (mg/kg dry matter) in fully developed leaves of agronomical crop plants (Amberger 1988)

Micronutrient	Deficiency	Adequate supply	Toxicity
Mn	<20	20–250	>500
Fe	<50	50–250	(>500)
Zn	<20	20–150	>400
Cu	<40	5–20	>40
B (monocots)	<2	2–5	>20
B (dicots)	<15	15–100	>200

Table 1.7.2. Critical toxicity of manganese ions in shoots of various higher plants. The "critical value" is that concentration at which the plant produces 10% less than its normal biomass (Marschner 1986)

Crop	Mn content (mg/g dry weight)
Maize	200
Soybean	600
Cotton	750
Sweet potato	1380
Sunflower	5300

present and, therefore, iron must be reduced in order to enter the plant (Fig. 1.7.2 A). This is accomplished by the enzyme **chelate reductase.**

Dicots and monocots (except grasses) are capable of increasing their capacity to reduce Fe^{3+} and thus take up iron more efficiently in iron-deficient situations. Chelate reductase is bound to the plasma membrane of the rhizodermis and the surface of this membrane is increased several-fold by formation of cell wall labyrinths of the so-called rhizodermal transfer cells (Fig. 1.7.3).

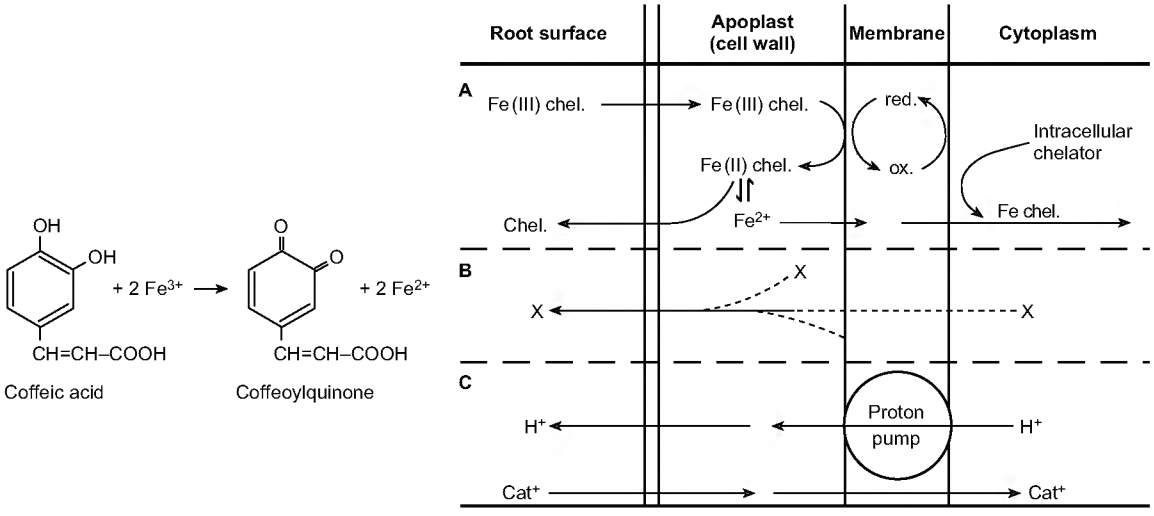


Fig. 1.7.2. Increased uptake of iron by iron "efficient" dicotyledonous plants, upon iron deficiency. A Increase in the reductive capacity. B Exudation of phenolic compounds. C Increase in proton excretion. *Chel* Chelator; Cat^+ cation; *X*, e.g. caffeic acid. (After Marschner 1986)

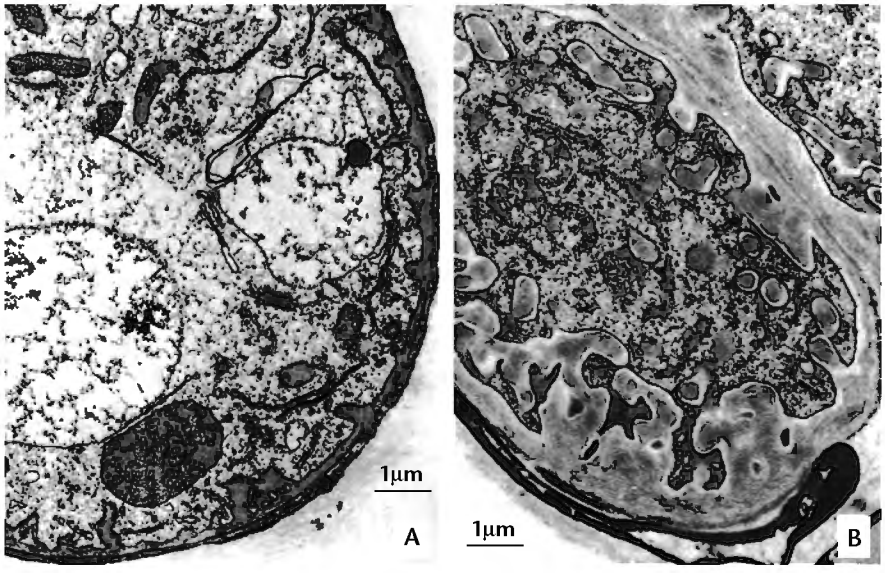


Fig. 1.7.3. Root cells with wall labyrinth (transfer cells). A Cross section through a root hair with weakly developed wall labyrinth. B Papillate rhizodermal cell (transfer cell) with amply developed labyrinth. (After Kramer et al. 1980)

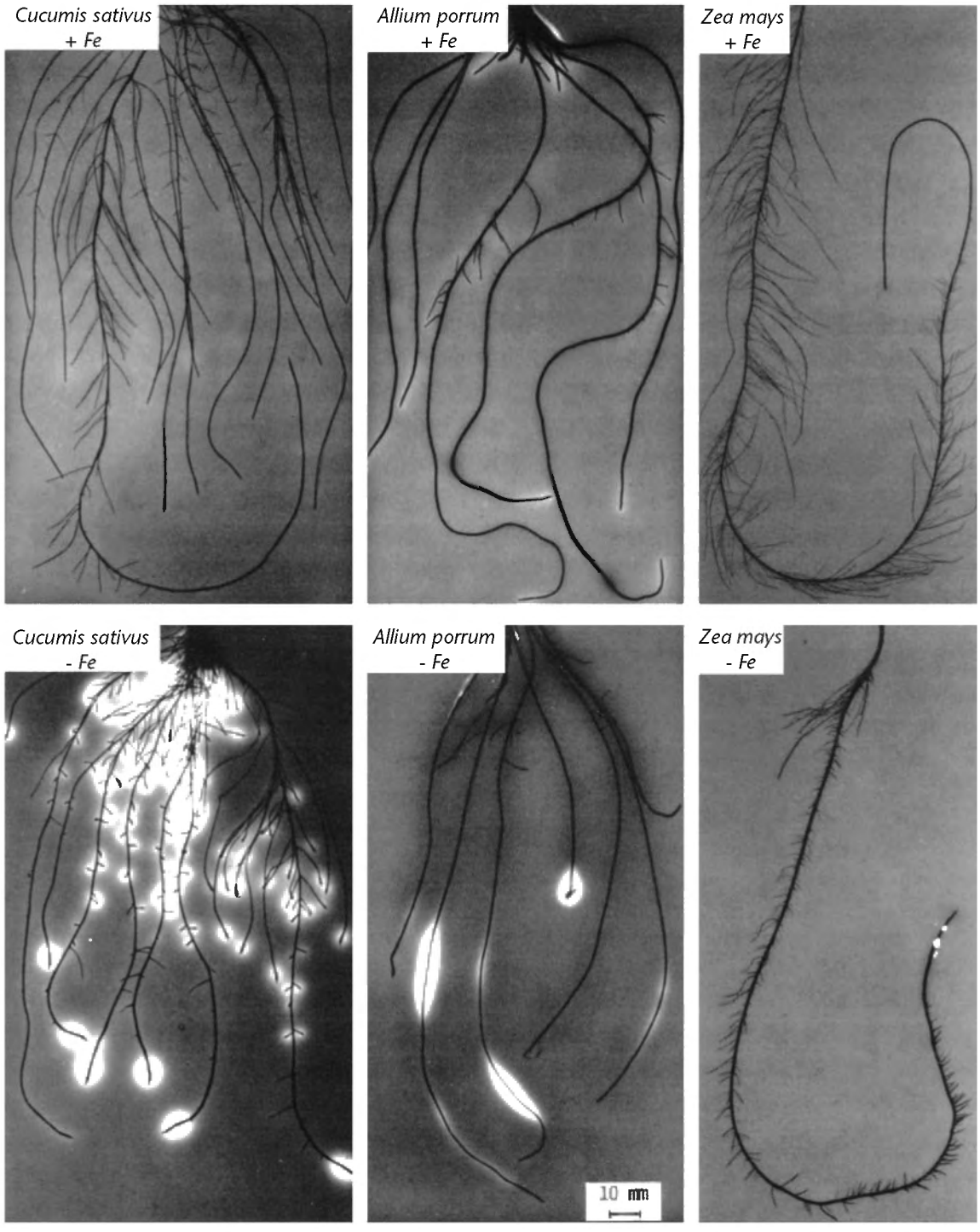


Fig. 1.7.4. Demonstration of proton excretion induced by iron deficiency in roots of different plant species. Roots of intact plants (pre-cultured in ca. 0.1 μM FeEDTA) were placed for 2 h in agar medium containing the pH indicator bromocresol (violet at pH 6.0); *yellow regions* (in the pictures *white*) show acidification of the agar medium to pH 4.5 and even below. (After Römheld and Kramer 1983)

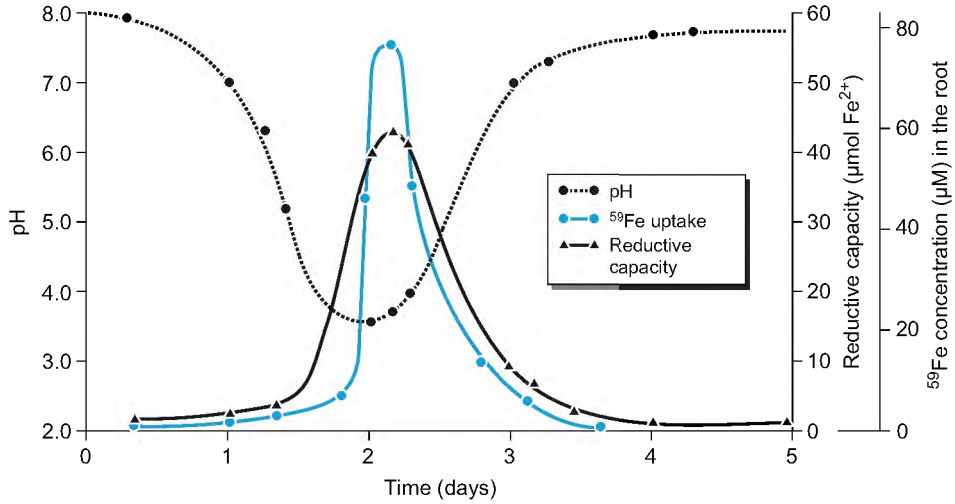


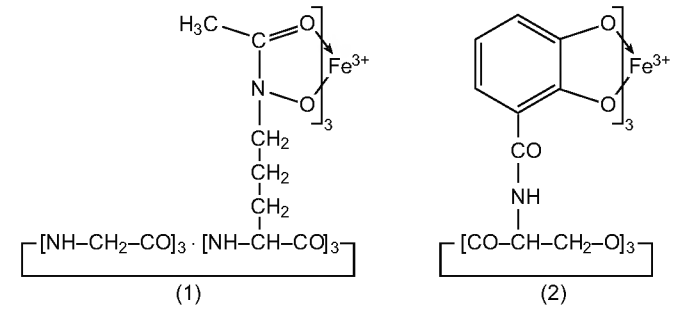
Fig. 1.7.5. Effect of Fe(III) supply on the pH of the medium and on the reductive capacity and uptake of iron, by sunflower, an iron-"efficient" plant. Such a plant, when placed in an Fe(III) solution of neutral pH, becomes iron-deficient within a short time. After only 1 day the plant commences to excrete protons and reducing compounds: the pH of the solution drops and the reduction capacity of the roots increases. With an increased iron(II) uptake the deficiency symptoms disappear and the root system returns to its normal state. The pH of the solution shifts towards the neutral point and the cycle starts again. Iron-"efficient plants" are mainly dicots, whilst many monocots, mostly grasses and cereals, cannot perform such a cycle. (After Amberger 1988)

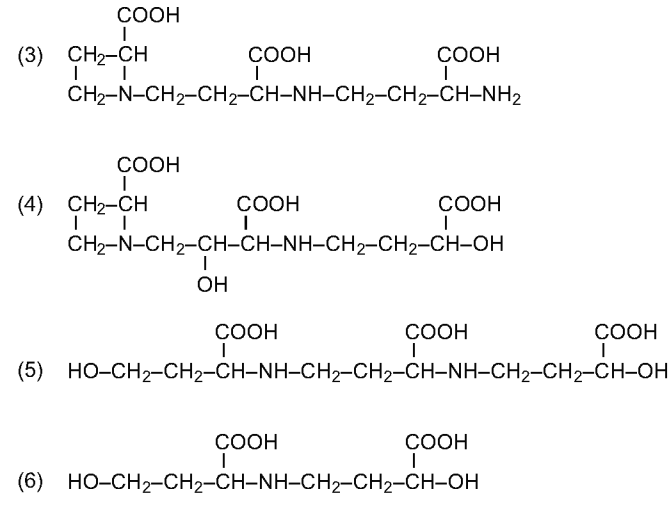
At the same time, the root exudes large amounts of substances which chelate trivalent iron and thus dissolve ferric ions from the soil-borne stock. Citric and malic acid are common **chelators** exuded from plant roots. Fulvic and humic acids, which originate in the soil, are also chelators for iron ions. In addition, in some cases, phenolic compounds, e.g. caffeic acid, can be involved in formation of soluble complexes, in their chemical transformation and in the reduction of the metal ion (see Fig. 1.7.2 B). Upon iron deficiency, root cells form large amounts of such phenolic compounds. Much improvement of low iron availability is achieved by an increase in the rate of proton extrusion by the H^+-ATPase of the plasma membrane which lowers the pH in the rhizosphere and increases the solubility of ferric ions (Figs. 1.7.2 C, 1.7.4 and 1.7.5). All these phenomena, which increase under iron deficiency, are most probably an outcome of the enormous increase in the surface area of the rhizodermal transfer cells. Likewise the rate of root-hair formation from rhizodermal cells increases, which also enhances the absorbing surface area of the roots.

At the outer surface of the plasma membrane, Fe^{3+} ions are released from the chelates and are reduced by a ferric chelate reductase to Fe^{2+} which is taken up by an Fe(II) transporter (Fig. 1.7.2 A). In order to protect the intracellular environment against the reactive species of iron (see below), iron ions are handled again as chelates, e.g. with nicotinamide. From such chelates iron is then incorporated into the target compounds. As soon as the iron supply of the plant normalises and the deficiency symptoms have disappeared, the rate of root growth returns to normal without the special amenities for iron uptake being formed. This status is maintained until iron deficiency comes up again. Cu deficiency also induces plasma membrane Fe-reductase activity (Cohen et al. 1997).

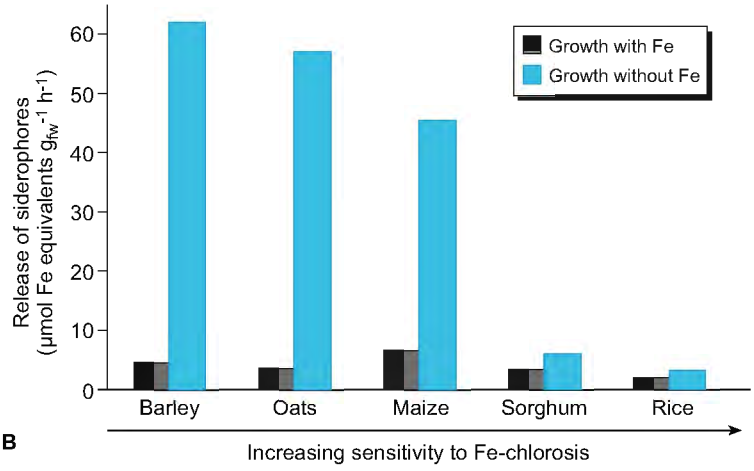
All these processes have been investigated in laboratory experiments with roots lacking any mycorrhizal symbiosis. In nature, however, roots of most plant species are associated with mycorrhizal fungi which can explore smaller soil pores than fine roots and considerably contribute to the supply of the plant with macro- and micronutrients.

Grasses possess a particular chemical mechanism for heavy metal uptake: The roots exude so-called **phytosiderophores** (Greek: *sideros* = iron, *pherein* = carry) into the soil. These compounds are complicated non-proteinogenic amino acids (Fig. 1.7.6 A), which solubilise iron from sparingly soluble complexes, and convert it to water-soluble Fe(III)–siderophore complexes from which it is available to plants. Fungi and bacteria also produce siderophores. The latter bind Fe(III) as hydroxamates, whilst plant side-





A



B

Fig. 1.7.6. A Chemical structures of microbial siderophores (*1* and *2*) and phytosiderophores (chelators) from higher plants (*3–6*). *1* Ferrichrome (cyclohexapeptide derivative of hydroxamates); *2* ferrienterobactin (cyclotris(*N*-2,3-dihydroxybenzoyl-L-serine) derivative of catechols); *3* nicotinamine; *4* muginic acid; *5* avenic acid A; *6* avenic acid B. After Schlee (1992). B Release of siderophores by various cereals upon sufficient Fe supply and Fe shortage. (After Mengel 1991)

rophores bind the ferric ion to the carboxy groups. A transporter mediating the uptake of Fe(III)–siderophore complexes has been recently demonstrated in maize. Cleavage of the siderophore complex, by ligand exchange or some other mechanism, occurs within the cell. Formation and exudation of siderophores are dramatically stimulated by iron deficiency (Fig. 1.7.6 B). The siderophore-mediated uptake of heavy metals has been termed "strategy II" in contrast to the uptake by chelate reductase and the ferrous transporter which is known as "strategy I".

In poorly aerated soils with a negative redox potential, iron usually occurs in its ferrous, easily soluble form. At identical pH, the solubility of Fe(II) oxide/hydroxide is by several orders of

Box 1.7.1 Redox potential of soil

The redox potential of soil results from the ratio of the oxidised to the reduced forms of metals:

$$E = E_O + \frac{RT}{nF} \ln \frac{a_{ox}}{a_{red}} \; [V]$$

$$E = E_O + \frac{0.059}{n} \log \frac{a_{ox}}{a_{red}}$$

where n denotes the number of electrons exchanged between the oxidised and the reduced form, a_{ox} is the activity of the oxidised form, and a_{red} is the activity of the reduced form.

Well-aerated soils have redox potentials of up to +0.8 V and poorly aerated soils within the level of ground water or peat soils of up to −0.35 V.

