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Part I
Review

Half a Century of Pursuing the Pervasive Proton

John A. Raven

Contents

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Abstract Acid–base regulation is probably a universal attribute of life, and energy coupling via transmembrane H^+ gradients is very widespread. Much of my academic career has been related to these two processes and to their interactions. Highlights from my studies of acid–base regulation are the quantitative resolution of the challenges for acid–base regulation in land plant shoots when metabolism involving net H^+ production (e.g. primary assimilation of NH_4^+, NH_3 or N_2) occurs there, quantitation of the energy costs of acid–base regulation for different locations and mechanisms of acid–base regulation for the assimilation of a range on N sources and the interaction of CO_2 concentrating mechanisms in aquatic photosynthetic organisms with acid–base regulation. Research on the significance of transmembrane H^+ gradients has included a significant contribution to the early development of chemiosmotic hypothesis of polar transport of indoleacetic acid, the evolutionary significance of chemiosmotic coupling and the role of H^+ leakage relative to other processes which consumed energy at an essentially constant rate regardless of the rate of light energy supply in determining the minimum photon flux density at which photolithotrophic growth can occur. On a global scale, work on the effects of anthropogenic CO_2 production on ocean acid–base balance has helped to set limits on the significance of this 'ocean acidification' for marine algae. A final point covered in the chapter is an analysis of the continuing attempts to determine precisely what is being regulated, e.g. the pH of the intracellular compartment or the ionisation state of one or more of weak electrolytes in the compartment.

1 Introduction

The role of pH in ecology and physiology has been understood for well over half a century when I began my PhD in 1963, and by this time both animal and plant physiologists recognised that acid–base regulation at the cell and organisms level was an important aspect of homeostasis. The plant work was led by crop physiologists who recognised the role of organic acid synthesis in acid–base balance (often termed charge balance) in plants growing with NO_3^- as N source. A role for protons in bioenergetics had been hinted at by R. N. Robertson, but it was the insight of Peter Mitchell of chemiosmotic coupling of oxidation–reduction energy and hydration–dehydration (ATP–ADP) energy (Mitchell 1961, 1966, 1968) that put protons centre stage in bioenergetics, not only with endergonic and exergonic fluxes of H^+ across proteolipid bilayer membranes involved in coupling of oxidation–reduction and hydration–dehydration energy but also, as we now know, endergonic solute fluxes coupled to exergonic H^+ fluxes and exergonic H^+ fluxes coupled to cell motility in archaeal and bacterial flagella. These ideas found an especially receptive home among those interested in plants since the publication of the Hill and Bendall (1960) 'Z scheme' for two photochemical reactions in series in photosynthetic electron transport from H_2O to CO_2, and a chloroplast research community less steeped in hypothesised chemical rather than H^+ electrochemical

potential differences across membranes, as 'high energy' intermediates, and with experimental material (thylakoid membrane preparations) in which it could be shown that H^+ energy gradients across membranes were a, if not the only, component of the interchange of oxidation–reduction and hydration–dehydration energy.

The 1960s were, then, an exciting time to work on plant energetics and solute transport, and my earliest pH related work was on the use of exogenous HCO_3^- as well as CO_2 in photosynthetic inorganic C assimilation in cells of the giant alga *Hydrodictyon africanum* (Raven 1968), although my PhD under the supervision of the incomparable Professor Enid MacRobbie was mainly about transport of Cl^-, K^+ and Na^+. A referee for that paper asked how direct effects of pH could be distinguished from effects on changed fractions of the various forms of inorganic C as external pH changes: this is a question which still bothers us today (Raven et al. 2005a; Raven 2011). The HCO_3^- work led to the review (Raven 1970) which attempted to relate the physiology of C_3 and C_4 land plants and of aquatic algae and embryophytes to the kinetics of Rubisco and to what was known of the transport of CO_2, HCO_3^- and H^+/OH^-. This attempt was premature, not least because the isolation methods for the Rubisco (not known as a Form IB Rubisco) from C_3 terrestrial plants did not yield enzyme preparations which showed the relatively high CO_2 affinity which is accepted today (Tcherkez et al. 2006). This certainly did not help my arguments that organisms like *Chlorella*, when acclimated to low CO_2, might need to accumulate CO_2, since if the then known in vitro kinetics of C_3 land plants were taken as representative of the in vivo state than a means of concentrating CO_2 would also be needed in those plants too in order to explain the dependence on external CO_2 in vivo. At least there was recognition of the possible widespread occurrence of what are now termed CO_2 concentrating mechanisms (CCMs) among photosynthetic organisms, but also the relation to acid–base regulation (not just 'charge balance') during HCO_3^- with subsequent assimilation of CO_2 (Raven 1968, 1970). This work set the pattern of a rather large number of review, synthesis and conceptual and quantitative modelling papers relative to primary data papers that has characterised my published output.

After discussing some aspects of my published work that relate to the roles of protons in plant biology, I address the question of what is being controlled in acid–base regulation in algae and plants without, alas, coming to a clear conclusion.

2 Acid–Base Regulation as a Function of Nitrogen Source

The work on inorganic C assimilation in *Hydrodictyon africanum* (Raven 1968) followed work (also in Professor MacRobbie's laboratory) by Andrew Smith (Smith 1967) on *Nitella translucens* in which he focussed on the products of assimilation of ^{14}C inorganic C and their distribution between cytoplasm and vacuole. However, it was not until Andrew had moved to Adelaide and I had moved to Dundee that we collaborated on acid–base regulation and related matters. Raven and Smith (1973, 1974, 1976b) set the scene with respect to the inadequacy

of 'passive' buffering, i.e. uptake of excess H^+ or OH^- by pre-existing weak electrolytes in intracellular compartments with pK_a values close to those of the set point pH of the compartment, for anything but short-term response to perturbation on the acid–base balance of a compartment. Net metabolic generation of OH^- (assimilation of exogenous HCO_3^-, NO_3^- and SO_4^{2-} in primary metabolism) or H^+ (net assimilation of NH_4, NH_3 and N_2 in primary metabolism) at the rates seen during growth, and observed rates of passive entry of H^+ in some organisms, demands the involvement of enzymes and/or integral membrane transporters in, respectively, the biochemical and the biophysical pH stats. Raven and Smith (1973, 1974, 1976b) point out the spatial restrictions on the occurrence of these pH stats: the biophysical pH stats need a large external phase to which excess intracellular H^+ or OH^- can be transported, which restricts this mechanism to aquatic organisms and the below-ground parts of terrestrial organisms. For the biochemical pH stat, the net synthesis of organic acids from a neutral ultimate precursor (atmospheric CO_2) can generate H^+ as means of neutralising excess OH^- in cells in any environment. This is especially important in land plant shoots, with accumulation of the resulting salt of the organic anion with the cations accompanying the NO_3^- and SO_4^{2-} up the xylem to the shoot in the shoot cell vacuoles or their transport to the roots in phloem for 'further treatment'. Metabolic production of OH^- is only quantitatively important as a means of neutralising excess H^+ when there is the salt of an organic anion which can be metabolised to CO_2 and OH^-, neutralising the H^+ and 'replacing' it with the cation which accompanied the organic anion up the xylem to the shoot, having been taken up by the root in exchange (in terms of charge, if not mechanism) for the H^+ produced in net organic acid synthesis in the shoots. Excess H^+ generated in the shoot cannot be transported to the roots in the phloem. The cases for the absence of significant H^+ transport in the phloem, and for the absence of OH^- generation in plants other than in assimilation of HCO_3^-, NO_3^- or SO_4^{2-}, or the catabolism of organic anions, have been further developed in, respectively, Raven (1977) and Raven (1986, 1988). Raven et al. (1990a) considered the evidence on acid–base regulation in symbiotically N_2-fixing vascular plants, while Raven and Farquhar (1990) have considered organic acid production in acid–base regulation in the context of the impact on the C stable isotope natural abundance of the plants. Raven and Farquhar (1989) present data from a 'null point' method of estimating leaf apoplasm pH. The energy and water costs of the various acid–base regulation processes related to N assimilation have been quantitatively modelled by Raven (1985) and most recently updated in Andrews et al. (2009).

These various predictions rationalising the distribution of N assimilation processes in algae and vascular plants in relation to acid–base regulation related to the assimilation of various N sources were, for NO_3^-, relatively well-quantified by experiment by the 1970s. However, some experimentation remained to be done for NO_3^-, and more for N_2 and, especially, NH_4^+ and NH_3. Some of these tests of predictions have been carried out, with general support for the predictions, by a series of excellent post-doctoral fellows. Acid–base regulation in *Hydrodictyon africanum* was investigated by Ida De Michelis and Hemal Jayasuriya (De Michelis et al. 1979; Raven and De Michelis 1979, 1980). Other work investigated the roles

of long-distance transport in acid–base regulation in NO_3^- and NH_4^+ assimilation in *Ricinus communis*, and in NO_3^-, NH_4^+ and N_2 (in root nodules) assimilation in *Phaseolus vulgaris*, by Susan Allen (Allen and Raven 1984, 1987; Raven et al. 1984; Allen and Allen 1987; Allen and Smith 1987; Allen et al. 1988). The problems posed by N_2-fixing symbionts in nodules on stems in air, rather than on roots in soil, in *Sesbania rostrata* was examined by Richard Parsons (Parsons et al. 1993, 1995). Acid–base regulation related to assimilation of gaseous NH_3 through shoots of two C_3 and one C_4 grass was investigated by Bernd Wollenweber and by Zu-Hua Yin (Wollenweber and Raven 1993a, b; Yin et al. 1996; Yin and Raven 1997, 1998), and the graminoid (Juncaceae) *Luzula sylvatica* was investigated by PhD student Paul Hill (Hill et al. 2001, 2002). Subsequent work on gaseous NH_3 has been less focussed on acid–base regulation and has a more ecological and environmental bias with PhD students Jennifer Carfrae and Matt Jones (Carfrae et al. 2004, 2007; Sheppard et al. 2004; Jones et al. 2007a, b, 2008).

The work described earlier has been followed up, with criticism and extension, by a number of workers (Sanders and Slayman 1982; Sakarno 1998; Kronzucker et al. 2001; Britto and Kronzucker 2002, 2005). Sanders and Slayman (1982) showed that oxidative metabolism was more important than the plasmalemma H^+ efflux ATPase in removing H^+ from inside cells, but in the long term there must be export of excess H^+ from the cells, though not necessarily via the H^+ ATPase (Raven 1986). Britto and Kronzucker (2005) make important points about the activity and regulatory properties of phosphoenolpyruvate carboxylase which do not readily fit the requirements of a biochemical pH stat, generating H^+ and neutralising OH^- generated in NO_3^- assimilation, and the clear anaplerotic involvement of this enzyme in nitrogen assimilation in producing the suite of carbon skeletons needed for amino acid and pyrimidine synthesis. However, it is clear that there is a role for additional organic acid synthesis from (ultimately) neutral substrates specific to NO_3^- as opposed to NH_4^+ assimilation in vascular embryophytes, especially when assimilation of NO_3^- occurs in shoots (Raven and Smith 1976b; Raven and Farquhar 1990).

An important point for the work on nitrogen assimilation but also relevant to the remaining sections is that work from the author's laboratory does not explicitly use the Strong Ion Difference procedures of Stewart (1978, 1981) but comes to the same conclusions.

3 Acid–Base Regulation in Algae and Aquatic Plants with an Emphasis on Carbon Source

The other main line of research on acid–base regulation concerns inorganic carbon acquisition. In the decade after Raven (1970) there were great advances in our understanding of the kinetics of Rubisco, including the finding that the enzyme has an oxygenase as well as a carboxylase activity, and culminating in a mechanistic

model of gas exchange in C_3 land plants (Farquhar et al. 1980), and the discovery of CO_2 concentrating mechanisms in a cyanobacterium (Kaplan et al. 1980) and a green microalga (Badger et al. 1980). Work with post-doctoral fellow Sheila Glidewell (Raven and Glidewell 1978) suggested that the C_4-like physiology but C_3 biochemistry of the freshwater colonial giant-celled alga *Hydrodictyon africanum* was due to intracellular accumulation of inorganic carbon in the cells based on active influx of HCO_3^-, but we did not attempt to measure the intracellular inorganic carbon concentration. Subsequent work involving the author has dealt with freshwater macrophytes and with marine macrophytes collected from the field and cultured for various periods in the laboratory and cultures of microalgae.

The work on freshwater macrophytes with post-doctoral fellows John Beardall, Howard Griffiths and Jeffrey MacFarlane showed that the red algae examined relied on diffusive entry of CO_2 (Raven and Beardall 1981a; Raven et al. 1982, 1994, 2000b, 2005a); MacFarlane and Raven 1985, 1989, 1990. For the red alga *Lemanea mammilosa* a detailed analysis of boundary layer effects under natural flow conditions of intracellular inorganic C transport and Rubisco kinetics was undertaken, with a satisfactory fit of the data to a mechanistic model (MacFarlane and Raven 1985, 1989; Raven et al. 2005a). The freshwater green alga *Cladophora glomerata* and the flowering plant *Ranunculus penicillatus* ssp. *pseudofluitans* (Raven et al. 1982, 1994) have CCMs and can use HCO_3^- as an inorganic carbon source and so share in the complications for acid–base regulation attendant on expressing a CCM (Raven 1999). PhD student Jonathan Newman investigated the mechanism of HCO_3^- use in *Ranunculus penicillatus* ssp. *pseudofluitans*: this seems to involve the conversion of HCO_3^- to CO_2 in invaginations in the radial walls of leaf epidermal cells (Prins and Elzenga 1989; Rascio et al. 1999). These transfer cell-like invaginations have carbonic anhydrase activity in the apoplasm, and presumably are acidified by a plasmalemma H^+ efflux pump (Raven 1976), with uptake of CO_2 (Newman and Raven 1993, 1999). This mechanism would be a variant on that found in ecorticate freshwater and brackish water characean algae and submerged flowering plants of the elodeid life form (Arens 1939; Walker et al. 1980; Price and Badger 1985; Price et al. 1985; Maberly and Madsen 2002; Ray et al. 2003).

The freshwater flowering plant *Crassula helmsii* exhibits Crassulacean Acid Metabolism (CAM) (Newman and Raven 1995), with its regulated variation of pH in the cell vacuole, a 'P' compartment in the sense of Mitchell (1966, 1968), i.e. a compartment into which H^+ is pumped and containing no functional nucleic acids and low diversity and concentration of proteins, which can tolerate larger excursions of pH than can the 'N' phases (Mitchell 1966, 1968) of cytosol, chloroplast stroma and mitochondrial matrix. More recent work on inorganic carbon assimilation by *Crassula helmsii* is that of Klavsen and Maberly (2009, 2010). Other work on submerged freshwater vascular plants involving post-doctoral fellows Howard Griffiths and Katherine Richardson confirmed that both *Isoetes lacustris* (a lycophyte at the pteridophyte grade of organisation) and *Lobelia dortmanna* (a flowering plant) took up much, or most, of their CO_2 through their roots and that *I. lacustris* uses CAM (Richardson et al. 1984). It was shown in work

with Professor Jon Keeley and Barry Osmond that the amphibious *Stylites* (= *Isoetes*) *andicola* took up most of its CO_2 through the root system and used CAM when submerged and when emerged, and did not produce functional stomata when growing on land (Keeley et al. 1984), while submerged seedlings of the flowering plant *Eriocaulon decangulare* took up much of their CO_2 through the roots and did not exhibit CAM (Raven et al. 1988). For the freshwater aquatic flowering plants this work is put into a broader context by Maberly and Madsen (2002).

For marine macrophytes the occurrence of diffusive CO_2 entry and of CCMs has been investigated in green, red and brown seaweeds and in an intertidal cyanolichen. The work of PhD student Andrew Johnston on the intertidal brown fucoid alga *Ascophyllum nodosum* showed that it had physiological characteristics of an alga with a CCM, apparently based on active transport at the plasmalemma of an inorganic C species (the alga can use HCO_3^-) although there is evidence of significant short-term incorporation of inorganic ^{14}C into dicarboxylic organic acids under some situations; the alga showed very substantial photosynthetic uptake of CO_2 from the atmosphere at natural CO_2 concentrations when emersed but still hydrated, and also exhibited very low-amplitude CAM, in part involving PhD student Misni Surif (Johnston et al. 1986a, b, c, 1987, Surif and Raven 1989a, b, 1990; Johnston 1991).

The occurrence of CAM was investigated in a range of brown algae and other submerged and intertidal macrophytes, and the low amplitude CAM was only found in a limited range of the Fucales (Raven et al. 1985, 1988, 1990a, b, 1995a, b, 1996; Johnston and Raven 1986c; Surif and Raven 1989b; Raven and Johnston 1991). However, in his excellent and comprehensive review of aquatic CAM, Keeley (1998) points out that the fate of the compounds labelled in the dark from inorganic ^{14}C in *Ascophyllum nodosum* is not consistent with typical CAM, so the role, if any, of the diel changes in titratable acidity and in malate in the fucoid brown algae in the carbon balance of the organisms is not clear. Metabolomic and genomic studies give no evidence of CAM-like metabolism (or C_4-like metabolism) in the (non-fucoid) brown alga *Ectocarpus siliculosus* (Cock et al. 2010; Gravot et al. 2010).

CCMs were found on the basis of physiological evidence, and correlated data on the natural abundance of stable isotopes, in all marine brown algae, in almost all of the green algae, and most of the red algae examined, as well as in the only marine lichen examined, *Lichina pygmaea* (Johnston and Raven 1986a, b, 1987; Raven and Samuelsson 1988; Surif and Raven 1989a, 1990; Raven et al. 1989, 1990a, b, 1994, 1995a, b, 2002, 2005a; Johnston et al. 1992; Maberly et al. 1992, 2009; Raven and Osmond 1992; Kübler and Raven 1994, 1995, 1996; Kübler et al. 1999; Sherlock and Raven 2001; Kevekordes et al. 2006; Hepburn et al. 2011; Marconi et al. 2011). The inspiration for the Johnston et al. (1992) and Maberly et al. (1992, 2009) work came from the 'pH drift' studies of Professor Stephen Maberly (1990). While a few of the organisms studied had been previously investigated, in most cases the data were first reported on inorganic carbon assimilation for the algae. The assignment of algae to 'CO_2 diffusion' or 'CCM' categories on the basis of pH drift and C stable isotope natural abundance measurements is generally robust, although there are some caveats (Raven et al. 2005a; Kevekordes et al. 2006; Midelboe and Hansen 2007a, b; Hepburn et al. 2011; Marconi et al. 2011; Moulin et al. 2011). The occurrence of CO_2 diffusion

macroalgae generally correlates with lower irradiances as predicted by Johnston et al. (1992), Maberly et al. (1992) and Raven et al. (2000a, 2002a, b), although this correlation is by no means perfect (Johnston et al. 1992; Maberly et al. 1992; Raven et al. 2000a, b; Kevekordes et al. 2006. Hepburn et al. 2011; Marconi et al. 2011; Moulin et al. 2011).

The work on cultured microalgae has a number of proton-related components. Post-doctoral fellows Richard Geider and Bruce Osborne showed that cultures of the diatom *Phaeodactylum tricornutum* at low (Geider et al. 1985) or very low (Geider et al. 1986) irradiances showed high yields of growth on the basis of absorbed photons. This is of interest in view of the 'photons and protons' predictions of Raven and Beardall (1981b, 1982) on the effects of H^+ permeability of thylakoid and other membranes becoming a multiplicative factor with other energy costs which are essentially independent of irradiance. These other energy costs are back-reactions in photosystem II, slippage in the ATP synthetase, leakage of CO_2 from the CCM if this machinery is present and expressed at low irradiances, and protein turnover and, with H^+ leakage, should restrict growth at low irradiances (Raven et al. 2000a, b; Quigg et al. 2006). The discussion earlier suggests that CCMs might be less common in algae adapted to low irradiances, and expression of CCMs might be decreased. Work by post-doctoral fellow Janet Kübler showed that CCM expression is decreased, at least when judged by the decreased affinity of cells for inorganic C, when they are cultured at low irradiances (Kübler and Raven 1994, 1995, 1996; see also Young and Beardall 2005). It would be helpful to follow up these studies with measurements of the intracellular:extracellular ratio of CO_2 as a measure of CCM function, as originally performed by Badger et al. (1980) and Kaplan et al. (1980) and some of their early followers (e.g. Beardall 1981; Beardall and Raven 1981; Beardall et al. 1982) and recently by Spijkerman (2011).

A clearly proton-related aspect of inorganic carbon acquisition by microalgae relates to a carbonic anhydrase (Cah3) expressed in the thylakoid lumen of *Chlamydomonas reinhardtii* and especially, in cells grown in low CO_2, in the part of the thylakoids which penetrate the pyrenoid (Karlsson et al. 1998; Moroney and Ynalvez 2007). Following the results and suggestions of Pronina and colleagues (Pronina and Semenenko 1988, 1990; Pronina and Borodin 1993), Raven (1997a, b) proposed a quantitative model of how HCO_3^- could move from the medium to the thylakoid lumen, where, catalysed by Cah3 and using the high H^+ concentration generated by the light-driven H^+ pumps located in the thylakoid membrane, a CO_2 concentration well in excess of that in the medium could be achieved, i.e. a CCM. The CO_2 could then diffuse through the thylakoid membrane to Rubisco in the pyrenoid. The proposed mechanism is essentially an internalisation of that proposed by Walker et al. (1980) with the addition of carbonic anhydrase (CA), as is now the case for variants of the Walker et al. (1980) model for characean cells and elodeid leaves (Price and Badger 1985; Price et al. 1985; Maberly and Madsen 2002; Ray et al. 2003). This mechanism is now part of the best-accepted model of the CCM of *Chlamydomonas reinhardtii* (Moroney and Ynalvez 2007; Markelova et al. 2009), although not all of the components have yet been identified. Furthermore, Cah3 has at least one more H^+-related role in photosynthesis, i.e. the enhancement of the rate of O_2 evolution by H^+ removal (Shutova et al. 2008). Finally, it must be

remembered that this kind of CCM is, as far as is known, only found in the well-investigated *Chlamydomonas reinhardtii* (Giordano et al. 2005; Roberts et al. 2007a, b. Raven 2010, 2011).

A final aspect of microalgal CCMs in relation to acid–base regulation is the interaction of carbon and nitrogen acquisition. Beardall et al. (1982) found that growth of *Chlorella emersonii* under nitrogen (supplied as NO_3^-) limitation with a high CO_2 concentration led to the expression of a CCM, just as did decreasing the CO_2 concentration at high (or low) nitrogen availability. This effect of nitrogen supply was related by Beardall et al. (1982) to the high nitrogen use efficiency (biomass increase rate per unit nitrogen in the cells) predicted for algae expressing a CCM, although this argument only strictly applies to growth in air levels of CO_2 (at lower CO_2). Regardless of the evolutionary explanation of this phenomenon, it clearly influences acid–base regulation to the extent that there is HCO_3^- influx as part of the CCM, adding OH^- efflux related to HCO_3^- entry followed by CO_2 assimilation to the OH^- efflux related to NO_3^- (and SO_4^{2-}) assimilation. A different situation occurs in a strain of *Chlamydomonas reinhardti* lacking the capacity to grow on NO_3^- and so grown with NH_4^+ (Giordano et al. 2003; see Raven 2001), where CCM expression (again judged from inorganic carbon affinity) is mainly regulated by nitrogen supply, with the lowest expression of the CCM under nitrogen limitation, paralleling decreased expression of a mitochondrial carbonic anhydrase. Here the decreased H^+ generation rate in the NH_4^+-limited cells is paralleled by a decreased rate of OH^- generation from HCO_3^- entry and CO_2 assimilation with decreased CCM expression. Also not related to a change in inorganic carbon supply, Giordano et al. (2007) examined metabolic responses of *Dunaliella parva* to a gradual change from NO_3^- to NH_4^+ as nitrogen source with a change from a net intracellular OH^- production to a net intracellular H^+ production from nitrogen assimilation against a (presumed) constant intracellular net OH^- production related to HCO_3^- entry in the CCM (Raven 2009, 2011).

4 An Acid–Base Dimension to Environmental Change

Increasing anthropogenic CO_2 production from fossil fuel burning and land use change has increased CO_2 input to the atmosphere over the last 250 years, and at least a quarter of this CO_2 has dissolved in the surface ocean. Through interaction with the existing inorganic carbon system in the ocean has produced a pH decrease of about 0.1 unit since 1750, with another 0.4 unit decrease predicted by 2100 (Raven et al. 2005b; Doney et al. 2009). My attention was focussed on this ‘ocean acidification’ in 2004 when I was asked to chair the Royal Society of London panel which produced the 2005 report (Raven et al. 2005b), although I had published on the effects of increasing CO_2 and temperature on microalgae (Raven 1991a, c; Raven et al. 1993) and macroalgae (Raven and Johnston 1991) as well as more general accounts (Beardall et al. 1998a, b). The predictions from the effects of increased CO_2 and temperature on extant genotypes show a variety of responses, with generally negative responses on growth of calcified algae and no effect, or a

stimulation, of growth for non-calcified algae (Raven et al. 2005b; Doney et al. 2009; Crawfurd et al. 2011; Gattuso and Hansson 2011; Raven et al. 2011, 2012).

A number of points need to be made. One is that 'ocean acidification' means a decrease in pH relative to the interglacial value before 1750; it is not predicted that surface ocean pH will fall below pH 7.0 even with continued fossil fuel burning (Raven et al. 2005b; Diaz-Pulido et al. 2007; Falkowski and Raven 2007; Doney et al. 2009; Gattuso and Hansson 2011). Another point is that the decrease in pH is not necessarily, or even probably, the major influence on photosynthetic aquatic organisms of the effects of increased atmospheric CO_2 on the CO_2–H_2CO_3–$HCO_3^-$$CO_3^=$–$H^+$–$OH^-$ system. For calcified organisms the decrease in $CO_3^=$ is very important, while for non-calcified organisms the increase in the concentration of the other inorganic carbon species is important. A third point is that very little of the currently available data used in modelling involves genetic adaptation (Collins 2011; Collins and Bell 2004, 2006; Collins and Gardner 2009; Collins et al. 2006a, b; Huertas et al. 2011). Such experiments are difficult to perform, but more are in progress. A fourth point is that few of the experiments involve changes to both inorganic carbon and temperature in factorial experiments (Hurd et al. 2009; Finkel et al. 2010; an exception is the work of Fu et al. 2007; Feng et al. 2008 and Fu et al. 2008). A related, and very important, point is that warming will mean a shoaling of the thermocline, with decreases in the nutrient flux from the deep ocean to the upper mixed layer and increases in the mean flux of ultraviolet and photosynthetically active radiation in the upper mixed layer (a point of relevance to phytoplankton but not phytobenthos) (Raven et al. 2011, 2012), requiring even more complex multifactorial growth experiments to provide data for modellers. Many models of future ocean primary productivity emphasise warming and the shoaling of the thermocline, with little or no account taken of the effects of ocean acidification (Steinacher et al. 2010).

These comments are NOT an attempt to underplay the importance of ocean acidification for the future of marine photosynthetic organisms or of inland water phototrophs. However, it is essential that there is a multifactorial approach to both experimentation and modelling, bringing in all relevant components of environmental change and the significance of genetic adaptation (Raven et al. 2011, 2012).

5 Acid–Base Regulation, Chemiosmotic Coupling, and the Last Universal Common Ancestor

Raven and Smith (1976a, 1981, 1982) and Smith and Raven (1978) suggested sequences of evolutionary events which could have occurred in acid–base homeostasis, bioenergetics and membrane transport, based on the then-popular 'chemoorganotrophy (= heterotrophy) first' hypothesis for the energetic basis of the earliest organisms on Earth. The scenario of Raven and Smith (1976a, 1981, 1982) and Smith and Raven (1978) has the Last Universal Common Ancestor (LUCA) of all extant organisms on Earth as a fermenting anaerobic organotroph with active, ATP-powered H^+ efflux related originally to acid–base regulation,

necessitated in part by acidic fermentation products. LUCA is now thought of by many scientists, including the author, as a chemolithotroph (Lane et al. 2010). However, intracellular acid–base homeostasis, in addition to redox homeostasis, would still have been an important factor in LUCA (Allen 2010).

6 Protons and Plant Growth Substances

Many natural plant growth substances are weak electrolytes with pK_a values close to the pH of intracellular and extracellular compartments (Raven and Rubery 1982). Since many of them also have relatively high lipid:water partition coefficients, the unionised form of, for example, auxin (indoleacetic acid), abscisic acid (ABA) and gibberellins would have significant lipid solution permeability through membranes and tend to be accumulated in alkaline compartments ('alkaline trap') as the anion. These effects must, to at least some extent, influence the distributions brought about by any other transport processes which exist for the growth substances.

In the case of auxin, the other transporters are those associated with the polar transport of auxin: this is now known (Goldsmith 1977; Estelle 1998) to involve the 'chemiosmotic' mechanism (Rubery and Sheldrake 1973, 1974; Raven 1975). Some commentators (e.g. Estelle 1998; Abel and Theologis 2010) are kind enough to give the author equal credit with Rubery and Sheldrake, despite the obvious lack of synchrony of publication. The proton efflux pump, the neutral auxin influx at the upstream end of the cell and the auxin anion channels at the downstream end of the cell are the essential components of the mechanism; none of these were well characterised when the mechanism was proposed.

For short-term regulatory ABA responses of stomata, Cowan et al. (1982) suggested that the increased stromal pH upon illumination acted as an 'alkaline trap' for the ABA anion. With a fixed (in the short term) ABA pool in the leaf, the trapping of ABA would decrease ABA in the rest of the leaf and, since ABA inhibits stomatal opening and promotes stomatal closing, stomata would open in the light. The reverse of this process could occur in the dark. Whilst this may not be a significant mechanism of stomatal control in diel (or sunfleck-shade) cycles, pH is a significant regulator in the role of ABA as a drought signal (Williamson and Davies 1997, 2002). Regardless of the role of pH, stomatal responses do provide very good regulation of water loss in transpiration per carbon gain in photosynthesis (Cowan 1977, 1986; Cowan and Farquhar 1977; Vico et al. 2011).

7 Circulating Currents Carried By H^+ and Their Role in Algal and Plant Biology

Currents circulate through and outside growing, polar, eukaryotic cells and organs, generally with positive charge influx near the extending tip and positive charge efflux in more mature regions (Raven 1991b). In most cases, and universally in

terrestrial rhizophytic plants, most of the current is carried by protons. The positive charge efflux in the more mature regions involves active H^+ efflux, while positive charge influx in apical regions involves a H^+ channel; buffered H^+ moves apically through the cytosol including, in multicellular structures, in plasmodesmata and basally in the aqueous medium.

What do these circulating H^+ do? In characeans, the acidic and basic zones on the internode, with H^+ efflux in the acid zones and H^+ influx in the alkaline zones, would produce circulating H^+ currents were it not for the intervention of the external inorganic carbon system. In the acid zones, H^+ plus HCO_3^- produce CO_2, while in alkaline zones removal of H^+ from HCO_3^- produces $CO_3^=$. The $CO_3^=$ in the alkaline zone then precipitates as $CaCO_3$, using half a Ca^{2+} which balances the HCO_3^- consumed in the acid zone; this half $Ca^{2=}$ carries a positive change as it diffuses into the alkaline zone, where it is used in $CaCO_3$ precipitation with the half Ca^{2+} which balances the now-deprotonated $HCO_3^=$ (Raven 1991b). A similar process occurs in polarised elodeid leaves.

Aside from inorganic carbon acquisition and calcification, circulating currents have been suggested, by setting up external electrical potential gradients (negative at the tip, positive in the more mature regions) to attract or repel microorganism which are galvanotactic (free-swimming cells, such as diazotrophic rhizobia) or galvanotropic (hyphae of mycorrhizal fungi such as glomeromycotes). So far, these suggestions remain without adequate testing, despite the best efforts of PhD student Andrew Miller (Miller et al. 1986, 1991). A further possibility is that it is power which is being transmitted, i.e. chemiosmotic energy flow with a long distance between the generator of the H^+ transmembrane energy gradient and the consumer of the H^+ gradient. An example is motility in filaments of motile (gliding) oscillatorean cyanobacteria. Calculation (Raven 1983) showed that earlier suggestions of long-distance (mm) transport of proticity along trichomes were exaggerated.

8 Intracellular Acid–Base Regulation: What Is Being Regulated?

8.1 Methodology

The methods for measuring the pH of various intracellular components are outlined in Table 1, showing that there are a range of methods available which are divisible into two categories. One is the use of pH-selective microelectrodes, which have high temporal and spatial resolution, can cover the whole pH range, and can be used best in larger cells. The other three methods involve the use of the degree of ionisation of weak electrolytes. The ionisation state, as a function of pH, of endogenous inorganic phosphates and phosphate esters are detectable with ^{31}P NMR. Exogenously supplied organic weak electrolytes can be detected by a

Table 1 Methods of measuring intracellular pH and their applicability

Method	pH range	Spatial resolution	Temporal resolution	Range of cell sizes	References
pH		microelectrode	0–14	Very good (vacuole, cytosol, plastid) in larger cells	Excellent
Better spatial resolution in larger cells	Davis (1974) and Spanswick and Miller (1977)				
^{14}C-labelled weak electrolyte distribution	1(−2) units above and below pK_a	Distinguish vacuole and cytoplasm in giant cells	Relatively poor	If cells are vacuolate, only fully applicable to giant cells	Walker and Smith (1975)
Weak electrolyte fluorescent imaging	1(−2) units above and below pK_a	Distinguish vacuole, cytosol, plastid in all but the smallest cells	Better then labelled weak electrolytes because applicable to smaller cell	All but the smallest cells	Dixon et al. (1989)
Weak electrolyte NMR (^{31}P)	1(−2) units above and below pK_a of endogenous inorganic and organic phosphates	None except through appeal to data from other sources	Depends on time needed to acquire sufficient data	All cell sizes	Roberts et al. (1980)

labelling with a radioactive tracer or by fluorescence imaging. The weak electrolytes can only be used effectively within a certain pH range close to their pK_a. As for terminology, a convention for the use of the weak organic acid DMO (2',2'-dimethyloxazalidine-2.4-di-one) in studies on giant algal cells is to distinguish the pH of the vacuole (determined by isolation of an aliquot) from that of the cytoplasm (Walker and Smith 1975). The cytoplasm here comprises the remainder of the protoplast, i.e. cytosol, nucleus, plastids, mitochondria and the endomembrane system other than the central vacuole, and its pH is determined by calculating the DMO content of the vacuole (minus that of the aliquot used to determine vacuolar pH) and subtracting this from the DMO content of the rest of the

cell, and using this DMO content and the cytoplasmic volume, with the external DMO concentration and external pH, to calculate the cytoplasmic pH. Note that this use of cytoplasm differs from the original use by cytologists who divided the protoplasm (everything contained by the cell wall of a walled cell) into the nucleus and the cytoplasm, i.e. the protoplasm minus the nucleus. The microelectrode technique measures the pH of the cytosol and the vacuole (Spanswick and Miller 1977) and, in some cases, that of the plastid stroma (Davis 1974). The same three compartments are also imaged and thus have their pH estimated using the fluorescent weak electrolyte technique (Dixon et al. 1989). The ^{31}P NMR technique shows differences in the pH of compartments containing the various phosphate compounds whose chemical shifts are measured, but the nature of the compartments has to be decided on the basis of other evidence, usually the range of phosphate compounds indicating a particular pH and value of the pH estimated. For a photosynthetic cell the typical pH values for the major compartments are plastid stroma > cytosol > vacuole, with inorganic and organic phosphates in the first two and only inorganic phosphates in the vacuole.

8.2 *External Influences on Cytoplasmic pH as a Means of Determining the Variable Being Regulated: General Considerations*

The techniques outlined earlier and in Table 1 give the pH of compartments (or, in the case of the 'cytoplasm', a combination of compartments) achieved by the various biochemical and biophysical mechanisms of acid–base regulation discussed by Smith and Raven (1979). The 'cytoplasmic pH' or, where separately measured, the plastid and the cytosol pH, is, for most of the plants, algae and cyanobacteria examined, a genotype- and environment-specific pH of between 7.0 and 8.0. For photosynthetic cells the cytoplasmic pH is higher in the light than the dark, and much of the increase in the light is related to the increased pH in the chloroplast stroma (Smith and Raven 1979; Raven and Smith 1980b). The vacuolar pH is almost invariably less than pH 6. Regulatory systems have, by definition, a value of the variable that they are controlling which is the one to which the variable is returned after a perturbation. This is known as the 'set point', and in general terms the set points for acid–base regulation are implicitly considered to be the pH value of the compartments which are measured under steady-state conditions of external pH and solute concentrations, light and temperature. Here we examine the extent to which this is the case, and the implications of regulating pH for pH-sensitive processes as a function of temperature and ionic strength. The importance of this procedure is that what is important for natural selection is not necessarily what we are accustomed to measure. This has, of course, been recognised for decades for marine invertebrates, based on the temperature effect on the pH of extracellular fluids and, to a lesser extent, the intracellular fluids. Here the effect of temperature on the pH of the body fluids suggests that the 'set point'

relates to keeping constant the ionisation state of histidine, the 'alpha stat' hypothesis (Wilson 1977; Hazel et al. 1978; Egginton et al. 1999; cf. Johnson et al. 1983).

8.3 Influence of External Factors: pH of the Medium

For all of the cells examined, changes in external pH within the range normally encountered by the cell cause, to a greater or lesser extent, a change in cytoplasmic pH in the same direction as the change in external pH, and usually with a change of not more than 0.1 pH units per unit external pH change (Smith and Raven 1979; Kurkdian and Guern 1989; Egginton et al. 1999; Rengel 2002) in the bulk medium, noting that the pH in the diffusion boundary layer is higher (in the light in photosynthetic cells) or lower (during respiration) than in the bulk medium (e.g. Smith and Walker 1980; Kühn and Raven 2008; Hurd et al. 2011; Flynn et al. 2012). The extent to which these changes reflect variation in the set point (Cram 1976; Walker 1976; Raven and Smith 1978; Raven and Geider 1988, 2003) for cytoplasmic pH with varying external pH, the best compromise that the organism can make between achieving a constant set point pH and the costs of that regulation in, for example, metabolic energy input, or the use of the variation in intracellular pH as part of the signalling loops which control the pH-regulation apparatus, is not clear. At all events, the observed variation means that such important intracellular ionisation states as $[H^+]$:$[OH^-]$, [Histidine]:[Histidine$^+$], $[CO_2]$:$[HCO_3^-]$ and $[H_2PO_4^-]$-$[HPO_4^{2-}]$.

8.4 Influence of External Factors: N Source

It has frequently been observed that intracellular pH, including cytoplasmic pH, is slightly higher when NO_3^- is the N source (assimilation producing excess OH^-) than when NH_4^+ is the N source (assimilation producing excess OH^-) (e.g. De Michelis et al. 1979; Raven and De Michelis 1979, 1980). As with the variation in intracellular pH with extracellular pH, there are three possibilities as to why this variation in intracellular pH occurs as a function of N source.

8.5 Influence of External Factors: Temperature

An important environmental factor which has been much less investigated in cyanobacteria, algae and plants than light–dark changes or variations in external pH is temperature. Temperature effects on cytoplasmic pH have been specifically addressed by Raven and Smith (1978) for giant internodal cells of the charophycean alga *Chara corallina*, using the distribution of the weak organic acid DMO. Raven and Smith (1978) found a decrease in cytoplasmic pH of 0.05 units for a

temperature increase from 5 to 15° and of 0.15 units between 15 and 25°, i.e. cytoplasmic pH control was more precise over the lower temperature range. In any case the variation with temperature was less than the 0.17 pH units decrease per 10°C temperature increase typical of the extracellular fluids of marine invertebrates and, with more variability, intracellular pH of these organisms (Raven and Smith 1978). The temperature effects on the body fluid and intracellular pH of marine invertebrates keep constant the ionisation state of water ($[H^+]/[OH^-]$) and of nitrogenous bases with pK_a values close to the body fluid or cytoplasmic pH, predominantly histidine, in the 'alpha-stat' hypothesis (see Raven and Smith 1978, and Wilson 1977; Hazel et al. 1978; Egginton et al. 1999). The variation in cytoplasmic pH with temperature in *Chara corallina* is much closer to what is required to keep the ionisation state of weak acids and their conjugate bases such as CO_2:HCO_3^- and $H_2PO_4^-$:HPO_4^{2-} as well as certain organic acids and phosphate esters cannot all be held constant as the environment changes. In any case, no single temperature dependence of the cytoplasmic pH set point can achieve constancy in each of $[H^+]$:$[OH^-]$, [Histidine]:[Histidine$^+$], $[CO_2]$:$[HCO_3^-]$ and $[H_2PO_4^-]$-$[HPO_4^{2-}]$.

Adduci et al. (1982) have also examined the effects of temperature on cytoplasmic and vacuolar pH, in this case for *Zea mays* roots using ^{31}P nuclear magnetic resonance. The cytoplasmic pH decreases by 0.5 units with a temperature increase from 4 to 28 °C, i.e. a decrease of 0.21 units per 10 °C temperature increase. This is higher than the values found for *Chara corallina* and is even higher than the value typical of marine invertebrates. However, in these experiments it is possible that the values at the higher temperature are compromised by restricted O_2 supply as a result of decreased O_2 solubility to the roots in the nuclear magnetic resonance tube, combined with the higher potential metabolic rate at the higher temperature, resulting in the possibility of hypoxia and production of lactic or malic acid as fermentation products (Felle 2005; Greenway et al. 2011). The reversibility of the temperature change effects (Adduci et al. 1982) does not rule out this possible amplification of the temperature effect, since post-anoxia metabolism of organic acids could occur. The work on *Chara corallina* would not be influenced in this way, since photosynthetic cells were investigated in the light (Raven and Smith 1978).

8.6 *Influence of External Factors: Temperature in an Ecological Context*

Regardless of the magnitude of the effect, the restricted data available show a decrease in cytoplasmic pH with increasing temperature in photosynthetic organisms, qualitatively the same as in marine invertebrates. This could have significant implications for phytoplankton cells in this time of increased atmospheric CO_2, resulting in increased CO_2 in the surface ocean water resulting in a

smaller relative (larger absolute) increase in HCO_3^- and a decrease in pH and CO_3^{2-} (Raven et al. 2005b; Doney et al. 2009). Among other outcomes, this would be expected to give a decrease in cytoplasmic pH. In parallel there is an increase in global temperature, with a very high probability that most of the temperature increase relates to anthropogenic production of greenhouse gases. The increased ocean surface water temperature would be expected to decrease the cytoplasmic pH (Raven and Smith 1978; Adduci et al. 1982). It is thus possible that these two effects of global environmental change, i.e. increased sea surface CO_2 and increased sea surface temperature, would have additive or multiplicative effects in decreasing cytoplasmic pH. However, it must be emphasised that the data on the effects on cytoplasmic pH of decreased external pH and of increased temperature were obtained without direct examination of interactions between the two factors. Experiments investigating the effects on intracellular pH of increased temperature and CO_2, singly and in combination, are required, such as have been carried out for the growth of a range of marine phytoplankton species (Fu et al. 2007, 2008; Feng et al. 2008). In this work it is important not only to use coastal marine phytoplankton, with significant temporal variations in external pH and temperature, but also to use oceanic species which are generally subject to smaller changes in external pH and in temperature.