The **reductive potential** of the soil is characterised by the **pe** value. The pe value (analogous to pH) is the negative log of the "concentration of electrons (n)" in the soil:

e.g. $pe = 2 \quad [e] = 10^{-2}$ M
$pe = -1 \quad [e] = 10^{1}$ M

The conversion factor between E and pe is: $pe = E\,(V) \times 16.9$

The pe values of a paddy rice field are between +4 (surface) and −3 (middle layer).

magnitude greater than that of the corresponding Fe(III) salts. At a soil redox potential of pe = 1 the solubility of iron ions is 10^{-5} M, but under aerobic conditions, at pe = 2–4, it is 10^{-16} M (for explanation of the pe value, see Box 1.7.1). In soils with a pe < 1, iron deficiency usually does not occur in plants. A soil redox potential of ca. +150 mV is required for the reduction of Fe(III) to Fe(II).

1.7.2.2 Physiological Consequences of Iron Shortage

Chlorosis caused by iron shortage shows that the biosynthesis of porphyrines is particularly sensitive to iron deficiency. This not only applies to iron as the central atom of heme, but also to the Mg-protoporphyrin-IX-monomethylester cyclase which forms the isopentanone ring of chlorophyll using oxygen from air. Such a monooxygenase requires a cytochrome P450 as cofactor which contains an iron ion. Thus an impact on chlorophyll synthesis by iron deficiency is conceivable. In addition, many ROS-scavenging enzymes (see Chaps. 1.3.5 and 1.6.2.2), e.g. peroxidases, contain heme and iron shortage limits their synthesis. This leads to an incomplete detoxification of ROS which, in turn, oxidise chlorophyll in a photodynamic, i.e. light-dependent, reaction. A lower rate of chlorophyll biosynthesis, concomitant with an increased rate of oxidative chlorophyll destruction, results in the phenomenon of **Fe chlorosis**. Lower chlorophyll concentrations require smaller amounts of chlorophyll-binding proteins. It is therefore not surprising that chloroplasts of iron-deficient plants have significantly less contents of pigments and proteins than those of plants sufficiently supplied with iron (Table 1.7.3).

Table 1.7.3. Effects of iron shortage in tobacco plants: iron limitation is particularly noticeable by reduced chlorophyll synthesis, which leads to chlorosis (bleaching) especially in the intercostal spaces (Marschner 1986)

Availability of iron(III) ions	Pigment concentration (mg/g fresh weight)		Protein content (mg/g fresh weight)	
	Chlorophyll a+b	Carotenoids	Chloroplast	Cytoplasm
Sufficient	0.98	0.45	8.6	12.8
Mild shortage	0.34	0.33	5.0	11.9

Physiological iron deficiency may also occur at a good supply of iron ions. At high internal phosphate concentrations, Fe(III) ions are sequestered as phytoferritin in the chloroplasts and are no longer metabolically available.

Phytoferritin is a hollow spheric protein that can store up to 5000 Fe(III) ions as alkaline iron phosphates $[FeO(OH)]_8[FeO(H_2PO_4)]$ (Casiday and Frey 2000).

The physiologically active form of iron is the Fe(II) ion, as a constituent of various complexes, e.g. in the heme or iron-sulfur proteins; only this form is able to transfer charges.

Many metabolic processes are more or less directly dependent on iron ions but discussion of an impact of iron deficiency on all of them is beyond the scope of this presentation. The reader is referred to textbooks on plant biochemistry, e.g. Buchanan, Gruissem, Jones: *Molecular Biology of Plants* (American Society of Plant Physiologists, 2000, Rockville, MD). Iron is a relatively atoxic heavy metal, required in larger amounts than other heavy metals in cell metabolism (see Table 1.7.1). However, the uncomplexed Fe^{2+} ion is toxic, as it catalyses Fenton reactions (see Box 1.3.8), which always result in the formation of ROS. Therefore, deposition of iron as ferritin, upon over-supply of iron, is important as a detoxifying process.

1.7.3

Stress by Heavy Metal Toxicity

Even metabolically essential heavy metal ions, e.g. Cu^{2+}, Mn^{2+} or Zn^{2+}, become toxic at concentrations exceeding the range of trace elements. Heavy metals such as Ag, Au, Cd and Pb which are not natural constituents of cells are toxic even at very low concentrations. However, heavy metal-tolerant plants are able to detoxify also such heavy metals which do not normally occur in a plant. Both phenomena will be discussed in the following section.

1.7.3.1 Copper

Copper belongs to those heavy metals which are essential for organisms, but required in only very small amounts (see Table 1.7.1). In the soil, Cu occurs almost exclusively as Cu(II), usually adsorbed to iron- and manganese-containing minerals, to soil colloids and as chelate with organic compounds. Copper ions are scarcely mobile in the soil, and because of the strong binding to soil particles copper concentrations in soil solutions are extremely low (ca. 0.01 mg/l). Even in such solutions copper occurs usually as a complex with organic low molecular weight compounds.

The copper content of untreated soils is in the range of 20–50 mg/kg soil, but in vineyards and hop fields, as well as citrus and coffee plantations, the concentration is considerably higher (>300 mg/kg soil) because of spraying with Cu-containing fungicides and fertilisation with sewage sludge and manure.

Because of the strong adsorption to soil particles, these high concentrations initially remain in the topsoil but ploughing displaces the copper-rich soil into the root region and thus may lead to copper intoxication of the crop (see Section "Copper Toxicity").

Copper uptake is in part similar to the uptake of iron, and as suggested by a pronounced ion antagonism, plants probably use metal-specific as well as general carriers for copper, iron, manganese and zinc. High affinity Cu uptake is specifically regulated by COPT genes. Amino acids and metallothioneins may be the relevant and often tissue- and age-specific transport metabolites to which Cu ions are bound.

Copper in Plant Metabolism

Relatively high Cu contents are found in carrots, potatoes, buckwheat, and in other soil-borne storage organs, less in above-ground plant organs. Buckwheat is also known as a copper-accumulating species. Obviously, mobility of Cu in plants is fine-tuned by a variety of Cu chaperones delivering Cu to the subcellular sites of demand.

Copper is well known as a component of electron-transporting proteins in photosynthesis (plastocyanin), in the respiratory chain (cytochrome oxidase) and in creating functional ethylene receptors (see Chap. 1.4). In plastocyanin, copper only mediates electron transfer whereas, in the cytochrome oxidase, copper takes part in electron transfer and, in cooperation with iron, in the binding of O_2. Oxidases, e.g. ascorbate and amino acid oxidases, peroxidases and phenol oxidases, often contain Cu. Copper proteins, like the above-mentioned cytochrome oxidase, frequently contain a second heavy metal: e.g. the copper-zinc-SOD where both heavy metals are linked by a histidine residue.

Copper Deficiency

Copper-deficient soils are characterised by a low sorptive capacity, such as well-aerated sand, heath (Podsols) and boggy soils. However, because of the higher plants' extremely low requirement for Cu (see Table 1.7.1), copper deficiency occurs mostly on boggy soils. Most of the typical symptoms of copper deficiency could only be recognised in pot culture experiments. Copper deficiency results in a reduced lignifica-

tion of the cell walls and a clammed sclerenchyma formation. This could be explained by the low contents or activities of phenol oxidases and peroxidases involved in lignin synthesis.

Copper Toxicity

In contrast to copper deficiency, Cu toxicity is widespread, less because of natural cuprous soils but more because of human activities: Rubbish tips, sewage deposits and agricultural areas with recurrent application of copper-containing chemicals, e.g. vineyards and orchards. Toxic copper concentrations give rise to many macroscopically and biochemically recognisable symptoms, which, however, develop after quite different time-spans. Similar to iron deficiency, copper toxicity causes chlorosis, as the Cu ion can readily replace the Fe ion in protein complexes and thus inactivate their enzymatic activities. In addition, Cu acts as a Fenton reagent and catalyses the formation of ROS and their reaction products which primarily attack the unsaturated fatty acids of membrane lipids and thus damage the cell membranes (see Chap. 1.3.3).

Such damage affects transmembrane ion transport, and in particular disturbs the intracellular K^+ relations which in turn control turgidity of the cell. This results in an inhibition of cell division. One of the fastest effects of toxic copper concentrations is the inhibition of the elongation growth of lateral and subsidiary roots. Instead of a well-structured root system, short, hairy, brown laterals are produced – a phenomenon which may be used to quantify copper damage. Such damage can be evaluated using an **EC system** (effect concentration: EC_{10} refers to an inhibition of the observed parameter by 10%, EC_{50} indicates 50% inhibition). However, EC and lethal concentration (LC) are not identical. EC measures the current inhibition, LC the irreversible damage. Concentrations which show no recognisable effects are called **NOEC** (no observable effect concentration).

At the cellular and subcellular level, increased Cu concentrations cause two major effects: removal of functional ions or central atoms from an enzyme, e.g. the replacement of Fe ions in cytochrome, or interference with the Ca^{2+} signalling system of the cell, on the one hand, and formation of cuprous sulfides in cysteine-containing enzymes, an example of which is the inhibition of nitrate reductase.

Substitution of an original metal ion by another excessively supplied heavy metal ion suggests a certain specificity which is probably defined in the protein. For example, ribulose bisphosphate carboxylase is much more affected by Mn^{2+} and Ni^{2+} than by Cu^{2+} or Zn^{2+}. On the other hand, Zn^{2+} replaces Mn^{2+} very effectively from the photosynthetic water oxidase. However, these are results of in vitro studies, showing possible reactions, but do not necessarily occur in planta: plants exhibit different ways to intercept heavy metals before their interaction with enzymes. The efficiency of such intercepting mechanisms, amongst other factors, may evoke different sensitivities of various plant species to copper and other heavy metals. It is assumed that the concentration ranges of heavy metal toxicity are shifted in metallophytes to higher concentration by such interception processes.

1.7.3.2 Cadmium

Cadmium is very toxic for any kind of organism. As far as is known, Cd is not a constituent of any metabolically important compound, i.e. there is – in contrast to copper and iron – no useful concentration of this ion in plants. Therefore, Cd is a true xenobiotic. Nevertheless, some plants may contain larger amounts of Cd (up to 100 mg/kg dry weight). Depending on their Cd content, plants are addressed as Cd accumulators or Cd avoiders.

Cd^{2+} accumulates in sewage sludge, waste water and river sediments from where it is taken up by plant roots. Cd can also be taken up from dust on the leaves. Cd^{2+} is readily distributed in the plant via the xylem stream, and then accumulates in all organs of the plant.

Plants are not defenceless towards Cd^{2+} supply by the substrate, as is shown in a comparison of Cd supply and Cd accumulation in leaves of various crop plants (Table 1.7.4). Beans belong to the excluders, but lettuce accumulates Cd. Thus plants show different sensitivities towards Cd (Table 1.7.5).

Toxicity of Cadmium

In soils, Cd rarely occurs as the only heavy metal pollutant as it is most frequently accompanied by Zn. Many toxic effects, therefore, result from multiple stresses by heavy metals or from ion replacement: In vitro substitution of Zn by Cd in the enzyme carbonic anhydrase leads to rapid inactivation (Table 1.7.6). The toxicity of

Table 1.7.4. Cadmium content of plants (mg Cd per kg dry weight) in relation to the cadmium content of the soil (Wallnöfer and Engelhardt 1984)

Plant	Organ	Cd content of the soil (mg Cd per kg soil)			
		1.4 (control)	4	10	30
Green cabbage	Leaves (old)	0.7	9.0	18	36
	Leaves (young)	0.4	2.3	5.6	21
	Shoot	0.5	3.1	4.5	9.6
	Root	0.7	2.3	6.7	8.3
Lettuce	Leaves (old)	1.2	9.6	26	44
	Leaves (young)	0.9	3.8	8.1	18
	Root	0.9	4.2	11	21
Red radish	Leaves	0.9	11	21	49
	Tuber	0.4	3.1	7.4	13
	Root	0.8	5.3	11	34
Leek	Leaves	0.6	3.5	16	28
	Stem	0.5	3.4	3.7	17
	Root	0.7	4.1	9.3	24
Bean	Leaves	0.2	0.4	0.5	0.9
	Seeds	0.2	0.2	0.2	0.2
	Shoot	0.5	0.6	0.9	1.6
	Root	0.9	2.8	8.2	14

Table 1.7.5. Toxicity of Cd for crop plants, measured as Cd content of the leaves that causes 10% reduction of the harvest (Wallnöfer and Engelhardt 1984)

Plant	Cd content of leaves (mg Cd per kg dry weight)
Bean	0.7
Leek	3.0
Green cabbage	>30
Radish	40
Lettuce	>40

Table 1.7.6. Loss of carbonic anhydrase enzyme activity (in vitro) after substitution of the original Zn ion in the purified enzyme by another heavy metal ion. The activity of the Zn enzyme is set at 100% (Ernst 1996)

Heavy metal	Activity
Zn	100
Co	55.9
Ni	4.9
Cd	4.2
Mn	3.9
Cu	1.2
Hg	0.05

Cd results from the strength of its links with cysteine residues in proteins.

A generally observed effect of xenobiotic heavy metals is the inhibition of photosynthesis caused by closure of stomata. This phenomenon is particularly pronounced with Cd (Fig. 1.7.7).

Inactivation of metabolically important enzymes results in a stimulation of catabolic reactions comparable to what is known as "wound respiration" (Table 1.7.7). At the subcellular level, swelling of mitochondria, vacuolization, i.e. demixing of the plasma, and deposition of Cd-containing granules have been described.

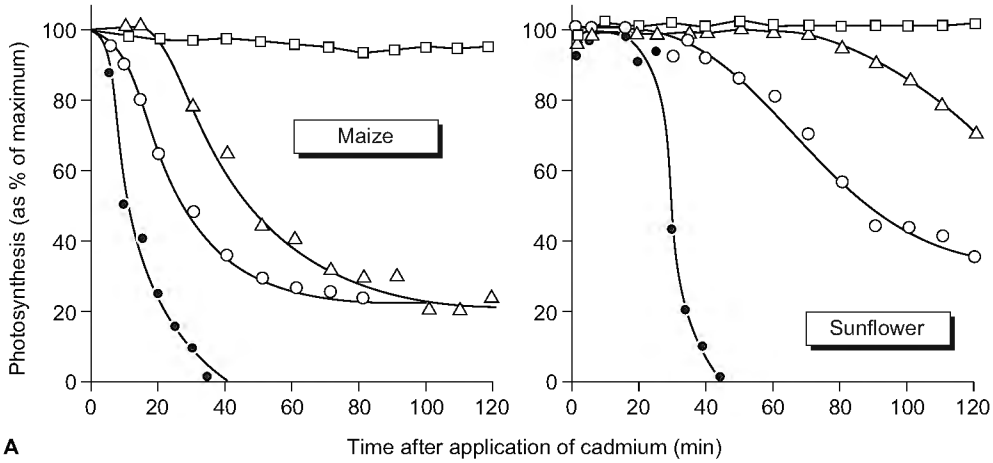
At the whole plant level, strong stress by Cd in addition to the reduced growth produces yellow streaked leaves (Table 1.7.5).

1.7.4

Reaction of Plants to Excessive Supply of Heavy Metals

At the same EC level sensitive as well as tolerant plants to stress by heavy metal generally show similar reactions when exposed to this kind of stress.

The first reaction complex may be considered an avoidance strategy, aiming at immobilisation of the heavy metals outside the protoplasts of root cells in order to prevent physiological effectivity. Complexing Cu^{2+} ions by root exudates in the soil, but also in the cell wall, on the one hand reduces the supply to the protoplast but, on the other, requires continuous root growth. Depending on the composition of the cell wall and the types and amounts of the exudates, the



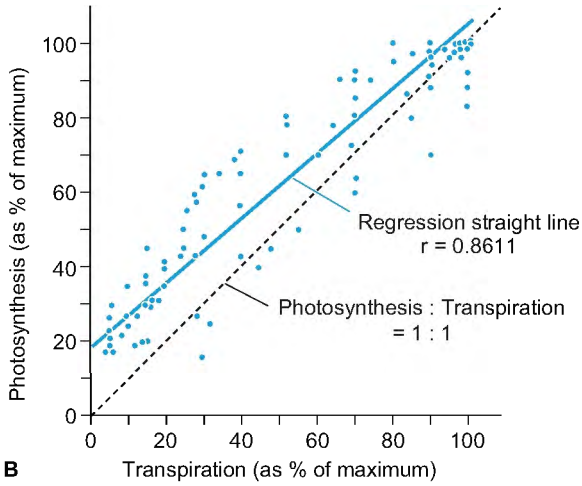


Fig. 1.7.7. Inhibition by cadmium of photosynthesis and transpiration of maize and sunflower. A Time kinetics of the influence of various Cd^{2+} concentrations on the rates of photosynthesis. □=control (27 mM KCl); △=4.5 mM $CdCl_2$; ○=9 mM $CdCl_2$; ●=18 mM $CdCl_2$. B Relationship between the inhibition of photosynthesis and transpiration by cadmium. The regression line shows a quasi-linear relationship between the two processes. As the regression line does not cross at zero, transpiration, i.e. stomatal conductance, must be inhibited to a greater extent than photosynthesis. This suggests that inhibition of photosynthesis by cadmium principally occurs through closure of stomates. The data points have been produced with both plant species. (After Bazzaz et al. 1974)

Table 1.7.7. Respiration and enzyme activity of leaves from soybean seedlings after 10 days growth at various concentrations of cadmium chloride. The measured activity is normalised to 1 g fresh weight. Respiration as well as activity of catabolic enzymes show a clear "injury effect" (Lee et al. 1976)

Activity	Cd^{2+} concentration (μM)			
	0 (Control)	0.45	0.9	1.35
Respiration (μl $O_2 \times g^{-1} \times h^{-1}$)	480	539	784	737
Malate dehydrogenase (μmol NADH$\times g^{-1} \times h^{-1}$)	230	250	400	390
RNase (μmol nucleotide$\times g^{-1} \times h^{-1}$)	3.0	10.1	6.4	9.8
DNase (μmol nucleotide$\times g^{-1} \times h^{-1}$)	1.2	3.6	3.6	4.8
Acid phosphatase (μmol phosphate$\times g^{-1} \times h^{-1}$)	190	260	400	410
Peroxidase (μmol $H_2O_2 \times g^{-1} \times h^{-1}$)	880	840	3440	4450

capacity of ion exclusion differs from species to species but less so with respect to the type of the heavy metal.

The second reaction complex is less well known. Competition of heavy metals for uptake has already been mentioned; that between copper and iron would also mean that copper uptake by the carrier is reductive. Apparently, Cu^{2+} can partially displace Ca^{2+} in biomembranes and thus changes the transport properties of the membrane. As far as is known adaptation to stress by excess copper reduces the K^+ efflux from cells. In the cytosol (reaction complex 3) Cu^{2+} is incorporated into the target enzymes or bound to polypeptides, oligopeptides or amino acids for transport into the vacuole and the xylem, respectively.

1.7.4.1 Polypeptides Induced by Heavy Metals

Two types of cysteine-rich peptides are effective in the sequestration of heavy metals in the vacuole: The larger **metallothioneins** (Fig. 1.7.8) and the smaller **phytochelatins** (Box 1.7.2). The role of both types will be discussed with respect to detoxification of elevated concentrations of heavy metals, because both types of polypeptides are synthesised in most plants in correspondence to the severity of heavy metal stress. However, their role in cell biology is still not completely understood.

Metallothioneins

Metallothioneins were originally discovered in microorganisms, animals and fungi, particularly in yeasts, and have recently been demonstrated also in plants; a Cu-binding metallothionein with a molecular mass of 8.5 kDa, the formation

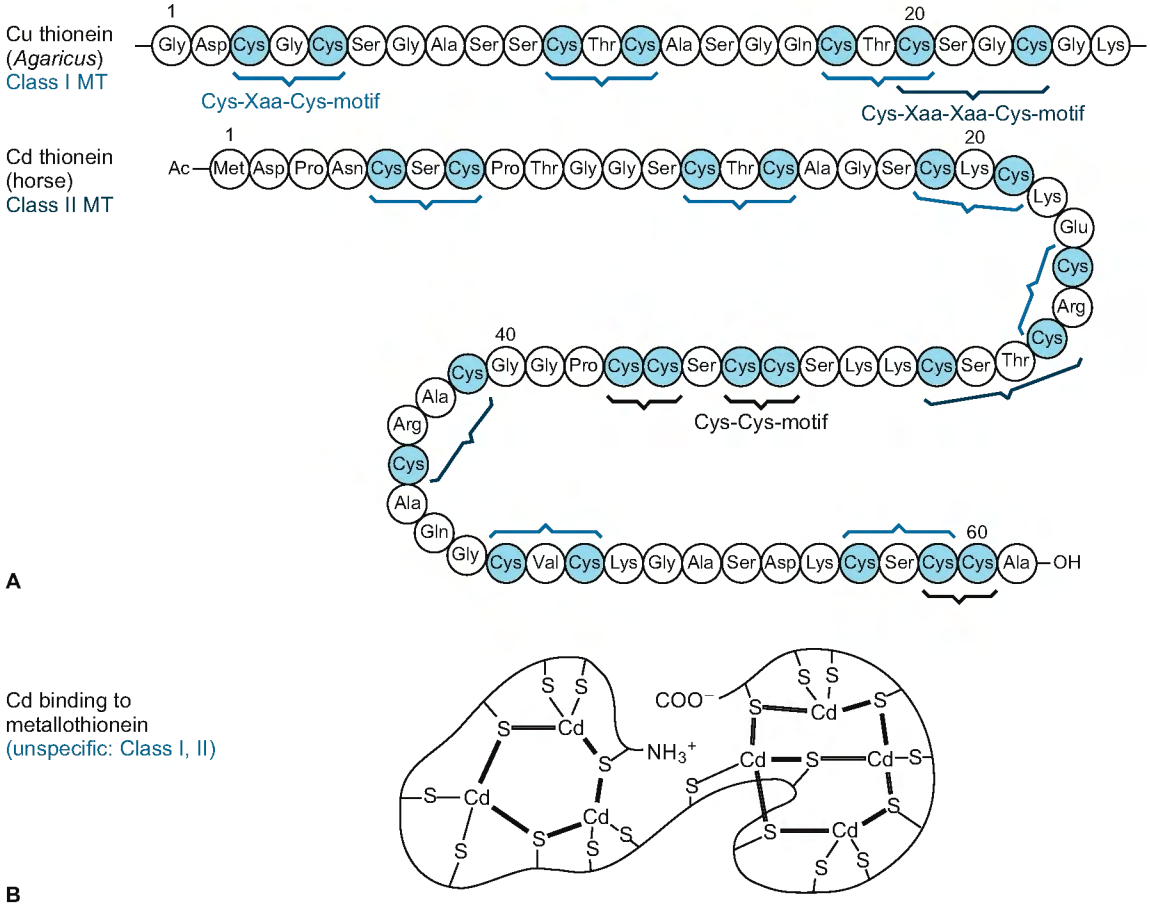


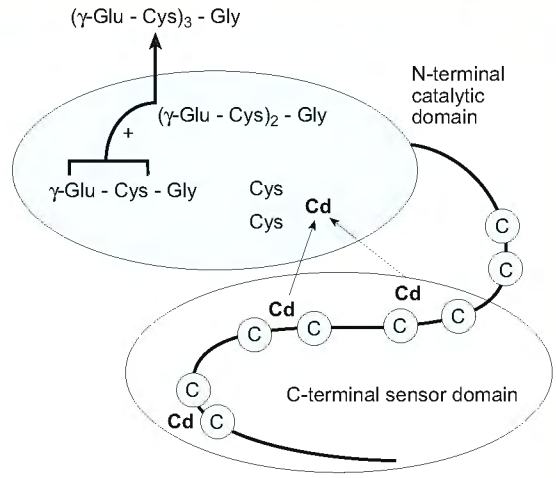
Fig. 1.7.8. A The heavy metal binding motifs of a metallothionein. B Binding of heavy metal ions (cadmium) to a metallothionein. (After Schlee 1992)

Box 1.7.2 Phytochelatins

Phytochelatins are derivatives of the tripeptide γ-Glu-Cys-Gly. This tripeptide is not a protein building block as the "peptide bond" is not between the carboxyl group next to the α-amino group (of Glu) and the amino group of Cys, but between the γ-carboxyl group of Glu, in other words, "at the wrong end of Glu". Phytochelatins are built along the following principle:

$$(\gamma\text{-glutamyl-cysteine})_n\text{-glutathione} \equiv (\gamma\text{-glutamyl-cysteine})_{n+1}\text{-glycine}$$

Biosynthesis of these molecules is by dipeptide transfer from glutathione and not en route of a normal polypeptide. The corresponding enzyme is the phytochelatin synthase (γ-glutamyl-cysteine dipeptide transpeptidase) a protein of M_r 4×25 kDa=~96 kDa:



Model of the phytochelatin synthase

$$2\ \text{glutathione} \rightarrow (\gamma\text{-glutamyl-cysteine})_2\text{–glycine} + \text{glycine}$$

$$(\gamma\text{-glutamyl-cysteine})_2\text{–glycine} + \text{glutathione} \rightarrow (\gamma\text{-glutamyl-cysteine})_3\text{–glycine} + \text{glycine}$$

and so forth.

Current understanding is that the Cys-rich C-terminal domain is a sensor for heavy metals; the cysteines bind the metal ions and concentrate them at the catalytically active N-terminal domain which thereby is activated (see Figure, after Cobbett 1999).

of which is specifically induced by a high supply of copper ions, was found in the roots of the cuprophyte *Silene cucubalus*. This is one of the few instances where heavy metal specificity appears to exist.

Metallothioneins are polypeptides of about 60 amino acids, whose chain is kinked in many places, and whose N- and C-termini are particularly rich in cysteine residues. It is remarkable that they do not contain aromatic amino acids and that the cysteines always appear in the sequence Cys-Xaa-Cys. This motif suggests the formation of metal sulfide clusters (Fig. 1.7.8 B).

The two cysteine-rich domains are separated by a cysteine-free central part, the so-called spacer, which comprises about 40 amino acid residues as seen primarily in mammalians (Klaassen et al. 1999) and in the metallothionein A from *Pisum sativum*. Such metallothioneins, referred to as class I type, have been found – at least as genes – in pea, maize, barley, wheat and *Mimulus*.

Metallothioneins of the class II type are characterised by slightly different cysteine motifs; in addition to -Cys-Xaa-Cys-, they contain the motifs -Cys-Cys- and -Cys-Xaa-Xaa-Cys- (see Fig. 1.7.8 A) as found in fungi, invertebrate animals and plants (soybean, *Ricinus* and *Arabidopsis*; Cobbett and Goldsbrough 2002).

As the genes of these metallothioneins are known (MT genes) analysis of their function can be performed, and the regulatory characteristics of their promoters can be studied using molecular biological methods. The promoters contain at least partly *cis* elements that bind transcription factors responsive to metals, but also ABA-responsive elements. In that respect they correspond to animal representatives whose expression is also regulated by heavy metal stress and internal factors. The spacer apparently gives structure to the cysteine-rich termini. Metallothionein knockout mutants show an increased sensitivity to stress by heavy metals.

This applies also to yeasts, where $CUP1_o$ cells are hypersensitive to copper stress, in contrast to the wild type. In "normal", i.e. heavy metal sensitive plants, metallothioneins are produced from single copy genes, but the genome of metallophytes contains multiple copies of MT genes. For yeasts, which produce metallothioneins correspondingly to the number of copies of MT genes, a connection between the content of metallothioneins and resistance to heavy metal stress has been shown. Plant MTs are classified into four types according to the arrangement of Cys residues. Type MT1 is expressed more in roots than in leaves, type MT2 primarily in leaves. The expression of MT1 and MT2b is mainly in the phloem and seems to play a major role in Cu homeostasis. MT2a and MT3 are mainly expressed in leaf mesophyll and strongly induced by Cu^{2+} in young leaves, root tips and ripening fleshy fruits. The occurrence of type MT4 seems to be restricted to developing seeds (Guo et al. 2003).

For plants, even though an increased transcription of MT genes has been observed under stress by heavy metals, it is not clear whether the corresponding proteins are formed in amounts sufficient for detoxification. In that context, other interpretations have been put forward:

- a storage function of metallothioneins, as one protein molecule is able to bind several heavy metal ions (see Fig. 1.7.8);
- a role as transporters for the sequestration of heavy metals into the vacuole (see Chap. 1.9.1.1).

Considering induction of metallothionein expression, as well as binding of heavy metal ions as "sulfides", it appears that metallothioneins are not very substrate-specific. The binding constants for Zn^{2+}, Cd^{2+} and Cu^{2+} are not dramatically different, even though Cu^{2+} is usually slightly stronger bound than the others. From that finding a type of cross-protection could be concluded, or, for tolerant species, a type of "cross-tolerance".

Phytochelatins

The second type of metal-binding peptides, the phytochelatins (sometimes also called metallothioneins class III), differs from metallothioneins in as much as they are not primary gene products, but are produced by enzymatic peptide transfer from tripeptides. Their molecular masses range from 2–10 kDa. Phytochelatins were discovered in cell cultures of *Datura innoxia* and *Rauvolfia serpentina* as well as in cultures of yeast; recently, they have also been found in cell cultures of tomato, tobacco and other species, usually upon stress by Cd and Cu. Also mosses, ferns and fungi are capable of producing phytochelatins (Grill et al. 1987).

Phytochelatins are derivatives of the tripeptide glutathione with the general formula $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ where n ranges between 2 and 11 (Box 1.7.2).

In phytochelatins of grasses glycine is replaced by serine, and in some members of the Fabaceae by β-alanine (**homophytochelatins**).

Phytochelatin is synthesised by phytochelatin synthase (a dipeptidyltransferase), which is constitutively expressed but is activated by heavy metals with high affinity to SH groups. Mercury, Cd, Cu, and Pb are particularly effective in that respect, while Fe, Mn, Mo, Cr, U and V have no effect on PC synthase expression.

Occurrence of metallothioneins and phytochelatins is not mutually exclusive. Upon heavy metal stress synthesis of both types of polypeptides is intensified. Metallophytes, however, produce significantly less phytochelatins than heavy metal-sensitive plants (Fig. 1.7.9).

This is the reason that heavy metal tolerance of metallophytes cannot be traced back to direct detoxification by phytochelatins. They probably play an important role in the sequestration of heavy metals to the vacuole (see Chap. 1.9.1.1);

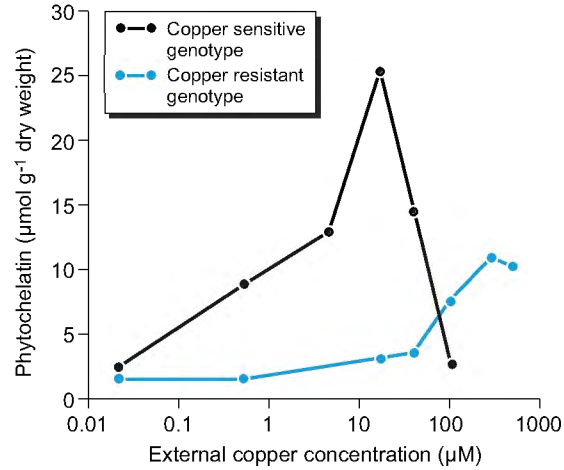


Fig. 1.7.9. Phytochelatin synthesis as related to the copper concentration in the root medium of copper-sensitive and -resistant genotypes of *Silene vulgaris*. (After Ernst 1996)

there, polymers of heavy metal phosphates can be formed, such as polymeric zinc phosphate in *Deschampsia* or *Lemna*.

More or less specific heavy metal binding proteins are usually interpreted as detoxifying elements. However, this would mean that the heavy metal–protein complexes or clusters would accumulate during the lifetime of a plant. This is not the case, although a correlation between the concentration of the heavy metal and that of the thioneins has been shown. However, the concentrations of the thioneins are much too low for such a function (less than 0.1% of dry weight). Rather than for direct detoxification heavy metal binding polypeptides may serve to maintain a heavy metal homeostasis of the tissue. Such a storage function could transiently contribute to a detoxification mechanism (Tomsett and Thurman 1988, but see also Cobbett and Goldsbrough 2002).

Heavy Metal Ion Pumps

Special consideration should be given to the discovery of **heavy metal ion pumps** in the plasma membranes of microorganisms, animals (including man), fungi and of the model plants *Arabidopsis thaliana* (AXA2p; Harper 1997), *Arabidopsis halleri* (Becker et al. 2004) and *Thlaspi caerulescens* (Assunção et al. 2003). Heavy metal pumps in plants belong to different types.

These enzymes are ATP-dependent pumps with a structure similar to that of K^+/Na^+-ATPases of the plasma membrane which remove heavy metals from the protoplast. Best investigated are the so-called copper ATPases (Solioz and Vulpe 1996). Two of these pumps have been described: CopA accomplishes the import of copper as the Cu^{1+} ion upon copper shortage. CopB functions in export, also of univalent copper. Defects in these copper ATPases lead to diseases in humans (e.g. Menke syndrome). These copper ATPases have certain structural features which are partly unique and partly similar to those of other metal

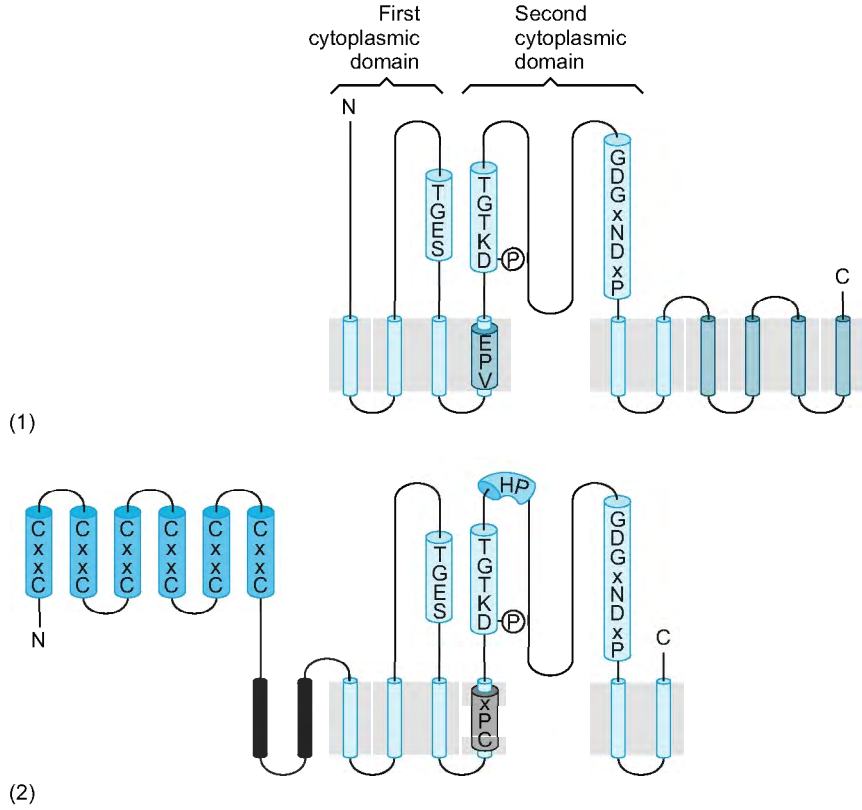


Fig. 1.7.10. Comparison of a Na^+/K^+-ATPase (1) with a heavy metal ATPase (2). The transmembrane helices and the conserved structural motifs of all P-type ATPases are in *light blue*. *TGES* Phosphatase domain; *DKTGT* aspartate kinase domain; *GDGxNDxP* ATP-binding domain. The sequences of such ion pumps that do not transport heavy metals are shown in *blue-grey*. The helices and sequences that only occur in heavy metal ion pumps are *grey*. The repetitive heavy metal binding domains are *in blue*. (Solioz and Vulpe 1996)

ion transporting ATPases (Fig. 1.7.10). The partial structures must fit into the transport cycle. All metal-transporting ATPases have in common an aspartate (D) in a very conserved sequence, TGTK**D**, which is phosphorylated by ATP. The phosphorylating domain is also conserved and is located in the sequence DKTGT. Dephosphorylation of the aspartylphosphates by a domain with phosphatase activity (TGES) forces a conformational change resulting in the transport of one to two metal ions across the plasma membrane. In addition to the structural elements of a Na^+/K^+-ATPase, the heavy metal ATPases possess an N-terminal extension, consisting of a series of Cys-Xaa-Xaa-Cys elements. In humans there are six repeats, in yeast only two. Further characteristic traits of copper ATPases are a conserved intramembrane motif: CPC, CPH or CPS 9 (so-called CPX motif), a His-Pro dipeptide and a smaller number of integral membrane peptides. The proline residue appears to be an essential part of a Ca^{2+}-binding domain, forming the channel or the transport domain. By the heavy metal binding domain the CPX-ATPases attain a

- certain degree of specificity for heavy metals,
- storage function for cytoplasmic heavy metals (CopA of *Pseudomonas syringae*, e.g. forms a CPX-ATPase, which stoichiometrically binds 11 copper ions).

It is to be assumed that the CPX-ATPases, as enzymes of the plasmalemma, play a major role in achieving homeostasis of essential heavy metals. However, this has not yet been shown in physiological experiments. Up- and downregulation of Zn transporters (ZIP family) seems to be responsible for zinc homeostasis in plants.

1.7.4.2 Cellular Response to Heavy Metal Stress: the Example of Cd

It was unclear for a long time whether thioneins or phytochelatins are produced as cellular response to Cd stress. The first Cd-binding polypeptides were found in Schizosaccharomyces and called "cadystins" because of the numerous cysteine residues in these compounds. Meanwhile these Cd-binding proteins have been identified as phytochelatins. In addition to the phytochelatins, Cd excretion by an ATP-driven $Cd^{2+}/2H^+$ exchange plays a role in Cd tolerance (Fig. 1.7.11).