In some coastal habitats there are larger changes in temperature and pH than are expected from global environmental change (Midelboe and Hansen 2007a, b). Particularly extreme are high intertidal rock pools which are flushed with seawater at high spring tides, but are isolated from exchange with seawater for about a week at neap tides. Such rock pools can have high densities of macroalgae. Typically, around the North Sea the dominant alga is a species of *Ulva* with the *Enteromorpha* life form of *Ulva* (Morris and Taylor 1983; Poole 1998; Poole and Raven 1998; Björk et al. 2004). The growth of the macroalgae can decrease the inorganic C concentration from 2 mol m^{-3} to less than 1.5 mol m^{-3} and increase the pH from just over 8 to pH 10 or slightly higher, with an increase in O_2 from about 0.22 mol m^{-3} to 0.7 mol m^{-3}, the asymmetry of inorganic C decrease and O_2 increase relative to the close to 1:1 stoichiometry in net growth relating to the asymmetry of CO_2 invasion from the atmosphere and O_2 ebulition from the supersaturated O_2 solution (Poole 1998; Poole and Raven 1998; Björk et al. 2004; Raven and Larkum 2007; see also Maberly 1990; Marconi et al. 2011). Superimposed on these trends over the course of emersion–submersion cycles of several days in high intertidal rock pools, there can also be diel temperature variations of 10 °C or more, as well as very significant variations in pH, O_2 and inorganic C (Morris and Taylor 1983; Poole 1998; Poole and Raven 1998; Björk et al. 2004). Assuming that the effects of changes in the environmental pH on the cytoplasmic pH of the *Enteromorpha* life form of *Ulva* are in the same direction as in the organisms discussed earlier, i.e. increasing cytoplasmic pH with an external pH increase and decreasing cytoplasmic pH with an increase in temperature, the diel changes in rock pool pH and in temperature would tend to offset one another. The increasing external pH in the day would tend to increase cytoplasmic pH, while the increasing temperature would tend to decrease cytoplasmic pH. The

reverse would happen as external pH and temperature decreased at night. Similar changes in pH and temperature (Maberly 1996), with similar implications for the cytoplasmic pH on photosynthetic organisms, occur in productive freshwater bodies.

8.7 *Influence of External Factors: Ionic Strength*

While the variations in salinity of high intertidal rock pools as a result of evaporation and rainfall are generally not large, it is of interest that there are significant effects of external salinity changes on the cytoplasmic pH of the green microalga *Dunaliella tertiolecta* which can grow over a wide range of salinities. Goyal and Gimmler (1989) showed that the cytoplasmic pH of cells grown in 170 mol m^{-3} NaCl was pH 6.82 in the dark and pH 7.36 in the light, while for cells grown in 770 mol m^{-3} NaCl the values were pH 7.00 in the dark and pH 7.50 in the light. Are these effects related to the cytoplasmic salinity? For the wall-less *Dunaliella* in the two media used by Goyal and Gimmler (1989) the intracellular osmolarity equals that in the medium, and most if not all of the increased cytoplasmic osmolarity in the medium of higher osmolarity is contributed by the compatible solute glycerol rather than by additional electrolytes, so that the salinity (or ionic strength) in the higher osmolarity medium is little higher than that in the lower osmolarity medium (Raven 1984, 1985). The effect of increasing ionic strength from 0 to about 300 mol m^{-3} of singly charged cations and anions is to decrease the pH at which $[H^+] = [OH^-]$ by about 0.15 pH units, with a further 0.05 pH unit decrease up to 600 mol m^{-3} (Harned and Owen 1958), so even if all of the intracellular osmolarity in the 170 mol NaCl m^{-3} medium was contributed all by ions the pH for water neutrality would be about 0.15 units less than that in pure water. A situation in which there must be a lower ionic strength in the cytoplasm is that of the effectively wall-less freshwater algae, mainly flagellate but some amoeboid and others non-motile, in which volume regulation involves a contractile vacuole, with an energy cost of volume regulation which increases as the square of the difference between the intracellular and extracellular osmolarity (Raven 1982, 1995). In some of these cells the total intracellular osmolarity, and hence the intracellular ionic strength, was as low as about 80 mol m^{-3} (Raven 1982, 1995). This would require a cytoplasmic pH at least 0.1 units higher than in a cell with a cytoplasmic ionic strength of 340 mol m^{-3} to give the same $[H^+]$:$[OH^-]$ ratio in the cytoplasm of the two cells at the same temperature. There are also ionic strength effects on the ionisation constants of the weak electrolytes found in the cytoplasm. For histidine in three model peptides, the pK_a increased by 0.12 units between 20 and 200 mol NaCl m^{-3} and by 0.08 units between 200 and 500 mol NaCl m^{-3} (Kao et al. 2000). For weak acids such as CO_2–HCO_3^- and $H_2PO_4^-$–HPO_4^{2-} the ionic strength effects are larger and there is a decreased pK_a with increasing ionic strength. For CO_2–HCO_3^- the pK_a decreases by about 0.3 units between 20 and 200 mol NaCl $m^{-3}$and a further 0.2 units between 200 and 500 mol NaCl m^{-3} (see references in

Raven 1984). These effects of ionic strength on pK_a values show that maintenance of cytoplasmic pH at the same value in cells with different cytoplasmic ion strength with values would give significantly different ionisation states of histidine and the weak acids at the different ionic strengths. Furthermore, different pH values for the two ionic strengths could maintain a constant ionisation state for histidine but not the weak acids, and vice versa.

Differences in ionic strength in the external medium and in the cytoplasm, such as is especially the case among the organisms mentioned earlier for *Dunaliella tertiolecta*, can have significant implications for the distribution of important metabolites. Raven and Richardson (1986) point out that CO_2 is significantly more soluble in glycerol than in isosmotic NaCl, so a slightly higher intracellular than extracellular CO_2 concentration could be maintained even with a net diffusive flux of CO_2 from the medium to Rubisco. However, it is clear that *Dunaliella* photosynthesis does not rely on diffusive CO_2 entry but rather on a CO_2 concentrating mechanism (CCM) (see Raven 2009). The different intra- and extracellular ionic strengths for cells grown at the high external osmolarity have implications for the speciation of inorganic carbon within the cells relative to outside. The pK_{a1} for inorganic C is higher at the low ionic strength inside the cell, so that the equilibrium $[CO_2]$:$[HCO_3^-]$ is higher inside the cell than outside the cell at a given pH, i.e. the energy used in making the intracellular solutes different alters the speciation of inorganic C. The cytoplasmic pH is less than that in the medium, and this too increases the equilibrium intracellular $[CO_2]$:$[HCO_3^-]$. By contrast, for freshwater organisms where the intracellular ionic strength is higher than that in the medium, the equilibrium $[CO_2]$:$[HCO_3^-]$ for equal internal and external pH values is lower in the cells than in the external medium. Raven et al. (2005a) point out that the energetics of CCMs functioning with active CO_2 entry at the plasmalemma are, in principle, no less energy costly than those which actively transport HCO_3^-. However, in those cases where the combination of the external and cytoplasmic pH and the difference in internal and external ionic strengths give higher steady-state CO_2 concentrations at the active site of Rubisco than would otherwise have been expected with the same active transport processes for the inorganic C species, the endergonic metabolic processes involved in the difference in cytoplasmic and external pH and ionic strength should be considered as contributors to energising the CCM.

8.8 *Influence of External Factors: Conclusions on the Nature of the Set Point Ion Acid–Base Regulation*

The change in the pH in a number of cytoplasmic compartments in response to changes in intracellular pH is usually less than 0.1 unit change in the pH of the intracellular compartment per unit external pH variation in the normal ecological

range. It is not clear whether these changes reflect variation in the set point for cytoplasmic pH with varying external pH, or the best compromise that the organism can make between achieving a constant set point pH and the costs of that regulation in, for example, metabolic energy input; the latter is probably more likely.

There is still doubt as to the temperature dependence of 'cytoplasmic' pH regulation in algae and plants, with a very small database. Without additional data it is not possible to say that there is constancy of the ionisation state of a particular molecular species within an intracellular compartment as an outcome of the effect of temperature on the pH of that intracellular compartment. Whatever ionisation state is held constant, the outcome will be different in organisms with an ionic strength in the intracellular compartment of the 'typical' value for cytoplasmic compartments of 300 mol m^{-3} compared to the situation in effectively wall-less freshwater cells with ionic strengths as low as 80 mol m^{-3}.

8.9 What Does Acid–Base Regulation Do?

It is clear that acid–base regulation does not maintain a constant pH in an intracellular compartment regardless of external pH, nitrogen and carbon source, temperature or intracellular ionic strength. If there is a weak electrolyte which is maintained at a constant ionisation state in acid–base regulation in photosynthetic organisms, it has yet to be identified. A related topic is that of what the cell or organism perceives of the acid–base status of the medium (Raven 1990).

9 Conclusions

My publications have not followed the route that is regarded as the norm for a scientist: hypothesis, experimentation to test the hypothesis, modified hypothesis, experimentation to test the modified hypothesis, and so on. While there have been elements of this sequence, the general approach has been of suggesting more hypotheses than have been tested, by me or by others.

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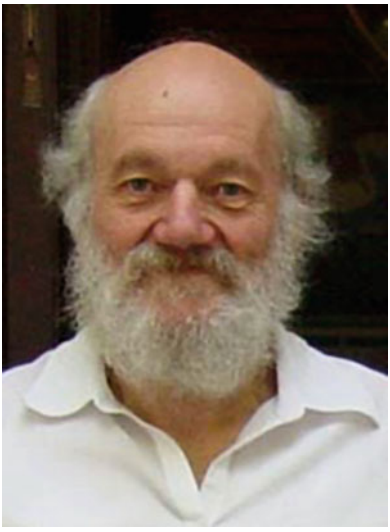
John Raven's Academic Career John Raven was born on 25 June 1941. He graduated with a BA in Botany from the University of Cambridge in 1963, and went on to postgraduate work (also in the University of Cambridge under the supervision of Dr (now Emeritus Professor) Enid MacRobbie FRS, graduating with a PhD in Botany (Plant Biophysics) in 1967. He continued to work in Enid MacRobbie's laboratory, having been awarded a Research Fellowship by St Johns College Cambridge in 1996, and an Official Fellowship by that College in 1970. He was also a University Demonstrator (untenured lecturer) in the University of Cambridge from 1968 to 1971. In 1971 he moved to the University of Dundee as a Lecturer in Biology; he became a Reader (Associate Professor) there in 1978, and a Personal Chair (Full Professor) in 1980. In 1994 he was appointed to the Boyd Baxter Chair of Biology at the University of Dundee, a position which he held until 2008 when he officially retired in 2008; he is now an Emeritus Professor associated with the University of Dundee's Division of Plant Science based at the James Hutton Institute in Invergowrie near Dundee. He is also an Honorary Professor at the Universities of Queensland and of Western Australia. In addition to research and teaching in the University of Cambridge and then the University of Dundee he has spent Sabbatical Terms in the Australian National University (1979) and Florida International University (1984), and has carried out laboratory and/or field work in Australia, Brasil, Canada, Italy, New Zealand, Peru, Sweden and the USA. He is a Fellow of the Society of Biology, a life fellow of the Botanical Society of Scotland, an honorary life member of the British Phycological Society, a corresponding member of the Australian Society of Plant Scientists and of the Botanical Society of America, holds an Award of Excellence from the Phycological Society of America, and was awarded an Honorary PhD by the University of Ümeå. He was elected to fellowship of the Royal Society of Edinburgh in 1981 and to fellowship of the Royal Society of London in 1990.



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Part II
Genetics

Gene Transfer in Legumes

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Contents

1 Introduction

In the last few decades, plant breeders, cell biologists and genetic engineers have joined forces and used a number of different techniques to unravel the secrets buried within the plant genome aiming at plant improvement. Breeders have traditionally relied on mass selection breeding following sexual crosses between

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the individuals possessing the best desirable characteristics and examining the best possible combinations within the resulting progenies over several years for the creation of new varieties. Cell biologists strive to develop tools for producing genetic novelties through the regeneration of plants (from protoplasts, cells, tissues and organs) following diverse in vitro selection treatments and then evaluate their conformity compared to the mother plants (wild type) as well as their specific biochemical, genetic and physiological status. Genetic engineers develop methods and techniques for the isolation and successful insertion of genes controlling desirable traits into plants by transformation.

Plant transformation may be defined as the sequence of delivery, integration and expression of foreign genes into the plant cells which will ultimately regenerate into a whole plant. This ability to introduce and express or inactivate specific genes in the plant genomes provides a new and powerful experimental tool for validating gene function, particularly in relation with various plant physiology mechanisms and processes that have not been resolved so far using other biochemical approaches. Another non-negligible application of this approach is that of obtaining and transferring genes that are not available to a given species due to sexual incompatibility from other plants, from microorganisms or even animals.

The process involves choosing a trait, identifying and isolating the gene(s) encoding it. To be functional, such gene(s) must include the regulatory regions ensuring their correct expression in the plant. Then, a reliable protocol must be devised and followed for the transformation of genes into plants, and the DNA sequences introduced must subsequently be integrated, expressed and maintained in the genome throughout subsequent cell divisions and progenies. Finally, transformed cells must be competent for regeneration into whole plants.

Gene delivery systems used to date can be divided into direct gene transfer (mediated by physical or chemical forces for delivery of the gene into plant protoplasts, cells and even tissues) and *Agrobacterium*-mediated gene transfer, where either *A. tumefaciens* or *A. rhizogenes* is used as vectors for introducing the foreign gene into the plant genome.

In this context, recombinant DNA technology has revolutionised biotechnology in such a way that plant transgenesis is now a relatively mature approach, and plant biotechnology provides today not only novel genotypes which carry agronomically useful genes for biotic and abiotic stresses, but also others that improve plant nutrition or increase yield components.

2 *Agrobacterium*-Mediated Transformation

In *Agrobacterium*-mediated transformation, the natural infecting capacity of the bacterium is used to transfect genes into plant cells (Chilton 2001; Somers et al. 2003; Sangwan et al. 2010), and this approach has become a tool used in plant breeding for crop improvement.

One significant limitation of the *Agrobacterium* gene transfer system is the fact that large groups of commercially important plants and some legumes among them are not hosts for *Agrobacterium* whereby this gene transfer system is not efficient for them.

A critical step for successful transformation with *Agrobacterium tumefaciens* is the co-cultivation of plant cells and bacteria to allow gene transfer, generally done by mixing plant cells with bacteria in vitro for a few days, after which bacteria are removed and cells or organs are regenerated to plants using adequate culture media. Alternatively, whole plants are dipped in Agrobacterium and subsequently allowed to grow under natural conditions, as in the floral dip transformation method of *Arabidopsis thaliana* (Clough and Bent 1998). This, unfortunately, has only been applicable to a few plant species to date including the legume *Medicago truncatula* (Trieu et al. 2000).

Due to the quite high recalcitrance of legumes to *Agrobacterium*-mediated transformation, numerous studies were focused on the optimisation of co-cultivation conditions (light regime, temperature, co-cultivation period, application of physical treatments such as sonication and vacuum, electroporation, mechanical pre-wounding or treatment by macerating enzymes). The co-cultivation step is a crucial and complex process where two different biological elements (plant explants and *Agrobacterium*) share the same space and conditions; thus many parameters should be tested to satisfy both partners and guarantee a successful outcome (Hansen and Wright 1999). A range of chemical substances which work for instance through the reduction of the resistance of the recipient cell (thiol compounds, anti-oxidants) or by facilitating their penetration through the cell wall (macerating enzymes) (Svabova and Griga 2008, and references therein) have been studied. A "classical" co-cultivation substance is acetosyringone (AS), a compound which is essential for induction of virulence genes. AS was used in several studies with legumes, e.g. De Kathen and Jacobsen (1990) found that AS did not increase the transformation ratio, Grant et al. (1995) used AS but did not compare its effect with the control, while Nadolska-Orczyk and Orczyk (2000) recorded a negative effect of AS but a positive influence of 5-azacytidine. A series of experiments with co-cultivation substances used previously for other leguminous (soybean, common bean, peanut) as well as non-leguminous crops (tobacco, sunflower, grapevine) were carried out by Svabova and Griga (2008). Subsequently, the effect of application of acetosyringone, L-cysteine, dithiothreitol, glutathione, cellulase and pectinase in various concentrations and combinations was examined. The only combination significantly improving responses was the addition of 100 μM AS and 50 mg L-cysteine to the co-cultivation medium.

In legumes, all methods for gene transfer are based on specific in vitro techniques used to foster the genetically modified cells to regenerate into plants, depending on the organ and the cells used for transformation. Regeneration either proceeds directly from meristems existing in the original explant or adventitiously, through organogenesis or somatic embryogenesis, which can both be direct or include an intervening callus phase, with such callus generating embryos or shoots following subculture on an appropriate medium.

Only a small fraction of the target cells are actually transformed. Thus, genetic modification requires a selection mechanism ensuring that the genetically modified cells are favoured to grow and divide over wild-type cells. Usually, this is achieved by coupling the gene(s) of interest to genes conferring resistance to antibiotics or herbicides, whereby adding antibiotics or herbicides precludes the division and growth of non-transformed cells. For most crops, such selection treatment is applied during a callus phase after which plants are regenerated via adventitious shoot formation or somatic embryogenesis, depending on the species. However, selection mechanisms are not always fully efficient with this procedure and hence regenerated plants may contain transgenic cells but also either a mixture of transgenic and wild-type cells (chimeras) or only wild-type cells (escapes). A proper selective concentration of selective substances should decrease the number of escape plants to a minimum. Several conclusions were obtained in experiments focused on the utilisation of various genes for resistance to antibiotics as selective genes, particularly kanamycin. Puonti-Kaerlas (1992), De Kathen and Jacobsen (1990) as well as Schroeder et al. (1993) found kanamycin less effective for selection than hygromycin and phosphinotricin. On the other hand, Grant et al. (1998) deemed kanamycin in higher concentrations and in combination with successive selection steps as effective. Similar results were published by Morton et al. (2000).

Generally, in large seeded legumes regeneration systems omitting callus culture are more effective than those where embryos or calli are initiated. The conversion of undifferentiated cell growth (calli) to the process of differentiation and induction of embryo- or organogenesis is a normally challenging task. A method of direct use of germinating seeds was first described on soybean by Chee et al. (1989). Such "Non in vitro-tissue regeneration" system, i.e. favouring the growth of pre-existing meristems in the explants, seems to be very suitable for application in transformation protocols of pea where, in spite of the chimaeric nature of the regenerated plants, the transgenes were transmitted to the progeny (Bean et al. 1997; Svabova et al. 2005, 2008).

3 Direct Gene Transfer Methods

Direct DNA transfer through physical or chemical methods provides an alternative to *Agrobacterium*, and it is the only way to introduce genes into the chloroplast genome (Clarke et al. 2011).

Examples of direct gene transfer methods are particle bombardment and electroporation, but also polyethylene glycol (PEG) treatment. In particle bombardment, small metal particles are coated with the sequences of interest and are shot into plant cells. In electroporation, plant cells and DNA are together in a solution and an electric stimulus is used to transfer DNA into the plant cells, whereas in PEG-mediated treatment protoplasts (and more rarely cells) are co-cultured with plasmids in the presence of PEG.

3.1 Electroporation

Electroporation is a simple and rapid method that involves applying electrical pulses to a suspension of protoplasts and DNA, placed between electrodes in a suitable cuvette. When a cell is exposed to an electric field, pores are formed through an enhancement of its transmembrane potential (Cole 1968; Neumann and Rosenheck 1973) which depends on the cell radius, the electric field strength delivered and the angle between the normal vector of the membrane and the direction of the electric field applied (Chang 1992). The transient pores in the plasmalemma allow the DNA to enter the cell and nucleus. The method has been used to introduce genes into protoplasts isolated from a range of different species and seems to be a universal method of gene transfer into prokaryotic and eukaryotic cells.

Developing procedures by which plants could be efficiently and successfully regenerated from single cells (protoplast) and organised tissues is the prerequisite for practical genetic engineering through electroporation-mediated protoplast transformation and for crop improvement. Protoplast regeneration requires techniques for efficient and reproducible protoplast isolation, induction of sustained protoplast proliferation in culture and shoot morphogenesis in resulting calli (Ochatt and Power 1992).

In pea, the major limitation of recovering stable transformants by protoplast electroporation was the requirement for an efficient protoplast-to-plant regeneration scheme. Puonti-Kaerlas et al. (1992) stably transformed protoplasts from two different pea cultivars (Belman and Filby) by direct gene transfer using electroporation; they recovered transgenic calli when hygromycin resistance was used as the selective trait, but no transformants were obtained when kanamycin resistance was used as selective marker. *Gus* gene was used to assess transformation efficiency using histochemical staining, and the transgenic nature of the calli selected for resistance against antibiotics was confirmed by DNA analysis. The effect of the field strength on survival and division rates of the protoplasts was also studied. Unfortunately, plants could not be regenerated from the transformed calli.

Lehminger-Mertens and Jacobsen (1993) and Böhmer et al. (1995) described the successful regeneration of plants from leaf and lateral shoot bud protoplasts of pea (*Pisum sativum*). Thus, protoplast-derived calli regenerated shoots but were unable to produce roots and could only be induced to develop fertile plants following grafting onto recipient pea seedlings as rootstocks. Later on, Ochatt et al. (2000) isolated and cultured viable protoplasts from five pea genotypes in an attempt to predict their expressable totipotency. The protoplast-derived tissues exhibited great differences in proliferation and their competence to regenerate shoots, both within and between genotypes. Flow cytometric analysis of DNA content of calluses at different developmental stages showed a correlation between endoreduplication and the ability to regenerate shoots, since fertile plants were regenerated only from calluses with a normal DNA level. This technique can serve as a tool for the early prediction of plant regeneration competence from protoplasts.

Electroporation-mediated gene transfer is a simple and rapid method involving application of electrical pulses to a suspension of protoplasts and DNA placed between electrodes in a suitable cuvette. However, it depends upon certain physical and chemical forces to introduce foreign genetic material into the host genome, including capacitance and field strength, duration and shape of electrical pulses, buffer composition and temperature, type of chimaeric gene constructs and the concentration and form of DNA.

During electroporation, weak electric fields are generally insufficient to create pores in the membrane and thus to promote incorporation of the plasmid and expression of the reporter gene in electroporated protoplasts. On the other hand, too strong an electric field will provoke irreparable plasma membrane damage, due to irreversible membrane breakdown, and thus causing extensive cellular death and virtually no transformation. Maximum transient gene expression has been reported under electric field strengths causing more than 50 % reduction in protoplast viability (Fromm et al. 1985; Hauptmann et al. 1987; Oard et al. 1990; Quecini et al. 2002).

Despite protoplasts being wall-less cells, which renders them sensitive to stress (particularly mechanic and osmotic ones), electroporation has been reported as a means of increasing a certain number of cellular processes in protoplasts and tissues. Stimulation of direct embryogenesis was reported in protoplast-derived *Medicago* tissues (Dijak et al. 1986). Likewise, electroporated protoplasts of a range of species, including legumes, exhibited an increased rate of cell division and faster and sustained microcallus growth compared to non-electropulsed protoplasts (Rech et al. 1987). Ochatt et al. (1988) reported a higher frequency of plant regeneration and root and shoot proliferation from electroporated protoplasts of the cherry rootstock Colt, and a similar response was observed with the medicinal species *Solanum dulcamara* (Chand et al. 1988). More recently, electroporation was shown to improve the production of embryos from isolated microspores of pea and other legumes (Ochatt et al. 2009) and also from intact anthers (Ribalta et al. 2012). Rech et al. (1988) observed a significant increment of DNA synthesis in electroporated protoplasts, while specific transgene amplification in shoots regenerated from electroporated protoplasts has also been reported in *Stylosanthes guianensis* (Quecini et al. 2002).

Transient gene expression following protoplast electroporation was reported only seldom in pea (Hashimoto et al. 1992; Puonti-Kaerlas et al. 1992; Ochatt et al. 2005), and the determination of the optimum electrical parameters permitting entry of the foreign information into protoplasts whilst permitting their subsequent viability and proliferation competence was a prerequisite for success in this domain. Conversely, there are quite a few examples of electroporation applied for transient and (less frequently) stable gene transfer in other legume species, as will be discussed crop-wise below.

3.2 Biolistic

Transgene delivery into pea plant cells by the biolistic approach was reported by several authors (Molnár et al. 1999; Warkentin et al. 1992). Recently, a series of

experiments were carried out with Biolistic® PDS-1000/He Particle Delivery System (Biorad laboratories, Hercules, USA.) in our laboratory to check the mean frequency of transformation. Alternatively, integrated transformation by using particle bombardment in combination with *Agrobacterium*-mediated approach (Droste et al. 2000) was tested. Transient *gus* gene expression after 7 days was shown by 6 % of explants after biolistic and by 33 % after composite approach. The limiting factor in this system was the necessity to use relatively little objects to put into the biolistic device. We had to use culture of apical meristems where the mere regeneration capacity is limited to 5 %, and a further decrease of explant viability was caused by application of the transformation protocol which led us to reject use of the biolistic method for mass production of transformants.

The use of the biolistic approach for successful stable transformation was first demonstrated with soybean (McCabe et al. 1988; Christou et al. 1989), and this approach has since been exploited on several occasions in this crop, as recently reviewed by Dickins et al. (2003). Likewise, particle bombardment has yielded transgenic plants of bean (Russell et al. 1993; Aragao et al. 1996; Aragao and Rech 1997), alfalfa (Filipe Pereira and Erickson 1995), peanut (Wang et al. 1998), faba bean (Ismail et al. 2001; Metry et al. 2007), chickpea (Kar et al. 1997) and *Vigna* species (Bhargava and Smigocki 1994). All these examples will be discussed individually in the sections devoted to each of these species below.

In the coming paragraphs we shall discuss the state of the art on *Agrobacterium*-mediated gene transfer of grain legumes in recent years and focus also on direct gene transfer in this group of species, aimed at both stable and transient genetic transformation.

4 A Brief Retrospective of Genetic Transformation of Legumes

Production of transgenic plants has been reported in a broad range of legume species (reviewed by Somers et al. 2003), including pea, and despite being generally regarded as recalcitrant to transformation (Ochatt et al. 2000, 2005; Svabova and Griga 2008), some appeared easier to transform than others (Somers et al. 2003).

Even when many of the available adventitious regeneration systems have been used for transformation of legumes (Somers et al. 2003; Ding et al. 2003), the most successful regeneration methods to date have been based on non-adventitious regeneration. This is probably so because, in legumes, adventitious methods have a very low efficiency (Ochatt et al. 2000; Somers et al. 2003), are only applicable to a few genotypes within a species (Trinh et al. 1998; Ochatt et al. 2000; Chabaud et al. 2007) or risk yielding plants of low quality due to somaclonal variation (Ochatt et al. 2001; Ochatt 2008).

Below, we give a brief overview of the methods and results of gene transfer in both forage and grain legumes.

4.1 Forage Legume Crops

4.1.1 Alfalfa

Alfalfa (*Medicago sativa* L.) is the most important forage pasture legume cultivated in the world and has been the object of a number of transgenesis studies (Table 1).

Atanasov and Brown (1984) reported a system for successful regeneration of plants from protoplasts from either leaf mesophyll or cotyledon-derived cell suspension cultures, which divided and formed colonies on Kao medium, followed by somatic embryo formation on a high auxin/low cytokinin medium. Monteiro et al. (2003) also reported plant regeneration from leaf-derived protoplast cultures of several alfalfa cultivars via somatic embryogenesis. Because of its high efficiency, this procedure can be used for electroporation-mediated protoplast transformation of alfalfa.

A preliminary study of transient gene expression in alfalfa protoplasts for functional analysis of *cis*-elements affecting expression of an elicitor-inducible bean chalcone synthase gene (*CHS*) promoter was carried out by Harrison et al. (1991). After electroporation of cell suspension protoplasts, they were able to identify the regulatory sequences for protein binding in CHS promoter using chloramphenicol acetyltransferase (CAT) assay.

Du et al. (1994) demonstrated *A. tumefaciens-mediated* transformation in highly embryogenic clones of alfalfa. Petiole and stem explants were transformed with different *A. tumefaciens* strains harbouring various vectors with *nptII* as selectable marker gene. Transgenic plants, from kanamycin-resistant callus, were verified for transgene integration by PCR and Southern hybridisation but no information on seed formation and progeny was provided. Petiole explants gave the best results and A281 was the most effective *A. tumefaciens* strain.

The first report on production of transgenic alfalfa expressing insecticidal protein was presented by Thomas et al. (1994). Petioles and leaf segments were inoculated with *A. tumefaciens* strain LBA 4404 with pAN70 plasmid, containing cDNA encoding the anti-elastase proteinase inhibitor (PI) from *Manduca sexta*. Transgenic plants developing from kanamycin-resistant callus were proven for their transgenic nature by Southern blot analysis. Western blot analysis confirmed the expression of anti-elastase protein, which was also evident by reduced thrip damage on transgenic plants. Mendelian segregation of *nptII* and *PI* gene was observed in progeny while transformation efficiency was 10 %.

Desgagnés et al. (1995) described a strategy for genetic transformation of commercial breeding lines of alfalfa. Leaf discs were co-cultivated with *A. tumefaciens* strains C58, A281, LBA4404 harbouring plasmid with *nptII* gene as selectable marker. Kanamycin-resistant plants, developed from callus via somatic embryogenesis were screened for transgene integration by PCR, Southern hybridisation and recallusing assays. When they crossed kanamycin tolerant T_0 parents, transgene inheritance to the progeny followed a segregation ratio near 1:1, consistent with a single pseudo-dominant gene being responsible for the kanamycin

Table 1 Alfalfa (*Medicago sativa* L.) transformation

Agrobacterium strain[a]	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
LBA4404	Regen-SY	Leaves	*GUS*		Samac et al. (2004)
A. rhizogenes NCPPB 1855.	Europe and Adriana	Roots	*Agropine/T-DNA*	–	Spano et al. (1987)
pUC19	*Not given*	Protoplasts	*CAT*	–	Harrison et al. (1991)
C58-R1000	Rangelander, Regen-S/C2-4	Petiole and stem	*T-DNA, NPT-II*	20	Du et al. (1994)
LBA4404	Moapa 69 and Cuf-lO1	Leaf and petiole	*nptII*	10	Thomas et al. (1994)
LBA4404	RYS1 and RSY27	Ovaries and leaves	*nptII*/GUS	–	Micallef et al. (1995)
LBA4404	Regen-SY-27	Leaves	*nptII*	20–50	Austin et al. (1995)
C58, A281, LBA4404	1.5, 8.8 and 11.9	Leaves	*nptII*	60	Desgagnés et al. (1995)
A28l and LBA 4404	Zajearska 83	Somatic embryos	*nptII* and/or *GUS*	30	Ninkovic et al. (1995)
pKANGUS and pFF19K	C2-4	Petioles and stems	*nptII/GUS*	–	Pereira and Erickson (1995)
LBA 4404, ABI and AGL1	Rangelander, *M. falcata* Ladak,	Leaves	*nptII/GUS*	60	Samac (1995)
A. rhizogenes 9402	Baoding, Rangelander	Hairy roots	*NPT* II	43–60	Lü et al. (2000)
LBA4404 and EHA101	*M. falcata* 47/1-5 and 47-150	Leaves	*nptII/GUS*	5–10	Shao et al. (2000)
LBA4404/pBI11 LBA4404/pTOK233	Zajecarska 83	Immature embryos	*hpt gene/GUS*	1–14	Ninkovic et al. (2004)
LBA4404	Regen-SY	Leaves	*nptII/GUS*	–	Samac et al. (2004)
vector pILTAB357	Regen-SY	Leaf disks	*npt*II	–	Tesfaye et al. (2005)
vector PSG529	RegenSY-27	Leaves	NptII	–	Calderini et al. (2007)
pPZP-hemL-nptII LBA4404	RSY1	Leaves	NptII	54	Rosellini et al. (2007)
LBA 4404	WL357HQ	Young seedlings	*GUS*	17.5	Weeks et al. (2008)
LBA 4404 vector p2GS-P5CS	Xinjiang Daye	Cotyledons, hypocotyls	*PPT*	6	Zhang et al. (2010)
LBA4404 and AGL1	RSY1	Leaves	*nptII/GUS*	10.4	Ferradini et al. (2011a)
AGL1	RSY1	Leaves	*MsGSAgr*	42–43	Ferradini et al. (2011b)

[a]All strains of *A. tumefaciens* unless indicated otherwise

tolerance. This study demonstrated that when genotypes are carefully screened for regenerability and when the best strain/genotype combinations are used, the success rates of alfalfa transformation could be maximised.

In order to observe the strain specificity of *A. tumefaciens* for alfalfa transformation, Samac (1995) inoculated greenhouse plants with several *A. tumefaciens* wild-type strains. The genotypes showed varying degree of susceptibility and resistance to these strains calculated by observation of stem tumour induced in inoculated plants. She then carried out in vitro transformation of leaf disc explants from different genotypes to verify the transformation capacity of disarmed *A. tumefaciens* strains i.e. LBA 4404, ABI and AGL1. Based on GUS screening, a significant genotype–strain interaction was observed, with more transformants when genotypes were transformed by strain LBA4404 than with either of the other strains.

In order to produce high levels of industrially important enzymes, Austin et al. (1995) developed genetically engineered alfalfa expressing bacterial and fungal proteins by inoculating leaf discs with *A. tumefaciens* strain LBA4404 containing plasmids with genes coding manganese-dependent lignin peroxidase (Mn-P) from the fungus *Phanerochaete chrysosporium* and alpha-amylase mature protein from bacterium *Bacillus licheniformis*. Transgenic plants (produced in 10–12 weeks with transformation efficiency ranging from 20 to 50 %) were screened for kanamycin resistance. Expression of *B. licheniformis* alpha-amylase was confirmed by alpha-amylase assay and that of *P. chrysosporium* Mn–P by western blot analysis. Field performance of the transformants was assessed. Mn–P was shown to segregate in sexual progeny derived after crossing transgenic plants with wild-type ones.

Micallef et al. (1995) compared the previously described alfalfa transformation method using leaf discs (Austin et al. 1995) with a new one using excised ovaries as explants. They co-cultured the explants with *A. tumefaciens* strain LBA4404 harbouring vector pDM4715 containing *nptII* under the control of the CaMV35S promoter and *gus* driven by the MAC promoter. Transgenic, kanamycin resistant, plants were obtained from callus induced from explants. Transformation of ovaries was less efficient and more time consuming in terms of callus production compared with transformation of leaf tissue. Backcrossing of transformants with a cultivated variety was carried out to improve transgenic alfalfa. Heritability studies of transgene by using *gus* as a dominant genetic marker suggested that three backcrosses were optimal as the progeny contained 94 % cultivar germplasm and could be used as parents of a new cultivar.

Use of biolistic approach for successful stable transformation of alfalfa was reported for the first time by Pereira and Erickson (1995), who bombarded calli derived from petiole and stem sections with vectors harbouring *nptII* and *gus* as selectable marker. Transformants developed from kanamycin-resistant calli were assayed for *GUS* gene expression by histochemical and fluorometric assays. PCR and Southern hybridisation confirmed transgene insertion and integration in the genome. The transgene was segregated in Mendelian fashion but regeneration and transformation frequency was considerably low.

First evidence of alfalfa hairy root transformation was reported by Spano et al. (1987) when plant stems from different alfalfa genotypes were inoculated with

A. rhizogenes strain NCPPB 1855, harbouring the agropine type Ri plasmid pRi 185. Adventitious hairy roots developed regardless of genotype and gave rise to calli. Auxin (2,4-D or NAA) was shown to inhibit hairy root growth while promoting callus formation directly from inoculated explants. Plant regeneration, via somatic embryogenesis, was only achieved with a highly regenerable genotype. When compared with non-transformants, T-DNA from Ri plasmid completely changed the root structure in transgenic plants. The same phenotypic alteration of transgenic plants from wild types was observed by Lü et al. (2000) when transformation of alfalfa was carried out with the aim of improving sulphur-amino acid content. Cotyledon explants were infected with *A. rhizogenes* strain 9402 with plasmid pBF649 containing a gene encoding protein of high sulphur-amino acid content (HNP) under CaMV35S promoter and *nptII* gene as selectable marker under control of nos promoter. Kanamycin-resistant plants derived from somatic embryos were proven for transgene insertion and integration by Northern hybridisation and recallusing assay. Assay of *HNP* gene showed that transgenic plants had significantly higher contents of sulphur amino acids compared with the control. They also demonstrated a negative correlation between age of hairy roots and embryogenesis frequency in alfalfa plants. Segregation of transgene in the progeny was Mendelian.

The susceptibility of alfalfa somatic embryos to *A. tumefaciens* infection was reported by Ninkovic et al. (1995). They selected somatic embryos as explants source for transformation of alfalfa via *A. tumefaciens* strains A28l and LBA 4404. Well-developed embryos were separated and cloned. Under constant kanamycin selection, clones were selected for proliferation by repetitive somatic embryogenesis. Transgenic nature of kanamycin-resistant clones was confirmed by *nptII* and/or GUS assay and Southern hybridisation. Unfortunately no seeds resulted from these transformants as all plants died due to adverse weather conditions when transferred to soil. By modifying and improving this protocol, Ninkovic et al. (2004) were able to regenerate high frequency of transformed alfalfa plants when somatic embryos were transformed with *A. tumefaciens* LBA4404 carrying the novel superbinary vector pTOK233. Based on hygromycin selection, up to 14 % transformation efficiency was achieved for *M. sativa* L. cv. Zajecarska 83. Such high transformation efficiency was attributed to the presence of an extra set of *vir* genes in superbinary vector pTOK233.

Shao et al. (2000) developed and compared two efficient regeneration protocols after transformation of tetraploid lines of alfalfa (*M. falcata* L.). Leaf explants transformed with LBA4404 and EHA101 harbouring different plasmids, all containing *nptII* as selectable, regenerated kanamycin-resistant plants either via direct somatic embryogenesis or via indirect production of somatic embryos from embryogenic callus. The former regeneration path proved to be more efficient but both systems led to production of transgenic plants as confirmed by recallusing assay on kanamycin, PCR and Southern blot analysis.

Samac et al. (2004) compared the activity of five constitutive promoters by *A. tumefaciens-mediated* stable transformation in the highly regenerable alfalfa clone Regen-SY. Transformation was carried out as described by Austin et al.

(1995). Two marker genes, i.e. *gus* and endochitinase gene (*ech42*) from biocontrol fungus *Trichoderma atroviride* conferring resistance against fungal pathogens were used to monitor the expression driven by promoters in different plant tissues. Based on GUS expression (measured by histochemical staining and quantified by fluorometric assay) and endochitinase activity, cassava vein mosaic virus (CsVMV) promoter was suggested to be useful for high level transgene expression in alfalfa. Although heterologous protein activity in tissues of transformed plants was several fold greater than in controls, none of the transgenic plants showed a consistent increase in disease resistance compared to controls when inoculated with *Phoma medicaginis var. medicaginis*, the causal agent of spring black stem and leaf spot in alfalfa.

Using the same protocol as Austin et al. (1995), the same endochitinase gene (*ech42*) fused in frame of a white lupin signal peptide acid phosphatase (APase) under CsVMV promoter was heterologously expressed in alfalfa by Tesfaye et al. (2005). Signal peptide caused the expression of endochitinase in plant rhizospheres. Relative RT-PCR was employed for determination of endochitinase mRNA transcript level in leaf and root tissue of transgenic alfalfa plants.

In order to exploit the potential to improve the quantity and quality of alfalfa forage, onset of leaf senescence was efficiently delayed in alfalfa when leaf disc explants were inoculated with *Agrobacterium* culture containing *ipt* gene translationally fused with the senescence-specific promoter SAG12 (Calderini et al. 2007). Transgene integration into transformants was determined by Southern analysis while expression of transgene was measured by RT-PCR. Transformed plants stayed green for a longer time hence providing forage for longer.

Rosellini et al. (2007) reported the development of an herbicide/antibiotic marker free genetic transformation system in alfalfa. A novel selectable marker gene (SMG) *hem*L encoding a mutant form of the enzyme glutamate 1-semialdehyde aminotransferase (GSA-AT) was transferred via *A. tumefaciens* [with the protocol by Austin et al. (1995)] and its efficiency was then compared with *nptII* selection system. Gabaculine (the selective substance for *hem*L gene) and kanamycin (the selective substance for *nptII* gene) were used to screen the transformants. Gabaculine-based system was more efficient than the conventional, kanamycin-based system. Inheritance of *hem*L gene in the progeny was Mendelian.

Since last 2 decades, an extensive research rendered the genetic transformation of Alfalfa possible, but it remains strongly genotype dependent and is restricted to a few highly regenerable genotypes. A simple, efficient, genotype independent and marker free *in planta* transformation protocol for successful alfalfa transformation was developed by Weeks et al. (2008). Young decapitated seedlings were immersed and vacuum infiltrated or vortexed vigorously in *A. tumefaciens* LBA 4404 suspension supplemented with sand. Rigorous vortexing significantly enhanced transformation frequencies over vacuum infiltration. Developing plants were assayed for GUS expression by histochemical staining and fluorometric analysis. This novel method has several advantages over conventional methods as it limits the time, materials and resources needed and is applicable to commercial varieties.

The capacity of regeneration of various local alfalfa cultivars was evaluated using different explant sources on different media by Zhang et al. (2010). Xinjiang Daye cultivar showed better frequency of callus formation while using hypocotyl explants. Frequency of callusing and shoot differentiation was further increased on medium with glutamine, shortening the period of regeneration. They then used this system to transform alfalfa via *A. tumefaciens* strain LBA 4404 containing vector p2GS–P5CS carrying two glutamine synthetase (GS) genes. Basta-resistant transgenic plants were proved for transgene integression by PCR analysis and Southern hybridisations. Effective expression of GS in transformants suggested it as a selectable marker in plant genetic transformation for screening of transformants hence avoiding the use of herbicides or antibiotic resistance genes.

Ferradini et al. (2011a) compared two different marker free *Agrobacterium*-mediated transformation methods in alfalfa, i.e. marker-less transformation and co-transformation. In the former, leaf explants were transformed with a vector carrying *nptII* and *gus* markers. Somatic embryos were regenerated without selection followed by kanamycin selection for the second cycle of regeneration from these embryos. The percentage of transgenic embryos was determined by GUS staining and PCR screening of T1 progenies. In the latter technique, explants were co-transformed with two vectors carried separately in *Agrobacterium* cultures. After crossing the primary transformants with a non-transgenic pollinator, marker-free segregants were obtained in T_1 progenies, confirmed by Southern hybridisation. Although the transformation efficiency using both marker-free approaches was low, these methods can be used as a tool for production of marker-free plants.

Instead of using bacterial-derived SMGs during genetic transformation, Ferradini et al. (2011b) used an alfalfa-derived mutant of glutamate 1-semialdehyde aminotransferase (*MsGSAgr*) gene and compared its efficacy with mutated *Synechococcus elongates GSA* (Rosellini et al. 2007). Inoculated explants produced green embryos in the presence of 30-μM gabaculine while all controls died on this media. Stable integration and expression of *MsGSAgr* were assessed by tailed PCR and gabaculine resistance. This novel plant-based marker system is promising for future studies.