As in the case of Cu^{2+}, binding of Cd^{2+} to phytochelatins does not serve as a long-term detoxification mechanism, but as short-term storage and probably plays a major role in intracellular Cd^{2+} transport. Mainly phytochelatins of

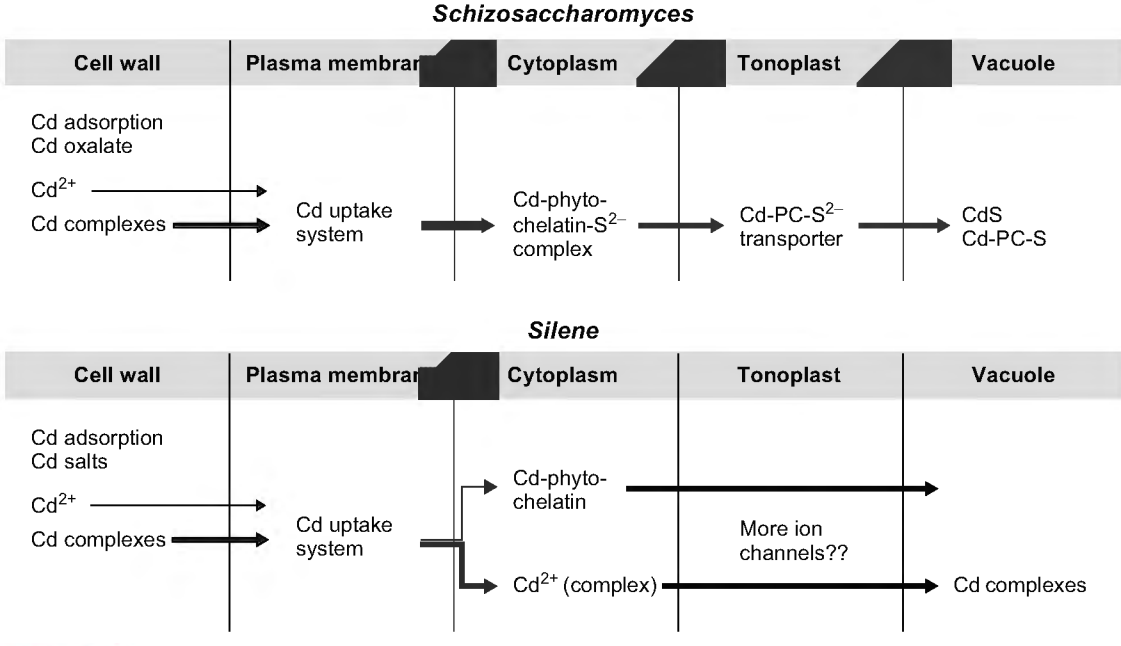


Fig. 1.7.11. Model of cellular and molecular detoxification mechanisms for cadmium in yeast and higher plants. (After Ernst 1996)

the (γ-Glu-Cys)$_3$-type and (γ-Glu-Cys)$_4$-Gly have been observed.

1.7.5

Heavy Metal Tolerance

Metallophytes, e.g. cuprophytes, use several strategies to counter the excess of heavy metals. However, they are not forearmed to cope with any concentration of these toxic ions, only the threshold at which damage occurs is higher. In principle, it is not possible to define a particular mechanism by which a plant achieves heavy metal resistance. Rather there is a gradual transition from metal-sensitive to metal-resistant plants, as the cellular responses to that stress are qualitatively similar and only differ quantitatively (Table 1.7.8). Simultaneous stresses from several heavy metals may readily overstretch the capacity even of strongly resistant plants. Such multiple supply often corresponds to real conditions, e.g. on ore outcrops, slag heaps from mines and in sewage sludge. Particularly in the latter, there are in addition to high copper and iron concentrations relatively high concentrations of cadmium or mercury, heavy metals that, in contrast to Cu, Mn and Fe, are true xenobiotics. How do plants cope with an excess supply of such ions?

1.7.6

Heavy Metal Extraction and Soil Decontamination by Plants (Phytomining, Phytoremediation)

Metal tolerance, and in particular heavy metal tolerance, of some plants offers two economically interesting possibilities: **Heavy metal extraction (phytomining)** and **soil decontamination** with the help of so-called **metal accumulating plants (phytoremediation)**. Plants can exploit substrates the mining of which is technically difficult and therefore unprofitable. Plants may also be used for the extraction of toxic materials, particularly heavy metals, organic pollutants and radionuclides from contaminated soils (soil remediation). For the latter possibility, five different procedures are used (Fig. 1.7.12):

- **phytoextraction**: toxin-accumulating plants extract the pollutant from the soil and accumulate it in the organs to be harvested,
- **rhizofiltration**: plant roots adsorb or take up toxins (particularly heavy metals) from water and sewage,
- **phytodegradation**: Plants and associated microorganisms decompose the pollutant (organic noxes),
- **phytostabilisation**: by synthesising complexes that bind the pollutant or by precipitation plants can decrease the bioavailability of the toxins,
- **phytovolatilisation**: plants detoxify the soil by production of volatile compounds (e.g. selenium).

Table 1.7.8. Heavy metal hyperaccumulating plants. Hyperaccumulating plants are able to accumulate heavy metals from 100 times to 10,000 times (e.g. *Haumaniastrum* for Co) the content of non-accumulating plants. The value of physiological tolerance of most heavy metals is at approx. 0.1% of plant dry matter. Exceptional values are for Zn (up to 1%), for Cd (0.01%) and gold (0.00001%). Surprisingly, some lichens, e.g. *Lecanora vinetorum*, also hyperaccumulate heavy metals (Brooks et al. 1998)

Element	Number of known hyperaccumulators	Examples (species)	Concentration (mg per g dry weight)	Biomass production (t per ha and year)
Ni	300	*Berkheya coddii*	17	18
Co	26	*Haumaniastrum robertii*	10.2	4
Cu	24	*Haumaniastrum katangense*	0.83	5
Se	19	*Astragalus pattersoni*	6	5
Zn	16	*Thlaspi calaminare*	10	4
Mn	11	*Macadamia neurophylla*	55	30
Ta	1	*Iberis intermedia*	0.3	8
Cd	1	*Thlaspi caerulescens*	3	4

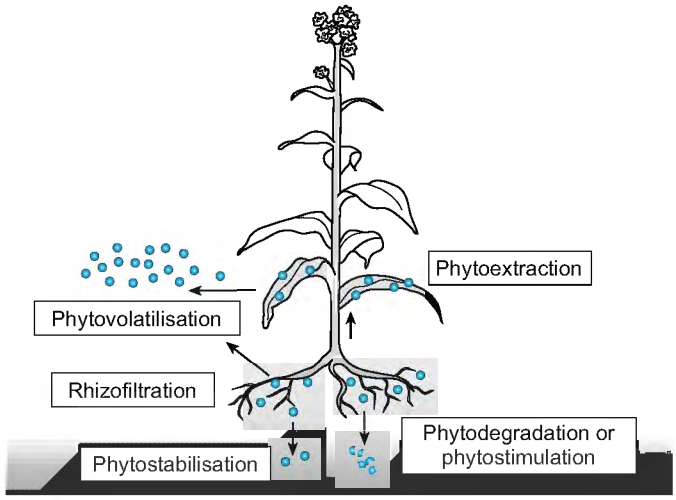


Fig. 1.7.12. The various strategies of phytoremediation. (After Pilon-Smits and Pilon 2000)

1.7.6.1 Phytoremediation

Plants which are able to extract large amounts of heavy metals from soil by accumulating them in an ample biomass even in a short vegetation period are particularly suited for **phytoremediation**. This technique is based on the work of the German botanist, Baumann, who in 1885 discovered accumulation of Zn in the mountain pansy (*Viola lutea* ssp. *calaminaria*), also called the calamine violet, and in an alpine penny-cress (*Thlaspi caerulescens*; Assunção et al. 2003). Two strategies of phytoremediation have been successfully employed: Continuous phytoextraction with hyperaccumulators and chelate-mediated extraction.

For the continuous removal of pollutants **hyperaccumulators** are preferred. Effective hyperaccumulation is obtained with many heavy metals, independent of whether they are metabolically active, or true xenobiotics, such as Cd. Hyperaccumulators may gain a kind of protection against biotic stress such as fungal infection as the pathogen is usually less resistant to intoxication by heavy metals; on the other hand, such protection is physiologically expensive, as it requires biochemical bulwarks mechanisms, e.g. synthesis of metallothioneins and phytochelatins, sequestration mechanisms to the vacuole or cell wall, excretion by salt glands and hydathodes, conversion of toxic amino acids, such as selenocysteine to the volatile methylselenocysteine, or repair after damage.

For **chelate-mediated extraction of pollutants**, annual plants with a high biomass production are particularly useful, e.g. large grasses, as long as the metal contamination of the soil is low or moderate. These plants that do not belong to the hyperaccumulators are allowed to develop to their full size on the contaminated soil. Heavy metal extraction is then initiated by solubilisation by applying artificial chelators such as EDTA to the soil.

An interesting possibility for **detoxification of mercury** has been described by Meagher (2000). Elemental mercury and mercury ions are released from gold mining, from industrial waste and upon burning of fossil energy carriers and medical wastes. Sulfate-reducing bacteria produce from such mercury-containing waste the extremely poisonous methylmercury ion $(MeHg)^+$, which accumulates in the food chain, and also in aquatic sediments. Gram-negative bacteria possess two enzymes which reduce methylmercury to elemental mercury, which is volatile and much less poisonous (about hundred times) than mercury ions. Organomercury lyase converts methylmercury to Hg^{2+} which by an NADPH-dependent mercurate reductase is reduced to mercury. *Arabidopsis* but also bigger plants, such as tobacco or *Liriodendron*, were transformed with the gene of one of these enzymes and crossed with a partner plant, into which the other gene had been transferred. The plants of the F_1 generation were considerably more resistant to mercury salts in the substrate, from which elemental mercury was evaporated. Transfer and expression of these genes also in aquatic plants are most desirable aims for the decontamination of the wastewaters especially from gold mines. However, phytovolatilisation is

not a solution, but a dilution of the problem, comparable with the construction of high chimneys in central Europe in the 1960s and 1970s to get rid of industrial sulfur dioxide locally, which was then transported predominantly to northern Europe.

Summary

1. About 25% of the heavy metals (Fe, Mn, Cu, Zn, Mo, Co) belong to micronutrients in plant nutrition; the majority of the heavy metals do not have a metabolic function in plants. Nevertheless, they are naturally present in all plants, but often in very low concentrations. They can be classified as xenobiotic heavy metals. Some of the micronutrients occur in several oxidation states and are therefore components of systems that transfer electrons (e.g. Fe in cytochromes). Free heavy metal ions in the reduced form (e.g. Fe^{2+}, Cu^{+}, Mn^{2+}) can easily react with oxygen and are therefore toxic Fenton reagents. Mobility of heavy metal ions in the soil and availability to plants depend on the redox potential and pH of the soil. Heavy metal deficiency occurs very rarely, in contrast to metal toxicity which is usually due to human activities. Plants which are capable of accumulating larger amounts of particular heavy metals in their tissues are termed metallophytes.
2. Upon heavy metal shortage, plants increase their capacity for uptake by producing a larger root surface (root hairs, labyrinth in the rhizodermis/transfer cells) and by enhancing secretion of chelators and protons (by plasma membrane H^{+}-ATPases) into the substrate, thus increasing the dissolved proportion of heavy metal ions. Because uptake of heavy metal ions frequently requires prior reduction (e.g. $Fe^{3+} \rightarrow Fe^{2+}$), the capacity of the plasmalemma chelate reductase is also increased. Grasses excrete so-called phytosiderophores instead of the chelators into their substrate. Phytosiderophores are non-proteinogenic amino acids which transfer heavy metal ions from insoluble into water-soluble complexes.
3. Heavy metal toxicity also occurs with plant micronutrients if the optimal concentrations are exceeded; of chemically reactive xenobiotic heavy metals already very low concentrations are toxic. Uptake of the latter is via the uptake system for micro- or macronutrients such as Ca. Heavy metal toxicity as well as deficiency result in chloroses which, however, are of different origin in the two cases. Chlorosis from toxicity results from bleaching of the photosynthetic pigments by ROS (heavy metals act as Fenton reagents). Chlorosis from shortage is caused by failing chlorophyll synthesis because of a lack of cofactors (e.g. cytochrome P450). Because heavy metal ions are also part of ROS-scavenging systems, chlorosis from shortage might also be the consequence of a less efficient anti-oxidative system. Apart from oxidative stress caused by heavy metals, they also very effectively replace functional ions from biochemically important complexes and form sulfides with the free sulfhydryl groups of proteins and therefore are toxic, even at very low concentrations. The mobility of heavy metal ions differs in the plant (Cu^{2+} is almost immobile, Fe^{2+} and Cd^{2+} are very mobile).
4. Sensitive and tolerant plants react in a similar way to heavy metal stress. On the one hand, enhanced exudation from the root cells traps heavy metal ions as insoluble complexes in the soil or in the cell wall and, on the other, the concentration of the cytosolic heavy metals is decreased by binding to specific oligo- and polypeptides.
5. Heavy metal binding polypeptides are metallothioneins and phytochelatins. Synthesis of both groups of peptides is enhanced under heavy metal stress. They are rich in cysteine residues by which several up to many heavy metal ions per polypeptide can be fixed. Metallothioneins are gene-encoded proteins with certain repetitive cysteine-containing motifs. The promoters of the MT genes contain elements for metal-responsive transcription factors and frequently also ABA-responsive elements. Metallothioneins are rather unspecific for heavy metals. They are considered as heavy metal storage proteins rather than as means for detoxification. In contrast to thioneins, phytochelatins are not oligo- or polypeptides arising from gene expression. They are synthesised by dipeptide transfer from glutathione. Their general structure is $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, where n ranges from 1 to 11. Glycine can be replaced by serine or β-alanine. They are synthesised by phytochelatin synthase, whose synthesis in turn is induced by the highly toxic heavy metal ions. Phytochela-

tins probably serve to sequester heavy metals to the vacuole and thus, though not representing direct detoxifying compounds, are involved in the relief of heavy metal stress. In the vacuole of some metallophytes polymeric heavy metal phosphates have been demonstrated. Recently, heavy metal pumps have been found in microorganisms, animals and yeast and in *Arabidopsis* as well. These enzymes are ATP-dependent pumps similar to the Na^+/K^+-ATPases, but with a cysteine-rich N-terminal extension.

6. Stress by heavy metals evokes multiple responses of plants, which are scarcely element-specific. Differences in tolerance between metallophytes and heavy metal sensitive plants are gradual. Protein denaturing by heavy metal ions can induce heat shock reactions (accumulation of chaperones).
7. Metallophytes can be used for phytomining and for phytoremediation. Phytomining is essentially a question of profitability and is only economically effective for rare heavy metals (Co, Ta, Ni, U). Heavy metal remediation of soils may be carried out by continuous extraction of the toxic compounds with perennial hyperaccumulators or by chelate-mediated removal of materials, using moderately heavy metal tolerant, annual plants with ample biomass production. These grow on the contaminated soils until maturity when the heavy metals are brought into solution by application of chelators. After the uptake of the metal the plants usually rapidly die and are then disposed of.

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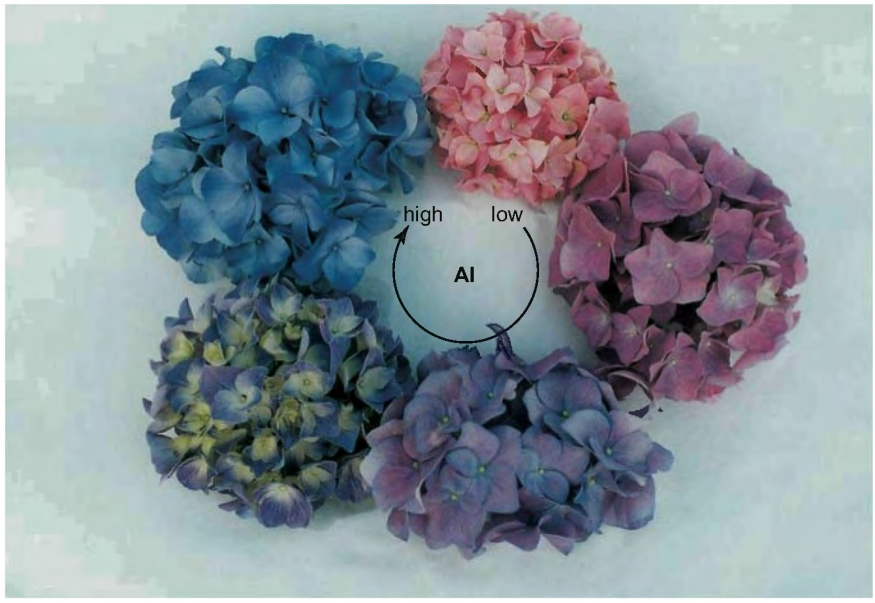
1.8

Aluminium



Aluminium ions form complexes with water-soluble vacuolar pigments. Gardeners use this to great effect in the ornamental plant *Hydrangea* sp., which at different Al^{3+} concentrations changes the colour of its flowers from white through red to deep blue. The colours in *Hydrangea* are the result of aluminium complexes with the anthocyanin delphinidin-3-glucoside and with chlorogenic acid (caffeoylquinic acid). The aluminium concentration in the flowers shown are (from red to blue) 51, 106, 640, 804, and 3959 mg Al^{3+}/kg dry weight. After Ma et al. (2001)

Recommended Literature

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Aluminium toxicity is a worldwide problem affecting growth of crop plants and yields on acid soils (see also Chap. 3.5.1 on forest damage): Al is the most frequent metal element in the earth's crust. In acid soils, i.e. at pH <5.5, the phytotoxic Al^{3+} ion becomes soluble to an extent which inhibits root growth and, as secondary effects, decreases uptake of nutrients and water and thus growth of the plant. In only slightly acidic or neutral soils aluminium forms insoluble oxides or silicates. More than 30% of the (potential) agricultural land has a pH of <5.5 with aluminium toxicity being a serious problem. The widespread acidic red soils, called oxisols, of the tropics and subtropics are particularly affected. Aluminium toxicity also occurs in temperate climates because of acid rain or mist.

However, there is growing awareness that on acid soils several factors in addition to Al toxicity may limit plant growth, such as high concentrations of iron and manganese ions or deficien-

cy in several essential mineral elements, in particular of phosphorus.

According to present knowledge, aluminium is not a trace element required for the nutrition of plants. Though not belonging to the heavy metals, it is considered a toxin because of its negative effects on plant growth. Nevertheless – as with most xenobiotics – inheritable resistance to Al ions in the root zone occurs, and possibly even tolerance of aluminium ions in the cell. Research into Al toxicity and resistance mechanisms has been made possible by breeding of almost isogenic lines, e.g. of wheat, which differ only in their sensitivity to Al. Because of the multiplicity of secondary effects of Al toxicity, the interpretation of the physiological effects of Al ions is still very controversial. However, methods of analysis have become more sophisticated and hence interpretation is clearing up.

1.8.1

Forms of Aluminium Available to Plants

There are three classes of aluminium ions available to plants: The **mononuclear** forms of Al^{3+}, **polynuclear aluminium**, and **complexed aluminium** (Macdonald and Martin 1988). The latter occurs usually in complexes with low molecular weight compounds, but, particularly at neutral pH, macromolecules are also able to bind Al ions.

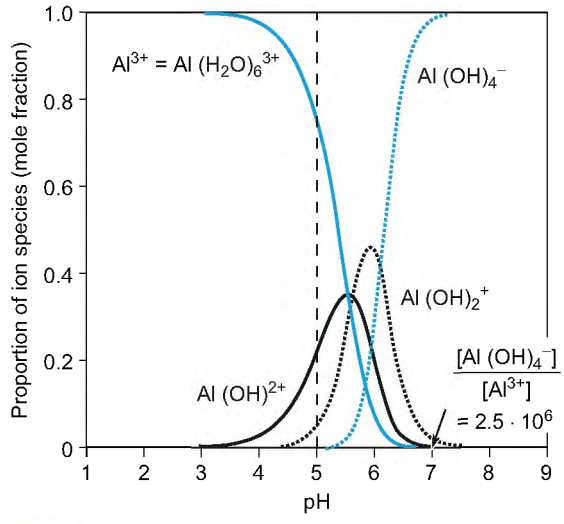


Fig. 1.8.1. Dependence on the pH of the proportion (mole fraction) of soluble, mononuclear aluminium ion species in aqueous solution. At higher concentrations "Al_{13}" = $[AlO_4Al_{12}(OH)_{24}(H_2O)_7]^{7+}$, the triskaidea-aluminium is formed. (After Macdonald and Martin 1988)

In acidic solutions (pH < 5.0), mononuclear Al^{3+} occurs as the hexahydrate, $Al(H_2O)_6^{3+}$, conventionally called the Al^{3+} ion (Fig. 1.8.1). With increasing pH this hexahydrate is progressively deprotonated producing $Al(OH)^{2+}$, then $Al(OH)_2^+$, and, finally, at neutral conditions, the insoluble $Al(OH)_3$, also called gibbsite, is formed. In an alkaline milieu, the aluminate ion $[Al(OH)_4^-]$ is produced, which is again soluble, but as an anion has different sites of attack than the cation Al^{3+}.