4.1.2 Barrel Medic

Successful genetic transformation of *Medicago truncatula* was reported for the first time just under 20 years ago (Thomas et al. 1992; Table 2). *M. truncatula* was proposed as a model system for legume genomics (Cook 1999), taking advantage of the superior in vitro regeneration (via somatic embryogenesis) and transformation characteristics of a few genotypes, among which are Jemalong 2HA, M9-10a and R108-1. Indeed, the limiting step in the entire process is generally the in vitro regeneration of transformed cells into transgenic plants. Transformation protocols can be classified according to the nature of the regeneration process either via embryogenesis or organogenesis. The most efficient protocols currently available use embryogenesis. However, the efficiency of somatic embryogenesis is highly

Table 2 Barrel medic (*Medicago truncatula* Gaertn.) transformation

Agrobacterium strain[a]	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
EHA101	Jemalong	Leaves	*nptII*	1 plant recovered	Thomas et al. (1992)
LBA4404	Jemalong A17	Cotyledons	*Bar, nptII*	3.11	Trieu and Harrison (1996)
LBA4404	Jemalong A17, 2HA1, 2HA3-9-10-3	Leaflets	*nptII, GUS*	2–29	Chabaud et al. (1996)
A281	R 108-1, Ghor, 131-1, 139-2, E4258	Hypocotyls, roots, petioles, leaflets	*GUS*	4.5–5	Hoffman et al. (1997)
EHA105	R108-1(c3)	Young leaves	*GUS*	2–3	Trinh et al. (1998)
EHA105	Jemalong A17	Young seedlings, flowering plants	*nptII, bar*	4.7–76 % (flower infiltration) and 2.9–27.6 (seedling infiltration)	Trieu et al. (2000)
A. rhizogenes A4T pLP100 pMtENOD11–gusA binary vector	Jemalong J5	Hypocotyls, epicotyls and seedling radicles	*nptII, GUS*	33–63 (hairy roots)	Boisson-Dernier et al. (2001)
EHA105	R108-1 (c3)	Young leaves	*GUS*	Not given	Scholte et al. (2002)
EHA105	R-108-1, Jemalong J5	Floral organs	*nptII, GUS, GFP*	High but not precised	Kamaté et al. (2000)
LBA 4404, C58pMP90, C58pGV2260, AGL1	Jemalong 2HA	Leaflets	*nptII, GUS*	26	Chabaud et al. (2003)
EHA105 or AGL1 with pCB302-phas-GUS	A17	Cotyledons	*PPT, GUS, GFP*	3–15	Zhou et al. (2004)
EHA105	Jemalong M9-10a	Leaflets	*nptII, GUS*	25–45	Araujo et al. (2004)
LBA4404	R-108-1	Embryo clusters from cotyledons and petiole base	*nptII, GUS*	9	Iantcheva et al. (2005)
AGL1, C58C1, EHA105, LBA4404	R-108	Root segments	*bar, GUS*	10.6–41.3	Crane et al. (2006)
EHA105	Jemalong M9-10a	Leaves	*GFP, GUS*	15.7	Duque et al. (2007)
EHA105	R-108	Leaves	*nptII, GUS*		Scaramelli et al. (2009)
EHA105	Jemalong M9-10a	Leaves	*nptII, ipt*	7.8–11.7	Confalonieri et al. (2010)

[a]All strains of *A. tumefaciens* unless indicated otherwise

genotype dependent. Thomas et al. (1992) and Rose et al. (1999) selected and used genotype 2HA, which belongs to *M. truncatula* ssp. *truncatula*, the same as genotype M9-10a used by several authors (Santos and Fevereiro 2002; Araujo et al. 2004; Confalonieri et al. 2010), while Trinh et al. (1998) worked with genotype R108, sometimes assigned to *M. truncatula* ssp. *tricycla* (Chabaud et al. 2007). Being the genotype used for genome sequencing and for mutagenesis and TILLING programs, Jemalong is also the genotype of choice when requiring genetic crossing of transgenic plants with characterised mutants.

In addition to the use of such highly embryogenic genotypes, using hypervirulent strains of *A. tumefaciens*, such as AGL1 or EHA105 (see above), can also significantly increase transformation efficiency. Several agents have been reported for the selection of transformed cells including kanamycin, hygromycin or phosphinothricin (Chabaud et al. 2007).

Hashimoto et al. (1992), Trinh et al. (1998), Scholte et al. (2002), among others, obtained barrel medic transformants using leaves as explants and subsequently regenerating the produced callus into plants through somatic embryogenesis. An efficient transformation procedure for barrel medic has also been developed by Kamaté et al. (2000), where flower parts were used as explants followed by regeneration through embryogenesis, while Trieu and Harrison (1996), aimed at reducing the tissue culture work involved, developed a new method based on cotyledonary node explants, followed by regeneration of multiple shoots from the pre-existing meristems. Some time later, Trieu et al. (2000) reported two *in planta* procedures for transformation based on infiltration of flowers and seedlings but were later unable to reproduce them, nor were they corroborated by other laboratories.

4.1.3 Lotus japonicus

Lotus japonicus is a wild legume that has become a model plant for genome studies in legumes, particularly in reference to rhizobial and arbuscular mycorrhizal symbiosis. As such, several teams have undertaken its genetic transformation over the last 20 years (Table 3).

Handberg and Stougaard (1992) optimised the regeneration protocol of *L. japonicus* and then assayed tissue susceptibility to *A. rhizogenes* and *A. tumefaciens*. Hypocotyls challenged with *A. rhizogenes* gave hairy roots capable of regeneration. When transformed with various disarmed strains of *A. tumefaciens*, 90 % of hypocotyl explants gave to transgenic callus based on kanamycin/hygromycin resistance. Hygromycin-based selection was more efficient and rapid. When the wild-type plant was pollinated with pollen from transformed plants, 25 % of plants in the progeny were hygromycin resistant.

In order to study the behaviour of maize transposable elements in *L. japonicus,* Thykjaer et al. (1995) carried out *A. tumefaciens*-mediated transformation with transposable element *Ac* following the protocol reported by Handberg and Stougaard (1992). Kanamycin-resistant calli showed variegated sectors after GUS

Table 3 *Lotus japonicus* L. transformation

Agrobacterium strain[a]/method	Genotype/Cultivar	Explants	Selection agent/marker genes	Transformation efficiency (%)	References
LBA4404, C58, or GV2260	Gifu B-129, B-177	Hypocotyls, leaves	Hygromycin, kanamycin	Not given	Handberg and Stougaard (1992)
SLJ1931 T-DNA	Gifu B-129	Cotyledons	*nptII, GUS*	42	Thykjaer et al. (1995)
C58Cl	Gifu	Plantlet stems at cotyledon	*nptI1*	Not given	Oger et al. (1996)
LBA4404	Gifu B-129-56	Hypocotyls	*GUS*	70	Stiller et al. (1997)
MSU440	Gifu	Roots, hypocotyls	*GUS p35S-hpt-tml*	23.5, 13.3	Martirani et al. (1999)
AGL1	Gifu B-129	Hypocotyls	*Bar*	70	Lohar et al. (2001)
EHA101	Gifu B-129	Hypocotyls	*GUS/HPT*	11–20	Aoki et al. (2002)
A. rhizogenes LBA1334	Gifu B129	Hairy roots	*GUS*	60	Kumagai and Kouchi (2003)
AGLO and AGL1	GIFU B-120	Roots	*Hpt/GUS*	58	Lombari et al. (2003)
LBA 4404/GPTV	Gifu	Hypocotyls	Geneticin	90	Edwards et al. (2004)
LBA 1334	Gifu	Hypocotyls	*GUS*	62	Díaz et al. (2005)
LBA4404	MG-20	Hypocotyls	*GUS*	Not given	Kato et al. (2005)
A. rhizogenes	Gifu ecotype F9	Roots	*Hygromycin/GUS*	Not given	Lombari et al. (2005)
A. tumefaciens	Not given	Hypocotyls	*Hygromycin/GUS*	Not given	Tirichine et al. (2005)
A. rhizogenes, A. tumefaciens	Not given	Hypocotyls	*nptII, hptII, bar*	Not given	Udvardi et al. (2005)
EHA105, AGL1, LBA4404	Gifu B-129	Hypocotyls, cotyledons	*Hygromycin*	0.9–12.8	Wang et al. (2010)

[a]All strains of *A. tumefaciens* unless indicated otherwise

staining, hence showing somatic activity of *Ac* in *L. japonicus* calli. Regenerated primary transformants were selfed and GUS expression in the cotyledons of the progeny suggested that maize transposable elements were mobile in *L. japonicus* and that the transposition mechanism operating in *L. japonicus* was similar to that in maize. Mobility and reinsertion of *Ac* element were also proved by Southern hybridisation.

Oger et al. (1996) developed a novel method of genetic transformation based on a hormone independent regeneration system. They inoculated decapitated cotyledons with a disarmed *A. tumefaciens* strain harbouring plasmid carrying nopaline synthase (*nos*) gene and *nptII* gene conferring kanamycin resistance. Nopaline presence was investigated by high voltage paper electrophoresis analysis. The transgenic nature of the nopaline positive kanamycin-resistant plants was confirmed by PCR and Southern hybridisation but no information about progeny was provided.

The susceptibility of *L. japonicus* to a large number of wild-type strains of *A. rhizogenes* for production of hairy roots was evaluated by Stiller et al. (1997), and strains 9402 and AR10 proved to be most virulent. To assess the applicability of the hairy root system for molecular analysis of plant genes involved in nodulation, hypocotyl explants were inoculated with these strains carrying binary vectors with *uidA* and *luc* as selectable marker genes driven under CaMV35S promoter. About 80 % of hairy root lines regenerated fertile transgenic plants. Expression of *gusA* and *luc* was investigated through histochemical staining and luciferase assay. Seventy percent of *A. rhizogenes*-induced hairy roots were capable of normal nodulation. Martirani et al. (1999) used the high frequency hairy root induction ability of *A. rhizogenes* for T-DNA tagging of nodulation and root-related genes in *L. japonicus*. Wounded root explants were inoculated with *A. rhizogenes* with vector p35S-gusA-int. The study of wound sites indicated that transgenic roots developed via direct organogenesis from the primary root near the wound site. Plant lines expressing intense GUS activity in the root meristem and the vascular tissues in nodulation-specific manner were developed from hairy roots induced from inoculated explants. Kumagai and Kouchi (2003) used a hairy root-based transformation system for studying the post-transcriptional gene silencing of genes expressed in roots and nodules of *L. japonicus*. After inoculation with *Mesorhizobium loti,* transgenic plants showed GUS expression in root nodules driven either by CaM35S promoter or lj27 promoter (specific for nodule-infected cells). Expression of *gus transgene* in nodules and other root parts was silenced by transforming them with *A. rhizogenes* strain LBA1334 harbouring pHKN30 or pHKN31 plasmid expressing hairpin RNAs with sequences complementary to the *gus* coding region. *Gus transgene* was either completely silenced or its activity was significantly decreased in transformed tissues when its expression was observed in root nodules of hairy roots as compared to control plants. From these results, Lohar and Bird (2003) suggested *L. japonicus* as a powerful model legume to study functional genomics of nodule formation and plant–nematode interactions. The above protocols work well but it took long time for rooting of transformants, hence production of T_0 seeds. To overcome this, Díaz et al. (2005) developed a modified protocol for rapid production of transgenic plants, thus facilitating the study of root

nodule regulating genes. After transformation with *A. rhizogenes,* hairy roots were induced that could be inoculated with *M. loti* after 2 weeks. The nodules thus emerged were comparable to those of wild type, and expression studies of genes involved in nodulation could be completed within 2 months.

Lohar et al. (2001) reported a simple *A. tumefaciens*-based protocol for genetic transformation of *L. japonicus* using herbicide resistance marker. Hypocotyl explants were transformed with *A. tumefaciens* strain AGL1 equipped with binary vector pTAB10 with *bar* as selectable marker gene driven by CaMV35S promoter. PPT-resistant plants checked for transgene insertion by PCR were morphologically normal. The transgene was segregated in the progeny as demonstrated by chlorophenol red assay and phosphinotricin spray.

An optimised *A. tumefaciens*-mediated transformation system of *L. japonicus* based on antibiotic selection and efficient regeneration was developed by Aoki et al. (2002) using strain EHA 101 harbouring binary vector carrying *GUS* reporter gene and two selectable marker genes, i.e. *nptII* and *hpt* conferring resistance to kanamycin and hygromycin, respectively. Hygromycin-resistant shoots were regenerated from calli. This optimised protocol ensured survival of only the transformed plants on selection medium, as GUS activity was observed in all of the transformed shoots while all non-transformants were dead.

Lombari et al. (2003) obtained transgenic *L. japonicus* plants when regeneration-competent dedifferentiated root explants were inoculated with AGLO and AGL1 strains of *A. tumefaciens* containing *hpt* as selectable marker and *gus* as reporter gene. Transformation efficiency as demonstrated by GUS expression was several fold than that with the transformation procedure using hypocotyl explants. It took 4 months to get transformed plants. After selfing the primary transformants *hpt* segregated in Mendelian fashion.

Edwards et al. (2004) used the transformation protocol developed by Thykjaer et al. (1995, 1998) for structural manipulation of a plant cell wall polysaccharide by downregulating the Galacto-mannan glycosyltransferases (GMGT) responsible for its polymerisation. *L. japonicus* GMGT cDNA cloned in various orientations driven under dual 35 S promoter was expressed in seed endosperm of transformed plants. Geneticin-based antibiotic selection was employed to obtain transgenic plants. Transgenic lines exhibited galactomannans with higher mannose/galactose values in their seeds, consistent with post-transcriptional gene silencing.

Kato et al. (2005) modified the *A. tumefaciens*-based transformation method for *L. japonicus* genotype MG-20 by optimising hormone concentration (auxin and cytokinin), and used this method for heterologus expression of an allergin protein used in immunotherapy, in mature plants. Hypocotyl explants were transformed with *A. tumefaciens* strain LBA4404 with pDerf1 plasmid containing *Derf1* gene from American house dust mite *Dermatophagoides farina*. Based on Southern hybridisation results, expression of Derf1 protein in transformants was confirmed by RT-PCR. The size of plant synthesised Derf1 protein was exactly comparable to that of native Derf1 purified from *D. farina* proved by Western blotting.

An *A. tumefaciens*-based protocol with improved regeneration was reported by Tirichine et al. (2005). After co-culturing explants with *Agrobacteria*, hypocotyl

callus was selected for geneticin resistance. Shoots were induced from transgenic calli on media with cytokinin (BAP) and were rooted on auxin (NAA) containing media.

Lombari et al. (2005) compared several methods for genetic transformation of *L. japonicus* using various explants and regenerating putative transformants on different media. When using dedifferentiated roots as explants for *A. tumefaciens*-based transformation, the advantage laid in the availability of explants that can be stored at 4 °C for long time and are hence readily available when needed. When using *A. rhizogenes*-based hairy root transformation method, the study of root or nodulation-related genes is facilitated.

In some previous protocols, transformation frequency remained low due to quite a high rate of false positives. Wang et al. (2010) employed an efficient screening system based on hygromycin followed by regeneration on medium supplemented with auxin (IAA) to efficiently select and regenerate the transformants. They then tested three strains, i.e. LBA4404, AGL1 and EHA105 for their susceptibility to transform *L. japonicus*. Among them, EHA105 which had never been tested previously for *L. japonicus* gave a tenfold higher transformation efficiency than the other two strains. This improved transformation, selection and regeneration system was then used for silencing of different *CYCLOIDEA* genes in *L. japonicus* through RNAi technology.

4.2 Miscellaneous Forage Legumes

Trifolium repens, the white clover, a forage legume native to Europe, North Africa and West Asia is among the first legume species where *A. tumefaciens*-mediated transformation was successfully carried out (White and Greenwood 1987). Stolon internode segments were transformed with two non-oncogenic *A. tumefaciens* strains, LBA4404 and GV3850. Kanamycin-resistant calli were assayed for in situ neomycin phosphotransferase II activity. Transgenic nature of cells and shoots was further assessed by the presence of nopaline from *nopaline synthase* gene as well as *nptII* specific probe hybridisation to DNA fragments. In order to improve the nutritional quality of legumes, Ealing et al. (1992) followed the same protocol to overexpress a chimaeric gene encoding the pea albumin 1 (PAl) protein rich in sulphur amino acids under 35 S CaMV promoter into the white clover mediated via *A. tumefaciens*. In transgenic plants, the abundance and stability of the PAl protein was assessed by immunoselection of in vivo [^{35}S] Na_2SO_4-1abelled plant proteins.

Medicago varia, a forage legume also known as bastard alfalfa, is also among the first forage legume species whose successful *A. tumefaciens*-mediated transformation was reported (Deak et al. 1986). Kanamycin-resistant transformed plants were regenerated through somatic embryogenesis from callus induced from stem cuttings. The transgenic nature of developing plants was confirmed by neomycin phosphotransferase activity and southern hybridisation.

Astragalus sinicus, a model forage legume generally known as Chinese Milkvetch, is very widely grown as a green-manure-cum-forage legume. Tryptophan (Trp) is an essential amino acid since it is not synthesised by animals and must be obtained in the diet of non-ruminants such as pigs, poultry and humans. In order to increase the Trp contents in legumes, the *A. rhizogenes*-mediated hairy root transformation of *A. sinicus* was carried out by Cho et al. (2000), overexpressing Trp feedback-insensitive *ASA2* gene. Northern-blot hybridisation demonstrated constitutive expression of p35S-*ASA2* gene in the transgenics. Expression of the transgene resulted in a 1.3- to 5.5-fold increase in free Trp. Overexpression of this gene in *A. sinicus* can be used as a new tool for studying the regulation of Trp biosynthesis in legumes.

Stylosanthes guianensis, the Brazilian lucerne, is a forage legume cultivated in tropical and subtropical regions of the world. In order to enhance foliar blight resistance, Kelemu et al. (2005) carried out *Agrobacterium-mediated* transformation of rice chitinase gene under the control of CaMV35S promoter in leaf segment explants. Transformed calli were selected on medium containing kanamycin, and regeneration was via somatic embryogenesis. Ten-week-old plants were artificially inoculated with sclerotia and were then evaluated for their reactions to *Rhizoctonia solani*, starting 6 days after inoculation. Thus, transgenic plants had a higher level of resistance to *R.solani* than did control plants. DIG-labelled chitinase probe during Dot blot analysis confirmed the stable inheritance of rice-chitinase gene to the progenies along with resistance to *Rhizoctonia* foliar blight disease.

Lotus corniculatus, the Bird's-foot trefoil, is a perennial, fine-stemmed, leafy legume native to grassland temperate Eurasia and North Africa with and increasing importance in agriculture as pasture and hay crops in recent years. Tanaka et al. (2008) obtained transgenic *L. corniculatus* from Superroot-derived leaves via *A. tumefaciens*-mediated transformation. Transgenicity of kanamycin-resistant calli was verified by GUS staining, but transformation efficiency was low and the process from gene transfer to PCR identification took 6 months. Jian et al. (2009) developed an improved and highly efficient *A. rhizogenes*-mediated transformation of Superroot-derived *L. corniculatus* by introducing the efficient Superroot regeneration system. Transgenic nature of plants was verified by GUS staining as well as GFP observation, with a frequency up to 92 % based on detection of GUS activity. Transgene integrity and stability were examined by Southern (DIG method) and Western blot analysis.

4.3 Grain Legume Crops

4.3.1 Soybean

Soybean was the first grain legume for which transgenic plants were produced via both *Agrobacterium*-mediated transformation (Hinchee et al. 1988; Parrott et al. 1989; Di et al. 1996) and particle bombardment (McCabe et al. 1988; Christou et al.

Table 4 Soybean (*Glycine max* [L.] Merr.) transformation

Agrobacterium strain/method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
pTiT37-SE	Century, Cobb, Douglas, Harosoy, Heilongjiang I0, Heilongjiang 26, JI03, Lee, Manchu, Manitoba Brown, McCall, Peking, PI 283332, Williams 82	Cotyledons	*nptII*	6	Hinchee et al. (1988)
LBA4404, EHA101		Immature embryos	*nptII*	0.03	Parrott et al. (1989)
Plasmid pZA300 electroporation	Clark 63	Immature cotyledon protoplasts	*Hpt, GUS*	0.29–0.68	Dhir et al. (1991)
Biolistic—pMON 10026 or pMON 13671 plasmids	Williams 82, Fayette	Shoot tips from immature green seeds, embryogenic cell suspensions	*nptII, GUS*	0.4	Sato et al. (1993)
Biolistic—vector pIC35/A with *BtCrylAC* gene	Jack	Somatic embryos	*Hph, BtCrylAC*	Many plants recovered no precise figure given	Stewart et al. (1996)
Biolistic—12 plasmids	Fayette	Embryogenic suspension	*HPH, GUS*	Not given	Hadi et al. (1996)
Z707		Cotyledonary nodes	*nptII*	1.25	Di et al. (1996)
Biolistics—plasmids 35 S-SGFP-TYG-nos (pUC18) and HBT-SGFP-TYG-nos (pUC18)	Chapman	Embryogen suspension	*Hygromycin B, GFP*	Not given, no fertile plants recovered	Ponappa et al. (1999)
AGL1	Bert	Axillary buds, cotyledonary nodes	*bar*	0.7	Olhoft and Somers (2001)
EHA101	Bert	Cotyledonary nodes	*hph*	16.4	Olhoft et al. (2003)
EHA101		Cotyledonary nodes	*bar*	2–6	Paz et al. (2004)

(continued)

Table 4 (continued)

Agrobacterium strain/method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
	Bert, Harosoy, Jack, Peking, Thorne, Williams, Williams 79, Williams82, Clark, Delsoy 5710, Essex, Ogden				
EHA105	Nannong88-1, Nannong18-6, Yu23, Nannong 87C-38	Cotyledonary nodes	*nptII, GFP*	1.39–2.20	Xinping and Deyue (2006)
EHA105, LBA4404, KYRT1	Hefeng 25, Hefeng 35, Hefeng 39, Heinong 37, Heinong 43, Dongnong 42, Lefeng 39	Embryonic tips	*Bar, GUS*	4.29–18	Dang and Wei (2007)
EHA105	Kariyutaka	Cotyledonary nodes	*Bar, GFP*	1	Sato et al. (2007)
EHA105	Kariyutaka	Cotyledonary nodes	*Bar, GUS*	4.4	Yamada et al. (2010)

1989), and the plant recovery approaches used ranged from somatic embryogenesis from immature seeds to non-adventitious organogenesis from cotyledonary nodes of seedlings or germinating seeds (Table 4). Nevertheless, genetic transformation of soybean remains non-routine, as most methods show a reduced efficiency of 0.2–2 % and, very rarely, up to 5–6 %. The low efficiency of T-DNA transfer itself and the regeneration systems used to recover transformed plants are the two main limiting factors. The first was improved through the use of more virulent *Agrobacterium* strains (Torisky et al. 1997) and addition of e.g. L-cysteine, dithiothreitol (DTT) and sodium thiosulfate during co-cultivation of explants with the bacteria (Olhoft and Somers 2001; Olhoft et al. 2001, 2003). Conversely, progress made in improving regeneration (Somers et al. 2003) is scanty, as somatic embryogenesis is highly genotype specific and associated with extensive somaclonal variation among regenerated plants (Parrott et al. 1989). Based on proliferation of meristems pre-existing in the cotyledonary node, organogenesis is less genotype dependent and was thus adopted by several groups (Dan and Reighceri 1998). However, meristems are complex structures where meristematic cells represent only a very low proportion of the total cells in the explant, and the recovery of transgenic plants capable of transmitting the target genes to subsequent generations is very low with this method (Christou et al. 1990).

Dang and Zhi-Ming (2007) developed an optimised method for *Agrobacterium*-mediated transformation for expression of binary insect resistance genes, where they examined the effects of several factors by measuring transient expression levels of β-glucuronidase and the number of resistant explants with phosphinothricin selection. The hypervirulent *A. tumefaciens* strain KYRT1 proved to be better than EHA105 and LBA4404. Improved transformation efficiencies were obtained when embryonic tips were incubated with an *Agrobacterium* suspension for 20 h, in an acidic medium, and then co-cultivated at 22 °C in the dark for 5 days. By combining the best treatments, transgenic plants of seven soybean cultivars were obtained, most of which were fertile, with a transformation frequency ranging from 4.3 to 18 %. Analysis of T1 plants showed inheritance and stable integration of transgenes, coupled with a high resistance to cotton bollworm. More recently, Yamada et al. (2010) used cotyledonary nodes wounded with a micro-brush whereby they increased the frequency of transformation.

Direct transformation by particle bombardment has been reported on soybean (Dickins et al. 2003). The first transgenic soybean plant developed by direct gene delivery was created in 1988 (McCabe et al. 1988). The first commercially available transgenic Roundup Ready soybean was also developed by particle bombardment (Padgette et al. 1995). Due to the importance of soybean as a crop, several other transformation methods were examined, including whole plant transformation, pollen tube and pollen transformation, *in planta* electroporation or protoplast-based transformation (Dickins et al. 2003, and references therein).

Paz et al. (2004) studied the conditions required for an efficient *Agrobacterium*-mediated transformation of cotyledonary node explants. They were able to increase the transformation efficiency up to threefold by using cysteine and DTT during co-cultivation. Similar results of increase in transformation efficiency were obtained

by Xinping and Deyue (2006) who transformed multiple soybean cultivars by infecting cotyledonary nodes with *A. tumefaciens* while adding thiol compounds during co-cultivation. Zeng et al. (2004) refined the use of antioxidants during co-cultivation and glufosinate selection in *Agrobacterium*-mediated transformation of soybean genotypes which are otherwise difficult to transform.

Sonication of explants has also been used before co-cultivation to improve the transformation efficiency. Santarem et al. (1998) reported an optimisation of transient gene expression following the sonication of immature cotyledons co-cultured with *Agrobacterium* strains.

Townsend and Thomas (1996) patented a method for the *Agrobacterium*-mediated transformation of cultured soybean cells. Among several factors identified, they discovered that temperature during co-cultivation of explants with agrobacteria is an important factor for efficient transformation. They showed that usual temperatures (26–28 °C.) for the culture of soybean cells are inappropriate for efficient transformation. Conversely, a lower temperature resulted in highly effective transformation.

4.3.2 Common Bean

Phaseolus vulgaris usually known as common/dry bean is amongst important food legume serving as an important source of protein and calories in the developing world. Veltcheva et al. (2005) reviewed the problems and progress for in vitro regeneration and genetic transformation of common bean (Table 5).

In 1991, McClean et al. demonstrated the susceptibility of a wide range of dry bean genotypes to infection by *A. tumefaciens* strains A 208, A 281 and LBA 4001. They also transformed cotyledonary nodes of *P. vulgarus* cv Othello with *A. tumefaciens* strain C58Z707 but were unable to regenerate plants from kanamycin-resistant calli. They also established kanamycin-resistant root cultures from hypocotyl tissues infected with the *A. rhizogenes* strain A4RS. Transgenic nature of calli and roots was confirmed by Southern hybridisation.

Russell et al. (1993) used electric-discharge-mediated particle acceleration to produce transgenic navy beans. Apical meristems were bombarded with DNA-coated gold particles under partial vacuum and high voltage discharge. Transgenic plants were recovered through de novo shoot formation. Since the DNA was delivered to organised tissue, many of the problems associated with recovering plants from protoplasts or callus were avoided. Multiple shoots were screened for GUS enzyme activity through histochemical staining followed by PAT activity by herbicide spraying to recover transgenic plants at a rate of 0.03 % germline transformed plants/shoot. DNA presence was confirmed by PCR and Southern blot analysis.

Particle bombardment for production of transgenic dry bean was conducted by Aragao et al. (1996). They co-transformed different genes linked (in the same plasmid) or unlinked (in different plasmids) coated on tungsten particles into embryonic axes using a locally built high pressure helium-driven particle

Table 5 Common bean (*Phaseolus vulgaris* L.) transformation

Agrobacterium strain/method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
A208, A281, LBA4001, C58Z707, A4RS	Pinto, black, white, kidney and Cranberry types	Cotyledonary nodes, hypocotyls	*nptII*	Not given	McClean et al. (1991)
Biolistic	Seafarer	Apical meristems	*bar, GUS, PAT*	0.03 germline transformed plants/shoot	Russell et al. (1993)
Biolistic	Olathe	Eembryonic axes	*GUS*	0.9	Aragao et al. (1996)
C58ClR	Admires, Great Northern Tara, Xan-159	Cotyledonary leaves	*nptII, aadA, GUS*	Not given	Kapila et al. (1997)
Biolistic	–	Embryonic axes	*bar*	03–0.8	Vianna et al. (2004)

acceleration device. Transformed plants were continuously selected on kanamycin and GUS activity. The average frequency of transformation was 0.9 %. The progeny of the self-fertilised transgenic plants was screened by PCR analysis and southern blotting. Seventy-seven percent of these transformed plants segregated in a Mendelian fashion.

Biotic stress plays an important role in reducing bean yields, and Aragao and Rech (1997) transformed apical meristems by the biolistic method with a construct containing viral antisense RNAs and regenerated them via organogenesis. The transgenic plants showed delayed and faded symptoms of Bean golden mosaic geminivirus (BGMV).

Development of an efficient transient gene expression system is indispensable in order to study the expression of different gene promoters and proteins. Kapila et al. (1997) optimised vacuum infiltration-based *A. tumefaciens*-mediated transient expression in bean leaves. Higher final *Agrobacterium* cell density ($OD_{600} = 2.4$) proved to be an important factor for the success of transient expression in bean leaves.

Optimisation of factors influencing *A. tumefaciens-mediated* transformation using several genotypes of common beans was studied by Zhang et al. (1997). When explants derived from mature seeds of susceptible genotypes were injured, precultured and then transformed with *A. tumefaciens* strain A2760, a transformation efficiency of 4 % was achieved as proven by GUS staining. Genotype, explant (age, type and source), pre-cultivation conditions, *Agrobacterium* strains, co-cultivation conditions, selection system and conditions for regeneration are important factors influencing *A. tumefaciens*-mediated transformation (Nagl et al. 1997).

Development of vector free genetic transformation system of legumes and hence avoiding the presence of antibiotic resistance genes is very desirable. Vianna et al. (2004) developed transgenic bean plants by introducing a 1.5-kb linear DNA fragment carrying the *bar* gene using the biolistic method of Aragao et al. (1996).

Transformation frequency was comparable with that using entire circular plasmid. Southern blot analyses revealed a similar pattern complexity in transgenic plants obtained with either the entire plasmid or a DNA fragment. Stable transmission of transgene to the progeny followed the Mendelian ratio (3:1). This method presented a novel approach to get transgenic legumes containing only the gene responsible for a desirable trait.

4.3.3 Peanut

Direct DNA delivery via microprojectile bombardment has become an established approach for gene transfer into peanut (*Arachis hypogaea* L.) (Table 6). Wang et al. (1998) bombarded embryogenic cultures from three peanut cultivars with two plasmid constructs containing a uidA gene controlled by either a soybean vegetative storage protein gene promoter or a CaMV35S promoter. GUS transient expression proved useful to predict stable transformation and confirmed that image analysis provides an efficient method for semi-quantitation of transient expression. They regenerated over 200 transgenic plants from 38 cell lines of which about half were fertile. GUS expression driven by the vspB promoter was modulated by chemical and positional information.

Sharma and Anjaiah (2000) developed a procedure for regeneration via adventitious shoot bud formation from cotyledons excised from mature seeds, and they combined it with transformation to produce one or more independently transformed shoots with an efficiency of up to 55 % of the explants. In parallel, Rohini and Rao (2000) co-cultivated wounded embryo axes with one cotyledon cut off, they were allowed to develop without selection pressure and, ultimately, primary transformants were transferred to the glasshouse with GUS used as reporter gene for identification of transgenic plants; they later used the same approach to generate transformants with variable responses towards leaf spot disease (Rohini and Rao 2001). This procedure subsequently inspired various teams, and Anuradha et al. (2006) used it for transformation with a *gus::npt*II fusion gene-based vector. Likewise, this methodology was also adopted by other teams working with several grain legume species other than peanut, including pea (Svabova and Griga 2008).

Yang et al. (2003) tested the bacterial mercuric ion reductase gene, *merA*, as an alternative selectable marker system. *MerA* reduces toxic Hg(II) to the volatile and less toxic metallic mercury molecule, Hg(0), and renders its source Gram-negative bacterium mercury resistant. A codon-modified version of the gene, *MerApe9*, was cloned into a plant expression cassette containing the ACT2 promoter from *Arabidopsis thaliana* and the NOS terminator. The expression cassette also was inserted into a second vector containing the hygromycin resistance gene driven by the UBI3 promoter from potato. Stable transgenic plants were recovered from somatic embryo tissues bombarded with the plasmid containing both genes. Expression of *merA* as mRNA was detected by Northern blot in leaf tissues of transgenic plants, but not in somatic embryos. Western blot showed production of the mercuric ion reductase protein in leaf tissues.

Table 6 Peanut (*Arachis hypogaea* L.) transformation

Agrobacterium strain/method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
Biolistic	Georgia Runner, Florunner, MARC-1	Embryogenic cultures	*Hph, GUS*	36.5	Wang et al. (1998)
C 58	J-11, ICGS-11, Robut-33-1, ICGS-76, ICGS-44	Cotyledon explants	*nptII, GUS*	55	Sharma and Anjaiah (2000)
LBA 4404	TMV-2	Embryo axes	*nptII, GUS*	3.3	Rohini and Rao (2000)
LBA 4404	TMV-2	Embryo axes	*nptII*	5.33	Rohini and Rao (2001)
C 58	JL-24	Cotyledon explants	*nptII, DREB1*	Not given, 71 confirmed transgenic plants analysed	Bhatnagar-Mathur et al. (2007)
EHA105	JL-24	Embryo axes	*nptII*	7 plants	Anuradha et al. (2008)
EHA101	JL-24	Cotyledon explants	*HptII*	17	Tiwari et al. (2008)
C 58	JL-24	Cotyledon explants	*Marker free*	24-34	Bhatnagar et al. (2010)
LBA 4404	Golden	Embryonic nodes	*Bar*	38	Iqbal et al. (2011)
LBA 4404	Golden, BARI-2000	Cotyledonary nodes	*Hpt*	42	Iqbal et al. (2012)
EHA101 LBA 4404	JL-24	Cotyledon explants	*Hpt, nptII, GUS*	81	Tiwari and Tuli (2012)

Qiusheng et al. (2005) studied the effects of antioxidants on the plant regeneration and GUS expression frequency in peanut explants co-cultured with *A. tumefaciens*. The explants cultured on medium containing different antioxidants were able to produce more shoots and showed no browning of callus and an increased rate of shoot formation as compared to controls. Moreover, antioxidants were able to promote buds and shoots to grow into plantlets. The transformation efficiency as observed through GUS histochemical staining was also significantly increased by using antioxidants.

Most recently, a Rice chitinase-3 under enhance version of CaMV35S was introduced into peanut through *Agrobacterium*-mediation transformation using strain LB4404 harbouring the binary vector (pB1333-EN4-RCG3) containing the chitinase (chit) and hygromycin resistance (hpt) gene as selectable marker (Iqbal et al. 2011, 2012). Putative transgenic shoots regenerated were examined for the presence of the integrated rice chitinase gene and hygromycin resistance and the integration pattern of transgene in the nuclear genome was confirmed through Southern hybridisation. Viable and fertile T0 transgenic plants were produced with over 42 % transformation frequency. T1 plants were tested for resistance against *Cercospora arachidicola* by infection with the microspores. Transgenic strains exhibited a higher resistance than the non-transgenic control, and chitinase gene expression in highly resistant transgenic strains was correlated with fungal pathogen resistance. Also very recently, Tiwari and Tuli (2012) optimised conditions for peanut transformation to reach a transformation efficiency of 81 %.

4.3.4 Faba Bean

As with the other protein legume species, improvement of faba bean using genetic engineering has been limited due to the difficulties in developing an efficient and reproducible regeneration system (Table 7). Nevertheless, in the last 2 decades several regeneration protocols were developed for *Vicia faba* L. (Di Antonio et al. 1988, Fakhrai et al. 1989; Khalafalla and Hattori 2000; Hanafy et al. 2005). Consecutively, faba bean transformation procedures were also developed, particularly those using *Agrobacterium* inoculation of meristematic tissues (Böttinger et al. 2001; Hanafy et al. 2005). *Agrobacterium*-mediated gene transfer with faba bean and related species counts a number of examples, and the subject was recently the object of a comprehensive review (Hanafy et al. 2008).

The first gene transfer studies with *Vicia faba* L. were focused on induction of tumours by *Agrobacterium* virulent strains carrying the Ti plasmid. During further culture of this faba explants, intensive root proliferation was observed (Di Antonio et al. 1988; Siefkes-Boer et al. 1995). Jelenic et al. (2000) inoculated the stems of seedlings of three broad bean cultivars with *A. tumefaciens* wild-type strains (A281 and B6S3), transconjugant strains (C58C1(pArA4abc) and C58C1 (pArA4b)), the B6S3 root and shoot mutants (GV3101(pGV2255), GV3101 (pGV2215) and GV3101(pGV2235)) and *A. rhizogenes* wild-type strains (8196 and 15834). Cultivars differed in susceptibility to strains and a plant genotype::

Table 7 Faba Bean (*Vicia faba* L.) transformation

Agrobacterium strain[a]/method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
A. tumefaciens strains A281, B6S3, GV3101, C58C1, *A. rhizogenes* strains 8196, 15834	Lobab Lippoi, Topolo, Oslje	excised cotyledons, leaves, internodal stem segments	–	20–90 % based on induced tumour	Jelenic et al. (2000)
EHA101	G 461, G 674	Cotyledons, Shoot apex	*nptII, GUS*	0–50 % based on Kanamycin resistance	Ismail et al. (2001)
EHA101, EHA105	Mythos	internodal segments	*nptII, GUS*	0.025	Böttinger et al. (2001)
EHA101, EHA105	Mythos,Albatross, Giza2, Giza 429, Giza blanka,Giza 716	embryo axes, internode segments, leaf explants	*nptII, bar*	0.15–2.0	Hanafy et al. (2005)
Biolistic	Giza2, G3, G40, G429, G461, G716, G834, Misr1, Sakha1, Nubaria1	Embryo axes, Shoot apex	*Bar, GUS*	2	Metry et al. (2007)
Biolistic	Otafuku	Leaves	*GFP*	Not given	Melhorn et al. (2008)

[a]All strains of *A. tumefaciens* unless indicated otherwise

strain interaction was detected but only unorganised tumour tissue was obtained. In vitro transformation with Ri plasmid-containing bacterium strains was similarly unsuccessful.

Böttinger et al. (2001) reported the first reliable transformation method for faba bean (*Vicia faba* L.), using stem segments from aseptically germinated seedlings as explants undergoing callusing and with transgenic shoots regenerated from such stem calluses at a frequency of 10–30 % of the initial explants used.

Using microprojectile bombardment, cotyledon and callus explants of *Vicia faba* L. were transformed (Ismail et al. 2001) and transformants were evaluated for NPTII presence by PCR Southern blot hybridisation and GUS assay. Transformation of *Vicia faba* L. using microprojectile bombardment with the plasmid pCG1258 containing *bar* and *gus*-intron genes was developed also using mature embryos (Metry et al. 2007). Faba bean cultivars were utilised to produce transgenic faba bean (2 %). Expression of the transgenes was evaluated by PCR, Southern blotting and GUS assay.

Hanafy et al. (2005) developed a system based upon direct shoot organogenesis after transformation of meristematic cells from embryo axes with *A. tumefaciens* strain EHA105/pGlsfa harbouring a binary vector with a gene encoding a sulphur-rich sunflower albumin (*SFA8*) linked to the bar gene, and strain EHA 101/pAN109 carrying the binary plasmid containing the coding sequence of a mutant aspartate kinase gene (*lysC*) from *E. coli* in combination with *nptII* gene. The coding sequences of *SFA8* and *LysC* genes were fused, respectively, to the seed-specific promoters *V. faba* legumin B4 (LeB4) and phaseolin. Seven phosphinothricin (PPT) resistant clones were recovered, with integration, inheritance and expression of transgenes confirmed by Southern blot, PCR, enzyme activity assay and Western blot.

Youssef et al. (2007) transformed embryogenic axis explants with *A. tumefaciens* strain LBA-4404 harbouring AFP plasmid which contains defensin gene under the control of CaMV 35S promoter, NOS terminator and *gus* intron as a reporter gene. The integration of the defensin transgene into the genome of faba bean plants was confirmed by PCR assay, and *gus* gene expression indicated that some of the putative shoots were indeed transformed.

In 2008, Melhorn et al. examined the competence of guard cells to synthesise ABA, using two Arabidopsis enzymes of ABA biosynthesis, p35S::AtNCED3–GFP and AAO3–GFP, that were introduced into guard cells of broad bean leaves. AtNCED3–GFP expression was detected at the chloroplasts, while GFP and AAO3–GFP were expressed in the cytosol. The stomatal aperture was decreased in AtNCED3–GFP and AAO3–GFP-transformed guard cells. This indicated that ABA biosynthesis is stimulated by heterologous expression of AtNCED3 and Arabidopsis aldehyde oxidase 3 (AAO3) proteins, which both seem to be regulatory enzymes for ABA biosynthesis in these cells. Stomatal closure by the expression of AtNCED3 and AAO3 also suggested that the substrates of these enzymes are present and native ABA-biosynthesis enzymes are active in the guard cells.

4.3.5 Chickpea

Chickpea regeneration is possible with varying degrees of success but, to date, there have been few successful reports of production of transgenic chickpea plants using Agrobacterium-mediated transformation (Fontana et al. 1993; Krishnamurthy et al. 2000; Polowick et al. 2004; Sarmah et al. 2004; Senthil et al. 2004; Tewari-Singh et al. 2004) and only one on biolistic transformation (Kar et al. 1997), as detailed in Table 8. Most of these articles showed variations in transformation efficiency, number of transgenic plants recovered, rooting efficiency and subsequent establishment of the plants in the greenhouse and inheritance of transgenes in the progeny. Thus, Fontana et al. (1993) claimed a 4 % success but obtained only 3 GUS positive transgenic chickpea plants, whereas several teams (Kar et al. 1997, Krishnamurthy et al. 2000; Tewari-Singh et al. 2004) reported a 0.4–4 % frequency of *Agrobacterium*-mediated transformation based on multiple shoot formation from embryo axis explants and coupled with recovery of transgenic plants. Of these, though, only Krishnamurthy et al. (2000) and Tewari-Singh et al. (2004) showed transgene inheritance in T1 generation of transformed plants.