At higher aluminium concentrations, particularly at neutral conditions, polynuclear forms occur, most frequently the triskaidea-aluminium $[AlO_4Al_{12}(OH)_{24}(H_2O)_7]^{7+}$, the so-called Al_{13}. All positively charged forms are phytotoxic, including Al_{13}. Mononuclear aluminium easily forms complexes with oxygen donor ligands, e.g. carboxyl groups, phosphates (inorganic P_i and polyphosphates) and sulfate.

1.8.2

Aluminium Toxicity

1.8.2.1

Aluminium Uptake

The kinetics of Al^{3+} uptake in wheat roots is biphasic, an initial fast and a subsequent slow phase. The fast phase results from the uptake into the cell wall and in the mucilage (the so-called mucigel) around the root tip. The slow phase represents the uptake into the symplast. Al^{3+} deposited in the cell wall, which either forms precipitates or is adsorbed, can be desorbed and solubilised with citrate. Three hours of exposure of wheat roots to citrate released between 50 and 75% of their Al^{3+} content, whereas in *Arabidopsis* roots only a quarter of the absorbed Al^{3+} could be extracted from the cell wall (Table 1.8.1). Aluminium-resistant cultivars absorb Al^{3+} more slowly and in much smaller quantities than sensitive species. This applies to uptake into the apoplast as well as into the symplast.

Al^{3+} enters the symplast either via cation carriers or by adsorption and endocytosis. In addition, in the acidic apoplast (pH $\approx$ 4.5), aluminium citrate complexes are uncharged and can slowly permeate the plasma membrane.

Since the radius of the Mg^{2+} ion is similar to that of the Al^{3+} ion (Table 1.8.2), it is assumed

Table 1.8.1. Cell-wall-bound Al^{3+}: roots of wild-type and an aluminium-resistant mutant of *Arabidopsis* were incubated for 0 and 3 h, respectively, in 25 µM $AlCl_3$ solution and subsequently washed for 5 min with either Al-free nutrient solution or (Al-free) nutrient solution containing citrate. The amount of Al^{3+} remaining in the roots was then measured. Somewhat more than 25% of the Al^{3+} absorbed by the roots could be extracted from the cell wall by forming a complex with citrate (Larsen 1998)

Plant	Extraction with water		Extraction with 0.5 mM citrate (pH 4.4)	
Treatment	0 h	3 h	0 h	3 h
		Remaining Al^{3+} [nmol/mg dry weight]		
Wild type	0.2±0.1	42.3±3.4	0.2±0.1	29.3±0.8
Mutant alr-104	0.3±0.1	28.6±1.6	0.4±0.2	31.7±0.9

Table 1.8.2. Toxicity of soluble aluminium ions can be ascribed to the similarity of their ionic radii with those of essential nutrient elements (arrows). Effective ion radii (in Å) are shown in relation to the coordination number (Macdonald and Martin 1988)

	Coordination number					
Ion	4	5	6	7	8	9
Be^{2+}	0.27		0.45			
Al^{3+}	0.39	0.48	0.54			
Ga^{3+}	0.47	0.55	0.62			
Fe^{3+}	0.49	0.58	0.65			
Mg^{2+}	0.57	0.66	0.72		0.89	
Zn^{2+}	0.60	0.68	0.74		0.90	
Gd^{3+}			0.94	1.00	1.05	1.11
Ca^{2+}			1.00	1.06	1.12	1.18

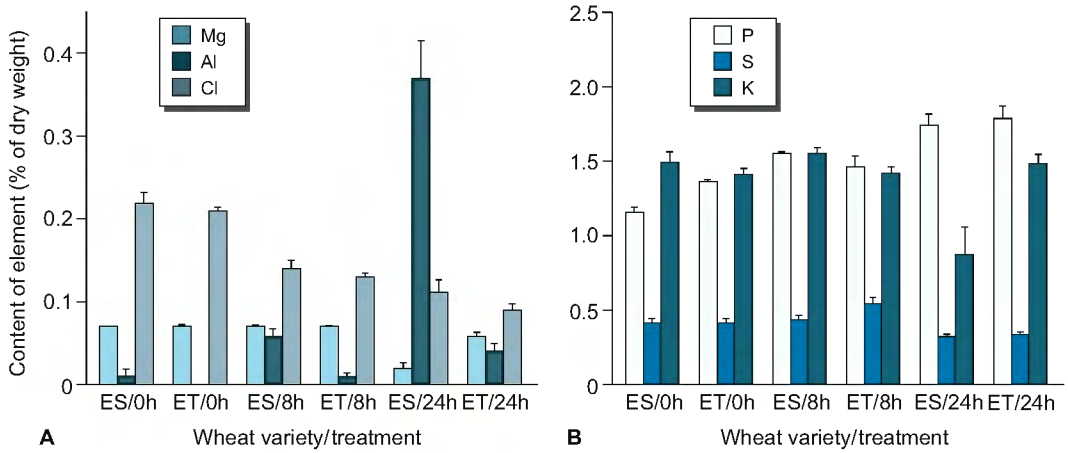


Fig. 1.8.2. Extrusion of ions from the root tips of Al^{3+}-sensitive and -tolerant wheat varieties by incubation of seedlings in 100 µM Al^{3+} solution for 0, 8 and 24 h. A Mg^{2+}, Al and Cl^- content of dried root tips. B P, S and K^+ content. *ES* Homozygous, sensitive line, *ET* homozygous, tolerant line. (After Delhaize et al. 1993a)

that the latter penetrates through Mg^{2+} channels, which can be seen by the concomitant reduction of Mg^{2+} uptake or by a decreased Mg^{2+} content (Fig. 1.8.2). Because of the similar ionic radius, the Al^{3+} ion might also use the siderophore uptake system for Fe^{3+}, which is well developed in grass roots.

Finally, it cannot be excluded that positively charged Al ions adsorb at the external side of the plasma membrane and reach the cytosol via endocytosis. Al^{3+} does not interact with K^+ channels and, considering the usually high K^+/Al^{3+} ratio, has therefore almost no influence on the membrane potential.

1.8.2.2
Phenomena of Aluminium Toxicity

In plants Al^{3+} is not very mobile and, therefore, usually remains in the root, which is therefore the organ where Al toxicity has its biggest impact and where toxic symptoms and resistance mechanisms have primarily been investigated.

The visible effect is a drastic inhibition of the elongation growth of the main and lateral roots, occurring within 1–2 h of exposure. Upon moderate stress elongation growth is resumed later on, but at a significantly lower rate. Therefore, sensitivity to Al^{3+} is highest in the elongation zone (Fig. 1.8.3); Al^{3+} accumulates in that zone and callose forms in response to the stress. In Al-resistant plants these phenomena are less pronounced.

Al^{3+} also inhibits cell division, but with a delay of hours with respect to cell elongation, and therefore inhibition of DNA synthesis cannot be the primary reason for the inhibition of root growth. On the other hand, inhibition of cell division activity is the reason for a long-term inhibition of root growth.

1.8.2.3
Symplastic Aluminium Toxicity

It was previously assumed that Al^{3+} binds to the weakly alkaline phosphate groups of DNA and thus inhibits cell division. However, this interpretation appears more and more unlikely. Likewise the earlier assumption that Al^{3+} predominantly affects the root cap could not be proven. It is

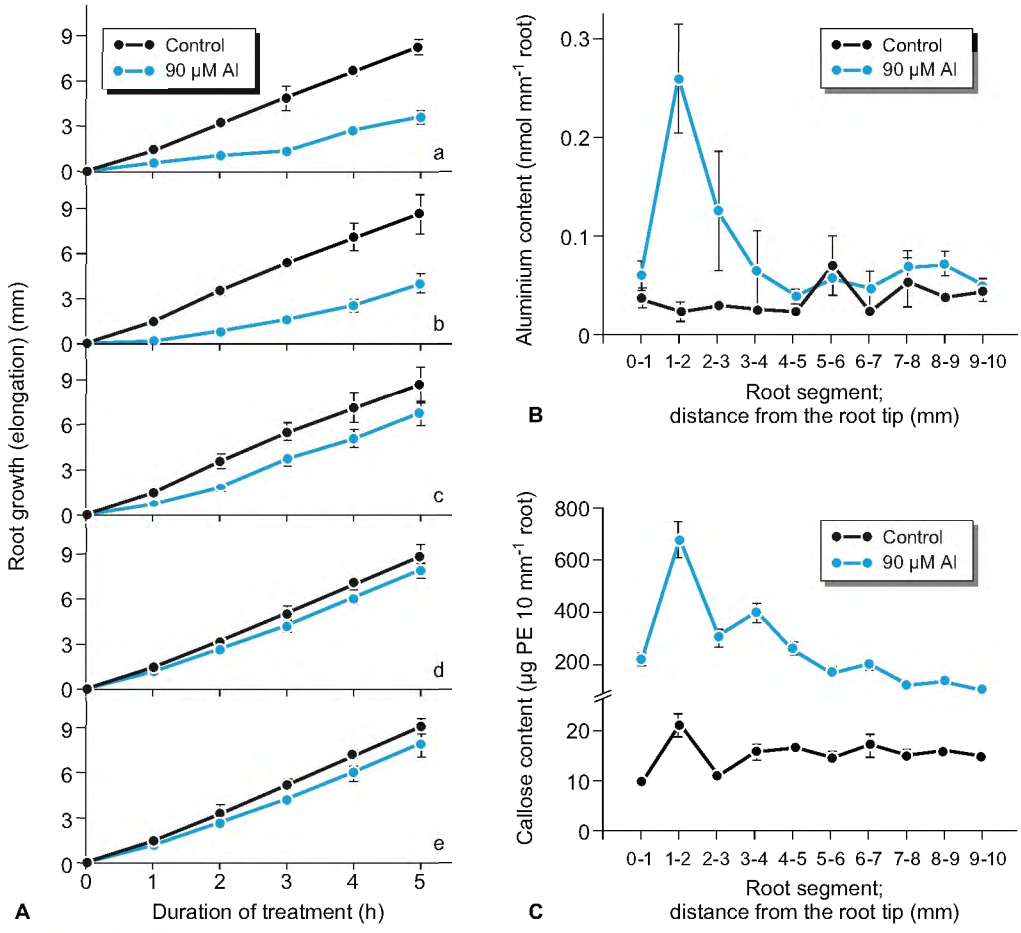
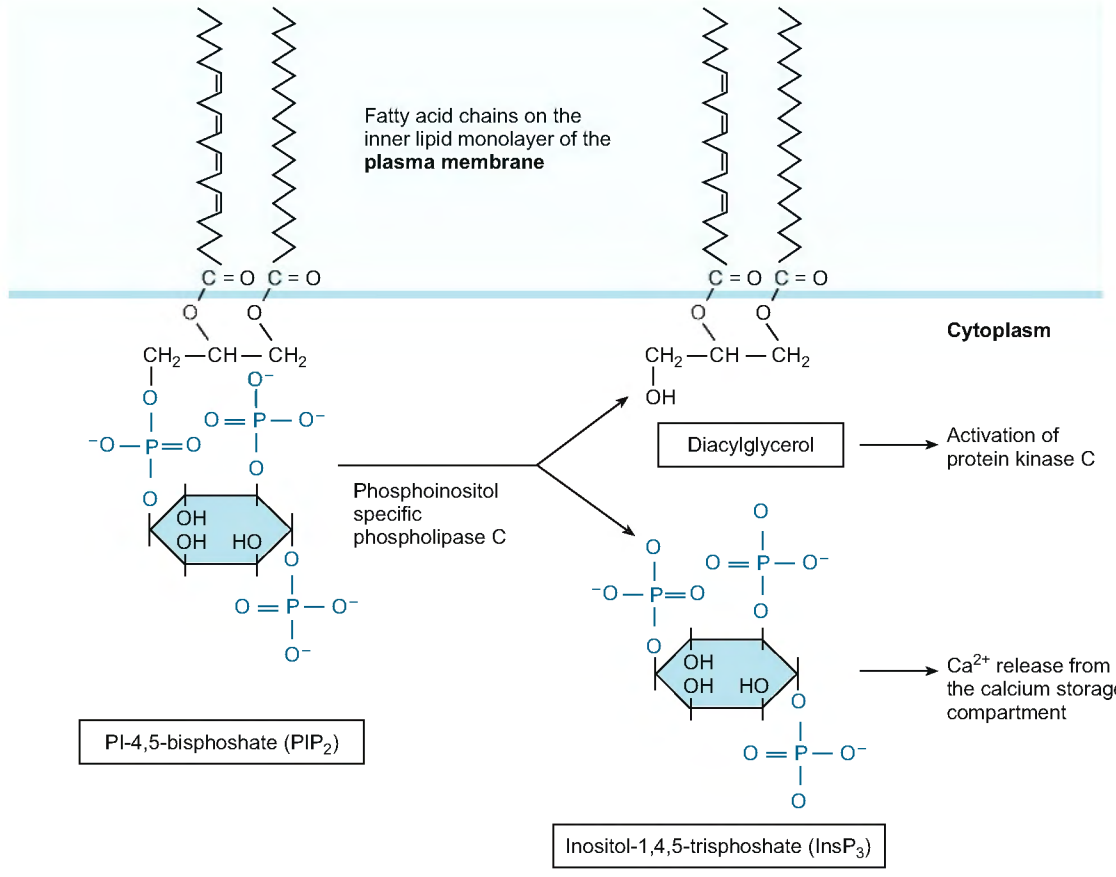


Fig. 1.8.3. A Effect of Al^{3+} ions (90 µM) on the elongation growth of individual 1-mm-long sections of an intact maize root. These sections could be selectively exposed to the 90-µM aluminium solution. *a* 0–1 mm; *b* 1–2 mm; *c* 2–3 mm; *d* 3–4 mm; *e* 4–5 mm. The standard deviation refers to five replicates. B Aluminium content of the individual segments of the apex of a maize root after 1-h exposure to 90 µM Al^{3+}. C Aluminium stress causes deposition of the polyglucan callose [determined with aniline blue, in so-called Pachyman equivalents (PE)] in those zones where aluminium affects root growth. (After Sivagura and Horst 1998)

Box 1.8.1 Role of phospholipase C (PLC) in the intracellular signal network

PLC liberates inositol-1,4,5-trisphosphate ($InsP_3$) from the membrane lipid inositol-diacylglycerol. $InsP_3$ controls the cytosolic level of Ca^{2+} which acts as second messenger. The other component of the PLC reaction, diacylglycerol, autocatalytically activates PLC. $InsP_3$ is the target of Al^{3+} which in this way interferes with the Ca^{2+} metabolism of the cell.



now thought to interact with the phosphorylated proteins of the cell nucleus. In dicotyledons, $Al(OH)^{2+}$ and $Al(OH)_2^+$ have a stronger toxic effect than Al^{3+}, in grasses (monocotyledons) Al^{3+} is more toxic. However, the soluble complexes with sulfate, fluoride and with organic acids (chelates) as well as insoluble salts such as $Al(OH)_3$ or $AlPO_4$ are not toxic. Because of the similarity of the radii of their ions (Table 1.8.2), Al^{3+} interferes predominantly with the Mg^{2+} and Fe^{3+} metabolism. Increase in Mg^{2+} ion concentration thus counteracts aluminium toxicity. Inhibition of Ca^{2+}-dependent reactions was also observed, but this is not yet understood at the molecular level.

The molar fraction of positively charged Al ions depends on the pH of the solution (see Fig. 1.8.1). While an acid pH is common in the apoplast, the cytosol is usually neutral to weakly alkaline (pH >7). In this pH range almost the total aluminium occurs as aluminate and the concentrations of Al^{3+} and its derivatives are in the nanomolar to picomolar range. In addition, several chelating agents in the cytosol are able to decrease the concentration of Al^{3+}.

Despite the low concentrations of cytosolic Al^{3+}, malfunctions occur, e.g. of reactions which are controlled by Mg^{2+}. The Al^{3+} ion replaces Mg^{2+} from the ATP–Mg complex, as the Al–ATP

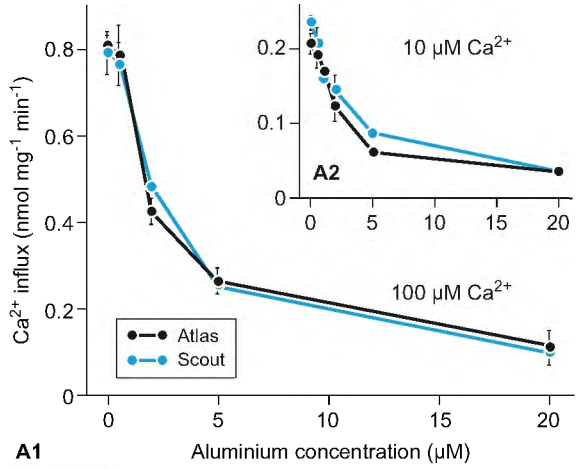


Fig. 1.8.4. Influence of various aluminium concentrations on the voltage-dependent ($E_{membrane}$ = −100 mV) Ca^{2+} uptake by plasma membrane vesicles, isolated from roots of Al-sensitive (Scout 66) and -resistant (Atlas 66) wheat cultivars. A1 Inhibition of Ca^{2+} uptake at a concentration of 100 μM Ca^{2+} in the solution. A2 Inhibition of Ca^{2+} uptake at a concentration of 10 μM Ca^{2+} in the solution.
(After Huang et al. 1996)

complex binds 1000 times stronger to hexokinase than does the Mg–ATP complex. In addition, Al^{3+} inhibits Ca^{2+} uptake into the cell by inactivating the voltage-gated Ca^{2+} channel, even though it does not interact with the membrane potential (Fig. 1.8.4). Interference with calcium is also obvious from the derangement of the intracellular signal transduction (Buchanan et al. 2000).

Al^{3+} prevents the signal-induced increase in inositol-1,4,5-trisphosphate ($InsP_3$) and the subsequent release of Ca^{2+}: Either Al^{3+} binds to the Mg^{2+}-binding site of the G-protein or it reacts directly with phospholipase C; direct binding of Al^{3+} to $InsP_3$ is, however, not likely (Jones and Kochian 1995) any more than a reaction with calmodulin and an interference with its function in the control of Ca^{2+} metabolism.

Because of the inhibition of root growth, with concomitant thickening of the root tips, it is to be expected that Al^{3+} also reacts with the cytoskeleton, the **microtubules** and **actin filaments**. Biochemical studies have shown that Al^{3+} inhibits the Mg^{2+}-dependent association of the tubulin molecules with the microtubule and the Ca^{2+}-dependent dissociation. As microtubules have a dynamic structure with a growing (+) and a decreasing (−) pole, the interaction of Al^{3+} with microtubules leads to a fixation of the cytoskeleton. In microtubule association, Mg^{2+} binds to the GTP and GDP receptor sites. The association constant of Al^{3+} with this site is 3×10^7 higher than that of Mg^{2+} and an Al^{3+} activity of 40 nM replaces Mg^{2+} at millimolar concentrations!