Recently, Polowick et al. (2004) also showed successful *Agrobacterium*-mediated transformation of chickpea, at a 1.3–3.1 % frequency of recovery of rooted transgenic plants but these were obtained after a prolonged tissue culture period, which could lead to somaclonal variation. Senthil et al. (2004) showed 2.0–13.3 % transformation efficiency, but performance of in vitro rooted plants in the glasshouse was inconsistent, requiring grafting of shoots, which was very laborious. Finally, Bhattacharjee et al. (2010) transformed four different chickpea genotypes with three different strains of *Agrobacterium* (EHA105, AGL1 and LBA4404) harbouring the binary vector pCAMBIA1301 with reporter genes (*gus*, *hpt*) driven by CaMV35S promoter and efficiently regenerated rooted plants whose T2 progeny expressed both reporter genes in the expected 3:1 inheritance.

4.3.6 Lentil

Lentil (*Lens culinaris* Medik.) is a grain legume produced in Asia, the Middle East and parts of North and South America as a source of protein in human diets. This crop has also been studied within the context of gene transfer by a number of groups (Table 9).

In order to assess the susceptibility of lentil to crown gall transformation, Warkentin and McHughen (1991) used four strains of *A. tumefaciens,* i.e. C58, Achh5, GV3111 and A281. All these strains were capable of inducing tumours at a high frequency on shoot apex explants when infected in vivo and on excised shoot apices in vitro, which were capable of growth on hormone free medium, a characteristic of tissue transformed with oncogenic *Agrobacterium* strains (Braun 1958). Their results suggested that disarmed versions of any one of these strains could be suitable for the recovery of transgenic lentil plants from shoot apex explants. As

Table 8 Chickpea (*Cicer arietinum* L.) transformation

Agrobacterium strain/ transformation method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
A281, R1601	Pusa-256	Leaf, stem	*nptII*	Not given	Srinivasan and Sharma (1991)
LBA4404	Local ecotype (Italy)	Embryo axis lacking apical meristem	*nptII, GUS*	4	Fontana et al. (1993)
LBA4404	ICC- 4918	Immature cotyledon	*GUS*	Not given	Ramana et al. (1996)
LBA4404	Red chickpea Canitez 87 MB-10	Shoot primordial of mature embryo	*nptII, GUS*	Not given	Altinkut et al. (1997)
C58C1, EHA101	PG1, PG12, Chafa	Embryo axes	*nptII, bar,GUS*	0.4	Krishnamurthy et al. (2000)
LBG66	CDC Yuma; Kabuli-type	Sliced embryo axis	*nptII, GUS*	3.1	Polowick et al. (2004)
EHA101	P-362, P-1042, P-1043	Embryo with 1/2 cotyledon	*bar, nptII, GUS*	0.7–1.2	Tewari-Singh et al. (2004)
C58, LBA4404, AGL1	Kabuli (ICCV5), Desi (H208, ICCL87322, K850 and Annigeri)	Embryo axis slices	*bar, GUS*	5.1	Senthil et al.(2004)
GV 2260, GV 3850, LBA 4404, EHA 105	C 235, BG 256, Pusa 362, Pusa 372	Cotyledonary nodes	*nptII, GUS*	1.12	Sanyal and Amla (2008)
LBA4404	K850	Embryogenic axis	*nptII, GUS*	0.3	Ignacimuthu and Prakash (2006)
Biolistic	Chaffa PG12 (MPKV, Rahuri), ICCC37 and ICCC32	Epicotyl	*nptII, GUS*	18	Indurker et al. (2007)
LBA4404	ICC 10943, ICC 10386	Decapitated embryo axis	*hptII*	9–26	Pathak and Hamazah (2008)
GV3101	C-235	Embryo with 1/2 cotyledon	*GUS*	3	Patil et al. (2009)
AGL-1	ICCV 89314	Embryo with 1/2 cotyledon	*nptII*	0.066	Chakraborti et al. (2009)
C58	C 235 (desi type)	Axillary meristem explants	*nptII, GUS*	65	Bhatnagar-Mathur et al. (2009)
EHA105, AGL1, LBA4404	Pusa-256, KWR-108, Pusa-1003	Cotyledons, cotyledonary-nodes	*Hpt, GUS*	7.56–25.56	Bhattacharjee et al. (2010)
LBA4404	Desi (P-362)	Cotyledonary nodes	*nptII*	1.69–2.77	Mehrotra et al. (2011a)
LBA4404	Desi (P-362)	Mature embryonic axes	*nptII, GUS*	3.6	Mehrotra et al. (2011b)

Table 9 Lentil (*Lens culinaris* Medik.) transformation

Agrobacterium strain/ method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
C58, Ach5, GV3111, A281	Laird	Shoot apices	–	81–100 based on tumour induction	Warkentin and McHughen (1991)
Biolistic	Laird, CDC 599-23	Cotyledonary node	*bla, ALS*	3.1	Gulati et al. (2002)
A281	Emre 20, Malazgirt, Akm 565, Akm 302, Akm 362 and so on (total 21 genotypes)	Stem, leaf	*GUS*	0–82 based on tumour induction	Khawar and Ozcan (2002)
GV2260	Sultan-1	Cotyledonary node	*nptII, GUS*	95 based on transient expression	Mahmoudian et al. (2002)
LBA4404	Barimasur-2, Barimasur-4	Cotyledonary node, decapitated embryo, immature embryo, epicotyl	*nptII, GUS*	Not given	Sarker et al. (2003)
A281, 15834	Sultan, Erzurum 89, Akm 565, 93Cl003	Cotyledon node, epicotyl, shoot meristem	*GUS*	0–90 based on tumour induction	Dogan et al. (2005)
EHA105, C58C1, KYRT1	Sultan-1	Cotyledonary node	*nptII, GUS*	2.3	Akcay et al. (2009)
EHA105	L-4076	Whole seed	*nptII, GUS*	0.9	Chopra et al. (2011)

strain A281 induced the heaviest tumours, Khawar and Ozcan (2002) used this hypervirulent strain for inoculation of several explants from 21 genotypes of lentil. Based on their responses in terms of different parameters including % of tumour formation, tumour diameter and tumour weight, three were best when leaf explants were used, four with stem explants and 14 formed tumours on both leaf and stem explants. Low but reproducible levels of GUS expression could be obtained for confirmation of such induced tumours.

Warkentin and McHugen (1993) studied the regeneration of cotyledonary nodes and reported very low potential of these explants for transformation by *A. tumefaciens*. In order to overcome this difficulty, Gulati et al. (2002) developed a reproducible system for lentil transformation using highly regenerable cotyledonary node meristems by biolistic method. Rooting of shoots was achieved through grafting. They bombarded herbicide resistance gene acetolactate synthase (*ALS)* into lentil cotyledonary nodes. Putative primary transformants and selfed progeny plants were screened by leaflet painting using metsulfuron herbicide while transgene insertion was confirmed through PCR and Southern hybridisation.

As vacuum infiltration has been reported to enhance transformation frequency in *Agrobacterium*-mediated gene transfer in some legumes (Trieu et al. 2000), Mahmoudian et al. (2002) used it for transfer of *Agrobacterium* cells into lentil cotyledonary nodes without affecting the regeneration potential of shoots. Transient expression of *gus* transgene on agro-infected explants was demonstrated through histochemical staining 3 d after co-cultivation.

Sarker et al. (2003) tested a number of explants such as cotyledonary nodes, decapitated embryos, immature embryos and epicotyls for their regeneration ability following *A. tumefaciens*-mediated transformation. Histochemical staining showed that epicotyl explants exhibited highest transgene expression followed by decapitated embryos, which were found to be more effective in formation of multiple shoots and were thus suggested as suitable explants for lentil transformation. Unfortunately, as various other authors, they were also unable to root the transformed shoots.

Bayrac (2004) investigated the regeneration of several tissues on various media via indirect organogenesis. He then carried out *A. tumefaciens*-mediated transformation using peeled cotyledonary nodes, cotyledonary petioles, shoot tips and roots as explant. Shoot tips showed the highest percentage of GUS expression. Root formation was only achieved in media with NAA/IAA.

Dogan et al. (2005) compared tumour and root formation ability of several tissues from different lentil cultivars after inoculation with *A. rhizogenes* and *A. tumefaciens* strains. The frequency of tumour formation from cotyledon node explants was higher compared than from shoot meristems. Rooting was only observed in cultivar Erzurum 89 while *A. rhizogenes*-mediated hairy roots were induced only in dark.

Production of disease-resistant lentil would help to increase its production as it is susceptible to many biotic stresses. Fungus-resistant lentil was developed by Hashem (2007) by transforming decapitated embryos with one cotyledon with *Ri-pgip* gene coding polygalacturonase inhibitory protein, conferring resistance

against fungal pathogens followed by an optimised regeneration system, with a very high transformation efficiency of about 35 %. Micro-grafting was used for rooting transformants. She was among the first to develop a marker free transformation system in legumes, by removing *bar* gene and PGIP gene was kept in T-DNA cassette before carrying out transformation. Fungus-resistant marker free plants were demonstrated via semi-quantitative polygalacturonase-inhibition assay.

For optimisation of lentil transformation, Akcay et al. 2009 used a combination of several treatments with three *A. tumefaciens* strains, i.e. EHA105, C58C1 and KYRT1 to deliver T-DNA into cotyledonary node tissues. As compared to EHA105 and C58C1, KYRT1 was found about threefold more efficient for producing transient GUS expression on cotyledonary petioles. Among several treatments used, wounding of explants, use of an optimised transformation protocol with the application of acetosyringone and vacuum infiltration and gradual selection resulted in a high percentage of stably transformed shoots. Fertile transgenic plants were obtained through grafting transgenic shoots on rootstocks. The transgene insertion and expression were confirmed through PCR, RT-PCR and Southern hybridisation, and the transgenes were segregated in Mendelian fashion.

During production of transgenic lentil, micrografting has been extensively used to recover transformed plants, and there is a need to develop an efficient and reproducible regeneration protocol that can lead to root induction from developing shoots without passing through the laborious work of micrografting. Chopra et al. (2011) developed a simple and genotype independent in vitro regeneration system of lentil capable of root induction. They then used it for transformation using sonication-assisted *A. tumefaciens* (SAAT) transformation (first report of this method in lentil). A supervirulent *A. tumefaciens* strain EHA105 was employed for transferring the T-DNA containing *nptII* and *uidA* genes into whole seeds using sonication and vacuum infiltration, and 40 % of the kanamycin-resistant transfected shoots produced through direct shoot organogenesis were able to root on a medium with IBA and kanamycin. Transgene insertion and activity in leaves and roots were detected by PCR and GUS histochemical assay, respectively.

Most recently, in order to enhance drought and salinity tolerance, Khatib et al. (2011) introduced *DREB1A* gene driven by the rd29A promoter into lentil decapitated embryo explants followed by shoot regeneration from the apical meristems and cotyledonary buds via direct organogenesis. Subsequently, basta-resistant putative transgenic explants were micro-grafted onto non-transformed rootstocks to establish transgenic plants. Transgene insertion and inheritance to the progeny were evaluated through PCR and Southern blot analysis. Expression of *DREB1A* gene in transgenic plants was induced by salt stress and was confirmed through RT-PCR.

4.3.7 Pea

Studies of the interactions between *Agrobacterium tumefaciens* and *A. rhizogenes* and pea started in the late 1980s and early 1990s (Hobbs et al. 1989; Hussey et al.

1989; Schaerer and Pilet 1991). Simultaneously, the first complete transgenic pea plants were regenerated from transformed protoplast cultures by Puonti-Kaerlas et al. (1989) soon followed by De Kathen and Jacobsen (1990, 1995) who regenerated transgenic pea plants from epicotyls and cotyledonary nodes.

Thenceforth, a number of protocols have been reported for *Agrobacterium*-mediated gene transfer in pea (Bean et al. 1997; Grant et al. 1998; Nadolska-Orczyk and Orczyk 2000; Pniewski and Kapusta 2005; Polowick et al. 2000; Schroeder et al. 1994; Svabova et al. 2008), as listed in Table 10. In the procedure described by Polowick et al. (2000), segments of the embryogenic axis are used. Bean et al. (1997) and Nadolska-Orczyk and Orczyk (2000) used cotyledonary nodes, whereas Grant et al. (1995, 2003) used immature cotyledons as starting material. In all procedures, multiple shoots are formed after infection with *Agrobacterium tumefaciens*, some of which are genetically modified. In these systems, about 2–5 % of the initial seeds used for transformation formed transgenic plants. Limitations of the currently available transformation protocols are associated with the regeneration systems used, which either result in polyploidy and sterility, or in the production of high numbers of escapes and chimeric plants, or have a low repeatability (Puonti-Kaerlas et al. 1990; De Kathen and Jacobsen (1990); Davies et al. 1993; Schroeder et al. 1993; Bean et al. 1997; Nadolska-Orczyk and Orczyk 2000; Polowick et al. 2000; Grant et al. 2003).

Despite the fact that successful pea transformation was reported 20 years ago (Puonti-Kaerlas et al. 1990), the efficiency of transformation protocols is still relatively low (in the range 0.1–6.5 %—Davies et al. 1993; Schroeder et al. 1993; Bean et al. 1997; Jones et al. 1998; Polowick et al. 2000; Grant et al. 2003; exceptionally over 10 %—Nadolska-Orczyk and Orczyk 2000). Particularly, co-cultivation additives were not studied systematically (only sporadic data on acetosyringone use are available—De Kathen and Jacobsen 1990; Lulsdorf et al. 1991; Grant et al. 1995; Nadolska-Orczyk and Orczyk 2000; Svabova et al. 2005, 2008).

Krejcι et al. (2007) also reported *Agrobacterium*-mediated gene transfer in pea. They compared three different methods for pea transformation by using stem segments, axillary buds and embryonic segments as explants, respectively. When using stem segments as explants, no shoot or plantlet regenerated from callus. Conversely, a large number of regenerated shoots were obtained from the other two methods. They were able to get some transient expression of *uid*A gene but they could not achieve the stable incorporation of transgene in the plants.

The importance of producing transgenic peas (as for other grain legumes with a high content of seed protein) increased with the demand to develop plant-made vaccines. Expressing recombinant proteins in transgenic plants has been actively sought for the past 20 years, resulting in a fast and flexible production system (Penney et al. 2011). Plant-derived pharmaceuticals can be used against various human diseases, including cancer (Pujol et al. 2007), hepatitis B and C, measles, cholera (Mishra et al. 2008) or AIDS (Floss et al. 2008), as well as for veterinary purposes. In peas, there are several reports about pea-derived vaccines against rabbit haemorrhagic disease virus (Mikschofsky et al. 2009), intestinal infections

Table 10 Pea (*Pisum sativum* L.) transformation

Agrobacterium strain/ method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
GV3101	Filby, Petra, Puget, Stivo, Vreta	Axenic shoot culture and epicotyl	*nptII*	28 plants	Puonti-Kaerlas et al. (1990)
C58C1, A281, A8683	Madria	Epicotyl segments and nodes	*GUS, nptII*	5	De Kathen and Jacobsen (1990)
C58/3	Puget	Germinating seeds	*nptII, GUS*	1.44	Davies et al. (1993)
AGL1	Greenfeast, Rondo	Immature seeds	*nptII, bar*	1.5–2.5	Schroeder et al. (1993)
AGL1	Bolero, Trounce, Bohatyr, Huka	Immature cotyledons	*nptII, bar*	1.47	Grant et al. (1995)
AGL1	94-A26, Bolero, Hadlee, Crown, Courier, 89T46.UK	Immature cotyledons	*nptII*	0.8–3.4	Grant et al. (1998)
EHA105	Puget	Cotyledonary nodes	*bar*	1.1 ± 0.43	Bean et al. (1997)
AGL1, KYRT1	Greenfeast, Laura	Immature Seeds	*αAI-1, αAI-2*		Morton et al. (2000)
LBA4404, C58C1, EHA105	Laser, Heiga	Cotyledonary nodes	*uidA, nptII, hpt, dhfr, bar*	4.2–3.6	Nadolska-Orczyk and Orczyk (2000)
EHA105	Greenfeast, CDC Vienna, S2-90-25E, 93-4-18G, MP1338, MP1382, AWPNZ66, AWP1512	Embryogenic axis segments	*uidA, nptII, pat.*	0.6	Polowick et al. (2000)
EHA105	Puget, BC1/17	Cotyledonary meristems	*bar*	0.13–0.8	Welham and Domoney (2000)
AGL1 ,KYRT1	Bolero, Lincol, 97-B19	Immature cotyledons	*nptII, GUS*	0.2–13	Grant et al. (2003)
AGL0, AGL1, EHA105	Several edible and fodder Polish cultivars	Immature embryos	*bar*	4.1	Pniewski and Kapusta (2005)
EHA105	Adept, Komet, Lantra, Olivín, Oskar, Tyrkys	Cotyledonary nodes, seeds	*nptII, uidA*	18.1 % for cotyledonary node, 31.6 % for seed GUS expression	Svabova et al. (2005)
EHA105	Vladan, Havel, Ctirad, Zazrak, Cezar, Puget	Stem segments, Axillary buds, embryonic segments	*Bar, nptII, GUS*	0.5	Krejcι et al. (2007)
EHA105 with plasmids pGT89 and pBIN19	Adept, Komet, Menhir	Cotyledonary nodes	*Bar, nptII, GUS*	0.1–1.0	Svabova and Griga (2008)
Electroporation	Belma, Filby	protoplast	*Hph, GUS*	1–2.2	Puonti-Kaerlas et al. (1992)

in pigs (Novoplant 2007; http://www.gmo-safety.eu) and coccidiosis in chickens (Zimmerman et al. 2009).

For efficient and large-scale production of recombinant proteins in plants, transient expression by agroinfection has a number of advantages over stable transformation, as simple manipulation, rapid analysis and high expression efficiency are possible. In pea, Fan et al. (2011) using the pea early browning virus converted a virus-induced gene silencing system into an efficient agroinfection system by converting the two RNA genomes of the virus into binary expression vectors for *Agrobacterium* transformation. They vacuum infiltrated germinating pea seeds with 2–3 cm roots with Agrobacteria carrying the binary vectors, and expression of the gene for GFP as marker and the gene for the human acidic fibroblast growth factor (aFGF) was obtained in 80 % of infiltrated developing seedlings. Maximal production of recombinant proteins was achieved 12–15 days after infiltration, i.e. half the time for the production cycle of plants for harvesting the recombinant protein. Thus, compared to the leaf injection method, vacuum infiltration of germinated seeds is highly efficient and allows large-scale production of plants transiently expressing recombinant proteins. The synthesised aFGF was purified by heparin-affinity chromatography and its mitogenic activity on NIH 3T3 cells was shown to be similar to a commercial product.

4.3.8 Lupin

In one of the first examples of gene transfer with lupin, Molvig et al. (1997) succeeded in recovering plants of narrow-leafed lupin *Lupinus angustifolius* L. that expressed a sunflower albumin gene and thus had an enhanced nutritional value with an increase in the methionine levels (Table 11).

Working with the same lupin species, Pigeaire et al. (1997) produced transgenic plants from co-cultivated shoot apices, with an average transformation frequency of 0.4–2.8 %, depending on genotype. Similarly, Li et al. (2000) generated transgenic yellow lupin (*Lupinus luteus* L.) shoots, developed from existing meristems in the embryo axes explants, but these failed to root in vitro and had to be grafted onto rootstocks from seedlings of non-transgenic *L. angustifolius* L. Transformation efficiency was low (0.05–0.75 %).

Babaoglu et al. (2000) found that transformation competent cells from which buds developed were located at the periphery of the apical meristems and, based on this, they used a procedure for regenerating transgenic plants of *Lupinus mutabilis* following *A. tumefaciens*-mediated gene delivery. Kanamycin-resistant plants obtained expressed β-glucuronidase activity, and integration of the *nptII* and β-glucuronidase genes into the genome was confirmed by non-radioactive DNA–DNA hybridisation.

Pniewski et al. (2006) developed an improved transformation protocol with seedlings and hypocotyls of yellow lupin, reaching 44 % efficiency rate, which they used to produce calli and tumours that produced a small surface antigen of

Table 11 Lupin (*Lupinus* sp.) transformation

Agrobacterium strain[a]/method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
A. tumefaciens	Warrah	Cotyledon with sliced embryonic axis	*Bar, GUS*	0.01	Molvig et al. (1997)
AGL0, LBA4404, K61, EHA101	Unicrop, Illyarie, Yandee, Wandoo, Danja, Gungurru, Yorrel, Warrah, Merrit	Embryonic axis	*Bar, GUS*	2.8 % for Unicorp, 0.4 % for Merrit	Pigeaire et al. (1997)
Strain 1065 based on strain LBA 4404	Potosi	Part of hypocotyl and epicotyl with apical meristem, extreme tip of the apical dome	*nptII, GUS*	0.04	Babaoglu et al. (2000)
AGL0	Teo, Teo101, Wodjil, Popiel, Motiv 369, Juno, WDT 6174, WDT 6179	Lower hypocotyl and radicle wa	*Bar*	0.05–0.75	Li et al. (2000)
Derivatives of *A. rhizogenes* strain A4T	Ultra	Radicle sections	*GFP, GUS*	27–32	Uhde-Stone et al. (2005)
C58, A281 77, Ach5, GV3101, EHA105 and LBA4404	Ventus	Truncated seedlings	*nptII, GUS*	44	Pniewski et al. (2006)
A. rhizogenes strain A4TC24	Ultra	Hypocotyl region	*GUS*	Not given	Zinn et al. (2009)

[a]All strains of *A. tumefaciens* unless indicated otherwise

Hepatitis B Virus, the final goal being the production of an oral vaccine that could be administered as a portion of plant tissue.

Finally, Zinn et al. (2009) produced transgenic white lupin and Arabidopsis and studied deletion and mutation constructs linked to the β-glucuronidase (*gus*) reporter gene, in order to understand the structure of the LaSAP1 promoter and explore the role of the P1BS motif. In this context, Uhde-Stone et al. (2005) had shown that white lupin transformation provides a homologous system to directly study gene expression in proteoid roots, since transgenic white lupin roots have a similar morphology to normal roots, as both develop proteoid rootlets and express P-deficiency responsive genes.

4.3.9 Cowpea

Cowpea (*Vigna unguiculata* L.) is a major food legume for the poor in Asia and especially in Africa, where a number of experiments on gene transfer have also been performed (Table 12).

The first report on the susceptibility of cowpea to *Agrobacterium* was by Garcia et al. (1986, 1987), who demonstrated that *A. tumefaciens* was capable to transform cowpea cells derived from leaf callus that became kanamycin resistant, but they were unable to generate transgenic plants from them. Transgenic cowpea callus cells were also obtained by Penza et al. (1991) following co-culture of mature embryos with hypervirulent *A. tumefaciens* A281. Based on GUS expression, they showed that transformed cells were mostly located in the subepidermal regions of the plant stems.

Muthukumar et al. (1996) also tried to produce transformed cowpea by indirect DNA transfer using *A. tumefaciens* strain LBA4301; 15–19 % of the co-cultivated explants produced shoots on selection medium supplemented with hygromycin. Although transgene insertion and integration in primary transformants were confirmed by Southern hybridisation, there was no germination of their seeds and transgene inheritance.

Several years later, a biolistic approach was used to transform embryonic axes by Ikea et al. (2003). Putative transgenic plants were obtained after bombardment with a construct containing *nptII* and *gus* driven by CaMV35S promoter. Plants developing under constant kanamycin screening were assayed for GUS expression. Southern hybridisation confirmed the T-DNA insertion in primary transgenics. Though the transformation efficiency was low, this was the first report to demonstrate transgene inheritance to the progeny, but it did not follow Mendelian rule nor was transgene expression very stable.

Different conditions that significantly affect genetic transformation in cowpea were optimised by Popelka et al. (2006) using different plant tissues as explant. They then used this knowledge to transform cotyledonary nodes via *A. tumefaciens*. Nodes were grown on auxin-free medium in early stages and were transferred for shoot initiation to medium with low cytokinin level. A strict phosphinotricin-based selection procedure was followed to ensure that there were no escapes from the selection regime. Transgene insertion and functioning were verified using Southern

Table 12 Cowpea (*Vigna unguiculata* L.) transformation

Agrobacterium strain/ method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
C58CI	Not mentioned	Leaf discs	*nptII*	–	Garcia et al. (1987)
LBA4301	C-152	De-embryonated cotyledons	*hpt*	15–19 % based on Hygromycin resistance	Muthukumar et al. (1996)
Biolistic	IT83D-442	Embryonic axes	*bar, GUS*	0.57	Ikea et al. (2003)
AGL1	Sasaque, Holstein, Ebony	Mature and immature embryos, shootapex, embryonic axes with cotyledons	*bar, nptII, hpt, GUS*	0.05–0.15	Popelka et al. (2006)
EHA105	IC-202786, IC-257438, IC-259159, IC-243501, V-240, V-130, V-585	Cotyledonary nodes	*nptII, GUS*	0.76	Chaudhury et al. (2007)
Biolistic	Paraguacu, Pitiuba, Rouxinol, CE-11	Embryonic axes	*GUS, ahas*	0.90	Ivo et al. (2008)
LBA4404	Pusa Komal	Cotyledonary nodes	*nptII, GUS*	1.67	Solleti et al. (2008a)
LBA4404, GV2260, EHA105, AGL1	Pusa Komal, Pusa Safed, Pusa Sam-pada, Rambha, V-16, V-240, V-130, V-585	Cotyledonary nodes	*nptII, GUS*	1.64	Solleti et al. (2008b)
pGV2260, pGV3850	IT86D-1010	Embryos	*bar, GUS*	2.5–3.9	Adesoye et al. (2010)

hybridisation and PAT assay with a transformation efficiency of 0.05–0.15 %. This protocol was the first where transgene inheritance to the offspring followed the Mendelian fashion. Co-culture, a critical step during transformation, was further enhanced by Chaudhury et al. (2007) by wounding of cotyledonary node explants prior to inoculation with *A. tumefaciens* EHA105 harbouring a binary vector pCAMBIA2301, which contains *gus* and *nptII* genes both driven by the CaMV35S promoter, whereby they reported an improved transformation efficiency of 0.76% of explants and coupled with transgene inheritance to the progeny.

Ivo et al. (2008) used a novel selection system based on imazapyr, a herbicidal molecule that provides tolerance to *ahas* gene (a mutated gene from *Arabidopsis thaliana* used as a marker), and is capable of systemically translocating and concentrating in the apical meristem of the plant. They mixed this novel selection system with gus staining to screen transformants when embryonic axes were bombarded through the biolistic method. Transgenic plants expressing *gus* and *ahas* genes were obtained with ameliorated transformation efficiency of 0.9 % and transmitting traits to the progenies following Mendelian segregation.

Solleti et al. (2008a) transferred alpha-amylase gene to cowpea, with an inhibitory effect for certain pest attacks, in what is probably the first report on heterologous expression of a gene that confers a desirable agronomical trait in cowpea. The transformation method was improved by introduction of additional virulence genes in *A. tumefaciens* strain LBA4404 for efficient T-DNA delivery to regenerating cells. An improved regeneration system with optimised hormonal balance and geneticin selection was employed, which reduces the escapes and promotes regeneration of proliferating transformed cells. All these factors when combined boosted transformation efficiency to 1.67 %. Solleti et al. (2008b) then compared different *A. tumefaciens* strains for their ability to transform various cowpea genotypes employing this same approach.

Adesoye et al. (2010) used vacuum infiltration when embryo explants were inoculated in *Agrobacterium* liquid culture to enhance transformation, as vacuum infiltration had been reported to improve the transformation efficiency in pea (Svabova and Griga 2008). Co-cultivation of explants was carried out on selection-free medium. Double selection using hygromycin and phosphinotricin resulted in development of GUS positive plants with a much higher transformation efficiency, i.e. 2.5–3.9 %, than previously reported.

4.3.10 Mungbean

Mungbean (*Vigna radiata* L. Wilczek) is an important protein-rich grain legume, principally grown in tropical and sub-tropical countries. The very first assay for transformation of mungbean was reported by Pal et al. (1991), who regenerated shoots from cotyledon explants of Agrobacterium susceptible genotypes via organogenesis (Table 13). Based on GUS assay, 4–5 % of the kanamycin-resistant shoots were transgenic, but rooted and fertile transgenic plants were not recovered.

Table 13 Mungbean (*Vigna radiata* L. Wilczek) transformation

Agrobacterium strain/method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
Not given	Not given	Cotyledons	*nptII, GUS*	4–5	Pal et al. (1991)
Biolistic	Marechal	Cotyledons, mature embryos	*nptII, GUS*	Not given	Bhargava and Smigocki (1994)
LBA4404, EHA105, C58C1	K-851, Pusa-105, ML-5, ML-323, ML-337, PDM-11, PS-7	Hypocotyls, primary leaves, cotyledonary nodes	*nptII, GUS*	0.9	Jaiwal et al. (2001)
K599, EHA105	Kamphaeng Saen 1 (KPS 1), Suranaree 1 (SUT 1)	Cotyledons, cotyledonary nodes, cotyledonary roots	*nptII, hpt, GUS*	Only one shoot regenerated	Suraninpong (2002)
K599, EHA105	Kamphaeng Saen 1 (KPS 1), Suranaree 1 (SUT 1)	Cotyledons	*hpt, GUS*	31.25 based on gus staining	Suraninpong et al. (2004)
C58C1	NM92, NCM 209, NM98	Hypocotyls, primary leaves, roots, cotyledonary nodes	*nptII, GUS*	40–80 based on GUS staining	Tazeen and Mirza (2004)
C-58	K-851, LGG-407	Primary leaves	*hpt, GUS*	2	Mahalakshmi et al. (2006)
EHA105	PUSA-105	Cotyledonary nodes	*nptII, GUS*	1.51	Sonia et al. (2007)
LBA4404	BINA mug-5	Cotyledonary leaves, cotyledon attached with embryonal axis (CAEA)	*nptII, GUS*	Not given	Islam and islam (2010)

Gulati and Jaiwal (1994) reported rapid multiple shoot formation at high frequency from cotyledonary nodes using varying combination of growth regulators but regenerated plants were generally stunted, unbranched and flowered precociously. Bhargava and Smigocki (1994) used biolistic to introduce *gus* and *nptII* genes into ungerminated mature embryos of different *Vigna* species. GUS expression was observed in cotyledonary meristematic regions 18 h after bombardment. They were able to root some kanamycin-resistant transformed shoots but no information on seeding of these plants was provided. Nagl et al. (1997) reviewed the progress made with the regeneration and genetic transformation of *V. radiata*.

Establishment of optimised conditions necessary for regeneration and efficient *A. tumefaciens*-based transformation is a prerequisite for production of transgenic plants of *V. radiata* as reported by Jaiwal et al. (2001) for the first time. They co-cultivated hypocotyl and primary leaves with *A. tumefaciens* strains LBA4404 (pTOK233), EHA105 (pBin9GusInt) and C58C1 (pIG121Hm), all containing *nptII* and *gusA* marker genes under CaMV35S promoter. Stable fertile transformants were identified within 4–6 weeks from kanamycin-resistant GUS positive calli and node explants. Molecular analysis of putative transformed plants by PCR and Southern blot revealed the integration and expression of transgenes in primary transformants and their progeny. Choice of the explants, transformation vectors and use of selective agent were the most important factors. Cotyledonary nodes were the best explant source, as shoot regeneration occurs through direct shoot organogenesis avoiding somaclonal abnormalities.

Mungbean production is commonly affected by different insect pests. In this context, Suraninpong (2002) made an attempt to transform various mungbean tissue sources with *A. rhizogenes* K599 and *A. tumefaciens* EHA 105 harbouring plasmid pCAMBIA 1301 containing cholesterol oxidase gene (*choA*), coding a potent insecticidal protein active against boll weevil larvae. GUS expressing transformed hairy roots developed from cotyledonary root explants could not be regenerated into plants. In 2004, Suraninpong et al. improved the same method to get a transformation efficiency of 31.25 % in hairy roots based on GUS staining; only one, sterile, GUS-positive shoot could be regenerated from such roots.

Tazeen and Mirza (2004) studied different factors to standardise the *A. tumefaciens*-mediated transformation protocol for *V. radiata* using *A. tumefaciens* strain C58C1 harbouring a binary vector p35SGUSINT containing *NPTII* gene as selectable marker and GUS as a reporter gene. Kanamycin-resistant explants and regenerated calli were screened by transient and stable GUS expression, respectively, achieving up to 70 % transient GUS expression at pH 5.8 after 3 days of co-culturing in 2-day-old explants. Higher transformation efficiency (80 %) was achieved using primary leaves than hypocotyl (60 %) or root (40 %) explants but they were also unable to regenerate shoots from calli.

The lack of an efficient and reliable regeneration system for successful acclimatisation and growth of mungbean plants to maturity has slowed its improvement via tissue culture selection and transformation. Mahalakshmi et al. (2006) obtained the first stably transformed mungbean plants after developing a genotype independent, high frequency plant regeneration protocol from primary leaf

explants. Hygromycin-resistant transformed plants were morphologically similar to seed-germinated plants. Stable integration of the transgene in the primary transgenics and stable inheritance to progeny was confirmed through PCR and Southern hybridisation.

Successful production of insect resistant phenotypically normal and fertile mungbean expressing insect resistant gene was reported for the first time by Saini et al. (2007). They transformed cotyledonary node explants with *A. tumefaciens* EHA105 harbouring α-amylase inhibitor gene of *Phaseolus vulgaris* with insecticidal nature, and *bar* as a selectable marker, and obtained plants via direct shoot organogenesis. Stable integration and expression of the *bar* gene in T_0 plants was shown by PCR-Southern analysis and PPT leaf paint assay, respectively. Presence of the α-amylase inhibitor gene was also confirmed by Southern blot analysis while inheritance of both transgenes to the progeny was evidenced by PCR. Among different conditions, preculture and wounding of the explants, use of acetosyringone during co-cultivation and PPT-based selection of transformants played vital role for achieving an enhanced transformation frequency of 7.81 %.

Islam and Islam (2010) compared cotyledonary leaf and cotyledon attached with embryonic axis (CAEA) as explants for transformation of local mungbean varieties using *A. tumefaciens* strain LBA4404. Based on kanamycin selection and GUS assay, CAEA showed better response towards transformation than the cotyledonary leaf, obtained after 45 min of infection with Agrobacterial suspension having an OD_{600} of 1.3 and 3 days of co-cultivation.

4.3.11 Blackgram

Vigna mungo, blackgram, is a large-seeded grain legume grown in developing countries of southeast Asia, notorious for its recalcitrance to in vitro regeneration and, not unexpectedly, this has adversely affected attempts at its genetic transformation (Table 14).

Transformed blackgram calli obtained by Karthikeyan et al. (1996) after co-culture of segments of primary leaves with two strains of *A. tumefaciens,* i.e. LBA4404 and EHA105 exhibited neomycin phosphotransferase activity but were unable to regenerate plants.

Saini et al. (2003) established an efficient plant regeneration method through direct multiple shoot organogenesis from cotyledonary-node explants without cotyledons, which they used for *A. tumefaciens*-based transformation of *V. mungo*. An optimal selection system enabled them to produce kanamycin-resistant and GUS-expressing transgenic plants; however, the transformation frequency remained low, i.e. 1 %. They proposed that this low yield of transformants using cotyledonary node explants was due to a limited number of meristematic cells whose capability for regeneration was short lived. In this context, the current trend in genetic transformation of recalcitrant grain legumes has been to target meristems as a source of totipotent cells (Somers et al. 2003). Saini and Jaiwal (2005) first reported *A. tumefaciens-mediated* transformation of the shoot apical meristem in

Table 14 Blackgram (*Vigna mungo* L.) transformation

Agrobacterium strain/method References	Genotype	Explants	Selection marker and	reporter genes	Transformation efficiency (%)
LBA4404, EHA105	Co5	Primary leaves	*nptII*	10–23 % Kanamycin-resistant callus	Karthikeyan et al. (1996)
EHA105	Pusa-1	cotyledonary nodes with and without cotyledons	*nptII, GUS*	1	Saini et al. (2003)
EHA105	PS-1, Pusa-2, Vamban-1, Co-5,T-9	shoot apices,	*nptII, GUS*	1–6.5	Saini and Jaiwal (2005)
LBA4404	Vamban 3	Immature cotyledonary nodes, shoot-tips	*nptII, bar, GUS*	2.6–7.6	Muruganantham et al. (2007)
EHA105	PS-1	cotyledonary nodes	*nptII, GUS*	4.31	Saini and Jaiwal (2007)

legumes by establishing an efficient regeneration system from mature seed-derived embryo shoot apices. Optimisation of co-culture conditions and wounding of explants led to an efficiency of 6.5 %, and they later (Saini and Jaiwal 2007) optimised the conditions affecting *A. tumefaciens-mediated* transformation of blackgram. They concluded that inoculation of pre-cultured and mechanically injured cotyledonary node explants for 30 min with *A. tumefaciens* at a density of 10^8 cells/cm^3 followed by co-culture on SR medium for 3 days was most beneficial.

Muruganantham et al. (2005) introduced a different efficient plant regeneration system from immature cotyledonary nodes from 18 DAP seeds, later used (Muruganantham et al. 2007) to get transformed blackgram plants from both cotyledonary-node and shoot-tip explants from immature seeds. After selection on phosphinothricin, transformation efficiency was 7.6 %.

For transformation via either *A. tumefaciens* or biolistic method, embryogenic cultures can serve as good target tissues because such cultures provide a high level of cell exposure. Muruganantham et al. (2010) used primary leaf explants of *V. mungo* for the induction of embryogenic callus. Liquid shaken culture of embryogenic calluses led to plant regeneration via somatic embryogenesis. This regeneration method can be applied for genetic improvement of this crop through transformation.

4.3.12 Pigeonpea

Pigeonpea (*Cajanus cajan* L.), the main food legume of the semi-arid tropics because of its richness in protein, has also been genetically transformed (Table 15). Geetha et al. (1999) first reported its *A. tumefaciens*-mediated transformation using shoot apices and cotyledonary nodes as explants followed by direct regeneration,

Table 15 Pigeonpea (*Cajanus cajan* L.) transformation

Agrobacterium strain/method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
LBA4404	HyderabadC	Shoot apices, cotyledonary nodes	*nptII, GUS*	45–62 % Kanamycin-resistant shoots	Geetha et al. (1999)
GV2260	Pusa 855	Embryonic axes	*nptII,*	1	Lawrence and Koundal (2001)
Biolistic	ICPL 87, ICPL 88039, ICPL 87119, ICPL 85063, ICPL 88009, ICPL 87091, ICPL 2376, ICPL 87051, ICPL 91011, ICPL 332, and ICPL 84031	Leaves	*nptII, GUS*	50 % of selected plants	Dayal et al. (2003)
LBA4404	T-15-15	Decapitated mature embryo axes	*nptII, GUS, GFP*	1.7–6.7 % Kanamycin-resistant shoots	Mohan and Krishnamurthy (2003)
LBA4404	ICPL 87	Seed-derived calli	*nptII, GUS*	20–25 % based on randomly selected plants	Thu et al. (2003)
C-58	ICPL 87	Axillary meristem	*nptII, GUS*	54–70 % based on regenerated plants	Sharma et al. (2006)
LBA4404	LRG 30, ICPL 87, ICPL 85063	Plumules, nodes, cotyledons	*nptII, GUS*	36–80 % explants based on GUS staining	Surekha et al. (2007)

albeit at very low frequency, of transformed plants and coupled with integration of T-DNA into the genome of transgenic plants confirmed by Southern hybridisation.

Transgenic pigeon pea plants resistant to chewing insects, expressing the cowpea protease inhibitor gene, were successfully obtained by Lawrence and Koundal (2001) through indirect regeneration from callus derived from embryonic axes. Expression of transgene was verified through Northern hybridisation. The frequency of transformed shoots (T0) was less than 1 %.

A significant improvement was achieved through an efficient plant regeneration method (>90%) from leaf explants of pigeonpea by Dayal et al. (2003), who also showed genetic transformation of leaf explants using biolistics; 90% of the bombarded explants exhibited transient expression of the uidA gene and 50% of the selected plants that were transferred to the glasshouse showed positive gene integration.

An efficient regeneration protocol for pigeonpea based on callus induction and differentiation on high concentration of cytokinin from seed explants was developed by Thu et al. (2003). They transformed pigeonpea using both *A. tumefaciens-mediated* gene transfer and biolistics. Stable transmission and expression of transgene in the progeny was confirmed through GUS assays, PCR and Southern hybridisation.

Mohan and Krishnamurthy (2003) reported efficient *A. tumefaciens*-mediated transformation of pigeonpea based on a reliable regeneration method by direct organogenesis from mature embryo-derived explants using *nptII* as selectable marker and GUS and GFP as reporter genes. Southern hybridisation of plants confirmed the stable integration of *GFP* gene in transgenics. In parallel, calli were also induced from those Agro-infected explants. Transformed callus showed GFP and GUS expression but were unable to develop plants.

The first record on the successful production of pest-resistant pigeonpea was reported by Sharma et al. (2006) who transformed axillary meristem explants with the *Bt cry1Ab* gene driven by double-enhanced CaMV35S promoter, also containing fused *uidA* and *nptII* genes driven by CaMV35S promoter as selectable marker genes. Transformed organogenic tissues differentiated into shoot buds. GUS staining and southern blot analysis proved the transgenic nature of the progeny. Expression of the transgene in progeny was proved using RT-PCR. Stable expression of Cry1Ab protein was observed even in the T2 generation using ELISA. Here, 60 % of the independently transformed plants showed positive gene integration and expression and 65 % of the transformants showed single copy inserts of the introduced gene.

Surekha et al. (2007) showed effects of numerous pigeonpea genotypes, using various explant sources and different *A. tumefaciens* strains on pigeonpea transformation frequencies. Transformation frequency was evaluated using GUS staining and it depends upon genotype, explant and *A. tumefaciens* strain. They were also the first to prove endogenous glucuronidase activity in pigeonpea that is restricted to the root meristems region.

A very informative review on the progress of tissue culture and genetic transformation research in pigeon pea [*Cajanus cajan* (L.) Millsp.] was recently published by Krishna et al. 2010.

4.4 Miscellaneous Grain and Medicinal Legume Species

Lathyrus sativus L., grasspea, is an important grain legume cultivated extensively in tropical and sub-tropical parts of the world and is well adapted to adverse agricultural conditions (Vaz Patto et al. 2006). Despite all these features, only limited efforts have been made to exploit the potential of this grain legume because of the presence of a neurotoxic amino acid that causes neurolathyrism in humans with prolonged consumption (Ochatt et al. 2007). Barik et al. (2005) developed an efficient and reproducible procedure for Agrobacterium-mediated genetic transformation of grasspea using epicotyl segments as explants. Two different strains of *Agrobacteria*, i.e. EHA 105 and LBA 4404 were used, and up to 36% transient expression was achieved based on the GUS histochemical assay. Southern hybridisation of genomic DNA of the kanamycin-resistant GUS-expressive shoots proved the integration of the transgene. In T1, the plants segregated in a typical 3:1 Mendelian ratio.

Parkia timoriana (DC.) Merr., popularly known as "tree bean", is a very large legume tree distributed from northeast India to Irian Jaya and is the most widely distributed species of the Indo-Pacific region. Its pods are consumed as vegetable and salad while branches and wood are used as firewood or as timber. The first report of successful in vitro regeneration and establishment in the field for transgenic *P. timoriana* came from Thangjam and Sahoo (2012). They used cotyledonary node explants for transformation experiments with *A. tumefaciens* strain EHA105 harbouring a binary vector pCAMBIA2301 which contains β-glucuronidase (GUS) with an intron in the coding region and neomycin phosphotransferase (nptII) gene, both driven by CaMV35S promoter. Explants were grown on constant kanamycin selection. Transient GUS expression in cotyledonary explants was scored after 3-day co-cultivation using histochemical GUS assay. Transformants were verified by PCR and southern hybridisation.

Mucuna pruriens, known as velvet bean or cowitch, is a tropical legume used in agriculture and horticulture that has a range of medicinal properties. Sathyanarayana et al. (2012) reported a simple and reliable method for the genetic transformation of *M. pruriens* mediated via *A. tumefaciens*. Leaf discs were used as explants and regeneration from callus was achieved through somatic embryogenesis. Transformed plants were confirmed through histochemical GUS staining and PCR.

Sesbania drummondii, known as Rattlebush, Rattlebox and Poison bean, is a medium-sized perennial shrub native to coastal areas of the United States. It is an important source for phytopharmaceuticals. Padmanabhan and Sahi (2009) developed an efficient method for its *A. tumefaciens* genetic transformation followed by

successful regeneration of shoots through organogenesis from cotyledonary node explants. GUS staining followed by PCR and Southern blotting was done to evaluate the integrity of transgene.

5 Concluding Remarks and the Way Forward

From the recent advances in genetics and genomics it is clear that gene transfer is emerging as a major player in the understanding of gene function and its validation, and also that it may play an important role for the generation of genetic novelties that, pending appropriate legislation and stringent control of the performance of transgenic plants and inheritance of introduced traits once in the field, should find their way into the breeding strategies for a number of crops. Progress achieved to date in the use of transgenic legumes worldwide is rather limited and this contrasts markedly with the expectations of both farmers and breeders (Dita et al. 2006; Eapen 2008). Legumes will still require a large research input worldwide until efficient protocols permitting their routine transformation are available.

To date, transgenic plants of grain legumes have mostly been produced up to the T0 generation levels only and more rarely up to later progeny levels (T1, T2, etc.). This would, however, be a major prerequisite for the successful commercialisation of any transgenic pulse crop, as the stability and inheritance of transgenes introduced must be ascertained under field conditions. The fact that most pulse crops are mainly cleistogamous reduces the risk of transgenes being horizontally transferred to related species but does not entirely preclude it, and all proof in this context will have to be provided if pulse crops are to find their way into commercial exploitation as both food and feed.

With the novel post-genomic tools, new opportunities exist now for a better understanding of many metabolic pathways and for the identification of genes involved therein. This will undoubtedly contribute to the development of grain legumes with a higher nutritive value, but also to an improved yield and tolerance to various biotic and abiotic stresses. In this respect, the study of transcription factors involved in the development of the embryo and in seed filling will play a crucial role and contribute to improving the nutritional value of grain legumes, i.e. by suppressing anti-nutritional compounds and toxins. However, transgenic legume research must still gain pace and the commercialisation of genetically modified legume crops will still require several years before it becomes a reality.

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Physiology of the Potato–*Potato Virus Y* Interaction

Polona Kogovšek and Maja Ravnikar

Contents

Abstract Many morphological, physiological, and phytochemical changes in plants are caused by biotic stress. Response of plants to various pathogenic microorganisms and pests is described at different levels: changed morphology of the plant and

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development of symptoms like lesions and necrosis; alterations in hormone concentrations; signaling molecules; secondary metabolism; pathogenesis-related proteins; and mRNA or gene expression. Due to its small size and therefore early description of the genome, *Arabidopsis thaliana* is a model plant in which metabolic processes are well described and constitutes a basis for research on other plants (Dardick et al. 2000; Pieterse et al. 2009; Simon et al. 2010; Wan et al. 2002). Nevertheless, plants from other families possess specific metabolic pathways not observed in this model plant (e.g., tuberization is specific for potato), which gives urgency to research on a wider variety of plant families. There are only limited reports on the responses of potato plants, especially to virus infection. The availability of the potato genome sequence opens up the possibility of advancing the understanding of the physiology of potato response to virus infection.

Interaction between potato plants and *Potato virus Y* (PVY), the most devastating potato virus, will be described, starting with the biological variability of PVY, available detection methods, viral movement, and its accumulation in plants. The response of potato plants to PVY infection will be described, first the symptoms' development from different points of view, from macroscopical to cytological, and second the main metabolic pathways involved in response to infection. Alterations in photosynthesis, hormonal pathways, defense and signaling mechanisms, and other pathways will be demonstrated. The most important methods enabling disclosure of potato response mechanisms and pathways will be described.

1 Introduction

When plants come into contact with viral pathogens, they first react at the molecular level, which leads to the expression of a broad spectrum of physiological changes observed as alterations in respiration, photosynthesis rate, hormone concentrations, and the accumulation of pathogenesis-related proteins and signal molecules. The variations in the biochemical balance, as a result of physiological changes, influence cellular morphology, such as degeneration of chloroplasts, thickening of the cell wall, accumulation of secondary metabolites, changes of plasmodesmata, and cell death. Eventually these changes can be recognized macroscopically as symptoms like mosaic, vein clearing, chlorosis, and necrosis. The latter type of symptoms can also be observed in potato plants of different susceptible cultivars infected with *Potato virus Y* (PVY).

PVY disease on potato plants, known also as one of the causes of potato degeneration, was recorded at the beginning of the twentieth century (Kerlan and Moury 2008). During the replication of PVY RNA genome, numerous errors can be introduced into the sequence, leading to evolution of new isolates (Nie and Singh 2003a). Moreover, RNA viruses are prone to exchange genetic material between isolates through recombination events, which also introduces variability in viral sequence (Lorenzen et al. 2008; Blanchard et al. 2008; Karasev et al. 2011). The

emergence of new isolates inducing more severe disease led to the observation of symptoms and their description. Uncontrolled spread of PVY has led to outbreak of the disease; the main reason for high-yield losses in potato production being the PVY isolates that induce severe symptoms on potato tubers of sensitive cultivars (e.g., PVY^{NTN}, $PVY^{N\text{-}Wi}$, $PVY^{N:O}$) (Kerlan and Moury 2008). To control virus spread, diagnostic detection methods were developed and resistant cultivars were introduced. To understand the successful potato defense mechanisms against PVY virus, studies on the impact of PVY on potato plant physiology are necessary. Physiological studies offer an insight into specific plant–pathogen interactions, revealing the knowledge needed for breeding and, possibly, molecular engineering of new, resistant cultivars.

2 *Potato Virus Y*

Potato virus Y (PVY) is a type member of genus *Potyvirus*, the largest plant virus group, and infects a wide range of species mainly from the *Solanaceae* family. The PVY genome consists of a single stranded (+) oriented RNA, approximately 9,700 nt long, which is expressed as one polyprotein. The latter is autoproteolytically cleaved into ten functional proteins: P1 (first protein), HC or HC-Pro (helper component protein), P3 (third protein), 6K1 (first 6 kDa protein), CI (cytoplasmic inclusion protein), 6K2 (second 6 kDa protein), Vpg (genome-linked protein), NIa (small nuclear inclusion protein), NIb (large nuclear inclusion protein), and CP (coat protein). On each end of PVY genome, there is a distal noncoding region, 5′NTr and 3′NTr (Kerlan 2006).

PVY is one of the most important potato viruses and is spread worldwide and is recognized as the fifth most important plant virus regarding its scientific and economic importance (Scholthof et al. 2012). It is transmitted in nature through infected "seed" materials, by aphid vectors, mechanically by contact or artificially in the laboratory. There are also reports of transmission by seeds (Kerlan and Moury 2008). PVY is aphid-transmitted in a nonpersistent manner by a wide range of aphid vectors from the Aphidinae. PVY is most effectively transmitted by *Myzus persicae*, but *Macrosiphum euphorbiae* also plays a significant role in PVY spread (Cervantes and Alvarez 2011).

Following the genetic characteristics, PVY isolates are classified into five strain groups—PVY^{N}, PVY^{O}, PVY^{C}, PVY^{Z}, and PVY^{E} (Singh et al. 2008). PVY^{O}, PVY^{C}, and PVY^{Z} cause hypersensitive reactions (HR) in resistant cultivars of *Solanum tuberosum* that bear *Ny* and *Nc* and proposed *Nz* resistance genes, respectively, but do not cause vein necrosis in tobacco. The group of isolates classified as PVY^{E} neither elicit any of the above-mentioned resistance genes, nor do they cause vein necrosis in tobacco. PVY^{N} isolates also do not cause HR in potato but result in vein necrosis in tobacco (Kerlan et al. 2011). Regarding the molecular characteristics, several strain groups of PVY are recognized, e.g., PVY^{NTN} (Beczner et al. 1984), $PVY^{N\text{-}Wi}$ (Charzanowska 1991), $PVY^{N:O}$ (Nie and Singh 2003b), and $PVY^{NA\text{-}NTN}$

(Nie and Singh 2003a). Isolates classified within the PVY^{NTN} and $PVY^{N\text{-}Wi}$ subgroups possess a nucleotide sequence similar to those of PVY^{N} and PVY^{O} isolates. Recently, a recombinant isolate with typical PVY^{NTN} features and HR reaction in an N*z* gene bearing potato cultivar was characterized at the biological and molecular levels and classified in PVY^{Z} strain group (Kerlan et al. 2011). Furthermore, recombinant isolates, like PVY^{NTN} and the novel PVY-NE-11 (Lorenzen et al. 2008), serve as parents in new recombinant events, producing a new recombinant PVY genome with unique biological, serological, and molecular properties, classified as PVY^{E} (Galvino-Costa et al. 2011). $PVY^{N\text{-}Wi}$ isolates share biological properties with PVY^{N} and serological properties with PVY^{O} isolates. The North-American isolate $PVY^{N:O}$ has been classified biologically in the $PVY^{N\text{-}Wi}$ subgroup (Singh et al. 2008); however, molecular analysis has shown that $PVY^{N:O}$ isolates closely resemble PVY^{NTN} isolates (Karasev et al. 2011). PVY^{NTN} isolates cause the most devastating disease of potato, i.e., potato tuber necrotic ringspot disease (PTNRD) (Fig. 1). $PVY^{N\text{-}Wi}$ are more infectious than PVY^{N}, but cause less severe symptoms in potato than the standard PVY^{NTN} isolates, although they have the ability to cause PTNRD occasionally (Piche et al. 2004). New recombination events and mutations within the PVY RNA provide new isolates, which make PVY one of the most divergent plant virus groups, whose exact classification still remains a challenge.

Emergence of new recombinant and mutated isolates (Lorenzen et al. 2008; Kerlan et al. 2011; Galvino-Costa et al. 2011) restrains the use of established diagnostic methods, which need to be simultaneously improved. The specific PTNRD inducing isolate could be detected if the region of the nucleotide sequence responsible for the development of PTNRD symptoms was known. So far, it is clear that it is within neither the CP sequence (Ohshima et al. 2000; Glais et al. 2002) nor HC-Pro gene (Schubert et al. 2007). Identification of nonrecombinant isolates inducing PTNRD present in Japan and North America (Ohshima et al. 2000) has indicated that the recombinant nature of the viral genome is not the only prerequisite for the necrotic pathotype. Furthermore, it was concluded that amino acid changes within several genes could contribute to the final symptomatic phenotype, in which neither all genes nor any particular sequences are essential (Barker et al. 2009).

Nevertheless, numerous serological and molecular methods have been developed to distinguish PVY isolates. Bioassays offer the most accurate assessment of pathogenicity of the virus isolate; however, they are labor intensive and time consuming. Enzyme-linked immunosorbent assays (ELISA) are more rapid and, using strain-specific antibodies, the PVY^{O} strain group can be distinguished readily from the PVY^{N} strain group (Boonham and Barker 1998; Ounouna et al. 2002). However, PVY^{NTN} and $PVY^{N\text{-}Wi}$ cannot be discriminated properly. A lateral flow device, based on the ELISA principle, allows the detection of PVY in a few minutes (Danks and Barker 2000). During the last decade, numerous molecular methods based on differences in genome nucleotide sequence have been developed to detect and distinguish all PVY isolates. Different variants of polymerase chain reaction (PCR), such as uniplex, duplex, multiplex, and three-primer PCR, have been developed (Weilguny and Singh 1998; Boonham et al. 2002; Moravec et al. 2003; Crosslin 2005; Glais et al.

Fig. 1 Potato tubers expressing typical symptoms of potato tuber necrosis ringspot disease

2005; Lorenzen et al. 2006; Rigotti and Gugerli 2007; Schubert et al. 2007; Chikh Ali et al. 2010). PCR coupled with restriction fragment length polymorphism (RFLP) (Glais et al. 1996) and an electrophoretic method detecting a shift in mobility of partially annealed RNA transcripts (Rosner and Maslenin 2001) were developed and optimized for discrimination of PVY^{NTN} and PVY^{N} isolates. Advantages of the PCR-based methods, namely speed, sensitivity, and specificity, have overcome drawbacks such as risk of contamination, sensitivity to inhibitors, and complexity, allowing their implementation in many diagnostic laboratories. For quantification, and to avoid agarose gel electrophoresis, which carries a high risk of contamination, as the endpoint analysis, the real-time PCR method was introduced. Real-time PCR overcomes the basic drawbacks of conventional PCR (contamination, absence of quantification), has wider possibilities of analysis (multiplex, quantification, point mutation identification), and requires fewer reagents and less time (Lopez et al. 2009). To further increase specificity and sensitivity of PVY detection, real-time PCR (Jacquot et al. 2005; Kogovšek et al. 2008), the AmpliDet assay based on nucleic acid sequence-based amplification (NASBA) (Szemes et al. 2002), and SNaPshot assay (Rolland et al. 2008) were developed.

Despite this, a fast, reliable, and inexpensive method for simultaneous detection and differentiation of all PVY isolates is still not available. The importance of the virus, coupled with contradictory data (mainly linked to the cause of PTRND disease), stimulated the worldwide scientific community to found PVY^{wide} organization (http://www.inra.fr/pvy_organization_eng), which aims to collect data on genome, biological, and serological properties of many PVY strains collected worldwide. On the basis of such new data, appropriate strategies for virus control and identification will be developed.

3 Potato Sensitivity/Resistance to PVY Infection

Soon after the introduction of potato and its cultivation for human consumption, viruses started to restrict its production. The practice of seeding the previous year's tubers, which could be infected, led to expansion of the diseases. At first, the virus-mediated diseases were termed "potato degeneration", observed as losses in crop production, and were assumed to be the consequence of the continuous vegetative propagation of potato or the unfavorable climate and soil conditions. At the beginning of the twentieth century, potato viruses were identified as a causative agent of the potato crop yield losses. Following new practices in potato seed production, first clonal selection and then virus-free planting material, obtained using micropropagation, were established, minimizing the probability of the infection and virus transmission.

Sensitive potato cultivars develop symptoms upon infection with PVY, leading to great reduction in yield. The need for resistant potato cultivars thus became urgent. With identification of genes harboring natural resistance in wild Solanaceae species and their introduction into the breeding process, production of potato cultivars resistant to PVY started. There are three types of resistant response of potato plants, namely resistance to infection, resistance to virus accumulation, and resistance to virus movement (Solomon-Blackburn and Barker 2001b). A few decades ago, modern genetic engineering approaches provided the opportunity to produce alternative resistant cultivars. In transgenic resistant cultivars, a gene originating from other organism (e.g., PVY) is incorporated into the potato genome, offering resistant reaction to PVY through gene silencing. There is also a report of successful protection against PVY by mutating a potato gene necessary for interaction with PVY (Cavatorta et al. 2011). Currently, a number of potato cultivars are on the market that harbors different levels and types of resistance. Nevertheless, development of the disease symptoms correlates not only with the potato cultivar and its level of sensitivity to infection but also with the particular virus isolate and environmental conditions (Scholthof 2007).

3.1 Natural Resistance to PVY

In potato plants, several types of natural resistance to PVY can be distinguished. They are encoded by distinct resistance genes (e.g., Ry_{sto}, N*y*) and act against either a specific virus strain or a broad spectrum of strains. Non-host plants prevent virus uncoating and translation and they do not develop any symptoms or support virus accumulation. Host plants can exhibit incompatible or compatible interaction with the virus (Fig. 2). In the plant that does not support virus multiplication and spread, the plant and the virus are in an incompatible interaction, while a compatible interaction is defined as an interaction allowing viral spreading (Valkonen 1994). In an incompatible interaction, the plants respond to viral infection with an extreme resistance (ER) or a hypersensitive resistance (HR) response. In both cases, necrotic

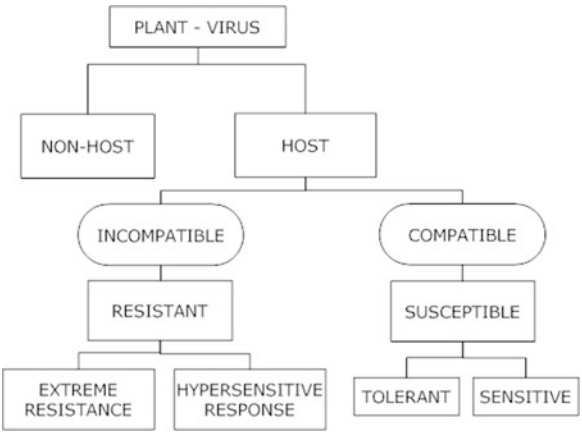

Fig. 2 Schematic presentation of possible reactions in plant–virus interaction

lesions limit the virus and prevent its further spread. In contrast, in the case of a compatible interaction, plants are susceptible to viral infection. A variety of symptoms can develop as a result of infection, because the spread of the virus and disease development are not prevented. The necrosis that forms in a compatible interaction does not stop the virus and has a tendency to spread during pathogenesis (Hinrich-Berger et al. 1999). Susceptible plants can be either tolerant or sensitive to infection with PVY. Tolerant plants can accumulate high titers of virus without symptoms or yield loss (cv. Pentland Squire) (Ravnikar 2005). Cultivars that respond to virus infection with development of the disease are sensitive cultivars (cv. Igor).

Extreme resistance of plants to viruses is defined by three criteria: localization of the virus to the primary infection site, limited virus replication, and little or no visible appearance of symptoms. Extreme resistance against PVY is supported by the activation of *Ry* genes that act against a broad spectrum of virus strains by limiting their accumulation (Valkonen 1994). Up to the present, three *Ry* genes have been mapped on potato chromosomes and originate from *S. tuberosum* subsp. *andigena* (gene Ry_{adg}) or the wild species *S. stoloniferum* (gene Ry_{sto}) and *S. chacoense* (Ry_{chc})—all are very important in breeding for PVY resistance (Szajko et al. 2008). In extremely resistant potato cultivars bearing the *Ry* gene, the resistance reaction stops movement and replication of the virus, thus preventing virus systemic spread. In that case, PVY can only replicate in the initially infected cells and move to a few adjacent cells (Hinrichs et al. 1998).

The hypersensitive reaction to PVY is strain specific and correlates with induction of *N* genes leading to induction of systemic acquired resistance (SAR). In plants bearing *N* resistance genes, PVY^{C} activates *Nc* gene, PVY^{O} activates expression of *Ny* gene (reviewed in Solomon-Blackburn and Barker 2001a), and the PVY^{Z} group of isolates induce a proposed *Nz* gene (Singh et al. 2008), leading to the development of necrotic lesions localizing the virus and making the plant resistant to virus infection. A novel gene, designated as *Ny-1*, was described as being responsible for HR in potato plants infected with common PVY^{O} and necrotic PVY^{N}, $PVY^{N\text{-}Wi}$, and PVY^{NTN} isolates, presenting an alternative to *Ry* genes in potato breeding (Szajko et al. 2008).

3.2 Genetic Engineering Based Pathogen Derived Resistance to PVY

The use of conventional breeding for introducing a resistance gene into the potato genome is a difficult, expensive, and long-term approach. An alternative is to make use of pathogen-derived resistance by genetic engineering. So far, the coat protein (CP) gene (Okamoto et al. 1996; Stanič Racman et al. 2001) and the P1 proteinase gene (Pehu et al. 1995; Maki-Valkama et al. 2001) have been expressed in potato plants of various cultivars, making sensitive cultivars resistant to PVY. pr17 protein from Luteovirus *Potato leaf-roll virus* (PLRV) was shown to block cell-to-cell movement of PVY in transgenic potato (Tacke et al. 1996). However, the strength and specificity of the resistance differed between the approaches. The exploitation of posttranscriptional gene silencing, induced by introducing the 3′ end of PVY CP dsRNA into the potato genome, offered resistance to PVY isolates from strain groups PVY^{N}, PVY^{O}, and PVY^{NTN} (Missiou et al. 2004).

3.3 Non-pathogen Derived Resistance to PVY

There is also a report of non-pathogen derived resistance to PVY. Eukaryotic translation initiation factor 4E (eIF4E) is known to be necessary for successful virus infection through binding with VPg viral protein. Potato plants transformed with a modified gene for eIF4E were shown to be resistant to infection with $PVY^{N\text{-}Wi}$, PVY^{O}, and PVY^{NTN} (Cavatorta et al. 2011). Pokeweed (*Phytolacca americana*) antiviral protein (PAP), a ribosome inhibiting protein found in the cell walls of pokeweed, was used in potato transformation (Lodge et al. 1993). Plants transformed with cDNA for PAP show resistant response to infection with PVY.

4 Spread, Localization, and Accumulation of Potyviruses in Plants

After entering a plant cell and initial replication at the site of infection, viruses can spread from cell to cell through plasmodesmata or, for long distances, via plant veins (Carrington et al. 1996). Potyviruses possess a unique mechanism of intercellular transport involving several proteins, none of which is dedicated to a movement function by itself. The formation of competent coat protein (CP) is assumed to be necessary for cell-to-cell movement, indicating a role for CP as a movement protein of *Tobacco etch virus* (TEV) (Dolja et al. 1994). Additionally, cylindrical inclusion (CI) protein was shown to be essential for virus cell-to-cell movement of *Plum pox virus* (PPV) (Gomez de Cedron et al. 2006). Subcellular localization of the viral proteins at early stages of infection showed accumulation of CI, together with CP, P3 protein, and

viral RNA, in close vicinity to plasmodesmata penetrating the cell wall. The latter observation supports the idea of potyvirus cell-to-cell movement as virions or as complexes containing CP proteins and viral RNA (Rodriguez-Cerezo et al. 1997).

Genome-linked protein (VPg) is also involved in cell-to-cell movement and controls accumulation and long-distance transport of *Potato virus A* (PVA) in potato leaves (Rajamaki and Valkonen 2002). Helper component—proteinase (HC-Pro)—was also shown to be essential for long-distance transport (Cronin et al. 1995). Another role of HC-Pro was identified by analysis of potyvirus aphid mediated transmission, where it was shown that HC-Pro binds to VPg (Yambao et al. 2003) and the N terminus of the CP (Blanc et al. 1997), which might serve as a connection between virus particles and aphid mouthparts. Atomic force microscopy revealed a protruding tip at one end of PVY virions that consisted of VPg and HC-Pro proteins, and it was proposed that the end of the potyviral particles containing VPg might be a regulatory switch modulating involvement of virus particles in different virus functions (Torrance et al. 2006).

By mechanical inoculation, the viruses most probably enter the plant through the leaf trichomes and spread from there to the epidermis, as was shown in the case of potato plants infected with *Tobacco etch virus* (TEV) fused with β-glucuronidase (GUS). Cell-to-cell spread, measured on a daily course, revealed gradual increase of the infected area from 1 to 10 cells within a period of 4 days (Hinrichs et al. 1998). Interestingly, a similar rate of cell-to-cell spread of PVY^{O} was observed in sensitive potato cv. Quarta and resistant cvs. Pirola and Betina up to the fourth day following inoculation. Later, the spread of the virus is stopped in a resistant cultivar whereas, in a sensitive cultivar, the virus spreads further (Hinrichs et al. 1998). The latter was also observed in various potato cultivars with different levels of sensitivity to PVY^{NTN} infection. From the fourth day after inoculation, the virus multiplied in inoculated leaves and then spread systemically (Mehle et al. 2004; Baebler et al. 2011).

The amount of virus and the severity of the symptoms in different cultivars do not always correlate, indicating that the titer of virus does not correlate with the severity of symptoms or type of resistance (Mehle et al. 2004). The dynamics of the viral multiplication appears to be more important, because the most pronounced differences between two phenotypes (symptomatic and asymptomatic) can be observed at the initial stage of viral multiplication and first development of local primary symptoms (Baebler et al. 2011).

For long-distance movement, viruses apply phloem and in some cases xylem (Dicenta et al. 2003; Ion-Nagy et al. 2006). Rates of viral long-distance movement to upper and lower parts of the plant appear to be equal and independent of the cultivar sensitivity (Mehle et al. 2004; Baebler et al. 2011). Three weeks after inoculation, the virus titer was approximately the same in all parts of the potato plant, with the exception of the roots (Mehle et al. 2004). In plants of the susceptible potato cultivar Russet Burbank, PVY^{NTN}, PVY^{O}, and $PVY^{N:O}$ have a higher tendency to move from the inoculation site to the lower parts of the plants. Three and four weeks after inoculation, amounts of virus detected by ELISA in lower leaves were higher than in upper leaves. The concentration of the virus in inoculated and non-inoculated leaves was affected by the age of the inoculated leaf at the time of the inoculation (Cervantes and Alvarez 2011).

Real-time PCR and electron microscopy negative staining methods were applied to analyze the distribution of PVY^{NTN} RNA and PVY^{NTN} particles, respectively, within systemically infected plants of highly sensitive potato cv. Igor (Kogovšek et al. 2011). PVY^{NTN} accumulates in all tissues and organs, including tubers, although the accumulation of viral RNA and particles was not uniform. Virus particles accumulated mostly in stem pith and in senescent leaf petiole, while viral RNA accumulated to the highest levels in all tissues of symptomatic leaves, in senescent leaf petiole, and in all layers of stem. The localization of viral RNA at the cellular level was also analyzed by in situ hybridization, where the distinctive signal was detected in leaf trichoma cells, in petiole, and in stem parenchyma cells. Uneven distribution of the virus indicates that tissue- or organ-specific mechanisms are employed by the virus or by the plant to determine the level of virus accumulation (Kogovšek et al. 2011).

PVY^{O} CP was detected immunologically by tissue printing in stem epidermal and phloem tissues of potato plants cv. Désirée and tobacco plants cv. Xanthi (Krzymowska and Hennig 1997). A dense signal of PVY^{O} CP was observed in epidermis and peripheral parenchyma and a relatively weak signal was observed in the phloem tissue of tobacco petiole. Double-sided labeling immunocytochemistry and light microscopy were used to localize PVY in the vascular tissue of inoculated tobacco leaf, where it was observed mainly in parenchymal cells, but not in companion cells (Ding et al. 1998).

The subcellular localization of typical *Potyviridae* protein inclusions from cytoplasmic inclusion proteins (CIPs), seen as pinwheels, scrolls, and laminated aggregates (Shand et al. 2009), was observed in tobacco cells infected with PVY using transmission electron microscopy. Pinwheels can be seen as semicircular chains connected together at one end, forming a windmill-like structure (Fig. 3a). Scrolls that look like concentric circles of chains (Fig. 3b) and parallel lines of chains are recognized as laminated aggregates (Fig. 3c). In potato plants infected with PVY^{NTN}, CIPs accumulated in the cytoplasm of parenchymal cells in close vicinity to epidermis, in epidermal and in trichome cells of leaves, and also in stem parenchymal cells (Pompe-Novak et al. 2001; Kogovšek et al. 2011).

5 Response of Potato Plants to Infection with PVY

5.1 Symptoms

5.1.1 Macroscopic Changes on PVY Infected Potato Plants

Potato plants can be infected primarily by aphid assistance or mechanically. In susceptible plants virus spreads to the whole plant, including tubers, giving rise to a systemically infected plant. Plants grown from infected tubers are secondary infected plants. At the site of primary infection, plants of a sensitive cultivar express

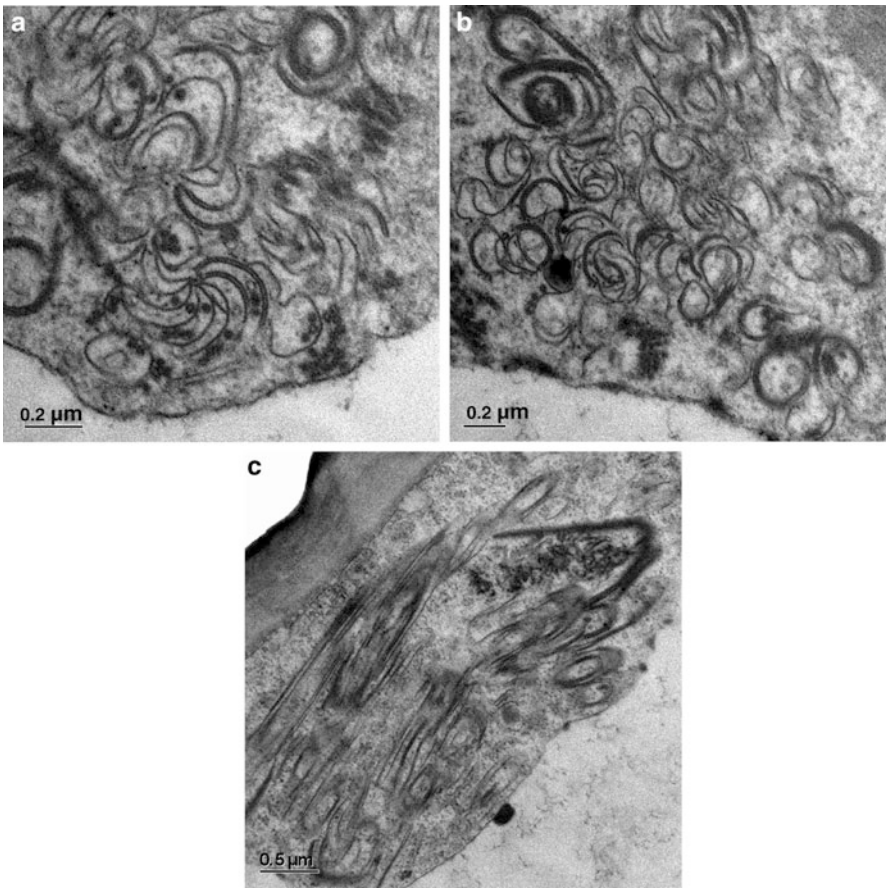

Fig. 3 Typical potyviral cytoplasmic inclusion proteins organized in pinwheels (**a**), scrolls (**b**), and laminated aggregates (**c**)

local symptoms, and symptoms expressed on non-inoculated parts of the plants are recognized as systemic symptoms. Local and systemic symptoms on primarily infected potato plants and symptoms on secondarily infected potato plants can differ, and their appearance is dependent upon several factors.

The rate of development of local symptoms, together with their strength, expressed as the appearance of chlorotic ringspots, local necrotic lesions, yellowing or dropping of inoculated leaves, and ring necrosis on tubers, was found to correlate with the level of sensitivity of the cultivar to PVY infection (Kus 1995; Mehle et al. 2004). However, the intensity and severity of the symptoms also depend upon environmental factors such as light intensity (Draper et al. 2002), temperature, humidity, and other prevailing conditions.

The highly sensitive potato cv. Igor develops severe local chlorotic ringspots on inoculated leaves, approximately 5 days after inoculation with PVY^{NTN} (Fig. 4). The surroundings of the chlorosis become yellowish and chlorotic ringspots turn

Fig. 4 Sensitive cv. Igor infected with PVY^{NTN} (left) and mock inoculated (right). Severe chlorotic ringspots develop approximately 5 days after inoculation with PVY^{NTN}. The surroundings of the chlorosis become yellowish, leading to total collapse of the leaf and eventually to leaf drop

into necrotic lesions, leading to total collapse of the leaf and, eventually, to leaf drop, the so-called palm tree symptom. At about the tenth day after inoculation, the plants harbor only upper non-inoculated leaves, which express systemic mosaic and leaf curling (Mehle et al. 2004).

Moderately sensitive cv. Quarta responds to infection with PVY^{O} with the development of local necrotic lesions in the intercostal region and vein necrosis at 4 dpi. The necrotic area expands with time and leaf often becomes yellow and dies prematurely (Hinrich-Berger et al. 1999). Similar leaf yellowing can be observed in tolerant-like cultivar Désirée infected with PVY^{NTN}, where inoculated leaves and upper non-inoculated leaves develop mild or no necrosis (Mehle et al. 2004; Baebler et al. 2011).

PVY^{NTN} infection does not induce any local or systemic symptoms in tolerant cv. Pentland Squire or extremely resistant cv. Sante (Mehle et al. 2004). On the other hand, PVY^{O} induces necrotic streaks along the leaf veins and small necrotic lesions on inter-veinal regions in extremely resistant cv. Pirola (Hinrich-Berger et al. 1999).

Secondary infected potato plants express mosaic, stem, and vein necrosis and leaf drop, and can develop stunted phenotype. Secondary infected tubers of sensitive cultivars usually show dramatic yield loss and intense ring necrosis (Kus 1994).

5.1.2 Microscopic Changes in PVY Infected Potato Tissues and Cells

Electron microscopy (EM) offers the only reliable approach for observing viral particles within plant tissue. In the plant sap, PVY virions can be observed as filamentous flexuous particles (Fig. 5a). However, within the tissue section, there

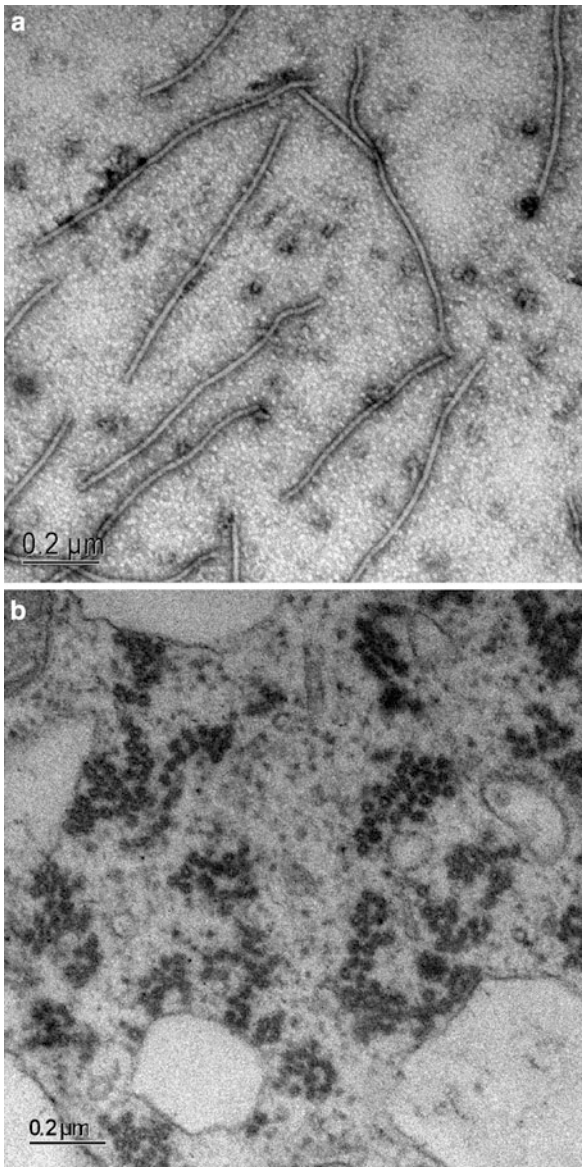

Fig. 5 (**a**) Filamentous flexuous particles detected in plant sap of infected potato; (**b**) Cross-section of tubular aggregates accumulated in PVY^{NTN} infected potato cell

are no reliable reports of PVY virion localization, although structures with a high probability of being viral particles were reported (Fig. 5b) (Kogovšek et al. 2011). Normally, within PVY-infected plant tissue, typical inclusions of CI protein can be identified by EM (Fig. 3). EM analysis of plant tissues embedded in resin also offers an insight into changes caused by viral infection at the ultrastructural level. On the other hand, light microscopy offers an insight into changes in the tissue at the cellular level.

Light microscopy observation of a necrotic area that developed following PVY^{O} infection of resistant cv. Pirola showed that epidermal and collenchyma cells were brown and that the pattern extended to the collenchyma cells on the periphery of the necrosis. Within the necrotic cells of leaves of susceptible cv. Quarta and resistant potato cv. Pirola, autofluorescence was observed and Aniline blue staining revealed a strongly fluorescent zone in the non-necrotic cells adjacent to the necrotic cells, indicating accumulation of lignin or other polyphenols and depositions of callose (Hinrich-Berger et al. 1999).

PVY^{NTN} infection has a strong impact on the ultrastructure of the chloroplasts in infected potato plants of sensitive cv. Igor. In non-symptomatic parts of the leaves, the chloroplasts of palisade cells were smaller than in the same type of cells in healthy plants. Close to the spot necrosis, the chloroplasts were even smaller and their swelling progressed towards the center of the necrosis. At the center of the necrosis, thylakoid structure loosened and the chloroplasts condensed, the cytoplasm was dense, no vacuoles were present, cells were shrunk, the cell wall was wrinkled and the intracellular spaces enlarged. Electron microscopy analysis of the infected tissue ultrastructure revealed total destruction of cells, cell wall thickening, and destruction of peroxisomes and chloroplasts within the necrotic lesion (Poljšak-Prijatelj and Ravnikar 1992). The alterations in ultrastructure caused by infection could be recognized as part of the apoptotic process, leading to cell death and development of the symptoms (Pompe-Novak et al. 2001). In epidermal cells, and in adjacent cells, which had just become necrotic, very dense cytoplasm with no vacuole was observed. Adjacent cells were collapsed due to plasmolysed protoplasts. On the edge of larger and older necrosis, protoplasts collapsed into a mass of membranous structures, organelles were destroyed, and wall oppositions could be observed between necrotic and living cells. Interestingly, the degree of hypersensitive reaction was, at the ultrastructural level, very similar, if not the same, in sensitive cv. Quarta and resistant cv. Pirola (Hinrich-Berger et al. 1999). At later stages of infection, when systemic symptoms developed, larger amounts of chloroplasts per cell were observed in green parts of the leaves, while in secondary infected plants grown in vitro, chloroplasts were smaller and had exvaginations. However, in neither system could any visible changes on thylakoid be observed, meaning that the reduced levels of photosynthesis are more likely to be the consequence of changes at the metabolic level (Pompe-Novak et al. 2001).

In stem and parts of the leaves with no visible symptoms, potyviral pinwheels, mitochondria, peroxisomes, chloroplasts, and large crystals were often situated tightly together (Poljšak-Prijatelj and Ravnikar 1992; Pompe-Novak et al. 2001).

As the virus spreads systemically it reaches tubers as well. Potato tubers of the resistant cv. Kuba developed small internal point necroses and black spots on the surface upon grafting-assisted infection with PVY^{NTN} (Blaszczak et al. 2005). Scanning electron microscopy revealed that, within those areas, cells were greatly damaged and sunken. In the case of sensitive potato cvs. Rosalind and Vineta, the PVY^{NTN} infection caused more severe symptoms, observed as brownish necrotic areas on the surface and also in deeper parts of the tuber, including phloem tissue. Within the necrotic tissue, strand-like structures concentrated, and cells were

damaged and dead. Infected cells contained no starch granules, which could be connected with virus-induced starch degradation into soluble sugars.

5.1.3 Cytological Changes in PVY Infected Potato Plants

The apical meristem of secondarily infected potato plants of sensitive cv. Igor was smaller, due mainly to reduced cell number, whereas the peripheral zone, involved in leaf primordial formation, was the most affected region. Additionally, the mitotic activity of the peripheral zone was lower, leading to lower numbers of leaves and smaller total leaf area. Lower numbers of leaves were also the consequence of the longer time needed for the development of the two subsequent leaves observed in infected plants. A low mitotic index was detected in mid-meristem, resulting in reduced development of stem tissues, observed as narrower stem diameter (Dolenc et al. 2000).

Mean tissue doubling time in healthy plants grown in vitro increases with time, meristem size decreases, and growth rate slows down. However, in secondarily infected potato plants the mean tissue doubling time and meristem size did not change (Dolenc et al. 2000). The latter effect could be connected to higher cytokinin levels in shoots of secondarily infected potato grown *in* vitro (see Sect. 5.2.3), which are necessary for induction as well as maintenance of the mitotic activity.

5.2 Changes in PVY Infected Potato at Metabolic Level

5.2.1 Functional Genomics Approaches Used to Study Potato–PVY Interaction

To understand the metabolic changes in potato plants infected with PVY, various approaches have been used offering different levels of insights into potato–PVY interaction. Various "omics" approaches were used, applying techniques like cDNA microarrays, two-dimensional electrophoresis (2DE), mass spectrometry (MS), and others. Transcriptomic approaches offer insights into changes in the plant at the level of mRNA expression. The inability to detect posttranscriptional regulation can be remedied by proteomics approaches that enable observation of the proteins in the plant. Posttranslational modifications, e.g. phosphorylation, can be analyzed using proteomic approaches. Nevertheless, the final result of the enzymatic changes has to be observed at the level of metabolites. The latter can be achieved with metabolomic approaches. Furthermore, phenomics profiling approaches are used to collect data on a certain set of morphological and developmental traits, which allow putting together a comprehensive picture of a plant's phenome.

Up to now, potato–PVY interaction has been analyzed mostly at the transcriptomic level, while only a limited number of experiments have been carried out at proteome and metabolome levels. The huge amount of data retrieved from the

high-throughput methods used in "omics" studies has to be analyzed with care. Bioinformatics and statistics are crucial for evaluating the results. By combining the results of all four types of approaches, a systemic insight into potato–PVY interaction is acquired.

For transcriptomic studies, quantitative real-time PCR, subtractive hybridization libraries, and cDNA microarray methods were used. Real-time PCR is a high-throughput, extremely sensitive method enabling observation of changes in gene expression. The method was used to validate the expression of specific genes from selected pathways obtained by analysis of the cDNA microarray results (Baebler et al. 2009; Kogovšek et al. 2010; Baebler et al. 2011). The only limiting factor is the number of genes analyzed simultaneously. Subtractive cDNA library is very useful for selecting the cDNA clones corresponding to mRNAs present in one sample, but not in another. The cDNA clones represent a library of mRNAs expressed at a certain time point in infected, but not in healthy plants and vice versa, and can be used in cDNA microarray preparation (Pompe-Novak et al. 2006) or can be correlated to cDNA microarray results (Baebler et al. 2009). Alternatively, cDNA microarrays enable simultaneous observation of very large numbers of genes, although the number of tested samples is the limiting factor. The first potato microarrays were custom cDNA microarrays, where the cDNA originated from subtractive hybridization libraries from PVY infected potato samples (Pompe-Novak et al. 2006; van Dijk et al. 2009). Later, commercially available potato TIGR cDNA microarrays were used in potato–PVY interaction studies, where expression of 10,000 potato genes could be observed (Baebler et al. 2009; Kogovšek et al. 2010), while formerly only 400 genes could be spotted. Long oligo microarrays designed by Potato Oligo Chip Initiative (POCI) are currently the most suitable ones for studying potato physiology having biologically and technically improved.