When maize roots were treated with 50 μM Al^{3+}, microtubules of the outer cortex cells showed fixation in the transverse direction and stabilisation after 1 h simultaneously with the onset of growth inhibition. Al^{3+} requires about 30 min to migrate through the outer three cortex layers.

Three to 6 h after application of Al^{3+}, the microtubules of the inner cortex layer changed their transverse orientation to a random distribution, giving rise to isodiametric cell growth and thus thickening of root tips.

Because of radial expansion, together with the fixation of the cytoskeleton in the external cortex layers, fractures and lesions occur and the outer cell layers peel off. The reorientation of microtubules in the inner cortex is most likely a secondary effect as indicated by the delayed incidence. Microfilaments also show a response to Al^{3+}, but not as strong as that of microtubules (Fig. 1.8.5). Microfilaments of the outer cortical layer reorient from random texture towards a longitudinal direction, whilst the random texture of the inner microfilaments is fixed.

1.8.3
Al^{3+} Resistance

The relative resistance of isogenic lines of plants towards Al^{3+} is usually based on several properties, the genes for which are found in one to several loci. Al resistance is almost always dominant, Al sensitivity on the other hand recessive, and in crossings the F_1 generation is therefore usually Al-resistant.

Resistance may be based on avoidance or tolerance. Avoidance means exclusion of Al^{3+} (Pellet et al. 1997), tolerance chelate formation. Exclusion, too, is mostly achieved via (external) chelates.

1.8.3.1
Al^{3+} Exclusion

Exclusion of the Al^{3+} ion is usually achieved by excretion of chelating acids such as citric, malic or oxalic acid from the root tip (Fig. 1.8.6). By

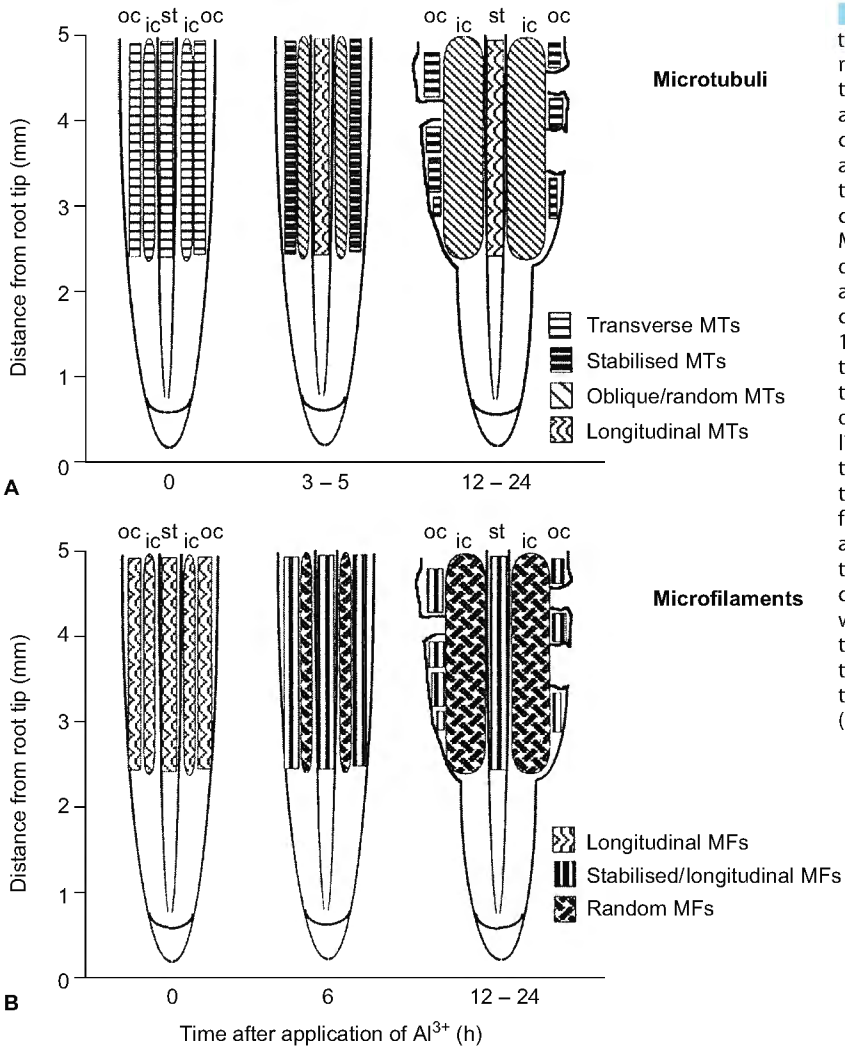


Fig. 1.8.5. Changes in orientation of A microtubules (MT) and B microfilaments (MF) in the elongation zone of a maize root following application of aluminium (Al^{3+} concentration = 50 µM). A One hour after application of Al^{3+}, the microtubules in the outer cortex (*oc*) became fixed in transverse direction. Microtubules with oblique to random texture were produced 3 h after Al^{3+} application in the inner cortical layer (*ic* inner cortex), and 1 h later in the stele (*st*). This alteration in the orientation of microtubules may lead to an expansion of cells of the ic and thus to swelling of the root. After 12 h, cells of the oc are deformed and lesions in the outer cell layers result. B Microfilaments become stabilised 3 h after Al^{3+} application; random texture and increased clustering of microfilaments in the ic and stele were seen 6 h after Al^{3+} application. Fixation and reorientation of the microfilaments occurred later than that of the microtubules. (After Blancaflor et al. 1998)

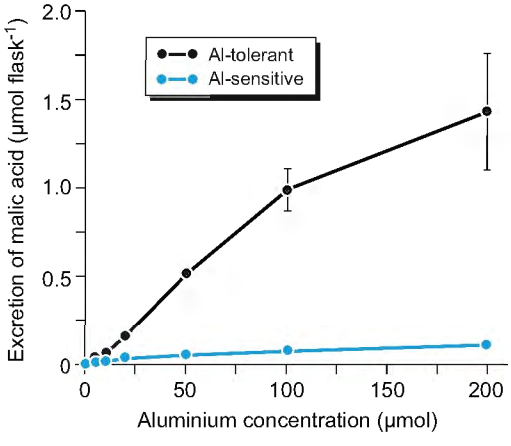


Fig. 1.8.6. Excretion of malic acid by seedling of Al-sensitive and -resistant wheat varieties. Six-day-old seedlings were exposed for 24 h to different concentrations of an Al^{3+} salt solution. (After Delhaize et al. 1993 b)

binding to these acids, the Al^{3+} ion is no longer available to the uptake system.

Malate excretion is a specific reaction to Al^{3+}, as it is not triggered by other trivalent cations (La^{3+}, Sc^{3+}), and also not by Al_{13}. Citric acid chelates strongest, succinic acid weakest (Fig. 1.8.7).

Under aluminium stress, Al^{3+}-sensitive cultivars excrete malic acid, too, but much less than tolerant cultivars (see Table 1.8.3). Scavenging of Al^{3+} by excretion of malic acid by Al-sensitive cultivars could be boosted by the addition of malic acid to the nutrient solution (Fig. 1.8.8).

Resistance of wheat is mainly due to excretion of malic acid, whilst maize reacts to Al^{3+} stress by excreting citric acid (Kollmeier et al. 2001). Al^{3+}-sensitive and -resistant cultivars of these crops likewise differ mainly in the amount of secreted acid. It is not the acidification of the rhizosphere

Table 1.8.3. Excretion of organic acids by Al-tolerant and -sensitive wheat seedlings after 24-h exposure to Al (5 seedlings each in 20 ml nutrient solution, containing 50 μM Al^{3+}). (After Delhaize et al. 1993 b)

	Excreted acid (nmol seedling^{-1} h^{-1})			
Organic acid	Sensitive –Al	sensitive +Al	Tolerant –Al	Tolerant +Al
Malic acid	0.08 ± 0.08	0.33 ± 0.00	< 0.08	3.57 ± 0.08
Succinic acid	0.08 ± 0.08	0.08 ± 0.08	0.08 ± 0.08	0.58 ± 0.08
Citric acid	0.17 ± 0.08	0.08 ± 0.08	0.08 ± 0.00	0.17 ± 0.00

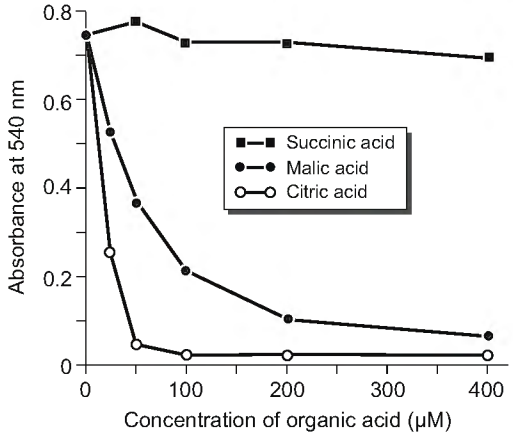


Fig. 1.8.7. Chelation of Al^{3+} by organic acids. Citric, malic and succinic acids were incubated for 1 h with a nutrient solution containing 50 μM Al^{3+}, 3.2 mM Na acetate buffer (pH 4.2) and 250 μM haematoxylin. The formation of Al–haematoxylin complexes was measured by absorption at 540 nm. The reduction of absorption in the presence of organic acids shows that organic acids chelate part of the aluminium which is then no longer available for the formation of a complex with haematoxylin. (After Delhaize et al. 1993 b)

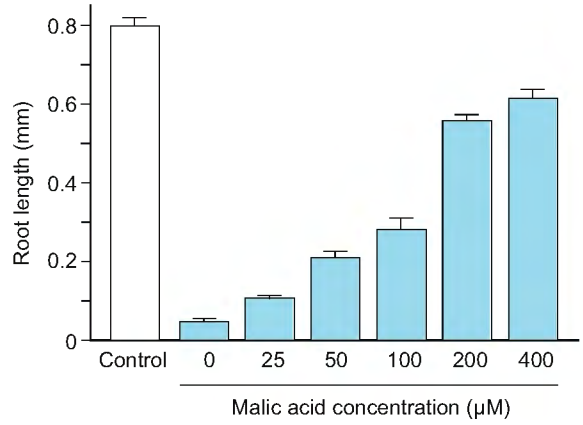


Fig. 1.8.8. Alleviation of the toxic effect of Al^{3+} by malic acid. Al-sensitive seedlings were grown in a nutrient solution containing 50 μM Al^{3+} and various concentrations of malic acid. Control seedlings were grown without aluminium and without malic acid. (After Delhaize 1993 b)

that finally effects aluminium tolerance, but the complexing of the Al^{3+} ion. Similarly, some Al^{3+}-insensitive species secrete phosphate by their roots, leading to the formation of insoluble $AlPO_4$.

Rapid, Al^{3+}-triggered excretion of chelating acids leads to the question how aluminium ions are perceived. Excretion of malic or citric acid can be blocked by inhibitors of anion channels. This, and the fact that malate is a common metabolite of the cytosol, suggest that the primary effect of Al^{3+} is the opening or activation of a malate-transporting channel. But how Al^{3+} gates this channel is still unsolved.

1.8.3.2 Significance of the Apoplast for Al^{3+} Resistance

Cell walls, particularly pectin-rich primary cell walls, carry many negative charges which are usually compensated by bivalent ions (Mg^{2+}, Ca^{2+}). It is assumed that Al^{3+} replaces such bivalent ions from their binding sites, and that the cell wall thus acts as an **aluminium trap**. Such binding sites would quickly saturate with Al^{3+} ions, but upon continuous root growth new ion-binding sites originate so that protection of the protoplast by the cell wall does not seem unrealistic. However, so far differences in cell walls of sensitive and resistant cultivars have not been shown.

1.8.3.3 Al^{3+} Exclusion by Alkalinisation of the Rhizosphere

Even slight changes in pH alter the composition pattern of the aluminium ions (see Fig. 1.8.1). It

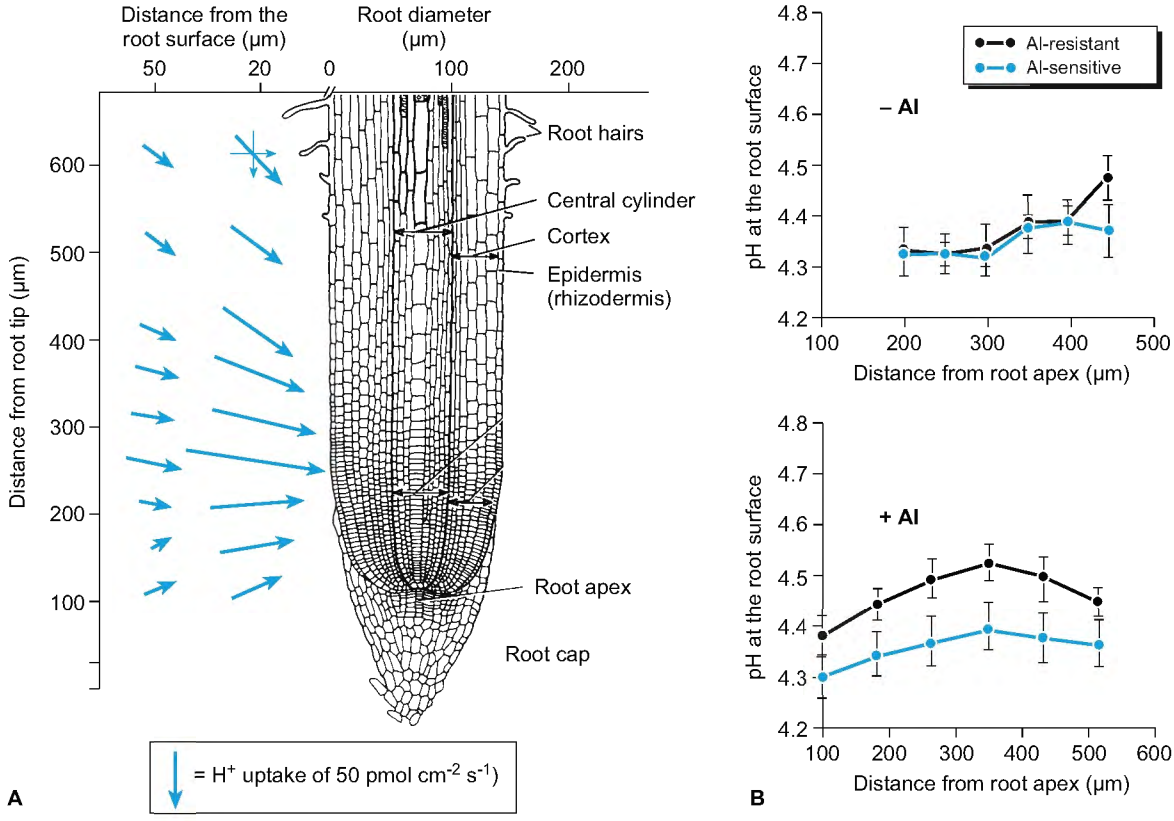


Fig. 1.8.9. A Diagram of the proton uptake by the tip of an *Arabidopsis* root. The length of the *arrow* indicates the extent of the proton influx. B Influence of aluminium ions on the pH in the rhizoplane ("root surface") of the Al-sensitive wild-type and the Al-resistant mutant alr-104. (After Degenhardt et al. 1998)

was therefore assumed that aluminium resistance, based on exclusion, could also be achieved by alkalinisation of the rhizosphere. Such a mechanism has been recently shown with Al-resistant *Arabidopsis* plantlets, using a pH microelectrode. This alkalinisation is achieved by proton uptake through the root tip leading to a small increase in alkalinity (ca. 0.1 pH units) of the medium at the root surface. Measurements of root growth confirm the significance of the seemingly small pH difference (Fig. 1.8.9). However, such an effect could also result from a reduced H^+ release from the root. This would then mean that Al^{3+} inhibits the proton pump of the root cell plasma membrane. The slight inhibition of root growth by Al^{3+} at pH 4.5 compared with that at pH 4.4 could also be due to a decrease in the mole fraction of Al^{3+} in favour of $Al(OH)^{2+}$ (see Fig. 1.8.1).

1.8.4

Al^{3+} Tolerance

Aluminium tolerance is imputed to species or cultivars that tolerate an increased intracellular aluminium concentration. Examples are old leaves of tea which accumulate up to 30 g Al per kg dry mass, or hydrangea, whose bracts (sepals) turn blue with the addition of a little Al^{3+} to the irrigation water and whose leaves accumulate up to 3 g Al per kg dry weight (Ma et al. 1997, see also figure introducing Chap. 1.8).

Corresponding to the high content of soluble Al^{3+} in acid soils of the tropics substantial concentrations of Al ions (up to 1 g/kg) occur in wild plants (*Melastoma*, *Vaccinium*) of rain forests. The Al tolerance of these plants could result from an effective sequestration (excretion by the plasma membrane or deposition in the vacuole) or from intracellular detoxification by chelating agents or Al-binding proteins. Buckwheat is an example of an Al-tolerant plant (the

Table 1.8.4. Concentrations of aluminium and oxalic acid in leaves, roots and in the cell sap of buckwheat. Plants were grown in nutrient solution (pH 4.5), which was exchanged every second day by a nutrient solution containing 0.5 mM $CaCl_2$ and 0 or 50 µM Al. Plants were harvested after 10 days of treatment (after Ma et al. 1998)

Plant organ	Al (mmol/kg fresh weight)	Water content (%)	Cell sap		
			Al (mM)	Al (%)[a]	Oxalic acid (mM)
Leaves					
+Al	2.01	87.5	2.03	88.4	51.08
–Al	0.02	87.4	0.01	–	46.79
Roots					
+Al	3.45	94.7	2.09	57.3	8.80

[a] Percent of total aluminium. Rest deposited in cell wall.

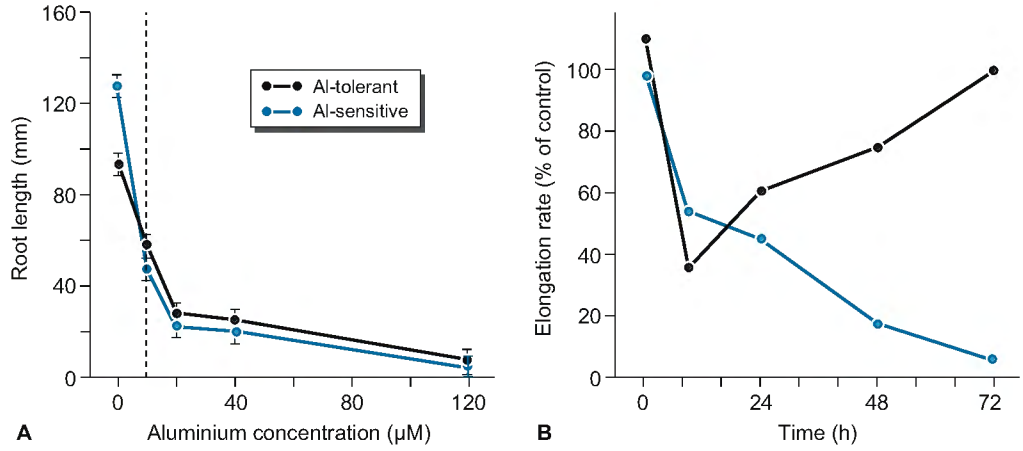


Fig. 1.8.10. A Inhibition of root growth of a sensitive and tolerant bean variety grown for 3 days in an Al^{3+}-containing nutrient solution. B Dependence of inhibition on the Al^{3+} concentration is the same in both cultivars. However, the tolerant variety recovers quickly from moderate stress (10 µM Al^{3+}), whereas root growth of the sensitive variety remains inhibited. (After Cumming et al. 1992)

cultivar *Jianxi* accumulates up to 0.5 g Al/kg leaf dry weight) which sequesters Al^{3+} from the cytosol into the vacuole where it forms complexes with oxalate (Al^{3+}/oxalate = 1:3; Table 1.8.4). In addition, Al-stressed, buckwheat roots excrete considerable amounts of oxalic acid, which complexes Al ions immediately in the rhizosphere. Exogenous Al oxalate (1:3 and 1:2) does not inhibit root growth, and thus is not toxic. Apparently, such complexes are so stable that under physiological conditions Al is not released.