Changes in the proteome composition can be observed by different variants of 2DE and MS. With 2DE proteins are separated on the basis of their mass and charge. Different staining methods are used for protein visualization, from classic silver and Coomassie blue to fluorescence-based dyes, which offer higher quantitative accuracy and sensitivity and are compatible with modern downstream procedures (Agrawal et al. 2005). Proteins separated on 2DE can be further identified and characterized by MS.

MS-based techniques are often used for identification, characterization, and quantification of proteins and peptides separated by 2DE, mixtures of peptides, plant metabolites, and volatiles. Various types of MS technique are available and the researcher can decide for the most suitable one, depending on the sample type and the analysis (Allwood et al. 2011). Nevertheless, more classical biochemical methods are also used for qualitative and quantitative evaluation of plant metabolic changes. Enzyme activity can be assayed spectrophotometrically, while high-performance liquid chromatography (HPLC) is used to separate and quantify hormones, photosynthetic pigments, and secondary metabolites.

One of the most demanding tasks of high-throughput "omics" studies is data analysis and interpretation. In order to reach reliable conclusions, statistical approaches must be applied, providing lists of components of biological system

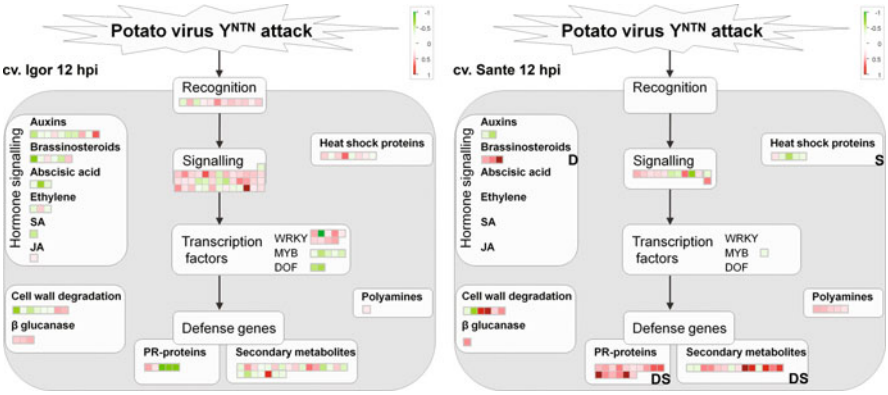

Fig. 6 Responses of two potato cultivars with different reactions to PVY^{NTN} infection at the level of genes related to perception and signaling. The figure is exported from the MapMan program. The visualization program MapMan is organized in BINs, where each BIN represents a biological function or pathway and can be divided into subBINs, representing subgroups of genes involved in the biological process. MapMan enables observation of changes in the expression of genes that are part of general metabolism, specific cellular functions (protein synthesis), biological responses to stress, or genes for enzyme families with unknown function (Baebler et al. 2009)

(genes, proteins, or metabolites) whose expression changed significantly. None of the statistical tests guarantee that the selected components are the most important in plant pathogenesis; however, by combining different statistical and data mining approaches, greater reliability of the results can be achieved (Rotter et al. 2008).

The last step in data analysis is interpretation of the results. Because the lists of significantly differentially expressed components are not necessarily informative and not easy to interpret, they need to be connected with their function/location in the cells—the so-called gene ontology. Potato ontology was prepared within the software MapMan (Fig. 6), which also offers visualization of metabolic pathways (Thimm et al. 2004), statistical evaluation of potentially regulated pathways by Wilcoxon rank sum test (Usadel et al. 2005), and adaptation and generation of new groups of interest (Rotter et al. 2007).

5.2.2 Silencing

RNA silencing is one of the most intriguing, however, the least explored, plant defense mechanisms. It consists of a number of interconnected pathways that limit the synthesis, stability, and translatability of RNA molecules. The common feature of these pathways is that they are sequence specific and this specificity is provided by small (s)RNAs that are complementary to sequences in the target molecule. In plants RNA silencing decreases gene expression in three ways: by degradation of transcripts, inhibiting translation of mRNAs, or by promoting targeted methylation of DNA to effect transcriptional gene silencing (reviewed in Carr et al. 2010).

RNA silencing appears to play roles in SA-induced resistance to viruses and the inhibition of virus gene expression and replication during the HR. Cellular RNA-dependent RNA polymerases, which are involved in plant RNA-silencing pathways, were shown to be necessary for the maintenance of basal resistance against PVY (Rakhshandehroo et al. 2009). The HCPro protein, the viral silencing suppressor protein, from *Tobacco etch virus* and *Plum poxvirus* was shown to be involved in SA-mediated signaling (Pruss et al. 2004; Alamillo et al. 2006). In potato–PVY interaction until now no such response was explored into detail; however, oligo microarray analysis shows that certain genes involved in gene silencing are differentially expressed (unpublished data). The importance of silencing in potato response to PVY is one of the assignments to be explored and revealed in future.

5.2.3 Photosynthesis and Photosynthetic Pigments

The impact of virus infection on photosynthesis is one of the most fully described metabolic changes. In general, virus infection of plants causes a decrease in photosynthetic activity and chlorophyll content, and downregulation of photosynthesis related genes, which have been observed in PVY infected plants, using a broad range of methods from HPLC (Milavec et al. 1999; Milavec et al. 2001) and infrared gas analyzer (Zhou et al. 2004) to cDNA microarrays and real-time PCR methods (Pompe-Novak et al. 2006; Baebler et al. 2009; Kogovšek et al. 2010; Baebler et al. 2011).

In contrast to the generally observed decrease in photosynthesis in virus-infected plants in immediate response to PVY^{NTN} infection, meaning 30 min after inoculation, an increase in expression of photosynthesis-related genes was observed in both sensitive cv. Igor and resistant cv. Sante by cDNA microarrays and real-time PCR (Baebler et al. 2009). A similar response was observed in two sensitive cultivars, namely Igor and Nadine, inoculated with two virus isolates, PVY^{NTN} and PVY^{N}, where genes related to photosynthesis were expressed more highly in plants of both cultivars infected with the more aggressive PVY^{NTN} than with the milder PVY^{N} (Kogovšek et al. 2010). The upregulation could be the plants' response to elevated energy demand needed for the first response to stress. Later the expression of photosynthesis-related genes was downregulated in all tested potato cultivars (Baebler et al. 2009; Kogovšek et al. 2010).

Symptomatic (sensitive cv. Igor and transgenic NahG-Désirée) and asymptomatic (non-transgenic Désirée) genotypes can be differentiated based on the expression of photosynthesis-related genes through the time scale. The expression of those genes was mostly correlated and exhibited similar dynamics (Baebler et al. 2011).

HPLC analysis of photosynthetic pigments revealed that PVY^{NTN} infection causes a decrease in the total amount of photosynthetic pigments per unit of dry weight of leaves of secondary infected potato plants grown *in vitro*, and revealed that those changes are cultivar specific (Anžlovar et al. 1996). In soil-grown plants of sensitive cv. Igor, no significant changes in accumulation of photosynthetic pigments were observed early (24 h) after inoculation, while later (few days after

inoculation)—but before development of any visible symptoms—the levels of all photosynthetic pigments decreased (Milavec et al. 1999). The effect was more pronounced in plants grown in autumn, which already contained smaller amounts of photosynthetic pigments, than in plants grown in spring. It appears that the plants with less pigments were more susceptible to infection.

HPLC analysis of photosynthetic pigments revealed a decrease in the total amount of photosynthetic pigments in symptomatic and senescent leaves (Milavec et al. 1999). In accordance with this, expression of genes involved in photosynthesis, metabolism of pigments, and photorespiration, measured by cDNA microarrays, was lower in systemically infected plants of sensitive potato cv. Igor than in healthy plants of the same cultivar (Pompe-Novak et al. 2006).

In the upper green leaves of infected plants of susceptible cv. Igor, photosynthetic pigment content increased, indicating that the plants accumulate the chlorophyll in order to compensate for the damage caused by virus replication in infected leaves. Changes in the total amount of photosynthetic pigments were due mainly to changes in chlorophylls, because the levels of carotenoids did not change in any group of the plants. When comparing the ratios of carotenoids to chlorophylls a significant increase in ratio was observed in symptomatic and senescent leaves, whereas the ratio of chlorophyll a to b did not change (Milavec et al. 2001).

In contrast, in sensitive potato plants of cv. Chunzao systemically infected with PVY^{NTN}, decreased photosynthesis rate was observed although no changes in chlorophyll content or photochemical efficiency could be detected in symptomatic leaves. However, it appears that the decreased photosynthesis rate was due mainly to alterations in Calvin cycle reactions leading to downregulation of electron transport. The electron transport is re-directed to alternative electron sinks such as nitrate reaction, photorespiration, and Mehler reactions (Zhou et al. 2004) (for the latter see Sect. 5.3).

5.2.4 Hormone Pathways

The involvement of jasmonic acid, salicylic acid, cytokinins, and some other hormones and signaling molecules in potato–PVY interactions has been analyzed in various cultivars expressing different reactions to infection. The results indicate that hormones have a very important role in potato pathogenesis, although their mode of action is unclear.

Jasmonic Acid

The involvement of jasmonic acid (JA) in potato–PVY^{NTN} interaction was observed in two different systems: secondarily infected plants of the sensitive cv. Igor grown in vitro (Petrovič et al. 1997) and primarily infected plants of the tolerant-like cv. Désirée and resistant cv. Sante grown in soil (Kovač et al. 2009). Higher concentrations of JA were detected in infected potato plants than in healthy

plants of all tested cultivars, regardless of the growth system and stage of infection. In infected potato plants of cvs. Désirée and Sante the concentration of the bioactive precursor of JA synthesis, 12-oxophytodienoic acid (OPDA), also increased (Kovač et al. 2009).

The precise role of JA in potato pathogenesis is still unclear. The involvement of JA in virus replication and spread can be deduced from the observation of lower PVY^{NTN} concentrations in plants grown in media with exogenously added JA (Petrovič and Ravnikar 1995). Moreover, a more pronounced increase in concentrations of JA and OPDA was detected in resistant than in moderately sensitive cultivars early after inoculation (Kovač et al. 2009), which could further support the above results and indicate the role of jasmonates in the very early resistant response of the potato. Secondary infection of potato plants of sensitive cv. Igor grown in vitro evoked an altered distribution of endogenous JA. In infected potato plants, JA accumulated predominantly in the roots but, in healthy plants, in the shoots (Petrovič et al. 1997). Infected plants also showed higher responsiveness to exogenous JA treatment, because addition of JA to medium affected the growth of infected potato plants more strongly than that of healthy potato plants. The effect was observed as an increase in length and fresh weight of shoots and a decrease in fresh weight of roots (Petrovič and Ravnikar 1995).

With potato cDNA microarrays, no relevant response was observed for genes related to JA pathway (Baebler et al. 2009).

Salicylic Acid

Potato plants contain very high levels of basal salicylic acid (SA) (Yu et al. 1997) and the relation between resistance and high SA levels was described in potato–virus interaction (Singh et al. 2004). However, in resistant potato cv. Sante lower basal SA levels were observed than in sensitive cv. Igor (Krečič-Stres et al. 2005), indicating that the SA basal level does not correlate with resistance in potato–PVY interaction. Moreover, at early stages of infection of susceptible cv. Igor, the concentration of SA increased (Krečič-Stres et al. 2005), although it appeared that the increase in SA was not sufficient to limit the virus replication and/or movement, which could be due to lack of signal transduction or some other component of the plant protection pathway. In contrast, no changes in SA concentration were detected in potato plants of the tolerant-like cv. Désirée and resistant cv. Sante a few hours after infection with PVY^{NTN} (Kovač et al. 2009). The role of SA in systemically infected plants could be deduced from the slight, but significant, increase in SA conjugate concentration in inoculated leaves of resistant transgenic cv. Igor (Krečič-Stres et al. 2005).

However, the importance of the SA pathway in potato resistance was shown in transgenic potato plants of tolerant-like cultivar Désirée with modified expression of *nahG* gene responsible for SA biosynthesis (NahG-Désirée plants). The transgenic cultivar was depleted in SA and after PVY infection developed severe symptoms, meaning that the mechanisms for tolerance were disrupted. Moreover,

in transgenic plants PVY multiplied and spread systemically (Baebler et al. 2011). On the basis of these observations, one might conclude that SA is involved in the inhibition of cell-to-cell movement and systemic spread of the virus and that it takes part in the tolerant-like response of the cv. Désirée. The importance of SA in viral multiplication and symptoms development was also shown through reversion of the disease development by spraying plants with SA analogue (Baebler et al. 2011).

Cytokinins

The role of cytokinins in potato–PVY^{NTN} interaction could be partially connected with symptoms development. Infected plants of sensitive cv. Igor grown in vitro expressed a symptom-less phenotype and levels of biologically active cytokinins in their shoots increased (Dermastia and Ravnikar 1996). On the other hand, in roots of symptomatic potato plants of sensitive cv. Igor grown in vivo, massive inactivation of cytokinins was observed, limiting utilization or transport of active forms of cytokinins to plant shoots. The latter was not observed in resistant potato cv. Sante, which expressed no symptoms. One might conclude that the symptoms of premature leaf senescence and leaf drop are, in part, the consequence of virus-induced reduction of active cytokinins in roots of potato grown in vivo (Dermastia et al. 1995).

Other Plant Hormones

Brassinosteroids are a group of plant hormones with a wide range of biological activity. Genes coding for brassinosteroids were expressed more highly early after inoculation with PVY^{NTN} in resistant cv. Sante. However, while no such response was observed in sensitive cultivars, the above-mentioned group of hormones has a role in resistant response (Baebler et al. 2009). The roles of other plant hormones, like ethylene, auxins, and abscisic acid, were not investigated in such detail in potato–PVY interaction. Nevertheless, the data from cDNA microarray studies showed that other groups of hormones likely have a role in potato response (Baebler et al. 2009); therefore their importance cannot be overlooked.

5.3 Perception and Signal Transduction

Expression of genes related to perception and signaling was analyzed with cDNA microarrays, real-time PCR, and subtraction libraries (Baebler et al. 2009). The response to infection with PVY^{NTN} was analyzed in two cultivars: extremely resistant cv. Sante and sensitive cv. Igor, at 12 h after inoculation. In cv. Igor, genes related to perception and signaling were expressed more highly in inoculated than in healthy plants, while no such response was detected in resistant cv. Sante at the same time point. Moreover, genes for transcription factors from WRKY family

were expressed more highly in inoculated than in healthy plants of sensitive potato cv. Igor, while in resistant cv. Sante no significant changes in expression were observed (Fig. 6). One might conclude, therefore, that the sensitive potato cultivar possesses a mechanism that activates a defense response, but the outcome of the response was not successful. One of the reasons for unsuccessful protection could be a delay in the response of the sensitive cultivar, where no changes in signaling and perception-related genes expression were detected in the resistant cultivars. The latter could be explained by an earlier response in the resistant cultivar, which was not detected at the measured time point (Baebler et al. 2009).

5.4 Defense Response

5.4.1 Antioxidant Metabolism

Antioxidant metabolism is one of the protective mechanisms, which is activated immediately after infection, preventing virus spread and disease development. In sensitive potato cultivars (Igor and Nadine), genes of different types of antioxidant-related enzyme were differentially expressed early after infection and at the time of symptoms development (Pompe-Novak et al. 2006; Kogovšek et al. 2010). Moreover, cDNA microarray and real-time PCR analyses showed a specific response to two different PVY virus isolates, indicating that potato plants recognized and responded differentially to the two virus isolates (Kogovšek et al. 2010) (Fig. 7). Differences were also observed in the responses in gene expression between the two sensitive cultivars, which emphasize the complexity of antioxidant metabolism and implies posttranscriptional regulation. Thus, enzyme activity and metabolite accumulation analyses offer a better insight into cell metabolism.

Peroxidases

The activity of different types of peroxidase was analyzed spectrophotometrically in potato cultivars with different reactions to PVY^{NTN} infection, namely sensitive Igor, tolerant-like Désirée, and resistant Sante (Milavec et al. 2008). In all cultivars, the activity of peroxidases increased; however, different peroxidase types were involved in the early response of each cultivar. The type of peroxidase correlated, in part, with resistance (Milavec et al. 2008). However, in different resistant cultivars time-dependent activation of different peroxidases was observed, indicating different types or mechanisms of activation of the resistance. Nevertheless, in the sensitive cultivar the elevated peroxidase activity did not offer protection against PVY^{NTN} spread and severe disease development. It appears that the expression and activity of peroxidases are or are not effective or that their activity is regulated further on posttranslational level. Plant peroxidases have been shown to be involved in various defense reactions against pathogens and in the formation of reactive oxygen species

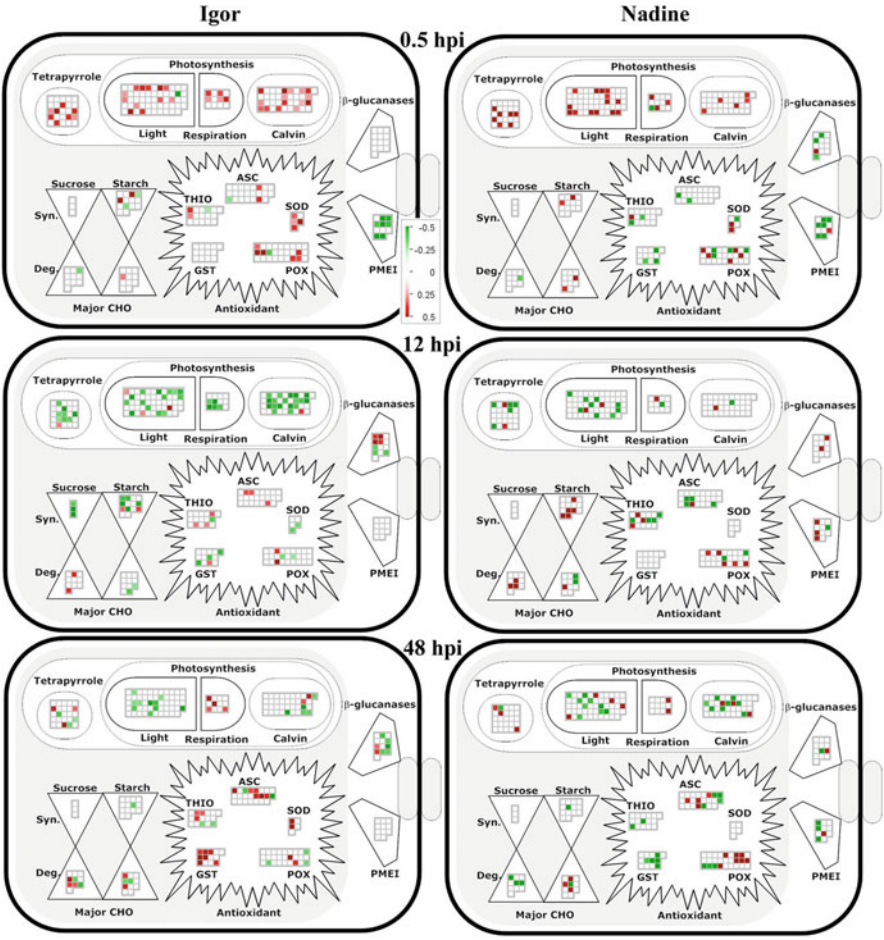

Fig. 7 Response of two sensitive cultivars to infection with two PVY isolates with different degrees of aggressiveness. Differences in the response of genes related to antioxidant metabolism can be observed between cultivars and in time course. The figure is exported from MapMan. Light—light reactions, respiration—photorespiration, Calvin—Calvin cycle, tetrapyrrole—tetrapyrrole synthesis; Major carbohydrates (CHO)—Syn.: synthesis, Deg.: degradation; Antioxidant—THIO: thioredoxin peroxidase, ASC: ascorbate and glutathione peroxidase, glutathione reductase, SOD: dismutases and catalases; GST: glutathion S-transferase, POX: peroxidases; PMEI: pectin methylesterase inhibitor. Each *square* presents log2 ration of the expression of one clone in PVY^{NTN} vs. PVY^{N} inoculated plants (Kogovšek et al. 2010)

and H_2O_2, a key signaling molecule. A strong and immediate response to PVY infection at the level of change in peroxidase activity indicates their role in signaling through H_2O_2 formation.

Hydrogen Peroxide Localization

The role of H_2O_2 as a signaling molecule, triggering expression of genes for localized cell death and inducing defense genes in adjacent cells, was shown in a resistant cultivar infected with two necrotizing PVY isolates. Accumulation of H_2O_2 was detected cytochemically on the surface of the epidermis and in adjacent mesophyll cells, in vascular tissue, and in leaf trichomes of infected leaves, at sites where local necroses later developed (Otulak and Garbaczewska 2010).

Using transmission electron microscopy, H_2O_2 was localized by the observation of precipitates of $CeCl_3$ (Otulak and Garbaczewska 2010). Precipitates accumulated along the cell walls of xylem tracheary elements, epidermal cells, and mesophyll. Additionally, the precipitates accumulated along the cell membranes of the mesophyll and phloem parenchyma, which could be connected to the role of H_2O_2 in modifying cell walls through peroxidase-assisted cross-linking of the polymers in the plant defense response.

Within the mesophyll cells, precipitates, indicating H_2O_2 accumulation, were detected in the endoplasmic reticulum, vacuoles, in the vicinity of chloroplasts, and in the nuclear envelope and perinuclear space (Otulak and Garbaczewska 2010). The localization of H_2O_2 in chloroplasts and nucleus indicates its possible involvement in redox homeostasis, which, in turn, acts as a signal influencing gene expression in both compartments. Changes in reactive levels of oxygen species are, in stressed cells, often accompanied by alterations in photosynthesis rate, where reactions connected to the photosynthesis constitute the main antioxidant detoxification system.

5.4.2 Pathogenesis-Related Proteins

The expression of genes for pathogenesis-related proteins (PR-proteins) was investigated with cDNA microarrays and real-time PCR in different cultivars with different reaction to PVY or PVA infection. As early as 12 h after inoculation with PVY^{NTN}, genes for proteinase inhibitors (PR-6) are strongly upregulated in resistant cv. Sante, while no such intense response was observed in sensitive cv. Igor (Baebler et al. 2009). In secondarily infected potato plants grown in vitro, the ratios between cysteine proteinases and their inhibitors were altered, indicating their potential role in the plant response to PVY-induced stress (Pompe-Novak et al. 2002).

β-1,3-Glucanases are classified into PR-2 family and are connected to virus spread through the degradation of callose, the physical barrier made by the plant to protect against pathogen penetration (Iglesias and Meins 2000). Three structural classes of β-glucanase have been identified so far in the *Solanaceae* family. Class I isoforms are basic proteins constantly accumulated in the cell vacuole, whereas class II and III isoforms are inducible acidic proteins secreted into the extracellular space (Ward et al. 1991). Genes of all three types of β-glucanase were differentially expressed in sensitive cvs. Igor and Nadine and resistant cv. Sante (Baebler et al. 2009; Kogovšek et al. 2010). Differential expression was displayed early after

infection with PVY^{NTN}, implying the involvement of those genes in the early response to virus infection. At later stages of infection, the expression of β-glucanase genes was induced in inoculated leaves before the first detection of viral multiplication, in non-inoculated leaves, and in secondarily infected plants (Pompe-Novak et al. 2006; Baebler et al. 2011). The exact role of β-1,3-glucanases in potato–PVY interaction is still not understood and at the moment cannot be distinguished whether β-1,3-glucanases are induced in response to viral infection or are under viral control to enable faster spread of the virus.

In resistant potato line infected with PVA and in tolerant-like cv. Désirée infected with PVY^{NTN}, expression of PR-1 associated genes was only moderately changed at 24 hours post infection, which can be explained by auto induction of defense-related genes (Vuorinen et al. 2010; Baebler et al. 2011). Analysis showed high basal expression of defense-related genes in resistant potato line (Vuorinen et al. 2010) and in tolerant cultivar, which is connected, in part, to higher concentrations of SA (Baebler et al. 2011). When comparing the symptomatic and asymptomatic genotypes, the response of PR-protein genes is stronger in symptomatic cv. Igor and NahG-Désirée infected with PVY^{NTN}. On the contrary, the response of defense-related gene group in non-inoculated leaves of asymptomatic cv. Désirée was not very different from either of the symptomatic genotypes (Baebler et al. 2011). In potato line, allowing virus multiplication in inoculated leaves and preventing systemic spread, no response on the mRNA concentrations of PR-protein associated genes was detected at 24 h after infection with PVA (Vuorinen et al. 2010). Altogether, one might conclude that the expression of PR-1 genes is not related directly to the type of response—resistant, tolerant, sensitive—to infection and that the expression of PR-1 genes is connected to SA concentrations.

5.5 Heat Shock Proteins

Heat shock proteins (HSPs) are well-known proteins involved in the response of plants to various stresses, when elevated expression is most often observed. The role of HSPs, especially HSP 70, in plant–virus interaction is most probably to fulfill the requirement for rapid protein maturation and turnover during a short virus multiplication cycle and in cell-to-cell and long-distance movement. Moreover, Hsp70 is assumed to be involved in localization/transportation of the viral replication proteins, the insertion of the replication proteins into intracellular membranes, and assisting the assembly of the viral replicase (reviewed in Nagy and Pogany 2010).

cDNA microarray studies revealed differential expression of genes related to HSP proteins in potato plants inoculated with PVY^{NTN}. Early after infection of sensitive potato cv. Igor, the expression of genes encoding different HSPs is mainly higher, while in resistant cv. Sante it is lower than in healthy plants (Baebler et al. 2009). Again, as shown in previous examples, the sensitive cultivar responds intensely to infection at the level of gene expression, although no effective protection is achieved.

5.6 *Secondary Metabolism*

The influence of PVY^{NTN} infection in secondary metabolism was analyzed by a cDNA microarray study of the responses of resistant cv. Sante and sensitive cv. Igor to infection (Baebler et al. 2009). Plants of the resistant cultivar responded to virus infection with elevated expression of genes involved in polyamine metabolism, 2-ODD, phenylalanine ammonia lyase, and other enzymes involved in lignin biosynthesis. On the other hand, changes of expression of these genes are not so profound in sensitive cv. Igor, indicating the involvement of secondary metabolism in successful protection against virus spread in the resistant cultivar.

6 Concluding Remarks and Perspectives on Studying the Physiology of the Potato–PVY Interaction

Analysis of potato–PVY interactions using different approaches has led to the conclusion that the timing of the response of plants to infection is crucial for the success of the final defense response. A delay in response can lead to ineffective protection and disease development (Baebler et al. 2009). However, different potato cultivars vary markedly in their response to infection with PVY. Cultivars that express similar phenotypic alterations upon infection can possess their own mechanisms of the response that can be observed at the biochemical and gene expression levels.

This observation raises more questions concerning the appropriate experimental setup to allow the observation of as many time points after inoculation as possible, in order to detect the plant's response. Furthermore, different parts of plants, or even tissues within the same plant organ, respond differently to virus infection. Analysis of smaller parts of tissues or even separate cells would offer an insight into the plant response at the single cell level. Moreover, non-targeted approaches, such as new generation sequencing for analysis of transcriptome, offer new possibilities in the analysis of the plant's physiological state. However, the cost and feasibility of the huge number of experiments required and of the interpretation of the resulting data place a firm limit on such investigations.

The next step towards understanding of potato–PVY interaction is testing the true role of selected genes or their products. Transgene potato plants with changed expression of genes, selected to be involved in response to infection, would be observed and evaluated with various phenomic approaches. Ionomics, temperature profile, photosynthetic and growth rate, root physiology, and other morphological and physiological characteristics would significantly contribute to better understanding of plant response to infection.

As commented above, bioinformatics and biostatistics play an important role in data interpretation. The development of tools for analyzing and interpreting the huge amounts of data obtained by various high-throughput methods is necessary.

Only a combination of different approaches will assist in obtaining a complete insight into potato–PVY interaction. Knowledge from biochemical, transcriptomic, proteomic, metabolomic, and phenomic studies would contribute to the construction of a model of potato response to PVY infection. A model would further allow prediction of the infection outcome, which could be useful in potato breeding.

Even though not widely described and discussed, endophytic microorganisms, present in or between the plant cells, have a role in plant's response to infection. Endophytic microorganisms can induce defense response through activation of systemic acquired resistance (Conn et al. 2008) or induced systemic resistance (Kavino et al. 2007). Studying and identifying the endophytic microorganisms would improve the understanding of the plant–virus interaction. Nevertheless, with disclosure of new partners involved in plant–virus interaction, the true complexity of the biological systems is revealed and therefore more questions are likely to be posed.

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Part III
Physiology

Cellular Mechanisms of Environmental Adaptation: Learning from Non-*Arabidopsis* Model Species

Dortje Golldack

Contents

Abstract Soil salinity is an abiotic stressor that severely limits crop yield and agricultural productivity. Currently, comprehensive elucidations of complex networks of cellular salt adaptation provide new opportunities for the breeding and engineering of improved plant tolerance to this environmental cue. Systematic analyses of cellular pathways of salt adaptation have been particularly performed in the glycophytic model plants *Arabidopsis thaliana* and rice by exploiting current state-of-the-art *omics* methods as well as a wide range of genetic tools. Despite the wealth of knowledge provided by these studies, detailed understanding of the complex networks of cellular salt adaptation requires, however, systematic analyses of salt-adaptive mechanisms in naturally halotolerant plant species. These studies on the molecular mechanisms of salt adaptation in halophytes are very limited due to the restricted availability of

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genetic techniques and resources in these species. Only recently, *Arabidopsis* relative model species (ARMS) and rice relative model species (RRMS) have been introduced into salt stress research allowing direct comparison with the glycophytic models *Arabidopsis* and rice. Particularly, cross-species transcriptome analyses allowed new insights into differences of stress regulating mechanisms in glycophytes and halophytes. Here, recent discoveries on *Arabidopsis* and rice relative model species are reviewed focusing on regulatory systems of salt adaptation as well as their biotechnological applicability.

1 Introduction: Environmental Changes and Current Goals of Plant Agricultural Biotechnology

Currently, agricultural production is facing new challenges due to constant global environmental changes in many areas worldwide. Rising temperatures, drought, and soil salinization steadily cause both significant losses of arable lands and increasing destruction of agricultural soils (e.g., Ashraf and Akram 2009). These environmental alterations become even more problematic in view of a constantly increasing world population that is expected to reach more than 9 billion in 2050 (Godfray et al. 2010; Tester and Langridge 2010). Thus, research in plant agricultural biotechnology is challenged to find solutions for meeting the food demands of a growing population facing increasing limitations of arable land. Particularly, identifying strategies to increase the yields of crop plants under conditions of water limitation and of irrigation with water of lower quality are of immense importance and interest.

Breeding and biotechnological attempts to improve tolerance of crops to the different environmental factors inevitably require detailed knowledge of the adaptive mechanisms of plants to diverse cues. An important and very interesting example is plant adaptation to the abiotic stressor salt. In this chapter current understanding of plant salt tolerance mechanisms is summarized that was built over more than two decades of intense research by a wide range of experimental approaches. In particular, focus will be on the contribution of different models systems to this research.

2 Salt Stress: An Inducer of Complex Networks of Cellular Responses

Intracellular uptake of excess salts results in ion toxicity and disturbance of cellular metabolism (Hasegawa et al. 2000). In addition, extracellular accumulation of ions causes hyperosmotic stress and cellular dehydration accompanied by excess generation of reactive oxygen species (ROS) and secondary oxidative stress (Miller et al. 2010; Jaspers and Kangasjärvi 2010). Besides salt-sensitive glycophytic species, salt stress research has investigated extremophile halophytes that tolerate salt concentrations up to 500 mM NaCl (Golldack 2004). It is known that plant salt adaptation in halotolerant species is mainly enabled by (1) synthesis and

accumulation of osmolytes to counterbalance the hyperosmotic component of salt stress, (2) by regulation of the intracellular ion homeostasis and vacuolar sequestration of excess and toxic anions and cations, and (3) by scavenging ROS (Hasegawa et al. 2000; Munns 2005; Abogadallah 2000).

Elucidation of these key mechanisms of plant salt tolerance has been mainly derived from physiological and molecular analyses focusing on differential regulations of salt-adaptive processes in halotolerant and glycophytic plant species (e.g., Bohnert et al. 1999; Zhang et al. 2004; Chinnusamy et al. 2006). Particularly the facultative halophyte *Mesembryanthemum crystallinum* has been studied as a halophyte model for many years and valuable knowledge on the physiology of plant salt adaptation is based on these analyses (Adams et al. 1998). However, work on natural halotolerant plant species became clearly limited when large-scale genetic screens and high-throughput *'omics'* tools were introduced in the plant sciences. Currently, analyses of plant adaptation to stress conditions are aimed at understanding the global regulatory networks that control abiotic stress responses (Golldack et al. 2011). Here, interactions of the different adaptive levels such as gene expression, protein synthesis, metabolite composition, and epigenetic processes are in the main focus. The ultimate goal of these studies is the identification of molecular hubs of salt adaptation in halotolerant species that might be potentially used in agricultural crops to improve the stress tolerance in the glycophytic crop species. Despite the extraordinary abilities of natural halophytes to adapt to a wide range of salt concentrations up to extreme hypersaline conditions, studies on salt-tolerant species have been significantly underrepresented due to the limited knowledge on genome sequence information on these plants (e.g., Møller and Tester 2007; Amtmann 2009). Instead, salt stress research has been focusing on the glycophytic plant models *Arabidopsis thaliana* and rice, exploiting technical approaches as forward and reverse genetics as well as diverse *omics* technologies (Urano et al. 2010).

3 The Model Plant *A. thaliana*: Pathways of the Salt Stress Response

In the glycophytic model plant *A. thaliana*, the first systematic analyses of salt stress responses on the transcriptome level indicated that approximately 3% of the whole genome responds to salt stress at the transcriptional level (e.g., Seki et al. 2002). Being a salt-sensitive species, data on the salt stress response of *A. thaliana* contribute to the understanding of salinity tolerance only in a limited manner. Particularly, biotechnological applicability of these studies to improve salt tolerance in crop plants is clearly limited based on the pronounced salt sensitivity of the model plant. However, the powerful approach of studying stress reactions on a transcriptome scale provided much novel information on how plants integrate different salt adaptation mechanisms in cellular networks. Thus, the work generated new insights into plant stress adaptation by identifying sets of salt-responsive genes that had not been linked with salinity adaptation before (Seki et al. 2001, 2002;

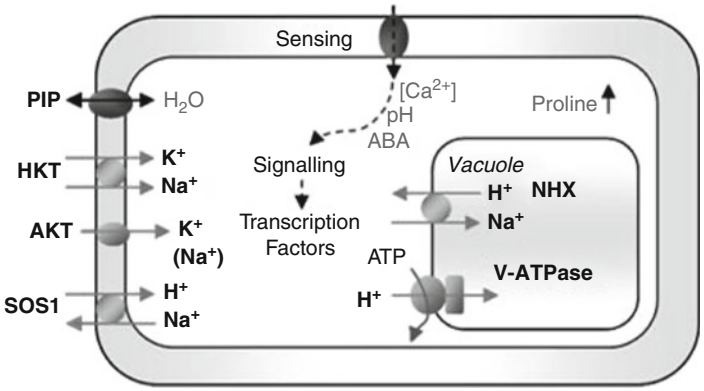

Fig. 1 Model of cellular mechanisms of plant salt adaptation (SOS1—Na^+/H^+-antiporter, HKT—K^+/Na^+-symporter or Na^+-transporter, AKT—voltage gated inward rectifying K^+ channel, PIP—plasmamembrane integrated aquaporin. V-ATPase—V-type H^+-ATPase, NHX—Na^+/H^+-antiporter, ABA—abscisic acid). See text for further detail

Rabbani et al. 2003). In addition, gene products involved in salt tolerance have been identified by systematic and comprehensive mutant screens in *A. thaliana*. Thus, the importance of the SOS (salt overly sensitive) pathway for the intracellular sodium homeostasis has been first described for *A. thaliana* but could be verified for other plant species later on (Shi et al. 2000; Olías et al. 2009; Fraile-Escanciano et al. 2010; Cuin et al. 2011). Within the SOS pathway, the Ca^{2+}-sensor protein SOS3 and the serine/threonine protein kinase SOS2 activate the plasma membrane proton/sodium antiporter SOS1 that regulates cytoplasmic sodium concentration (Chinnusamy et al. 2004).

Next to cellular extrusion of sodium by the SOS1 transporter, intracellular sequestration of excess sodium is another component of salt tolerance. Vacuolar sodium sequestration is mediated by NHX-type secondary active sodium proton antiporters that are energized by the vacuolar H^+-ATPase (V-ATPase) and the vacuolar H^+-pyrophosphatase generated proton motive force (Apse et al. 1999; Dietz et al. 2001; Silva and Gerós 2009; Adler et al. 2010; Krebs et al. 2010). Interestingly, in contrast to a halophytic species such as *M. crystallinum,* no salt-induced transcriptional regulation of V-ATPase has been found in *A. thaliana* (Kluge et al. 1999). This may indicate that cellular sodium extrusion rather than subcellular sodium sequestration plays the key function in the salt stress response of *A. thaliana* (Golldack and Dietz 2001; Krebs et al. 2010; Fig. 1). This view is further supported by recent studies on the function of the HKT-type potassium/sodium transporter in *A. thaliana*. For this transporter, function in exclusion of sodium from leaves and in shoot-to-root phloem recirculation of sodium has been shown (e.g., Horie et al. 2009). Interestingly, in *A. thaliana* there is good evidence that HKT1 recovers sodium from the xylem to xylem parenchyma cells (Sunarpi et al. 2005; Davenport et al. 2007).

4 Rice: The Crop Model of Salt Stress Research

Understanding the molecular responses of plants to the major environmental stress factor, salt, is of particular importance in the context of biotechnological applications to improve salt tolerance of monocotelydonous crop species. The monocotelydonous crops are evolutionarily distant from *A. thaliana* and *M. crystallinum*, and hence the applicability of knowledge on salt tolerance of these model species to crops is limited. To fill this gap, rice has been introduced into salt stress research. These studies were significantly facilitated by the availability of the full rice genome sequence and the applicability of genetic methods to rice (e.g., Rice Full-Length cDNA Consortium Kikuchi et al. 2003; Miki and Shimamoto 2004; Hiei and Komari 2008). In addition, next to highly salt-sensitive rice cultivars, there are several rice cultivars with a considerable salt tolerance available. Differences in salt tolerance among rice varieties are mostly due to differences in ion uptake and exclusion of toxic ions (Golldack et al. 2002). Thus, the sodium permeable potassium channel OsAKT1 showed transcriptional regulation in root epidermal and exodermal root cells in the sodium-excluding rice line Pokkali but not in the sodium-accumulating rice line IR29 proving the correlation of OsAKT1 expression with whole plant sodium selectivity in rice (Golldack et al. 2003). In addition, a sodium-selective HKT-type transporter mediates sodium exclusion from leaves via sodium removal from the xylem sap and is likely to be a key transporter in regulating cation homeostasis in rice under salt stress (Ren et al. 2005; Horie et al. 2009). This hypothesis obtained recent support from a transgenic study in barley. Here, overexpression of an HKT-type transporter enhanced both the translocation of sodium in the xylem and the salt tolerance of the transgenic plants (Mian et al. 2011).

The results suggest again that in glycophytic species, extracellular translocation and accumulation are more important for salt tolerance than intracellular ion sequestration. Understanding of rice salt tolerance mechanisms was significantly enhanced by cDNA microarray-based analyses of the salt-inducible transcriptome. Comparison with the salt-inducible transcriptome of *A. thaliana* indicated considerable overlap of the response to salt between the monocot and the dicot model (Rabbani et al. 2003). However, approximately 30% of salt stress inducible rice genes did not respond to salt stress in *A. thaliana* indicating the existence of distinct pathways of salt adaptation in these evolutionarily distant species.

5 A New Chapter in Salt Stress Research: *Arabidopsis* Relative Model Systems

Much information on cellular pathways of salt adaptation has been gained by exploiting *omics* methods and genetic tools in the models *A. thaliana* and rice. However, these analyses failed to elucidate all the processes that underlie the complex trait of extreme salt tolerance in natural halophytes. To improve the understanding of salt adaptation in the halophytes at the whole-genome level,

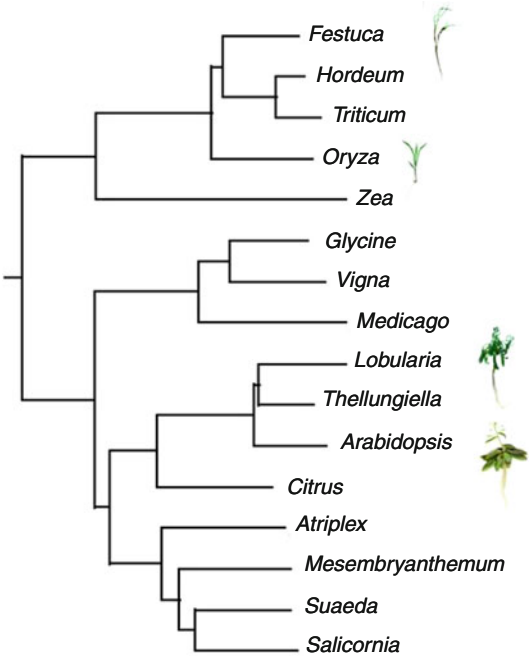

Fig. 2 Dendrogram showing the relationship of NHX-type transporters based on amino acid sequences (Popova and Golldack 2007; Diédhiou et al. 2009a)

establishment of new halophytic model species was highly necessary. Several requirements need to be met by these models: most importantly, accessibility of genome information and suitability for genetic analyses. Over the last years, several novel halophytic *Arabidopsis*-relative model systems (ARMS) have been identified and established.

A comparative screen of growth behavior under salt stress conditions in Brassicaceae identified several species with moderate-to-high salt tolerance including, for example, *Malcolmia triloba* and *Lepidium virginicum* (Orsini et al. 2010). These species are promising candidates for genome wide characterization of salt tolerance traits by using the wealth of genetic and sequence information available from their glycophytic close relative *A. thaliana*. As next steps, in-depth physiological studies of the salt adaptation strategies and assessment of the amenability of these species for genetic analyses will be required to select the most suitable models for systematic large-scale characterization of environmental adaptation.

The best-characterized halophyte among ARMS to date is *Thellungiella salsuginea* (*halophila*; Amtmann 2009). *T. salsuginea* is an extremophile species native to Central Asia and North America that shows considerable tolerance not only to salt but also to cold and drought (Zhu 2001; Wong et al. 2005). *T. salsuginea* shares high levels of nucleotide sequence identity with the model plant *A. thaliana* with 92% in average (e.g., Inan et al. 2004; Taji et al. 2010; Fig. 2). This close relationship enabled fast and easy cloning and functional identification of *T. salsuginea* genes based on the sequence information available from *A. thaliana*. Regarding the physiological strategies of salt adaptation, *T. salsuginea* mainly

utilizes the amino acid proline as an osmoprotective compatible solute (M'rah et al. 2007). *T. salsuginea* uses organ-specific regulation of potassium and sodium transporters as a means of ion homeostasis under salt stress (Inan et al. 2004; Taji et al. 2004; Kant et al. 2006; Volkov and Amtmann 200; Ghars et al. 2008).