In hydrangea the counter ion is citrate instead of oxalate. The corresponding complex contains aluminium and citrate at a ratio of 1:1 and accumulates in the vacuole. In contrast, in tea leaves, the bulk of Al ions seems to be bound to tannins.

The assumption that Al tolerance is inducible arises from experiments in which the initially inhibited root growth recovered completely after a few days (Fig. 1.8.10), indicating induction and achievement of Al tolerance.

Upon stress by Al, resistant wheat cultivars produced two additional proteins (51 kDa) in the plasmalemma fraction, which occurred only in the root tips and disappeared again when the stress was alleviated. Formation of these proteins was induced most effectively by Al^{3+}, to a lesser extent also by Cd^{2+} and Ni^{2+}, but not by Cu^{2+}, Zn^{2+} or Mn^{2+}. In the corresponding Al-sensitive cultivar only traces of these proteins were found under Al stress. One of these proteins could be a metallothionein. Another protein could be related to the production of "defence mucilage" by the outer root cap cells. Strong mucilage formation was observed upon incubation of root tips in solutions of Al salts. After excretion of the mucigel, root growth (of peas) recovered so fast that it was assumed that

the mucilage had adsorbed the aluminium ions and thus detoxified them (Hawes et al. 2000).

These examples show that Al stress may induce a more or less specific protein response, while the physiological role of such proteins still remains to be explained.

Summary

1. Aluminium is the most frequent metallic element in the earth's crust, where it appears in many, usually insoluble compounds and complexes. Three classes of aluminium ions available to plants are distinguished: Mononuclear Al^{3+}, polynuclear Al and complexed Al. At an acid pH (< 5) the mononuclear $Al(H_2O)^{7+}$ occurs, at neutral pH and at higher Al concentrations polynuclear forms develop, in particular the soluble $[Al_{13}]^{3+}$. All soluble Al ions are phytotoxic.
2. Aluminium is quickly taken up by adsorption to the cell wall and in the mucigel of the root tip. From there it enters into the cytosol via cation carriers (probably through a Mg^{2+} channel). Endocytotic uptake from the apoplast and, in grasses, uptake by the siderophore system are also discussed. Al^{3+} is almost immobile in the plant; aluminium damage occurs, therefore, predominantly in the roots.
3. Inhibition of root elongation growth by Al^{3+} is particularly striking; upon long-term stress, cell division is also affected. Because of similar ionic radii, Al^{3+} particularly affects Mg^{2+} (e.g. replacing Mg in the Mg–ATP complex) and Fe^{3+} metabolisms, and interferes with the regulation of the cytosolic level of free Ca^{2+}. By these effects and by the inhibition of signal transduction via an increase in inositoltrisphosphate, aluminium causes major changes in the regulation of cellular reactions. In addition, Al^{3+} leads to a reorientation and fixation of the cytoskeleton, resulting in anomalous growth in thickness of root tips and the incidence of numerous lesions.
4. (Relative) aluminium resistance may be based on avoidance or tolerance. Exclusion of aluminium ions (stress avoidance) is usually achieved by excretion of chelating acids (e.g. malic acid) which form aluminium complexes outside the root or the protoplast, thus rendering aluminium ions unavailable for uptake systems. Excretion of such chelators appears to be species-specific and is a typical reaction to Al^{3+}, but not to other trivalent cations. A pectin-rich cell wall, carrying numerous negatively charged carboxyl groups, may act as aluminium trap, normally displacing the cross-linking cations Mg^{2+} and Ca^{2+}. Al^{3+} in the rhizosphere may also be immobilised or made unavailable for plants by alkalinisation (via proton uptake or reduced proton release).
5. Aluminium-tolerant species are able to accumulate Al^{3+} in high concentrations. The most important mechanisms for this are export into the apoplast or sequestration into the vacuole. At the sites of deposition the aluminium ions must be complexed (e.g. by Al oxalate or with tannins) or fixed to Al-binding proteins to become inactivated.

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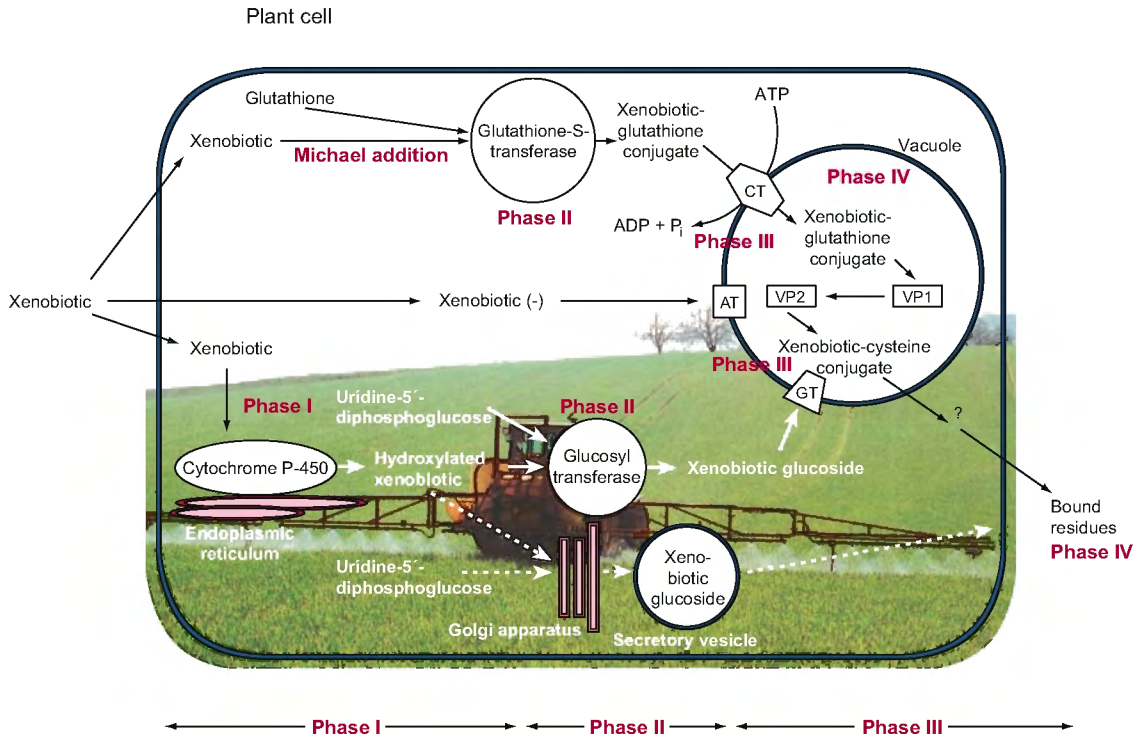
1.9

Xenobiotics



To detoxify xenobiotics organisms use reactions from their normal metabolism. With a clever combination of such reactions, foreign compounds can be detoxified and then deposited in the vacuole or in the cell wall in a form which is not harmful. Such detoxification passes through a sequence of four phases: Biochemical "opening" of the mostly lipophilic xenobiotic, formation of conjugates, sequestration of the conjugate into the vacuole or the secretory vesicle, and finally deposition in the vacuole or the cell wall via chemical modification of the conjugate (see also Fig. 1.9.5). After Coleman et al. (1997)

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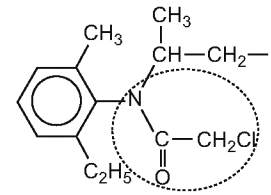
Box 1.9.1 Safeners and herbicides

Safeners are non-lethal xenobiotics which protect herbicide-sensitive (crop) plants from the effects of herbicides. If a plant is pre-treated with a safener, it can be sprayed with the herbicide and the "protected" plant will remain undamaged. In this way the selectivity of a herbicide can be indirectly increased. Safeners elicit a xenobiotic protective response, usually induction of glutathione-S-transferases (see Fig. 1.9.3). With the aid of these enzymes the herbicide is made "harmless". There are different classes of herbicide for which safeners have been developed. Two of these are shown. The corresponding safeners are, e.g., flurazole and dichlorimide.

Herbicide group chloroacetamides:

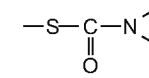
Dichloroacetamide $HCCl_2-C(=O)-NH_2$

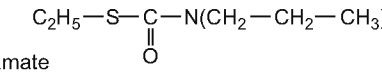
"Metolachlor"



2-Chloro-*N*-acetamide

Herbicide group thiolcarbamates:





e.g. Ethyl-*N*,*N*-di-*n*-propylthiolcarbamate

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Xenobiotics are chemicals (or, more generally, a chemical mix) which are not normal components of the organism which is exposed to it. However, xenobiotics are biologically active and may cause, dependent on toxicity and the amount taken up, defence reactions, damage or even death of an organism. With respect to plants, xenobiotics are, in addition to the previously discussed toxic heavy metals and aluminium, mainly pesticides and waste products, which enter soil, water and air – even in countries where waste management and control is well developed. Except for fertilisers, **herbicides** have the greatest share of the agrochemical market amongst all pesticides (insecticides, acaricides, nematicides, fungicides, rodenticides, herbicides) used for plant protection. Herbicides target plant metabolism and are well researched regarding their reactions in plants and in the soil.

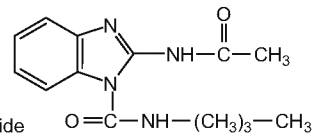
A particular problem of the pesticides is the **selectivity**. Herbicides are supposed to kill off weeds, but crops must be spared. Crops are commonly not able to detoxify herbicides (an exception is maize which detoxifies Atrazine) and therefore a modern trend is towards two-component systems: Herbicide and herbicide safener (antidotes or "immune chemicals", Box 1.9.1), with the latter activating the defence system of the crop so that it can cope with the herbicide. More difficult is the situation if the pesticide targets processes that are similar in the organisms to be protected and the pest (e.g. the use of fungicides in orchards). In many cases, the different sensitivity of the organisms or different time slots of a targeted process (e.g. mitosis in host and parasite) can be used to achieve some selectivity. Scab, mildew and grey mould (*Botrytis*) develop on mature leaves and fruits, so that toxins inhibiting mitosis, e.g. the fungicide "Benomyl" (a benzimidazole derivative) (Box 1.9.2 A), when not applied too early, only affect the parasite and thus render sufficient selectivity. Often the pests and the crop differ naturally in the mode of pesticide uptake or it is possible to formulate ("package") the chemicals in such a way that differential uptake is achieved. Differences in the structure or the chemistry of the plant surface of weeds and crops, as well as in their morphological charac-

Box 1.9.2 Herbicides

Some of the most important herbicides and their main effects (Fig. A).

Benzimidazole

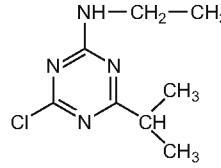
"Benomyl"



inhibits mitosis, fungicide

Triazines:

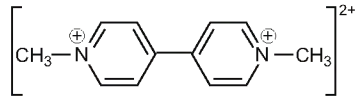
Atrazine



inhibits photosystem II

Methylviologen

"Paraquat"



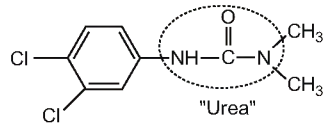
transfers the electrons from ferredoxin (photosystem I) to oxygen:

$e + MV + O_2 \rightarrow MV + O_2^-$

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

"DCMU"

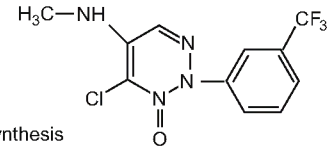
Dichlorophenyl-dimethylurea



inhibits, like atrazine, photosystem II

Pyridazinone

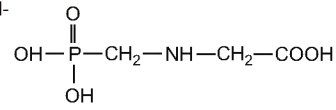
"Norflurazone"



inhibits carotenoid synthesis

N-Phosphonomethyl-glycine

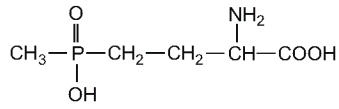
"Glyphosate" ("Round-up")



inhibits the synthesis of aromatic amino acids

Phosphinothricin

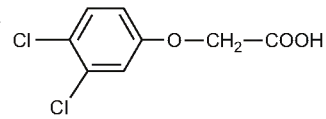
"Basta"



inhibits glutamine synthesis and thus amino acid metabolism

2,4-Dichlorophenoxy-acetic acid

"2,4-D"

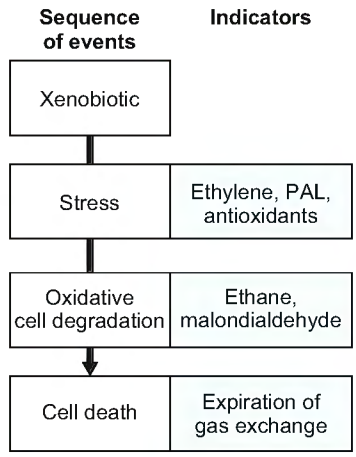


auxin analogue, leads to uncontrolled growth

A

Measurement of herbicide effects

Simple visual and analytical tests are differentiated. As in the assay of frost resistance (Box 1.3.6), a few days are usually required before damage has developed to a stage which allows quantification of yellowing or necroses (Box 1.1.1, Figs. 1–4). Analytical tests are more sensitive; for a first screening for herbicidal effects, algae are most suited, as the herbicide is in close proximity to the cells. In further rounds of screening the individual specific stress reactions can be examined, e.g. induction of PAL as the starting point for the formation of phytoalexins, or the release of ethylene from methionine (Fig. B). A relatively simple method of measuring damage is provided by chlorophyll fluorescence (cf. Box 1.1.1, Fig. 5).



B

Fig. B. Time course of poisoning by a xenobiotic and useful diagnostic parameters. *PAL*, increase in activity of phenylalanine ammonium lyase. (After Kuhnert 1996)

ters (e.g. leaf-form and orientation in the space), are important in this respect, resulting in differences in uptake.

In crop production, **toxicity** of xenobiotics and of their metabolites for humans and animals as well as the development of resistance of the pest to the pesticide are very important. Resistances develop very quickly if the non-resistant population is completely eradicated. Resistance inheritance is usually recessive. The population quickly looses the dominant targeted gene, if only homozygous recessive organisms propagate.

In the following, the effects of non-metallic xenobiotics on plants and in particular the reactions of plants to these compounds are exemplarily presented. Such reactions may be used for phytoremediation to clean and detoxify air, soil and water, an idea leading to the concept of the "green liver" (Sandermann 1994) in the sense that plants, in analogy to the livers of animals, are able to withdraw toxins (e.g. formaldehyde) from our environment and metabolise them to harmless substances.

The spectrum of xenobiotics is inexhaustible, but in contrast there are only few cellular mechanisms for detoxification. Most xenobiotics are lipophilic, and so diffuse easily into cells. They are also able to accumulate in biomembranes, decreasing their effectiveness and concomitantly the possibility of their metabolism.

1.9.1 Herbicides

Herbicides are the most used pesticides worldwide, constituting 49% of the agrochemical market; they accounted for 68% of total US pesticide sales at approximately US$ 6 billion.

Herbicides inhibit germination and growth of weeds but their use is only profitable if losses in yield were to exceed 5–10%. In general, a distinction is made between herbicides applied **before sowing** (pre-emergent herbicides) and those applied **after sowing** (post-emergent herbicides). Pre-emergent herbicides are applied directly to the soil, thus being also known as **soil herbicides**; they are designed to interfere with germination or to kill the seedlings. Post-emergent herbicides are sprayed on the standing crop (sometimes sprayed from aeroplanes). They interfere with photosynthesis or growth, and are therefore also called **leaf herbicides**.

1.9.1.1 Modes of Herbicide Action

By inhibiting specific physiological reactions herbicides should kill (Box 1.9.2A) unwanted plants. In the extreme case, when all plants are unwanted, so-called total herbicides are used, which are mostly **inhibitors of the photosynthetic light reactions**. Many **total herbicides** have a very specific effect, e.g. inhibition of plastoquinone reduction by blocking its binding site on photosystem II, e.g. by s-Triazine (s stands for symmetric Triazine), such as Atrazine or by urea derivatives, e.g. DCMU, or by production of ROS in photosystem I (e.g. by methylviologen = Paraquat).

Inhibitors of carotene biosynthesis, such as Norflurazon which inhibits phytoen-desaturase, also increase oxidative stress and thus stop the formation of shading and protective pigments.

Also **N metabolism** is a possible target. The chemical structure of Glyphosate resembles that of phosphoenol pyruvate and therefore this herbicide is bound very effectively by the enoylpyruvyl-phosphoshikimate synthase instead of the natural substrate, thus abandoning the synthesis of the aromatic amino acid. Similarly, "Basta" (=phosphinotricin) inhibits synthesis of glutamine which is the starting metabolite for amino acid synthesis in plants.

The herbicides listed above affect photosynthesis, carotenoid and amino acid biosynthesis, but other metabolic activities are also targets of herbicide design to achieve better selectivity and efficiency, e.g. mitochondrial respiration, lipid biosynthesis, nucleic acid synthesis, turnover of the cytoskeleton, phytohormone metabolism and steps in seed germination (pre-emergent herbicides). One of the most frequently used herbicides is 2,4-D, whose molecule is similar to that of the growth hormone auxin, wherefore it is able to disrupt its action.

Even if the principal effects of herbicides are known, e.g. the inhibition of a certain metabolic reaction, in many cases the molecular mechanisms still remain unknown, as the protein structures involved are themselves not known in sufficient detail. Of 2,4-D, e.g., the receptor (probably at the plasma membrane) is still not known. Many herbicides, particularly those affecting photosynthesis and respiration, produce oxygen- and other radicals, lipid peroxides and other particularly reactive substances, of which only the most basic chemistry is known. Fig-

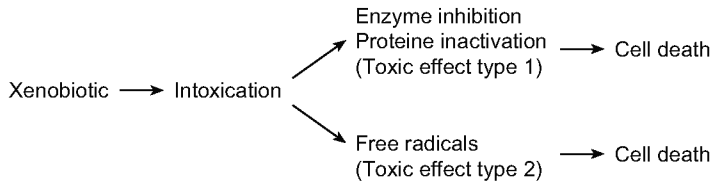


Fig. 1.9.1. Modes of action of total herbicides. In type 1 the toxic effect results from direct interaction of the xenobiotic with a "target" system whose inhibition causes the damage (e.g. inhibition of glutamine synthetase by the phosphinotricin "Basta", see Boxes 1.9.2 and 1.9.3). In type 2 mode of action, targets are also hit, but the damage is caused by the reaction of the plant (e.g. by the ROS response of the herbicide "Paraquat"). Likewise, inhibition of the synthesis of protective compounds (e.g. carotenoids by the herbicide "Norflurazon") leads to a type 2 toxic effect. (After Kuhnert 1996)

ure 1.9.1 shows an overview of the ways in which herbicides act.