When grown in soil culture, *T. salsuginea* (*halophila*) showed a LD_{50} at salt concentrations as high as 600 mM NaCl (Orsini et al. 2010). In hydroponic culture, the tolerance behavior of the species was obviously different depending on the amount of other ions, e.g., potassium and calcium in the media (e.g., Orsini et al. 2010). The plants continued growth at 200 mM NaCl in hydroponic culture but treatment with 250 mM NaCl was lethal for 50% of the plants within 3 days of stress (Volkov et al. 2003; Taji et al. 2004). *T. halophila* shows an ion uptake and accumulation behavior different from *A. thaliana* with less accumulation of sodium and higher contents of potassium compared with *A. thaliana* under salt stress (Volkov et al. 2003). According to these studies, *T. halophila* efficiently discriminates between sodium and potassium and achieves salt tolerance by exclusion of sodium due to specific features of root ion channels (Volkov and Amtmann 2006).

The notion that *Thellungiella* has adapted to salinity by evolving stress avoidance strategies rather than by adjusting cellular metabolism to hyper-sodic conditions is further supported by recent comparisons of physiological and metabolic responses of *A. thaliana* and *T. halophila* to desiccation and salinity (Arbona et al. 2010). Thus, *T. halophila* accumulated less chloride ions than *A. thaliana* under salt stress. Based on different metabolic compositions of the halotolerant and the salt-sensitive species, it might be hypothesized that *Thellungiella* is permanently "prepared" to balance dry and saline conditions (e.g., Gong et al. 2005). Accordingly, it might not require extensive transcriptional and metabolic re-programming to cope with environmental stress (e.g., Arbona et al. 2010).

This view was first attained in a comparative cDNA microarray study using cDNA populations of salt-treated *T. halophila* and *A. thaliana*. Thus, due to the high nucleic acid sequence identity of *T. halophila* and *A. thaliana* cross-species hybridization of *A. thaliana* cDNA arrays could be performed that enabled the investigation of *Thellungiella* salt stress responses at the transcriptome level (Taji et al. 2004). Interestingly, in *T. halophila* only very few salt-inducible transcripts were detected. However, several genes that are induced by salt in *A. thaliana* were expressed in *Thellungiella* at high levels even in the absence of stress. This constitutively high expression of stress-relevant transcripts is an important component of stress adaptation in *Thellungiella* (Taji et al. 2004).

6 *Lobularia maritima*: Characterization of a Promising *Arabidopsis* Related Model Species

As another *ARMS*, the facultative halophyte *Lobularia maritima* (L.) Desv. (*Alyssum maritimum* (L.) Lam., Brassicaceae) was introduced into the studies of abiotic stress tolerance (Golldack 2004). *L. maritima* is native to the coastlines of

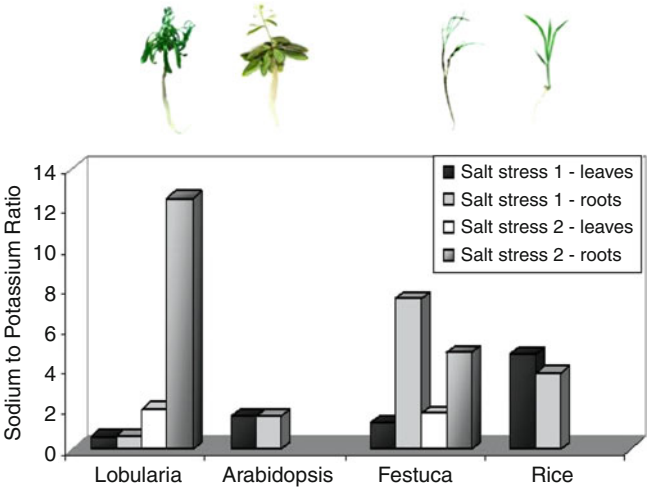

Fig. 3 Sodium to potassium ratio under salt stress. The halophytes *Lobularia* and *Festuca* were treated with 125 mM NaCl (Salt stress 1) and 500 mM NaCl (Salt stress 2) for 48 h. *Arabidopsis* were stressed with 125 mM NaCl for 48 h (Salt stress 1) and plants of the rice line IR29 were exposed to 150 mM NaCl for 72 h (Salt stress 1; modified from Golldack et al. 2002; Popova and Golldack 2007; Diédhiou et al. 2009a)

the Canary Islands and the Azores, and it was selected for comparative studies based on its close taxonomic and molecular relationships to *A. thaliana* (Popova and Golldack 2007; Fig. 2). In contrast to the sodium-excluding *Thellungiella*, *L. maritima* accumulates sodium while also maintaining high potassium contents under salinity (Fig. 3).

In accordance with a strategy of sodium inclusion, transcriptional upregulation of the vacuolar sodium/proton antiporter NHX1 and of the vacuolar ATPase in *L. maritima* indicates efficient mechanisms for intracellular sequestration of sodium (Popova and Golldack 2007). Comparative transcriptome analyses of *L. maritima* and *A. thaliana* allowed the identification of more than 100 salt-responsive transcripts in the halophyte. Many of these were related to known salt-adaptive mechanisms in halophytes such as regulation of ion homeostasis, synthesis of proline, and scavenging of ROS (Popova et al. 2008). In addition, a wide range of genes that had not been associated with salt stress responses before were found to be salt responsive in *L. maritima:* These encoded metabolic enzymes, proteins with functions in cellular defense and rescue, signal transduction factors, and regulatory components of gene expression. Regulatory proteins displaying different salt responsiveness in *A. thaliana* and in the related halophytes might open up novel strategies for engineering plant salt tolerance. As a first very promising example, expression of the bZIP-type transcription factor bZIP24 was transgenically modified in *A. thaliana* to mimic the pattern in *L. maritima*. As a fascinating result, salt tolerance in the transgenic plants was indeed significantly improved due to modified sodium/potassium homeostasis (Yang et al. 2009). These results clearly demonstrate

the usefulness of transcriptome comparisons between the glycophyte *A. thaliana* and related halophytes for an in-depth understanding of plant stress responses.

7 Salt Tolerance in Crops: Rice Relative Model Species

To identify regulatory elements and pathways of salt adaptation, studies in naturally halotolerant monocot species are also highly necessary. Accordingly, attempts have been started to identify rice-related halotolerant model species that have close taxonomic and genetic relationship to the monocot model rice. Thus, in the grass species *Puccinellia tenuiflora* that may grow on saline soils large-scale gene expression profiles in response to saline–alkali ($NaHCO_3$) stress were monitored by cDNA array analyses proving the feasibility of transcriptome analyses in naturally halotolerant monocot species (Wang et al. 2007). As an another example, the salt marsh species *Spartina maritima* share high nucleic acid identity with rice transcripts making cross-species microarray hybridizations of both species possible that might give fascinating insights into the complex traits of salt adaptation in a plant specialized to settle in an extreme environment (Chelaifa et al. 2010).

Even more advanced studies of comparative transcriptome analyses have been performed for rice and the salt marsh grass species *Festuca rubra* ssp. *litoralis*. The facultative halophyte *Festuca* tolerates 500 mM NaCl in hydroponic culture and accumulates sodium in leaf and root tissue by involvement of V-ATPase and the vacuolar sodium/proton antiporter (Diédhiou et al. 2009a; Fig. 3). Comparative cDNA array hybridizations with salt-responsive transcripts from rice and *Festuca* led to the identification of a complex network of differently regulated transcripts with functions ranging from ion homeostasis to metabolism and general stress defense that were transcriptionally regulated in the halophyte but not in rice (Diédhiou et al. 2009b). Among the transcripts with different transcriptional regulation in rice and the related halophyte, signaling elements with proposed key function in triggering the activation of the basic salt adaptation mechanisms are of main interest. These signaling elements might function as key regulators that activate the wide range of metabolic and cellular processes involved in successful plant salt adaptation. Accordingly, transgenic transfer of single signaling elements to glycophytic crops according to the model of the related halophytes might be sufficient to activate ionic and osmotic adaptations as well as scavenging of salt-induced ROS excesses by activating the complex network of adaptive processes via the master regulatory elements of the signaling pathways.

Thus, different salt-induced regulation of the serine/threonine protein kinase SnRK1b (sucrose non-fermenting-1-related kinase1) in *Festuca* and rice was detected and transgenic rice lines with overexpression of SnRK1b were generated (Diédhiou et al. 2008a). These transgenic rice plants showed increased salt tolerance and allowed identification of the vacuolar H^+-ATPase, the Na^+/H^+ antiporter NHX1, the Cl-channel CLC1, and a catalase as downstream regulated genes of SnRK1b (Diédhiou et al. 2008b). Interestingly, transgenic expression of the SUI-homologous

translation initiation factor eIF-1 conferred increased salt tolerance to rice as well (Diédhiou et al. 2008b). In transgenic eIF-1 overexpressing rice plants, salt tolerance was improved by modified regulation of ion homeostasis and redox status suggesting coordinated function of SnRK1b and eIF-1 in the same regulatory pathway (Diédhiou et al. 2008a, b).

Data of these studies provide evidence that comparative analyses of the salt-stress responses in glycophytes and related halophytes will be a promising strategy to identify transcription regulators with key function in plant salt tolerance.

8 Salt Stress Signaling: Identification in Glycophytic and Halophytic Model Plants

Systematic transcriptome-based comparisons of glycophytes and halophytes have identified signaling and regulatory elements as hubs within the complex traits of plant salt adaptation. Reprogramming of the regulatory pathways in glycophytes according to the model of halophytes might be powerful means to improve salt tolerance.

However, the current knowledge on intracellular processing and responses to salinity is still mainly derived from the salt-sensitive model species *Arabidopsis* and rice. Even in these models, signal perception mechanisms in response to dehydration and salinity are not understood in detail. Membrane integrity and modulation of lipid synthesis are likely to be involved in primary sensing (Kader and Lindberg 2010). Subsequent early signaling events include changes in plasma membrane H^+-ATPase and Ca^{2+}-ATPase activities that trigger concerted changes of Ca^{2+} influx, cytoplasmic pH, and apoplastic production of ROS (Beffagna et al. 2005). In addition, osmotic stress induced Ca^{2+} fluxes are linked to abscisic acid (ABA), and calcium-responsive protein kinases act as key regulators in drought and salinity-induced signaling cascades (e.g., Diédhiou et al. 2008a; Fig. 1).

Interestingly, although both drought and salt stress might result in intracellular accumulation of toxic amounts of ROS, hydrogen peroxide (H_2O_2) and nitric oxide (NO) also function as signaling molecules in ABA-mediated stomatal responses (Miller et al. 2008, 2010; Wilkinson and Davies 2010). Mutation of a cellulose synthase-like protein induced accumulation of ROS and it changed the sensitivity of mutant plants to salt stress and water deficit. Interestingly, regulation of plant osmotic stress tolerance via control of intracellular stress-induced ROS levels has been suggested (Zhu et al. 2010).

As convergent downstream targets of transcriptional regulation, many genes that are responsive to drought and to salinity belong to the ABA-responsive element (ABRE) and DRE/CRT (dehydration-responsive element/ C-repeat element) regulons (Yamaguchi-Shinozaki and Shinozaki 2005). In addition to this central regulon, a considerable number of transcription factors of diverse types with function in osmotic adaptation have been reported. Knowledge on integration of

these different transcription factor classes into the cellular signaling network of drought and salt adaptation is, however, only slowly emerging. Presumably, they have both specific and connective functions in linking primary and secondary signaling pathways to stress-adaptive transcription via ABRE and DRE/CRT pathways. As an interesting example, several NAC-type transcription factors improved drought and salt tolerance by transgenic overexpression (Peng et al. 2009; Yokotani et al. 2009; Xia et al. 2010; Yang et al. 2011). Interestingly, direct binding of the factors ONAC5 and ONAC6 to the promoter of stress-inducible genes as *OsLEA3* was shown to suggest rather restricted gene-specific regulatory function of NAC transcription factors at downstream positions within the cellular signaling cascades of salt adaptation (Takasaki et al. 2010). In contrast, AP2/ERF transcription factors regulate salt- and drought-responsive transcriptional responses of more than hundred target genes via ABA-dependent and -independent pathways. They are therefore likely to have key functions in the general upstream control of stress-responsive sub-transcriptomes (Golldack et al. 2011).

Recently, with the group-F bZIP transcription factor bZIP24 another very promising key regulator of salt stress adaptation was identified by differential screening of salt-inducible transcripts in *A. thaliana* and ARMS (Yang et al. 2009). Here, biotechnological applicability of this bZIP type factor for improving salt tolerance of the glycophyte *A. thaliana* has been demonstrated (Yang et al. 2009).

9 Perspectives

Elucidation of the complex network of salt adaptation mechanisms in naturally halotolerant plant species that are close relatives of the glycophytic models *Arabidopsis* and rice will provide an enormous wealth of knowledge for identifying the cellular hubs of stress responses. Particularly, cross-species comparisons of model glycophytes and related halophytes on the experimental levels of ionomics, transcriptomics, proteomics, and metabolomics will allow identifying the hierarchies of salt stress adaptive mechanisms. Here, understanding of cross talks of signal integration and signaling responses as well as knowledge of master and key factors in the salt stress responses will be valuable for identifying promising biotechnological strategies to improve salt tolerance in crop species.

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Epigenetic Flexibility Underlying Phenotypic Plasticity

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Contents

Abstract Phenotypic plasticity refers to the ability of an organism to produce different phenotypes under different environmental circumstances. The mechanisms underlying phenotypic plasticity received considerable attention in recent years. It has become widely acknowledged that plastic variation in phenotypes mostly take place by altering gene expression and eventually altering ontogenetic trajectory in response to environmental changes. Epigenetic modifications provide a plausible mechanism for the putative link between environmental variation and alterations in gene expression. While much attention is being paid to heritable epigenetic changes, little attention is being paid to swift and reversible epigenetic alternations, which mediate rapid plastic responses of the organism to environmental perturbation. This mechanism is particularly important to allow organisms with no/low genetic diversity to adapt to different environments, and is likely to be a favorable evolutionary response when organisms are exposed to stress periods that last shorter than a single life span.

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Studying epigenetic complexes in the real environment would allow us to get greater insights into the molecular machinery that interfaces the genotype and the environment.

1 Introduction

Phenotypic plasticity refers to the ability of an organism to produce different phenotypes under different environmental circumstances (Bradshaw 1965; Stearns 1989; Sultan 2000; Pigliucci et al. 2006). Phenotypic responses to environmental variation were formerly considered as anomalous "noise" that obscured the "true" genetic characteristics of the organism (Allen 1979; Pigliucci and Schlichting 1998; Sultan 2000). In recent years, however, it has become widely acknowledged that plastic responses could not only facilitate the expression of relatively well-adapted phenotypes under novel conditions and therefore allow a population to persist, but could also affect the performance and reproductive success of individual organisms, which in turn will impact the makeup of the next generation (Pigliucci et al. 2006; Richards 2006). Recent studies also suggest that phenotypic plasticity is important in buffering natural selection, generating novel trait combination, and promoting or degrading ecological speciation (West-Eberhard 2005; Fordyce 2006; Crispo 2008; Moczek et al. 2011; Thibert-Plante and Hendry 2011).

The mechanisms underlying phenotypic plasticity have received considerable attention in recent years (Schlichting and Smith 2002; Pigliucci 2005; Sultan 2005; Feinberg 2007; Bossdorf et al. 2008; Marfil et al. 2009; Morange 2009). Multidisciplinary approaches from the fields of ecology, physiology, genetics, and evolutionary biology have been taken to identify the complex relationships existing between the mechanisms that produce variable morphological, physiological, and behavioral traits during development, and to investigate the adaptive significance of phenotypic plasticity in ecological contexts (Reiber and Roberts 2005). Comparative studies of individuals exposed to different environments using post-genomic techniques have revealed that, although cued by the external environment, most phenotypically plastic responses are mediated by alterations in the internal environment via concomitant changes in gene expression and interaction (Schlichting and Smith 2002; Jaenisch and Bird 2003; Gilbert 2005; Wade and Archer 2006). Information on how gene expression is regulated in response to environmental change would thus provide clues to the molecular mechanisms underlying phenotypic alteration in response to environmental stimuli.

2 How Is Epigenetics Related to Phenotypic Plasticity

Recent studies have demonstrated that the epigenetic pattern of the genome is frequently correlated with particular states of gene activity (Jaenisch and Bird 2003; Grant-Downton and Dickinson 2005; Wade and Archer 2006; Bird 2007). Epigenetic regulation of gene expression represents the programming of the genome to express the appropriate set of genes in specific cells at specific time points in life,

which is accomplished by DNA methylation, histone modifications, chromatin remodeling, and the small RNA machinery. DNA methylation is now recognized as a primary and perhaps the most extensively characterized epigenetic mark.

Genomes contain two layers of information: genetic and epigenetic. Genetic information provides the blueprint for the manufacture of all the proteins necessary to create a living organism, whereas epigenetic information provides additional instructions on how, where, and when the genetic information should be used (Dunn et al. 2003; Astolfi et al. 2010). Both genetic and epigenetic information contribute to the phenotypic variation within a species and influence the success of organisms in their natural contexts. However, epigenetic information is of particular interest to the study of phenotypic plasticity because previous studies have indicated that phenotypic changes in response to environmental heterogeneity are associated with the on–off status or the quantitative expression level of genes, regulated by their epigenetic status (van Kleunen and Fischer 2005; Marden 2008). It is also known that epigenetic information can be directed by the environment at least partially independent of genetic information (Wheeler et al. 1992; Richards 2006; Lukens and Zhan 2007). Consequently, epigenetic variation can lead to inducible phenotypic changes under various environments, even in the complete absence of genetic variability (Kalisz and Kramer 2008). For example, epigenetic modification of *FLC* plays a critical role during the environmentally induced transition to flowering in *Arabidopsis* (Bastow et al. 2004). The alteration of DNA methylation state can affect the caste determination in honeybees (Kucharski et al. 2008; Elango et al. 2009).

Plants are constantly challenged by environmental perturbations, and thus have developed remarkable capabilities to modulate their physiological and developmental machinery through genome-wide gene expression changes in response to environmental perturbations (Zhou et al. 2007; Lopez-Maury et al. 2008; Wang et al. 2011). Many evidences indicate that epigenetic mechanisms play a crucial role in regulating gene expression in plant responses to environmental stress (Boyko et al. 2007; Choi and Sano 2007; Boyko and Kovalchuk 2008). Bossdorf et al. (2010) found that experimental alterations of DNA methylation not only affect the means and variances of ecologically important phenotypic traits in *Arabidopsis*, but also alter the degree of phenotypic plasticity of plants to different nutrient levels. Alternative phenotypes can be achieved through regulation of the expression of a specific gene or activation of an alternative genetic pathway (Schlichting and Pigliucci 1993; Pigliucci 1996). Though currently there is evidence that environment-induced epigenetic changes may be inherited by future generations (Richards 2006; Bird 2007; Verhoeven et al. 2010; Richards et al. 2010; Eichten et al. 2011), environmentally induced gene expression is not necessarily heritable, i.e., the gene is always transmitted but not necessarily its expression state, because environmental conditions often change rapidly and in unpredictable ways (Angers et al. 2010). Recent studies have clearly shown that epigenetic regulation of gene expression is highly flexible and reversible (Tsuji et al. 2006; Zhai et al. 2008; Wang et al. 2011). Epigenetic reprogramming triggered by environmental perturbations thus serves as a molecular basis underpinning the plastic responses of plants to environmental fluctuations, providing a mechanistic link between genes and environment.

3 How Flexible Is the Epigenetic Modification to Environmental Fluctuation

Although there is a growing body of evidence showing that epigenetic modifications play an important role in mediating environment-induced phenotypic plasticity, there is as yet little empirical evidence to demonstrate that epigenetic patterns are so sensitive to environmental stimuli that they can dramatically alter a phenotype within the life span of a single organism. It is unclear whether the epigenetic control system is flexible enough to provide a short-term strategy for organisms to respond to changes in the environment.

Alligator weed (*Alternanthera philoxeroides*) is native to South America and has now become a problematic weed in about 30 countries (Holm et al. 1997). The most striking character of this weed is its amphibian nature, resulting in thrive populations in both aquatic and terrestrial habitats. Ecological genetics analyses based on DNA fingerprint and common garden experiments indicated nearly no genetic differentiation among populations from different habitats (Geng et al. 2007), which is consistent with the dominance of asexual reproduction in alligator weed. Instead, alligator weed showed notable phenotypic plasticity in a collection of functional traits, including storage root allocation, stem diameter, stem pith cavity, and internode length (Geng et al. 2006, 2007). It has been proposed that phenotypic plasticity played a key role for this weed to colonize a wide range of habitats with different water availability (Geng et al. 2006, 2007; Li and Ye 2006; Pan et al. 2007) (Fig. 1).

To assess the relevance of epigenetic process to the phenotypic variation in alligator weed, and to detect whether changes in the epigenetic state occur as rapidly as the environmental change, we examined the genomic DNA methylation patterns of alligator weed in natural and manipulated environments using the methylation-sensitive amplified fragment length polymorphism (MSAP) technique. Considerable DNA methylation polymorphisms were found within and between natural populations. By transplanting plants from different source populations reciprocally into water-manipulated common gardens, we found that plants of different source populations not only underwent significant morphological changes in common garden environments, but also underwent a genome-wide epigenetic reprogramming in response to different water treatments (Gao et al. 2010). Plants raised in the same common garden, no matter whether they came from aquatic or upland habitats, exhibited a high level of DNA methylation similarity, demonstrating the environmental sensitivity and flexibility of the epigenetic regulatory system. This research not only provided evidence of the correlation between epigenetic reprogramming and the reversible phenotypic response of alligator weed to particular environmental factors, but might also help explain why genetically identical organisms can have dramatically different phenotypes in different environments. Previous studies also demonstrated the directional induction of DNA methylation patterns by experimental treatments (Wheeler et al. 1992; Wade and Archer 2006; Weinhold 2006).

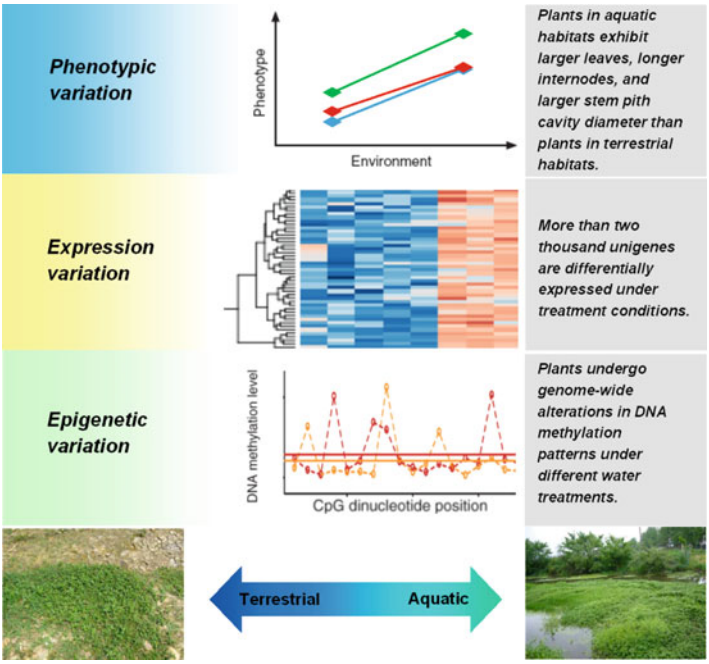

Fig. 1 Integrative research on environment-induced phenotypic variations in alligator weed

The epigenome serves as an interface between the dynamic environment and the inherited static genome (Szyf 2007). The relationship between the molecular process of epigenetic regulation and the ecological process of phenotypic plasticity has recently become a hot issue in ecological and evolutionary studies. However, much attention is currently paid to heritable epigenetic changes and their potential adaptive significances in natural populations. Little attention has been paid to swift and reversible epigenetic alternations, which mediate rapid plastic responses of the organism to environmental perturbation. Such flexible alterations can not only buffer environmental fluctuations by adjusting physiological capacities, but also enable epigenetically induced gene expression to generate specialized morphological adaptations, permitting persistence in varying environments and increasing the potential for evolution. This mechanism is particularly important to allow organisms with no/low genetic diversity to adapt to different environments, and is likely to be a favorable evolutionary response when organisms are exposed to stress periods that last shorter than a single life span.

4 How to Assess the Epigenetic Effects on Phenotypically Plastic Variation

Environmentally induced phenotypic variation ultimately results from environment-induced differential expression of "plasticity genes." The concept of "plasticity gene" has been raised early in conceptual and mathematical models for phenotypic plasticity (Pigliucci 1996; DeWitt et al. 1998), however, only a few plastic traits have been resolved at the molecular level, e.g., the shade avoidance response mediated by the light-sensitive plasticity gene (Callahan et al. 1999, 2005). Genes involved in the development of phenotypically plastic traits remain largely elusive in most non-model organisms, which greatly hampered past research on molecular mechanisms underlying phenotypic plasticity. Modern molecular tools such as the next generation sequencing technology are stimulating rapid progress in this area, allowing us to simultaneously monitor the expression of thousands of genes during induction and expression of phenotypic plasticity. When coupled with methylation sequencing of sodium bisulfite-treated DNA, knockout, and other technologies, it is now possible to identify the specific genes and pathways involved in plastic responses to the environment, and to make a causal link between methylation changes of plastic genes, ecological development ("eco-devo") processes, and particular plastic traits in non-model organisms (Fig. 2).

Given the sensitivity to the environment and potential reversibility of epigenetic modifications, the epigenetic machinery offers an important window to understanding the molecular mechanisms underlying plastic alterations in morphological and physiological traits in response to environmental stimuli. However, challenges remain in assessing the epigenetic effects on phenotypically plastic variation in natural populations. First, both genetic and epigenetic factors may contribute to phenotype modulation. The co-occurrence of genetic and epigenetic variation in natural populations often leads to problems in unequivocally separating DNA sequence-based effects from epigenetic effects and evaluating their relative importance for phenotypic variation. The simultaneous analysis of the phenotypic effects of interrelated genetic and epigenetic variation, and their interactions, currently constitutes a significant methodological challenge (Bossdorf et al. 2008; Johannes et al. 2008). Experimental designs to rule out the confounding effects of genetic factors thus deserve special attention in the research of epigenetic regulation and phenotypic variation in an ecological context. The plants of alligator weed exhibit extensive phenotypic diversity but little genetic variation in its introduced range, thus providing a suitable model to explore the relationship between epigenetic remodeling and phenotypic variation in changing environments.

Second, the epigenetic state is dynamic and is responsive to both developmental and environmental signals. During normal development, the epigenome is mainly sculpted by intrinsic developmental signals to shape the diversity of gene expression in different cell types of the organism. Differentiated tissues and organs within a given organism might have very different epigenetic profiles, and show significant epigenetic fluctuations at various stages of development. The epigenome is not

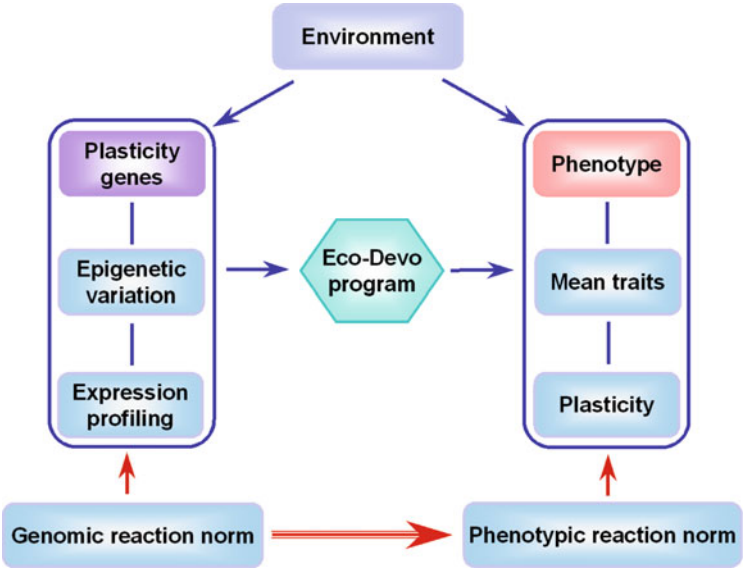

Fig. 2 An overview of the interacting networks through which environmentally induced changes in gene expression influence the development of plastic traits

exclusively influenced by intrinsic signals, however. Extrinsic environmental signals can also induce extensive epigenetic reprogramming of the genome, and modify the phenotype without changing the underlying DNA sequence. Integrative DNA methylation and gene expression analyses revealed that genes involved in responses to water treatments in alligator weed changed their methylation and expression patterns as well, in different organs at different time points of the treatment (unpublished data). The dynamic nature of epigenetic regulation and its link with both cell-intrinsic processes and cell-extrinsic cues make the epigenetic analysis of phenotypic variation more challenging than DNA sequence-based analyses. Systematic epigenetic profiling and comparative analysis are usually needed to explore which environmental factors, in what doses, and over what timescales, affect epigenomic markings. The time series approach to the transient transcriptional variation can help track the development of the plastic trait, and identify genes that are more vulnerable to alter epigenetic patterns in response to environmental changes and trigger off the plastic phenotypic changes (Aubin-Horth and Renn 2009).

The last challenge comes from the stochastic epigenetic variation in natural populations. Stochastic epigenetic changes are more common than environment-induced changes, which may occur at each mitotic process, and can result in significant epigenetic differences across cells and individuals (Petronis 2010; Bell and Spector 2011). Becker et al. (2011) showed strong evidences of spontaneous epigenetic variation in the *A. thaliana* methylome. We have also documented substantial interindividual epigenetic variation in alligator weed (Gao et al. 2010). Stochastic changes seem to provide a possible explanation for the observed

discordance between epigenetic variation and environmental differences. Xie et al. (2011) proposed that the statistical assessment of methylation entropy simply enables the distinction between deterministic and stochastic patterns of DNA methylation. Although, currently, we have a poor understanding of the factors that contribute to interindividual epigenetic variation and the mechanisms underlying statistical epigenetic differences among individuals, increased stochastic epigenetic variation might function as a driving force of evolutionary adaptation, increasing fitness in a varying environment (Feinberg and Irizarry 2010).

5 Conclusion

Living organisms have developed various strategies to adapt to environmental changes. In contrast to the long-term strategy of generating new traits by selection, phenotypically plastic variation provides an efficient short-term strategy for organisms living in changing environments to sense their environment and to respond to it rapidly and flexibly (Boyko and Kovalchuk 2008; Chinnusamy and Zhu 2009). Plastic variation in phenotypes can occur at different levels, from morphological modifications to drastic changes in physiology, life history and behavior, and in nearly all classes of organisms (Tollrian and Harvell 1999; Gabriel et al. 2005). Although diverse, plastic responses mostly take place by altering gene expression and eventually altering ontogenetic trajectory in response to environmental changes (Schlichting and Smith 2002; Jaenisch and Bird 2003; Gilbert 2005; Wade and Archer 2006). Epigenetic modifications provide a plausible mechanism for the putative link between environmental variation and alterations in gene expression (Gilbert 2001; Schlichting and Smith 2002; Sultan 2005; Richards 2006). Studying epigenetic complexes in the real environment would allow us not only to get greater insights into the molecular mechanisms underlying phenotypic plasticity, but also to establish an integrated understanding of the molecular machinery that interfaces the genotype and the environment.

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Whole-Plant Physiology: Synergistic Emergence Rather Than Modularity

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Contents

Abstract Work on "whole-plant physiology" which culminated in the 1970s and 1980s is reviewed. With its major issues, such as root–shoot interaction in nitrogen and sulfur assimilation, phloem–xylem transfers and circulation of matter in the whole plant, and hydraulic signaling of water relations, this older work shows integration in plants as unitary organisms. It has essential messages for progress with a holistic view on "systems biology". The huge amounts of data of molecular cell biology of plants ("omics") are often considered as modules. The discussion of signaling, such as electric, hydraulic, and chemical signaling, helps to advance to an understanding of

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integration and of emergence in contrast to modularity. Source–sink relations and root–shoot interactions in the performance of the whole plant in its environment are elaborated as examples for emergence from the coordination of parts. Timely systems biology must develop a whole-plant view by following systemic interactions comprehending all relevant spatio-temporal scales.

1 Introduction

In the 1970s and 1980s "whole-plant physiology" was an important topic in plant biology. The major themes at issue in that period are reviewed in this essay because this can provide a basis for the quest of a holistic view on "systems biology". The era of molecular biology developed a consideration of systems in terms of "omics", first with genomics and transcriptomics followed by others. The idea is that a complete analysis and documentation of genes and transcripts, metabolites, and any other groups of traits would pave the path towards understanding the entire underlying living systems. Thus, the term "systems biology" was born, coming up as a seemingly new concept. This has a strong touch of thinking in terms of modularity where blocks of omics data are taken as modules. Plants are considered as modular organisms having semi-independent and totally independent parts (Haukioja 1991). The extreme view is that performance of whole plants is a by-product of responses of modules (de Kroon et al. 2005). This view culminated with the caricature that "a tree is not a tightly integrated organism but a by-product of its parts" (Haukioja 1991).

Such a position, however, conflicts with the phenomenon of emergence. This is the observation that an entire whole can have totally different properties than given by summing up the properties of all its parts or modules. From integration of the parts and their complex interactions unique innovations can result. Advocating the concept of emergence the physicist Robert Laughlin used as a metaphor a painting of the impressionist artist Claude Monet where apparently erratic non-correlated patches of paint quite clearly lead to the emergence of the beautiful view of a garden with flowers of iris (see Bitbol 2011).

In view of the systemic whole-plant physiology culminating in the 1970s and 1980s, the current term "systems biology" at the beginning of the third millenary was conceptually not all that new. This now facilitates bridging the omics era of thriving for huge modules of data towards an extended integrative conception of systems biology, including the physiological process level. One way of doing this is considering signaling as a prerequisite of integration and emergence. Observations of source–sink relations and root–shoot interactions in the performance of the whole plant in its environment provide a solid basis and a wealth of information for a new view on systems biology. The holistic view needs to overarch scalar levels with hierarchical integrations and considering spatio-temporal nonlinear dynamics (Hütt and Lüttge 2002; Sandermann and Matyssek 2004; Lüttge and Hütt 2009; Hütt 2012; Matyssek and Lüttge 2012).

2 Whole Plant Physiology in the 1970s and 1980s

2.1 Hierarchy of Information Systems

In the 1970s and 1980s whole-plant physiology showed a vigorous development. Outstanding advocates of a whole-plant perspective of integration were Pitman (1975) and Sutcliffe (1976a, b). The perspective was rooted especially in the field of plant mineral nutrition. The major reason was that this was the field particularly occupied with studying transport processes, viz. uptake and whole-plant distribution of mineral nutrients. Sutcliffe (1976a, b) had developed the highly rewarding model system of germinating seeds, i.e., the cereal grains of oats (*Avena sativa L.*) and the seeds of the garden pea (*Pisum sativum* L.), for studying source–sink relations and the partitioning of resources. This also might be much worth reactivating for application of all the modern technical approaches now at hand. In the very early stages of germination, these seed models are self-consistent in that–most likely with the only exception of water–they contain all required resources, minerals and carbon skeletons in the reserves of the seeds, the endosperm and the cotyledons, respectively. Sutcliffe and his group performed a lot of experiments showing the intimate regulation of the sources in the seeds and the sinks of the developing system of the whole plant with partitioning between roots and shoots (Sutcliffe 1976a, b; Lüttge and Higinbotham 1979) with the conclusion that "a vascular plant functions as an integral unit".

It was realized that there was a hierarchy of systems with relevance for whole-plant integrations or of signaling systems as would be the preferred current term. Information for integration in the whole plant is inherent to signaling systems at various levels (Cram and Pitman 1972; Pitman 1975; Lüttge 1974; Lüttge and Higinbotham 1979):

- Transport of material carries information in the source–sink relations of the whole plant. The arrival or not arrival of water and nutrients from the roots in the shoot and of photosynthates from the shoot in the roots bears information of the current physiological state and performance of the respective organ, i.e., root system and shoot, respectively.
- The energy status of organs within the whole plant can be signaled by their outputs, where also the transport of metabolites carrying redox and phosphorylation energy equivalents may be involved.
- High-sensitivity signaling systems involve propagation of electrical signals, hydraulic signaling, and chemical signaling carried by the transport of phytohormones.

In the following Sect. 2.2, I shall recall the major issues of the 1970/1980 whole-plant physiology as a historical basis and to cast a bridge towards the current concerns in research of electrical, hydraulic, and chemical signaling (Sects. 3.1–3.3) and source–sink relations (Sects. 4 and 5) in whole plants.

2.2 Major Research Issues of the 1970s and 1980s

2.2.1 Root–Shoot Interaction in Nitrogen Assimilation

Nitrate in the substratum or soil is one of the major sources of nitrogen for plants. Root and shoot interact in the whole plant for its assimilation. A model first described by BenZioni et al. (1971) developed to one of the major issues of whole-plant nutrition research in the 1970s. Nitrate is taken up by the roots either together with K^+ ions or in exchange for HCO_3^- ions for electrical charge balance. Nitrate is transported together with K^+ to the shoot where reduction equivalents produced by photosynthetic electron transport provide ample reducing power. Nitrate reduction to ammonia via nitrate and nitrite reductases leads to the production of stoichiometric amounts of OH^- ions. Alkalization of the cytoplasm of leaf cells is controlled by synthesis of malic acid which neutralizes the OH^- and where the remaining malate^{2-} anions are transported together with K^+ via the phloem into the roots. There the malate can serve as a substrate for energy metabolism. The K^+ ions can be recirculated via the xylem to the shoot together with NO_3^-. The model has subsequently been studied intensively. Availability of K^+ to the leaves has been shown to be important, but other cations can also participate (Frost et al. 1978). When scarcity of K^+ is given, NO_3^- is taken up in exchange for HCO_3^- generated from respiratory CO_2 and K^+ is xylem–phloem recycled (see Sect. 2.2.3). Modifications of the model have been suggested. When NO_3^- concentrations in the root are low, reduction can occur in the root (Kirkby and Knight 1977) and reduced nitrogen is transported to the leaves in the form of amino acids (Schobert and Komor 1990). Notwithstanding the various modifications (see Lüttge and Higinbotham 1979), the BenZioni model up to date is one of the most illustrative examples of root–shoot coordination in the whole plant.

Another excellent example to be mentioned here in brief is the root–shoot interaction in symbiotic di-nitrogen fixation by root nodules with long-distance transport of metabolites in two directions via xylem and phloem (Jeschke et al. 1985; Walsh et al. 1989; Werner 1992; Lüttge et al. 2010).

2.2.2 Root–Shoot Interaction in Sulfur Assimilation

The BenZioni model developed for nitrate can be similarly applied to sulfate (Lüttge et al. 2010). Roots have a weak ability to reduce sulfate (Clarkson et al. 1983). Sulfate taken up by the roots is transported in the xylem to the leaves. As for nitrate the reductive power of photosynthesis in the leaves serves assimilation of sulfate. Reduction is enhanced by light and occurs mainly in the leaves (Rennenberg et al. 1979). Reduced sulfur is distributed in the plant via the phloem in the form of glutathione (67–70%), methionine (27–30%), and cysteine (2–8%) (Rennenberg et al. 1979). Glutathione (GSH) produced in the leaves acts as a signal to control sulfur nutrition in the whole plants. It serves as a shoot to root signal mediating inter-organ regulation of S nutrition (Herschbach and Rennenberg 1994).

2.2.3 Recirculation in the Whole Plant via Phloem–Xylem Transfer

A central subject of whole-plant physiology meticulously advanced by the two researchers J. S. Pate and W. D. Jeschke and their collaborators in the 1970s was phloem–xylem cycling of solutes. While it was much more difficult to obtain phloem sap than xylem sap, the development of a phloem bleeding technique paved the way (Pate et al. 1975) for not only documenting the fact of phloem–xylem transfers as such but also for thoroughly quantifying this interchange. In an extraordinarily remarkable and persistent series of publications, Jeschke and Pate have studied phloem–xylem cycling of various compounds involved in whole-plant performance:

- the flow of inorganic nitrogen and malate (Peuke et al. 1996) as relevant for the BenZioni model (Sect. 2.2.1)
- partitioning of ions in general (Jeschke et al. 1985, 1987)
- modeling sodium and potassium flows via phloem and xylem and distribution in the whole plant in relation of adaptation to and tolerance of salinity stress (Wolf and Jeschke 1987; Jeschke and Wolf 1988; Jeschke and Pate 1991a)
- uptake, flow, and utilization of C and N (Jeschke and Pate 1991b)
- cycling of the stress hormone abscisic acid (ABA) as relevant for translation of hydraulic signals into chemical signals (Sect. 2.2.4).

Other authors have contributed to the understanding of cycling of organic nitrogen and sulfur compounds (e.g., Cooper and Clarkson 1989; da Silva and Shelp 1990; Larsson et al. 1991; Geßler et al. 2003). The role of the parenchyma rays for phloem–xylem exchange in stem tissue has been underlined by van Bel (1990).

2.2.4 Hydraulic Signals Translated Into Chemical Phytohormone Signals

A further central issue of root–shoot interaction in the whole plant in the late 1980s and especially early 1990s was and now continues to be (Sect. 3.3.2) the topic of root to shoot signaling of soil-drying. The basic question is if stomata can be alerted to close before the leaves experience water deficit. The major protagonist in this case was W.J. Davies with his group (Davies and Zhang 1991). The technique of split root systems has played an important role, where half of the root system could be subject to low water potential (Ψ) to elicit putative drought signals and the other half could be kept at high Ψ to maintain water supply to the leaves. It became evident that it was soil water status rather than leaf water status that predominantly controlled leaf gas exchange (Gollan et al. 1985; Turner et al. 1985; Davies et al. 1990; Wartinger et al. 1990; Zhang and Davies 1990). The major signaling substance responsible for the root to shoot signal was realized to be the stress phytohormone abscisic acid (ABA). Other chemical signals were found to participate (Davies et al. 1994), including cytokinin phytohormones (Bano et al. 1993) and also inorganic ions and xylem sap pH (Schurr and Schulze 1996). Hydraulic and chemical signaling is integrated (Tardieu and Davies 1993; Tardieu et al. 1993). Clearly the hydraulic signals can be translated into chemical signals which in turn may be translated into electric signals (Sect. 3.1).

3 Signaling

3.1 Electric Signaling

Ever since the discovery of electrical responses to stimulation in plants, obscure ideas about plants' neurology were brought up (e.g., Tomkins and Bird 1973). This was always strongly provocative when one considers neurology as the study of nerve systems inferring foresight, intention, and consciousness to be restricted to the realm of cognitive behavior of (higher) animals. Astonishingly using such terminology plant neurobiology quite recently even comes up as a new move in plant biology (e.g., Trewavas 2003, 2005; Baluska et al. 2004, 2005; Brenner et al. 2006; Gurovich and Hermosilla 2009, with a rebuttal by Alpi et al. 2007). All kinds of interactions and communications involving plant cells are called "synapses" (Baluška et al. 2005). However, plants do not have neurons as specialized cells transmitting nerve impulses.