A general principle seems to be that toxicity of a herbicide increases with its electrophilicity, as this determines its ability to react with nucleophilic biological macromolecules such as proteins and nucleic acids. Weakly polarised electrophilic agents react with weak nucleophiles of the cell, and strongly polarised ones with strong nucleophiles.

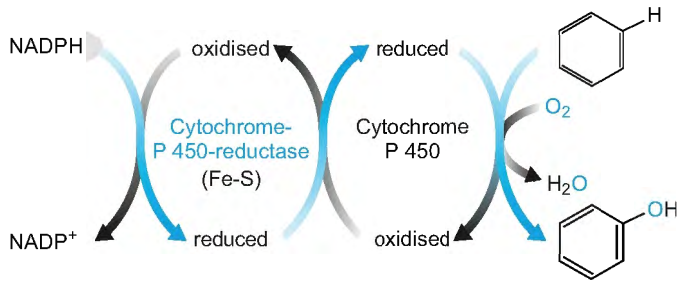
1.9.1.2 The Four Steps of Xenobiotics Detoxification by Plants

Detoxification of xenobiotics usually requires a reaction sequence which can be separated into four distinct steps (phases). Many pesticides and other xenobiotics are lipophilic compounds which are difficult to attack in the aqueous milieu of the plant cell.

1. **Phase 1** or **step I** of the **detoxification process** is therefore the introduction of a hydrophilic group. Oxidation, reduction or hydrolysis is the usual reaction to increase the compound's water solubility and to start detoxification. Aromatic rings are preferentially oxidised by monooxygenases (mixed function oxygenases; Fig. 1.9.2 A), which contain a cytochrome P450 and require molecular oxygen and a second electron donor. By oxidation electrophilic groups are introduced or created in the molecules which leads to an activation, i.e. increases their toxicity. Seemingly, the system of oxidases, monooxygenases and hydrolases exhibits little substrate specificity, but this is not the case: In *Arabidopsis thaliana* more than 60 genes coding for monooxygenases have been identified! The apparent non-specificity can be explained by the great number of relatively substrate-specific enzymes.
2. In **phase II** the now more hydrophilic compounds are conjugated, i.e. bound to other hydrophilic molecules. Conjugation is either by glycosylation of the newly formed functional groups or by formation of a thioether ("Michael-addition"; Fig. 1.9.2 B) with glutathione and its homologues (Fig. 1.9.2 C). Glycosylation is catalysed by glucosyl transferases with uridine diphosphate-glucose as the substrate, whereas thioethers are produced by the so-called glutathione-S-transferases (GSTs). The latter group of enzymes can use many natural and artificial compounds as substrates. Amongst these are many herbicides (Atrazine, 2,4-D, Metolachlor), but also typical plant pigments (anthocyanins), growth substances (auxin) or defence substances (salicylic acid; Marrs 1996).

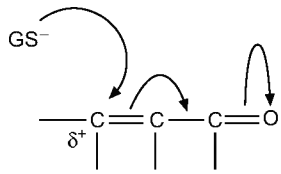
   As with the glycosyl transferases the apparent non-specificity of GSTs results from the concomitant presence of a larger number of very specific enzymes. Thus the selectivity of many herbicides is based on the occurrence of certain, substrate-specific GSTs in crop plants and the lack of such (iso-)enzymes in weeds. Also, the effects of "safeners" (antidotes) may be achieved via the induction of a specific GST. However, specificity of conjugation of xenobiotics depends on the specificity of GSTs for natural substrates, e.g. tannins and flavonoids (pigments) or auxins. Usually, safeners effect the induction of such transferases. Infections may also lead to the induction of GSTs. As well as GSTs other transferases are also able to increase the water solubility of xenobiotics by conjugation (Table 1.9.1).
3. In **phase III** the conjugated product is sequestered, either in the vacuole or in the cell wall,

Introduction of a hydrophilic group into an aromatic ring with the help of a C-450-dependent monooxygenase

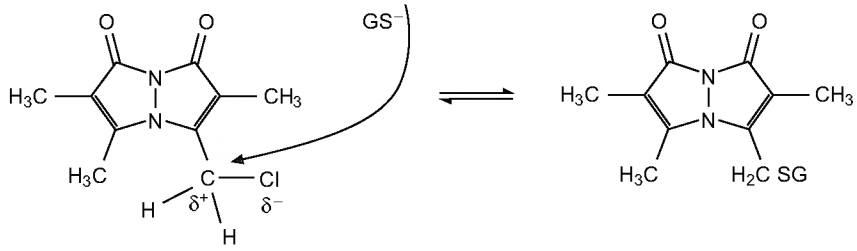


A

(i) "Michael addition" along a polarised double bond in αβ-unsaturated carbonyl groups



(ii) Nucleophilic replacement of relatively easily cleaved substitutes on saturated carbons

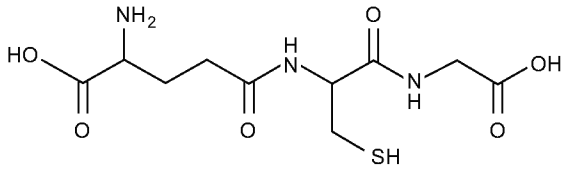


Monochlorobimane (non-fluorescing)

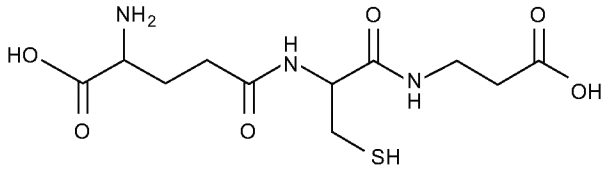
Bimane-glutathione (fluorescing)

B

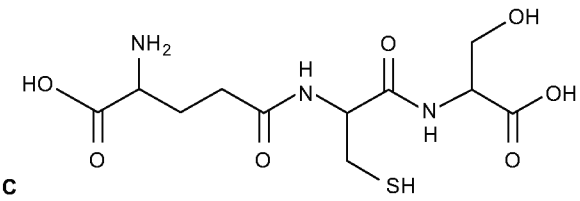
Structures of γ-glutamyl-cysteinyl tripeptides



Glutathione (γ-glutamyl-cysteinyl -glycine)



Homoglutathione (γ-glutamyl-cysteinyl-β-alanine)



Hydroxymethylglutathione (γ-glutamyl-cysteinyl -serine)

C

Fig. 1.9.2. Biochemistry of detoxification of xenobiotics (A from Lehninger et al. 1994; B, C from Coleman et al. 1997)

Table 1.9.1. Classes of plant enzymes that metabolise xenobiotics and their natural substrates. After Sandermann (1994)

Enzyme class	Xenobiotic substrate	Natural substrate
Cytochrome P450	4-Chloro-*N*-methylalanine	Cinnamic acid, pterocarpanes
Glutathione-S-transferase	Fluorodifen, alachlor, atrazine	Cinnamic acid
Carboxylesterases	Diethylhexylphthalate	Lipids, acetylcholine
O-Glucosyltransferases	Chlorinated phenols	Flavonoids, coniferylalcohol
O-Malonyltransferases	*β*-D-Glucosides from pentachlorophenol and from 4-hydroxy-2,5-dichlorophenoxyacetic acid	*β*-D-Glucosides from flavonoids and isoflavonoids
N-Glucosyltransferases	Chlorinated anilines, metribuzin	Nicotinic acid
N-Malonyltransferases	Chlorinated anilines	1-Aminocyclopropylcarboxylic acid, D-amino acids, anthranilic acid

again by enzymes of the natural secondary metabolism. Key enzymes are the so-called glutathione pumps. Sequestering is required because the GS conjugates can inhibit glutathione-S-transferases as well as glutathione reductases; and, in some cases, they themselves are potent toxins and must, therefore, be removed.

Glutathione Pumps (GS-X Pumps)

GS-X pumps transport the GS conjugates into the vacuole and are, thus, transport proteins of the tonoplast (Rea et al. 1998). They belong to the so-called ABC superfamily, a group of transporters which play an important role in animal, fungal and plant tissues, particularly in connection with secondary metabolism. These proteins have a "Walker" Box A and a "Walker" Box B, which are separated by a characteristic C-motif, hence the term ABC transporter. So far, more than 130 representatives of the superfamily are known, which translocate sugars, peptides, alkaloids, phenolic compounds and inorganic and organic ions.

This superfamily has two large subfamilies, multidrug resistance proteins (MDRs) and multidrug resistance associated proteins (MPRs), the latter being typical of plants. Their assembly is relatively uniform, even if the sequence homologies are, in part, below 40%. It is a large protein with several modular domains, namely one to two membrane-spanning domains (TMDs), two domains forming a nucleotide binding fold (NBF) and the regulatory unit R (Fig. 1.9.3).

These transporters are directly activated by MgATP, resulting in an "active" transport that is not dependent on a H^+ gradient (H^+-ATPase independent). Even though the mechanism is not fully explained, it is assumed that an intermediary acyl phosphate (perhaps with glutathione) is formed, indicated by the very strong inhibition by vanadate (formation of an acyl vanadate instead of the acyl phosphate). Remarkably, these transporters translocate glutathione conjugates, as well as GSSG (oxidised glutathione), but not reduced glutathione (GSH). So far, three such transporters which differ in substrate specificities have been described from *Arabidopsis thaliana* (AtMRP).

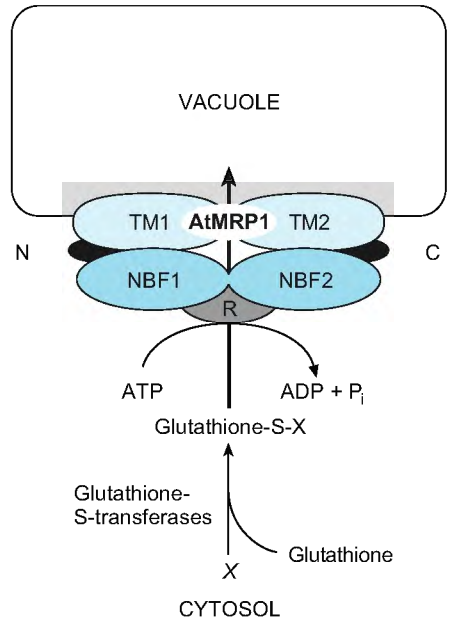


Fig. 1.9.3. Composition and mechanism of a plant ABC transporter from the multidrug resistance associated protein (MRP) family. After the compound X is conjugated with glutathione by a glutathione-S-transferase, it can be transported by the MRP into the vacuole. *N*, N-terminus; *C*, C-terminus; *TM* (*1,2*) trans-membrane domains; *NBF* (*1,2*) nucleotide binding pockets; *R* putative regulatory domain. (After Rea et al. 1997)

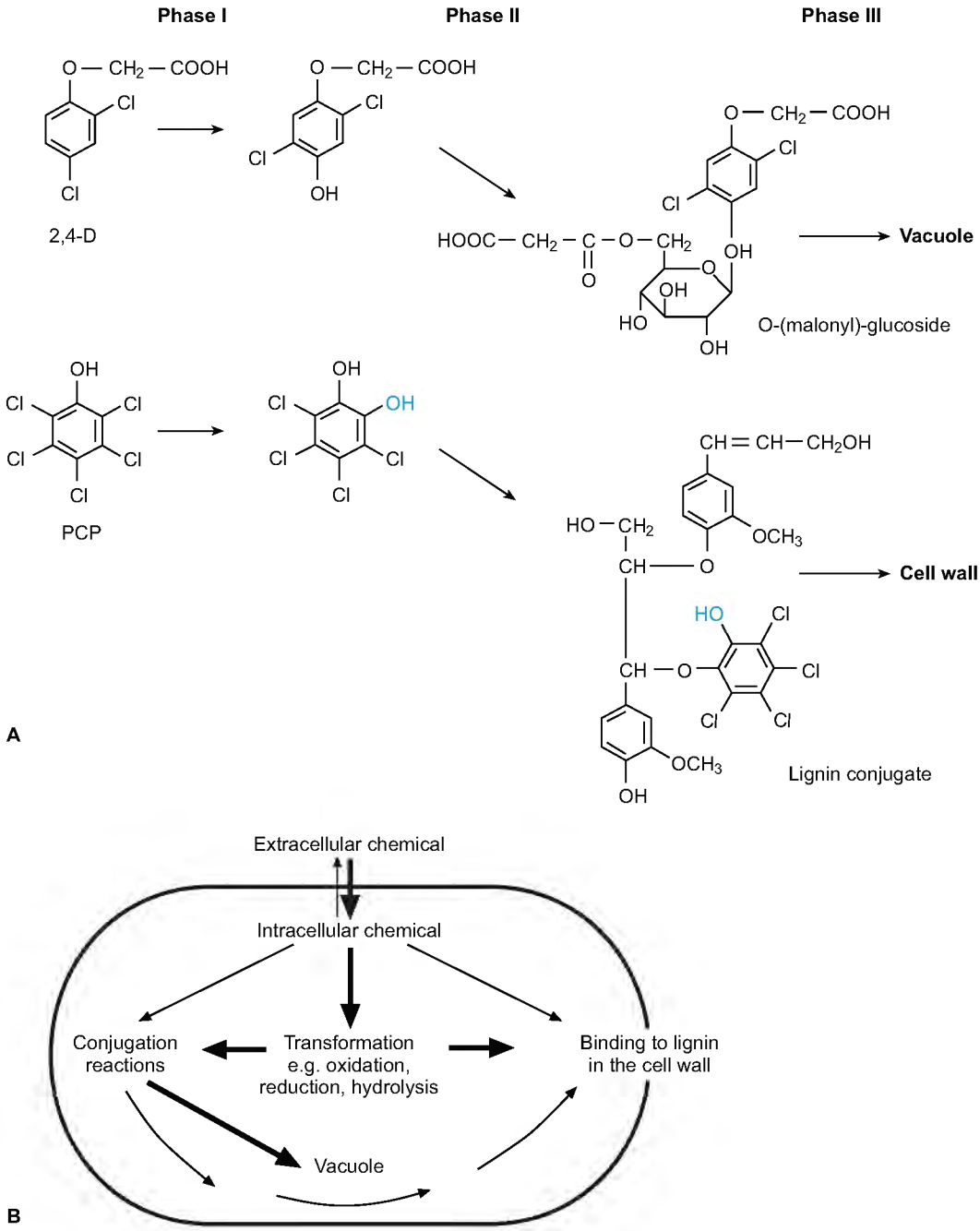


Fig. 1.9.4. Sequestration of xenobiotics, after conjugation, in the cell wall or vacuole. A Hydroxylation and conjugation of 2,4-dichlorophenoxyacetic acid and pentachlorophenol. B Map of reactions which result in the sequestration of xenobiotics after uptake into the cell. (Sandermann 1994)

Natural Substrates of GS-X Pumps

Of course, organisms did not develop GST-GS-X pumps for xenobiotics. Their natural substrates are nucleophilic substances of secondary metabolism: anthocyanins (flavonoids), auxin, salicylic acid, cinnamic acid, hydroxy fatty acids and degradation products of DNA. The Michaelis constant of AtMRP 1 and 2 for cyanidin-3-glucoside-GS conjugate (an anthocyanin) is ca. 50 µM and V_{max} is $\approx$ 5 nmol/mg protein per minute, for example. Similar kinetic data were reported for glucosyl conjugates, e.g. of degradation products of chlorophyll.

4. In **phase IV** the conjugates are immobilised in the vacuole. Conjugates with glutathione are often further metabolised in the vacuole in order to avoid their diffusion back into the cytoplasm. Thus, for example, a carboxypeptidase (exopeptidase) may cleave the glycine residue from glutathione. A vacuolar dipeptidase is able to remove the amino terminal glutamic acid, with the consequent formation of cysteine conjugates. The final fate of these cysteine conjugates is unclear. One possibility would be that they leave the vacuole again via the Golgi system and become irreversibly incorporated into the cell wall as "bound residues" (Fig. 1.9.4).

1.9.1.3 Immunochemicals

Some xenobiotics, e.g. 2,6-dichloroisonicotinic acid and its methyl ester, not only induce the three to four step detoxification system, but also PR proteins (pathogenesis related proteins), e.g. chitinases and β-glucanases (see Chap. 1.10.2.1), serving to combat pathogens (although their mode of action is frequently not known). Such xenobiotics are called **immunochemicals**. They are often formed to such a low extent that detection in cell extracts is hardly possible. When screening for a potential immunogenic effect of a xenobiotic a trick is generally used: Instead of the protein, the activation of the gene encoding for it is examined: Since several of these genes are known, activity of their promoter as induced by a potential immunochemical can be investigated. Fusion of the promoter with a reporter gene – e.g. the GUS-(glucuronidase-)gene of *E. coli* – provides a DNA probe. This probe can then be introduced, via the Agrobacterium system or the particle gun, into the genome of the test plant. After spraying a plant with the potential inducer, induction of the PR promoter by the herbicide can be seen as a stained product from the action of the glucuronidase.

The development of crop plants which respond to immunochemicals is considered as better than development of more herbicides (Box 1.9.3), because immunochemicals do not kill the weed, but rather increase the resistance of the crop. Thus, protective treatment with fungicides and insecticides could be strongly reduced. Treatment with a chemical would, however, still be required, but would induce the natural defence of the plant in the battle against detrimental organisms.

1.9.2 Gaseous Air Pollutants

The most important gaseous pollutants damaging plants are sulfur dioxide, ozone, nitrogen oxides (NO_x), peroxyacetyl nitrate (PAN), ethylene, and various other substances which are adsorbed on dust particles (Table 1.9.2).

Particularly noxious are the reductive smog (London type) which contains mostly SO_2, and the oxidative, photochemical smog (Los Angeles type), which is characterised by PAN. The large number of air pollutants cannot be covered here, but it is clear that sulfur dioxide results mainly from burning of S-containing fossil fuels and from smelting of metal sulfide ores. The main component of photochemical, oxidative smogs, PAN, is produced in a complicated chain reaction from nitrogen oxides and unsaturated hydrocarbons during incomplete burning of motor fuels.

Increasing use of oil and gas as domestic fuels and desulfurisation of industrial emissions have reduced the SO_2 concentration in industrial and heavily polluted areas to less than 10% of that in the immediate post-war years (Fig. 1.9.5). However, concentrations of nitric oxides rose or stagnated in many countries, despite the use of catalytic converters in cars. In addition to industrial and car exhaust gases many other gaseous pollutants accumulate in houses, e.g. formaldehyde (see below).

Of course, such gases are not only problematic to humans, but when present at higher concentrations may also damage plants. Forest decline in recent years (Fig. 1.9.6; see also Chap.

Table 1.9.2. Airborne substances, toxic to plants. From Elstner (1984)

Oxidative smog	NO_2, PAN, O_3 (Los Angeles type)
Reductive smog	SO_2 (London type)
Acids	HF, H_2SO_3, H_2SO_4, HCl
Dusts	Most importantly cement dust and blown soil particles
Phytoeffectors	Ethylene, airborne herbicides and plant protective agents
Allelopathic effectors	(e.g. terpenoids)