Reference is made to Charles and Francis Darwin. They discovered and in controversy with the dominating German plant physiologist Julius von Sachs, they rightly maintained that the kalyptra of the root tip is the site of perception of the gravitational stimulus eliciting gravitropical bending of roots (Darwin 1880, 1909). It is now known that electrical signaling is involved. The so-called geo-electric effect first described by Brauner and Bünning (1930) was confirmed by Stenz and Weisenseel (1991, 1993). An electrical field is built up in the root under gravitational stimulation and the root is bending towards the lower physical side which is more positive. Very detailed studies by the group of Andreas Sievers (e.g., Behrens et al. 1985) then unraveled the interaction of cushions of endoplasmatic reticulum with the amyloplasts serving as statoliths in the cells of the kalyptra. This generates an asymmetric distribution of membrane potentials and a polarization forming electrical fields and eliciting electrical signals followed by gravitropical bending. While it is evident that perception and primary signaling reside in the kalyptra, it is much too far reaching to conclude that the root tip functions like a brain (Baluška et al. 2004) and to appeal to Charles Darwin for support of such a postulation. Charles Darwin tended to compare roots and also moving plant tendrils with earthworms having tiny brains in their tips. However, evidently the Darwins were not aware of the current progress in genuine neurobiology. Therefore it is not fair to quote them as authorities for plant neurobiology. Electrical phenomena are a basic and general property of all living proteo-lipid biomembranes. Clearly membrane-electrical properties evolved earlier than the organs of a nervous system (Volkov 2000). However, the crevasse between the functions of electrical signaling in plants and bona fide neuronal activities in (higher) animals is so deep that it is misleading to draw analogies (Alpi et al. 2007). Electrical signaling per se is not neurology. On the other hand, by no means whatsoever this permits defending the error of taking plants as merely modular organisms with denying them the quality of being unitary individuals (Haukioja 1991). Rejecting the term "plant neurobiology" does not at all distract from the fascination inherent in much work that is currently performed on electrical signaling in plants and its importance for integrated whole-plant functioning.

Plant electrophysiology is a well-researched field which cannot be comprehensively reviewed here. A recent review is that of Fromm and Lautner (2007) and good overviews are found in the introductions of research papers (e.g., Dziubińska et al. 2001; Volkov et al. 2010). Of particular interest with respect to whole-plant systems biology is the systemic propagation and spreading of electrical signals. There are two well-known types of electrical impulses, i.e., action potentials (AP) and variation or slow wave potentials (VP) (Davies 2004; Dziubińska et al. 2001; Fromm and Lautner 2007; Zimmermann et al. 2009; Oyarce and Gurovich 2011). Recently Zimmermann et al. (2009) have added a third one which they name system potentials (SP).

The APs are propagated with high velocities over large distances in whole plants. Velocities reported range from just below 1 mm s^{-1} and up to 50 mm s^{-1} (Fromm and Eschrich 1993; Grams et al. 2009; Oyarce and Gurovich 2011), and there is even the quote of a very high velocity of up to 40,000 mm s^{-1} (Volkov 2000). Action potentials travel much faster along the veins than within parenchyma tissue (Grams et al. 2009). In the veins they are propagated within the phloem rather than the xylem (Fromm and Eschrich 1993; Fromm and Fei 1998; Volkov 2000; Dziubińska et al. 2001; Lautner et al. 2005; Fromm and Lautner 2007; Grams et al 2009; Shabala et al. 2009). The rates of the volume or mass flow of solutes in the phloem are generally at about 0.15–0.30 mm s^{-1} (Canny 1975). Thus, the propagation of APs quite evidently is much faster and APs run electrotonically along the plasma membranes of the sieve elements also involving ion channels (Fromm and Eschrich 1993; Grams et al. 2009).

The VPs are more localized and propagated over shorter distances with lower velocities in the range of 0.5 mm s^{-1} (Gil et al. 2008).

The SPs differ in their properties from both APs and VPs (Zimmermann et al. 2009). They are propagated at a rate of 1–3 mm s^{-1}. Zimmermann and Felle (2009) have described systemic signals which are not in causal relationship with voltage changes but with ion fluxes, especially of protons affecting apoplastic pH so that pH may function as the systemic signal. Such ion movements are the result of SPs. Propagation of SPs does not involve ion channels (as it is the case with APs) but is mediated by pumps especially the plasma membrane H^+-ATPase (Zimmermann et al. 2009).

The systemic function of the long-distance electrical signals is in root–shoot regulation of physiological plant performance including source–sink relations (see Sect. 4), respiration and activity of photosynthesis (Dziubińska et al. 1989; Filek and Koscielniak 1997; Lautner et al. 2005), and responses to wounding and herbivore attack (many of the references given in this section). Electrical signals elicited by various treatments of roots, such as with phytohormones indoleacetic acid (IAA), cytokinins, and ABA, or by addition of nutrients affect chlorophyll fluorescence, photosynthetic CO_2 uptake, and transpiration in leaves (Fromm and Eschrich 1993). The effects on photosynthesis may be indirect via control of stomatal aperture (Kaiser and Grams 2006). However, recently rather close interactions have also been described, where AP signaling is directly transferred to thylakoids (Koziolek et al. 2004; Lautner et al. 2005). This is mediated by changing H^+-fluxes across the plasma

membrane affecting cytoplasmic pH and the pH gradients across thylakoid membranes (Grams et al. 2009). A direct correlation of electrical events at the plasma membrane, cytoplasmic pH, photosynthetic electron transport in the thylakoid membranes, and thylakoid acidification has been suggested early by Pallaghy and Lüttge (1970) and Hope et al. (1972; see also Lüttge and Higinbotham (1979). It was proposed to explain the transient changes of the electrical potential at the plasma membrane of green cells elicited by dark–light–dark changes. Originally this was received with reservation. It was much studied later by other authors and basically confirmed up to quite recently (Bulychev and Turovetsky 1983; Vanselow et al. 1988; 1989; Bulychev and Kamzolkina 2006a, 2006b, see Grams et al. 2009).

3.2 Hydraulic Signals

Systemic hydraulic signaling is essential for whole plant life to secure adequate supply and partitioning of water to plant organs. Therefore, it can function via a rather direct coupling of signal and response when aboveground organs, i.e., leaves and shoots, experience water limitation before the belowground organs, i.e., the root system. The water potential gradient ($\Delta\Psi$) between the plant organs and the atmosphere can serve as a direct signal to the roots since it is the driving force for water movement or transpiration stream in the xylem. The situation is much more complex if the root system is sensing water stress before the shoot system with the leaves. There is a huge body of literature showing that stomatal closure in the leaves is an early response to low soil Ψ much before the leaves experience the stress by drought (reviews Davies and Zhang 1991, in the Introduction of Gil et al. 2008).

Propagating hydraulic signals can be pressure changes and hydraulic waves (Matyssek et al. 1991; Tang and Boyer 2003; Grams et al. 2007) with the movement of water columns in the xylem (Cermak et al. 1993). Due to the very low compressibility of water, considerable pressure can build up in the solution of the xylem conducts and shock waves can arise leading to rather fast signaling (Matyssek et al. 1991). Conversely, the movement of water columns usually appears to be too slow for fast systemic signal propagation. Hydraulic signals can be transformed to chemical signals which are also slow (Sect. 3.3.2). Obviously, in whole-plant physiology the limitation by the low velocity of propagation is mainly overcome by translation of hydraulic signals into the very rapidly propagated electrical signals (Lautner et al. 2005; Zimmermann and Felle 2009). Indeed, it is observed that hydraulic and electric signals are complementary to each other. They have distinct roles and may act independently of each other (Grams et al. 2007). Hydraulic surges and sudden turgor pressure changes are typically translated into the electrical signals of action, variation, or system potentials (APs, VPs, SPs, see Sect. 3.1). Hydraulic signals interact with chemical signals such as the phytohormone ABA (Comstock 2002; Sect. 3.3.2). Both hydraulic and chemical signals can translate into electrical signals.

Hydraulic signals can be the various components of water relations equations. The thermodynamic factor water potential (Ψ) per se cannot be perceived, but it can be sensed via the underlying parameter osmotic potential (π) and via its effects, i.e., water-potential gradients ($\Delta\Psi$) functioning as driving forces for water transport and determining volume flow. In the plant cells membranes, particularly the plasma membrane and the tonoplast, are the sites of sensing the turgor pressure (P) which is determined by Ψ and π ($\Delta P = \Delta\Psi + \Delta\pi$). Coster and Zimmermann (1975) have developed an electrochemical model for explaining membrane functions regulated by voltage and pressure. It is now often assumed that stretch-activated ion channels play a role, but it needs to be cautioned because turgor acts perpendicular to the membranes whereas stretch is a lateral force (Lüttge et al. 2010). Mechanosensitive, stretch-responsive ion channels and ion pumps in the living cells adjacent to the xylem conducts are involved in the signal propagation within the xylem (Stancović et al. 1997, 1998).

3.3 Chemical Signals

3.3.1 Systemic Chemical Signaling

Chemical signals are important in systemic signaling chains and networks. Their propagation is slow. Therefore in the whole-plant signaling networks they often translate into electric signals (Sect. 3.1) for faster propagation. However, they constitute very essential knots in the system. Chemical signals can be anything such as also the information inherent in distribution and partitioning of resources as already mentioned above (Sect. 2.1). There is a vast inflation currently of chemical compounds being nominated to function as secondary messengers in signal transduction networks. Hydraulic and chemical signals and others interact (Comstock 2002). Nitrate availability in the medium was shown to act locally as a signal controlling root hydraulic properties in plants, and thus, to translate the local chemical information of NO_3^- into a hydraulic signal (Sect. 3.2) coordinating responses at the whole-plant level (Gorska et al. 2008a, b).

The basic chemical signaling compounds in plants, however, are the primary messengers of the classic groups of phytohormones. The major ones in systemic signaling are the stress hormone ABA and the growth regulator IAA. At least it appears that their systemic functions are most intensely studied and they shall be looked at more in the following two subsections (3.3.2 and 3.3.3). Other phytohormones exert similar whole-plant actions. Root–shoot electrical signaling interacts with cytokinin signaling (Shabala et al. 2009).

Herbivore and pathogen defense reactions comprise whole-plant hormonal regulations. Defense signaling (Kessler and Baldwin 2002) particularly involves the phytohormones salicylic acid and jasmonic acid and the gaseous pheromone ethylene (von Dahl and Baldwin 2007; Koornneef and Pieterse 2008). Bursts of the stress hormone jasmonic acid elicited in leaves by herbivore attack lead to

short-term reductions in root growth (Hummel et al. 2009). The production of ethylene upon wounding by herbivores operates in the whole plant in a signal chain with jasmonic acid. This can lead to changes in resource allocation and partitioning, e.g., bunkering in the roots for making leaves less attractive for the herbivores and as resource for regrowth once the herbivores have left (von Dahl and Baldwin 2007). Induced resistance to herbivores and pathogens is often elicited by wounds and acts systemically in the whole plant (Koornneef and Pieterse 2008). It is noteworthy in this respect that wounds regularly trigger electrical signaling (Sect. 3.1).

The essential feature of whole-plant hormonal signaling is that all classical phytohormones are mobile in both long-distance transport pathways, i.e., the xylem as well as the phloem, so that two-directional signaling can occur along the length-axis of plants (Heil and Ton 2008, see also Zimmermann et al. 2009). This also allows whole-plant circulation of phytohormones as it is known, for example, from many studies on ABA (Wolf et al. 1990; Peuke et al. 1994; Jeschke et al. 1997a, b; Sauter et al. 2001; Hartung et al. 2002). It was elegantly demonstrated in grafting experiments with tomato. When the root stock was from an ABA-deficient mutant and the shoot scion from the wild type, ABA was detected in the root xylem but could have only originated from production in the shoot and transport to the root via the phloem (Holbrook et al. 2002). Phytohormones therefore systemically regulate growth, development, and differentiation as well as short-term functions of plants including immediate stress responses. There is a vast amount of literature which in the following two subsections can only be touched upon for demonstrating basic principles.

3.3.2 Abscisic Acid (ABA)

One major topic of the early whole-plant physiology was root signaling of limiting water supply to the shoot by xylem transport of ABA as a signal produced in the roots (Sect. 2.2.4). Such signaling of the water status of the soil can lead to stomatal closure in the leaves independent of leaf water potential (Ψ) (Sauter et al. 2001; Hartung et al. 2002). However, ABA is not the sole signal involved. It is often shown to be too slow and translates into electrical signals which are rapidly propagated via the phloem (Sect. 3.1) and elicit stomatal responses in the leaves (Gil et al. 2008). Thus transmission of the soil drying message may operate like in a relay race.

However, the maintenance of a whole-plant status of information is even more complex and the signaling system is not a linear relay chain. Reciprocal grafting experiments with ABA-deficient mutants and wild type of tobacco showed that root-produced ABA is not the exclusive factor. It is not only this but ABA even is not necessary at all in all cases (Holbrook et al. 2002). Stomata closed in response to soil drying regardless of the root genotype, i.e., also when the roots were unable to produce any ABA. A different signal must have been transmitted from the roots to the leaves and it is speculated that this may have been a chemical ABA precursor

(see Hartung et al. 2002) for the biosynthesis of ABA in the leaves, another phytohormone, such as cytokinin, or xylem sap pH as soil drying elicits an increase of pH in the xylem sap (Wilkinson and Davies 1997; Wilkinson et al. 1998). ABA was produced in the leaves effecting stomatal closure and even transported back to the roots via phloem circulation (see above).

This brief look into ABA signaling shows that relations of this stress hormone can provide a colorful portray of integrated systemic whole-plant functioning.

3.3.3 Auxin (Indoleacetic acid, IAA)

In comparison to signaling and coordination of responses in reactions to stress events under the mediation of the stress hormone ABA (Sect. 3.3.2), the morphogenetic signaling ruled by the growth regulator auxin is a different distinct type of hormonal action ensuring harmony of systemic whole-plant development and operation. Auxin forms gradients in plants ensuring development with characteristic whole-plant pattern formation (Friml 2003; Benková et al. 2003; Friml et al. 2003).

Auxin is the only phytohormone subject to polar transport (Friml and Palme 2002). In his experiments on the bending of coleoptiles of the grass *Phalaris canariensis* in the light, Charles Darwin (1880) already observed that there was a growth-regulating signal transported from the top to the bottom. The observation of polar transport of auxin from top to bottom of shoots was made in 1926 (Went 1926) leading to the development of the coleoptile Went-test for IAA (Went and Thimann 1937; Went 1939, 1974). The conception of the chemi-osmotic theory of polar transport followed several decades later (Rubery and Sheldrake 1974; Raven 1975). As a weak acid, IAA is dissociated to IAA^- at the slightly alkaline pH of the cytoplasm. At the basal end of cells along the plant axis it is exported to the apoplast by IAA^--efflux carriers specifically located there. Due to operation of the H^+-transporting ATPase of the plasma membrane, the apoplastic space is acidic (pH about 5) and the auxin is protonated to IAAH. At the upper end of the cells, IAAH-transporters are located for uptake into the next cells and so on. Thus, by the asymmetric arrangement of the IAA^--efflux and the IAAH-uptake transporters, the polar chemi-osmotic transport is mediated.

Then there came the discovery that the directed IAA transport is regulated by the members of a gene family, the so-called *PIN*-genes, named after the seedlings of *PIN*-deficient mutants showing a non-branched needle-like growth because they cannot produce leaves (Paponov et al. 2005; Grieneisen et al. 2007). The PIN-proteins are auxin-efflux facilitators (Friml et al. 2003; Paponov et al. 2005). Their asymmetric distribution in cells, together with a tissue and organ-specific expression of different members of the gene family, ascertains a concerted action in morphogenetic pattern formation. Different PIN-proteins are responsible for shoot–root translocation, symmetric distribution of IAA in the root tips, and for transport in the return direction back towards the shoot (Benková et al. 2003; Blilou et al. 2005).

This is a model exceptionally well illustrating whole-plant performance. It is a most wonderful example of the continuity of research from very early observations of polar IAA transport up to the molecular revolution of plant biology to embrace biology of the whole-plant system.

3.3.4 Pheromones and Other Signaling Volatile Organic Compounds

Chemical signals dissolved in the aqueous media of plants are often propagated too slowly, and they are therefore translated into electrical signals (Sect. 3.3.1). However, the restriction of slow movement of chemical signals is also evaded by volatile so-called pheromones, such as ethylene, methyl-jasmonate, and methyl-salicylate, and a plethora of secondary signaling volatile organic compounds (VOCs), diffusing in the gas phase and distributed by air movements. These compounds are not only essential carriers of information in the communication between different individuals and species of plants and with other organisms in complex ecological systems (Baluška and Ninkovic 2010; Ninkovic 2010, Sect. 5). They also can function—besides electrical signals—in very fast within plant signaling and systemic whole-plant responses when they diffuse via the gas phase between parts of the shoot system (Heil 2010).

4 Source–Sink Relations

For terrestrial higher plants it is the shoot with the leaves that provides their carbon via photosynthesis and it is the root system that provides their minerals via uptake of ions and water. Basically this is trivial and commonplace. However, the details are extraordinarily complex. Structural and functional shoot–root correlation is the example par excellence of whole-plant integration and much complex regulation is behind it. Considering such source–sink relations I recall aspects of root–shoot interactions in N and S nutrition and recirculation in the whole plant studied in the 1970s and 1980s (Sects. 2.2.1–2.2.3).

More recently, this is becoming essential again in the debate of the relative importance of shoot and root functions, viz. photosynthesis and mineral nutrition, respectively, in whole-plant performance. Körner (2012) emphasizes that it is not the source activity of photosynthesis that determines the buildup and activity of sinks but vice versa the activity of sinks that exerts a feedback on source activity so that sink activity determines the source activity of photosynthesis. Briefly said, typically growth does drive photosynthesis but photosynthesis does not drive growth. The buildup of sinks strongly depends on the supply of mineral nutrients where, according to Körner, nitrogen is the dominating and determining factor. Considering soil resources we should not hesitate to add phosphorus in view of the often delicate problem of P-nutrition (Bünnemann et al. 2011; Vance and Chiou 2011). The net rate of CO_2 uptake by leaves is reduced at low concentrations of NO_3^-, P_i, and K^+ (Longstreth and Nobel 1980).

5 Root–Shoot Interactions and Performance of the Whole Plant in Its Environment

New labeling techniques make use of stable isotopes, e.g., modified ^{13}C levels in the atmospheric CO_2 even within tree canopies of a forest (Grams et al. 2011). This opens new avenues for assessing qualitative and quantitative whole-plant relations of resource partitioning under the influence of environmental cues with increasing sophistication (e.g., Ritter et al. 2011). It is recently strongly criticized by Christian Körner (2012) that for many decades the consideration of the ecophysiological performance of plants in laboratory and field studies was highly carbon and photosynthesis biased. In his opinion this was due to the early introduction of infrared gas analyzers (IRGA) becoming the very instrument of many plant ecophysiologists and leading to an extraordinary one-sidedness. Soil resources, i.e., mineral nutrients, must match photosynthesis. In the "stoichiometry of life" sensu Körner, the carbon cycle cannot be separated from the nutrient cycle.

The Thornley model is relevant for understanding how mineral nutrition-dependent shoot–root interactions affect partitioning of photosynthetic assimilates. The model proposes that nutrient deficiency favors photosynthate partitioning to the roots (Thornley 1972). It has been verified for N and P but not for Mg and K, so that there are element-specific effects due to the physiological roles of the different elements (Marschner et al. 1996). It is noted that growth and ion accumulation are co-regulated and not simply correlated (Cheeseman and Wickens 1986).

There is a rich literature on the significance of plant nutrition for plant growth and functioning (Sects. 2 and 4). In ecological terms evidently N and P together with C are the basis of biomass and hence the construction of all plant organs with all their sinks which via their demands exert feedback regulation on photosynthesis. With limited supply of N and P, protein synthesis is hampered, and growth is reduced. Thus, the products of photosynthetic CO_2-assimilation cannot be utilized, which causes feedback inhibition of photosynthesis. As a consequence the plants suffer photoinhibition when the energy of irradiance exciting the light harvesting complexes cannot be used for photochemical work of carbon reduction.

It is useful to return to Sutcliffe's germinating seed model (see Sect. 2.1) to illustrate the balance between source provision and sink consumption. Figure 1 shows that in germinating oat seedlings among the three major elements of the plant biomass indeed N is more rapidly mobilized from the endosperm than C and P, and P is more rapidly mobilized than C underlining an important role of these mineral elements for the beginning independent life of the seedlings, and thus, supporting Körner's views. Particularly rapidly mobilized in relation to both C and N is K^+ in agreement with the basic requirement of K^+ up to levels of around 150 mM in the cytoplasm for metabolic functions (Leigh and WynJones 1984; Lüttge and Clarkson 1989). Conversely Mg^{2+} is slowly mobilized in relation to C and N. It is a very essential element but not at very high levels in the plant.

That plant ecophysiology is not so one-sided based on carbon is also documented by discussions in a large body of literature on whole-plant resource allocation

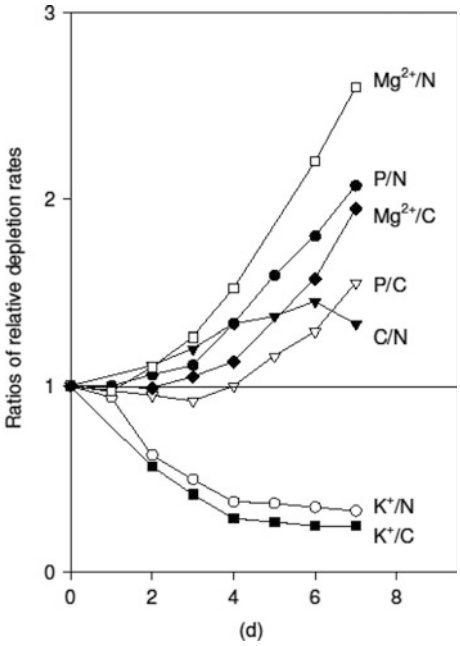

Fig. 1 Ratios of relative depletion rates of various elements from the endosperm during the first 7 days of germination of oat seedlings (*Avena sativa*) calculated from the changing contents in the endosperm after Sutcliffe (1976a). For normalization, initial contents of all elements were set equal to 1 and the relative contents were calculated for the various days of germination. Of these relative contents the ratios for pairs of elements were obtained as indicated. These are a measure of the ratios of rates of depletion from the endosperm where the ratio $=1$ means that both elements are mobilized from the endosperm at the same rate, ratios >1 mean that the element in the enumerator is mobilized more slowly than the element in the denominator, and ratios <1 mean that the element in the enumerator is mobilized more rapidly than that in the denominator. C is standing for dry mass, i.e., mainly carbon

(e.g., Matyssek et al. 2005, 2012), the role of water relations of photosynthesis, water-use efficiencies (WUE) and stomatal control with hydraulic, hormonal, and electric signaling at the whole-plant level (Sects. 3.1–3.3). Körner clearly has a point of actuality, but we need to bring things into context. The dominant role of nitrogen is assessed in much work on the high nitrogen demand of the construction of the photosynthetic apparatus (see Lüttge 2008). Nitrogen relations are particularly relevant in acclimations and adaptations of the photosynthetic apparatus to different regimes of irradiance, viz. sun and shade plant phenotypes (Chow et al. 1988; Evans 1988; Anderson and Thomson 1989; Fetene et al. 1990; Warren et al. 2000; Kitajima and Hogan 2003, see Lüttge 2008). Nitrogen-use efficiency (NUE) of photosynthesis is referred to in many studies (Evans 1988; Field 1988; Reich et al. 1994). WUE is determined by mineral nutrition. It is found to be closely correlated to leaf mineral or ash content across a range of species (Masle et al. 1992) and NUE shows distinct patterns among species and communities (Reich et al. 1994).

There is nutrient signaling from the root system in the soil to the leaves in the atmosphere. Adding minerals to the roots elicits electrical signals which affect photosynthesis (Sect. 3.1, Fromm and Eschrich 1993). In the opposite direction photosynthesis is involved in the control of root ion transport (Rao et al. 2002). The entire organism is involved in the control of transport processes in the roots (Shabala et al. 2009). Irradiance regimes interact with nutrient acquisition by the root system mainly via the energy status of the roots given by the supply of substrates maintaining their sugar pool (Shabala et al. 2009). Here I recall the point made above (Sect. 2.1) that the transport of material includes signaling and that supply of substrates for metabolism and energy status are, of course, correlated. In addition, rapid signaling will also be involved because abrupt changes in irradiance can affect root ion transport within seconds to minutes (Shabala et al. 2009).

The focus of this essay is on individual plants as unitary organisms. Nevertheless, it is worth briefly mentioning the embedding of the single whole-plant organisms at the hierarchically higher spatio-temporal level, i.e., in their environment, and ultimately as interacting components of ecosystems. At these levels similar processes with signaling and regulation of hierarchical order and similar functionality as within the plant again are operating. Aboveground signaling and interactions regulate the occupation of space (Grams and Lüttge 2010). Belowground signaling and interactions regulate the organization of root systems with recognition of self and non-self (Schenk et al. 1999; Gruntman and Novoplansky 2004; Novoplansky 2009; Grams and Lüttge 2010) where allelopathic chemical signals are involved and chemical signals are messengers for the establishment of symbioses such as mykorrhizas and nitrogen-fixing root nodules.

Responses to herbivore attack comprise relations both systemic within the whole plant (Sect. 3.3.1) and associated with plant communities in their occupied space (Sect. 3.3.4). The gaseous signal ethylene acts a pheromone. Furthermore, other phytohormones, such as jasmonate and salicylate, are volatile in their methylated forms (methyl-jasmonate, methyl-salicylate; Ninkovic 2010) and can be considered as pheromones. These agents also trigger the emission of secondary gaseous defense molecules, i.e., VOCs such as terpenoids, by plants attacked (for excellent overviews, see Baluška and Ninkovic 2010; Bruce 2010). These gaseous signals then function in plant-to-plant signaling within communities and can elicit intra- and interspecific preparedness of other plants for the defense of attacks to be expected (Ruther and Kleier 2005; Von Dahl and Baldwin 2007).

6 Whole-Plant Imaging

Signaling inherently is a matter of action in time. Therefore, in the electrical, hydraulic, and chemical signaling discussed in Sect. 3 the emphasis is on dynamics in time. Integration in addition requires the notion of space. One neither should nor can ever completely separate dynamic temporal and structural spatial aspects in the consideration of spatio-temporal systems biology of whole plants. The question

arising here is if it is actually possible to visualize integrated organization in whole plants. This is achieved by whole-plant imaging where the accent is on organization in space. A good example of the intimate relation of imaging with functional studies is inherent in the ways that led to understanding of whole-plant organization by IAA (Sect. 3.3.3) where imaging essentially contributed to unraveling spatial localization and expression of *PIN*-genes (Grieneisen et al. 2007, Sects. 3.3.3 and 6.2). Current methodology leads to vigorous development of imaging approaches covering all levels from subcellular to cells, tissues, organs, and whole-plant nesting in the environment (Schurr et al. 2009).

6.1 Early Imaging by Autoradiography

Early imaging of whole-plant functional integration was achieved by whole-plant autoradiography. Radioactive tracers were used to study the physiology of glands. After application of labeled substrates, autoradiographs were obtained from pressed and freeze-dried plants using x-ray film. In this way the processing of nectar by reabsorption of some secreted compounds via the nectary glands was demonstrated (Ziegler and Lüttge 1959; Lüttge 1961). The absorption of substrates by the glands of carnivorous plants and the systemic distribution of the labeled compounds in the whole plants could be depicted (Lüttge 1963, 1965). Long-distance transport of assimilates was studied, e.g., in the interesting primitive conducting elements of the shootlets of the bryophyte *Polytrichum commune* (Eschrich and Steiner 1967). Examples of the use of autoradiography in whole-plant mineral nutrition are given in the introduction of Rubio et al. (2004).

A new method developed for spatial mapping and imaging of radioactive tracer distribution in whole plants is digital autoradiography (Nielsen et al. 2002; Rubio et al. 2004). The samples are exposed to a photo-stimulatable storage phosphor screen which captures latent image formation produced by the ionizing radiation released from the radioactively labeled tissues. Blue phosphorescence is then generated by the screen in proportion to the amount of radioactivity. The great advantage is that this method also allows quantification. Rubio et al. (2004) have used this to study heterogeneity of ^{32}P-phosphate uptake along the length of roots, but it should also be applicable to labeling with other radionuclides.

6.2 Imaging with Reporter Genes

An approach which has widely proven to be extraordinarily efficient is the combination of imaging with molecular biology. Reporter genes are inserted in genomes replacing the coding DNA region of a gene of interest. Then the level of expression of the reporter gene and the production of the reporter gene protein reflect spatio-temporal action of the regulatory DNA-sequences of the original gene

of interest. If the products of the reporter genes generate staining, fluorescence, or luminescence, this leads to impressive whole-plant imaging of the localization and distribution of genes and their activities. Widely applied examples of such reporter genes are (1) β-glucuronidase which hydrolyses a glucuronide and sets free a blue product, (2) β-galactosidase the product of the *lac Z*-gene which hydxrolyses lactose and also produces a blue color when the cells carrying the reporter gene are incubated with an accordingly modified β-galactoside, (3) the product of the gene for a protein with green fluorescence from the jellyfish *Aequora victoria*, and (4) the luciferase gene from fireflies or bacteria producing bioluminescence in the presence of O_2, ATP, and the substrate luciferine (Alberts et al. 2002; Lüttge et al. 2010). There is a voluminous literature on various applications which cannot be covered in this essay and these molecular techniques can only be mentioned as an essential approach to whole-plant biology. The example of *PIN*-gene studies given in Sect. 3.3.3 may suffice.

6.3 Imaging with Optical Methods

Another important possibility of imaging whole-plant performance is chlorophyll fluorescence imaging. Recording chlorophyll fluorescence allows assessment of potential quantum yield, effective quantum yield of photosystem II, and relative photosynthetic electron transport rate. Photochemical and non-photochemical quenching of chlorophyll fluorescence indicate photochemical work and photoinhibition, respectively (Genty et al. 1989; van Koten and Snel 1990; Schreiber and Bilger 1993; Bilger et al. 1995; Maxwell and Johnson 2000). Thus, this technique provides deep insights into photosynthetic performance of plants. With the appropriate optical hardware, it produces images of photosynthetic activity at the level of whole leaves and entire shoot systems (Pieruschka et al. 2009). It is now very widely used, e.g., for depicting localized heterogeneity of photosynthetic activity (Rascher et al. 2001; Maddess et al. 2002; Rascher and Lüttge 2002; Duarte et al. 2005; Duarte and Lüttge 2007; Lüttge 2009), for following effects of stress which are not homogenous in leaves and whole plants (Osmond et al. 1999; Martínez-Peñalver et al. 2011; Sperdouli and Moustakas 2012) and pathogen attack (Meyer et al. 2001; Chaerle et al. 2004) including the consequences of virus infections (Osmond et al. 1998), for screening photosynthesis-mutants (Barbagallo et al. 2003), for crop improvement strategies (Baker and Rosenquist 2004), and for many other interests. Using fluorescence induced by powerful lasers, remote sensing is possible providing images of photosynthetic activity of whole plants in their environment (Hoge et al. 1983; Pieruschka et al. 2009).

Other optical methods allow detection of light absorption and reflection (Malingreau and Tucker 1987; Running 1990). Imaging by infrared thermography is also used (Chaerle et al. 2004; Wang et al. 2004). With sophisticated spectral resolution of light absorption, refinement can be attained so that even remote

sensing of biochemical properties, such as nitrogen levels, anthocyanin contents, and water status, of plants in canopies can be depicted (Asner et al. 2004; Asner and Vitousek 2005).

7 Conclusions

Whole-plant physiology was a strong way of thinking in the 1970s and 1980s. It was borne out by results from studying transport mechanisms leading to integration of functional organization on the various spatio-temporal levels of the whole plant. This is illustrated in this essay by reviewing pertinent examples. The discipline of whole-plant physiology is now almost lost to molecular cell biology with a strong tendency towards modularity rather than viewing plants as systemic highly integrated entities. However, evaluation of signaling has the power to cast a bridge. Hence, with the apprehension of signaling, such as the types of electric, hydraulic, and chemical signaling discussed, synergistic emergences in plant function can be characterized and understood. Thus, a new holistic view on "systems biology" is advocated for understanding plant life. Systems biology is nowadays frequently understood with a seemingly modular perception assessable by "omics" analyses. The current essay is juxtaposing this with observations on source–sink relations and their interactions for integration in the whole plant and its performance in its environment. Such work proves the systemic character of plant performance which underlines the need of dropping modularity for emergence. It urges the advancement from the scale of "omics" to the process scales on higher hierarchical levels of cells and organs in the whole plant and towards the stand and ecosystem levels. Thus, the plea for widening the view on systems biology of plants is supported, and there is a challenge for future experimental and theoretical research and work of modeling.

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Evo–Devo–Eco and Ecological Stem Species: Potential Repair Systems in the Planetary Biosphere Crisis

Ulrich Lüttge, Mario L. Garbin, and Fabio R. Scarano

Contents

Abstract We draw on well-established domains of the biology of evolution (EVO), development (DEVO), and ecology (ECO), particularly of plants, to develop the new concept of "stem species" based on "EVO–DEVO–ECO." In EVO the evolutionary

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theory of punctuated equilibrium of NILES ELDREDGE and STEPHEN JAY GOULD is thought provoking. These authors make use of spandrels, exaptations, and functional shifts to explain interruptions of stasis by punctuated speciation. In DEVO it is epigenetics where environment-induced chromatin methylations constitute heritable memories of experienced stress. In addition, spandrels, exaptations, and functional shifts shape the phenotypes emerging from reading the genome information. By feedback of development through the evolutionary selection of phenotypes, EVO–DEVO is more than the evolution of development. In ECO the thoughts dwell on the ecological impacts on development of phenotypes as well as the environmental pressure causing selection in evolution. EVO, DEVO, and ECO are nodes of a network with strong interactions between them. The "stem species" idea is issued by comparison with stem cells. In analogy to stem cells in organisms, "stem species" in ecosystems have multipotency and they fulfill repair functions in deteriorating and destroyed habitats. "Stem species" differ from invaders, nurse species, and pioneer species. This is exemplified. "Stem species" may strengthen optimism regarding self-repair and sustainability of the biosphere on Earth in a current time of extraordinarily irritating global changes.

1 Introduction

In the "Origin of Species" CHARLES DARWIN (1809–1882) above all developed the theory of EVOLUTION: EVO. He was much interested in DEVELOPMENT and growth on which many of his arguments were much founded (Friedman and Diggle 2011). He called DEVELOPMENT and embryology the most important aspects of natural history: DEVO. He did not outspokenly deal with ECOLOGY. ERNST HAECKEL (1834–1919) coined the term "ECOLOGY" in 1866, i.e., 7 years after the first publication of the "Origin" by DARWIN in 1859. However, the selective pressure of environmental conditions on organisms in evolution is an eminently ecological theme. This is the driving force of evolution considered in the "Origin": ECO. EVO–DEVO is well established in a large body of more recent literature. It is "the evolution of development" (Gould 2002). This can be extended as there is also feedback from development to evolution. Another extension is to take it a step further and add ECO arriving at an EVO–DEVO–ECO-concept (see Müller 2007; Gilbert and Epel 2009; Lüttge 2010a). An exegesis of the "Origin" could already bear out the entire EVO–DEVO–ECO-concept.

Ecology may lead us on to consider relations between environment and evolution on more global scales including the current planetary crisis. There have always been large ecological crises of global dimensions with waves of massive extinctions of species. We know of five large waves of massive species extinction during the last 450 million years occurring at intervals of 45–140 million years (100 million years on average), i.e.,

- 444 million years ago at the change from the Ordovician to the Silurian
- 364 million years ago at the change from the Devonian to the Carbonic
- 251 million years ago at the change from the Permian to the Triassic, when 90–95% of all existing species where extinct including the well-known trilobites
- 206 million years ago at the change from the Triassic to the Jurassic, when extinction was associated with the appearance of the dinosaurs
- 65 million years ago at the change from the Cretaceous to the Tertiary when 75% of all existing species were extinct and with them the ammonites and also the dinosaurs, which led to the tremendous proliferation of the mammals on Earth

(see Matyssek and Lüttge 2012).

All these waves of extinctions were due to environmental changes without any influence of man who did not exist at those times. We might call them natural environmental changes, but this would get us involved in the argument if we must not consider man as part of nature (Lovelock 1979, 2009; Wobus et al. 2010). Currently man is so severely affecting the planetary environment by output with overexploitation of natural resources and by input with pollution that we presently live in a sixth and evidently this time manmade planetary crisis.

The waves of extinctions were always followed or accompanied by innovations. New forms of life emerged with the expression of new traits making organisms fit for the changed environment. For examples of this feedback of ECO on EVO see Matyssek and Lüttge (2012).

Returning our view to DEVO we note that at the organismic level differentiation and development originates from omnipotent stem cells. If there are defects, omnipotent or pluripotent stem cells may also build up repair systems. This means that there is some potential of self-organization and self-sustainment in organisms, where stem cells are key elements in control and regulation. In this essay we ask the question whether there are mechanisms in habitats, ecosystems, or biomes up to the entire planet, which are similar with the only difference being scalar levels.

In 1979 Lovelock has conceived the entire biosphere of the planet including man as a supra-organism, which he named Gaia after the ancient Greek goddess of the Earth. He took Gaia as a self-organizing, self-sustaining entity stabilizing life. Indeed, notwithstanding large amplitudes of perturbations and the extinction waves and although survival of particular forms of life as such were never assured, life itself experienced sustainment throughout geological times. In another book 30 years later he proves much less optimistic (Lovelock 2009; Matyssek and Lüttge 2012; Lüttge 2012). In the current planetary environmental crisis, repair systems are needed for which particularly equipped species will be required. At the higher scalar levels of habitats and ecosystems such species should function in analogy to stem cells at the lower scalar level of organisms, so that we may call them "stem species" (Scarano and Garbin 2012).

We may reflect if the mammals following the dinosaurs after their extinction in the fifth of the waves named above as evolutionary innovations can be considered to have been such "stem species." However, after inspecting some basic properties of stem cells, we will rather explore the possible properties and nature of "stem species" in actual extant ecosystems. Action of "stem species" as part of natural repair systems strengthens the ECO-part of the EVO–DEVO–ECO-concept.

2 EVO–DEVO–ECO

2.1 Evolution

2.1.1 Gradualism and Punctualism of Evolution

In evolution the selection leads to fixation and establishment of genotypes. In contrast to a provocative view (Dawkins 1976) selection never works on individual genes (Gould 2002, and Sect. 2.2). CHARLES DARWIN considered selection to act on individual organisms while STEPHEN JAY GOULD (1941–2002) argued that selection is also acting on species (Gould 2002). Both may occur. The selection of stem cells and their functions in development and in organismic repair systems might have been shaped by a DARWINian mechanism of selection at the level of individual organisms. Stem species as elements of repair systems of ecosystems more likely are subject to GOULDian selection at the species level. Some exegesis of Gould (2002) and the distinction of DARWINian gradualism and ELDREGEian/GOULDian punctualism of evolution are very useful for developing ideas how "stem species" may function. Comparisons highlighted in Table 1 demonstrate the essence of differentiation between gradualism and punctualism. The key difference is that according to DARWIN new species evolve gradually, whereas ELDREDGE and GOULD propose a "punctuated equilibrium" where species remain stable for long periods of stasis in geological time ("equilibrium") and speciation is rather rapid interrupting or punctuating the equilibrium. In principle it appears that both concepts are not mutually exclusive and both might operate in different cases. However, punctuated equilibrium allows us to deduce some key elements of the "stem species" hypothesis we aim to develop in this essay.

2.1.2 Spandrels or Exaptive Surprise by Nonawaited "Stem Species?"

Spandrels and exaptation are in the core of the mechanism of punctuated equilibrium. Spandrels especially in ecclesiastical architecture are the very poetic and esthetic structural metaphor used by GOULD for explaining exaptation. There are functional elements in architecture, e.g., in a two-dimensional view arches in a linear row or in a three-dimensional view hemispherical domes mounted on a set of four rounded arches meeting at right angles to form a square as in GOULD's favorite example of the dome of the Cathedral of San Marco in Venice. These elements serve a distinct architectural and static purpose. However, unavoidably the two-dimensional arches leave triangular spaces between them, and similarly in the three dimensions curved triangular pendentives form as a structurally necessary side consequence under the arches supporting the dome. These spaces or spandrels arise as geometric byproducts completely nonadaptive to the actual function. However, such forms not explicitly chosen to serve a purpose may unexpectedly turn out essential for marvelous use, i.e., in the case of the architectural spandrels for the most artistic ornamentation by mosaics or frescos.

Table 1 Comparison of gradualism and punctualism of evolution

DARWIN: gradualism	ELDREDGE and GOULD: punctualism
New species evolve gradually without periods of stasis	New species evolve rapidly and are then subject to long periods of stasis
Within the lineages of organisms fossils should display many forms of transitions	Fossils should display few forms of transitions and the maintenance of given forms over long periods of time
New species originate from transformation of the whole parent population lineage	New species have their origin in the splitting of lineages
The parent population is completely integrated in the new species	A small sub-population is the origin of the new species
The entire geographic range of the species is included in speciation (sympatric speciation)	The sub-population giving rise to the new species is located in an isolated part at the periphery of the geographic range of the species (allopatric speciation)

Spandrels of architecture are equivalent to the exaptations of organisms. Like spandrels exaptations are not forms explicitly selected for adaptation and for serving a special purpose or function under the conditions of here and now. They are rather "structures co-opted for utility from different sources of origin . . . . . and not directly built as adaptations for their current function" (Gould 2002, p. 43). In other words, the nonadaptive property of exaptations allows a later or future cooption for utility, i.e., later becoming the prerequisite for success.

With respect to the underlying mechanism of punctuated equilibrium of evolution, we consider accumulation of neutral mutations or spandrels. They are neither of disadvantage or lethal, and thus eliminated by selection of the individuals carrying them, nor useful at the here and now, and thus positively selected as adaptations. Such neutral mutations accumulate and become useful when the equilibrium is punctuated by speciation *sensu* GOULD. These mutants may constitute an "exaptive pool" of traits which prove useful in changed or new environments. In addition there is the option of "functional shift" where traits adaptive to certain conditions can be found exaptive for different functions under different conditions.

With this brief exegesis of some aspects of the voluminous and great pace-making book of STEPHEN JAY GOULD (Gould 2002), we realize that "stem species" are an outcome of EVO–DEVO–ECO. GOULD guides us when we attempt to underline the important features of "stem species". They must have exaptive pools and they must have capacities of functional shift. Unlike adaptation which responds to given conditions, exaptation provides flexibility for future changes or with the precision given by GOULD: adaptation has function, whereas exaptation has effect (Gould 2002, p. 1233). This is exactly what we expect from "stem species": they must have effect.

2.2 Development

CHARLES DARWIN proposed that transformation of organisms over time in addition to natural selection is due to modification of development (Friedman and Diggle 2011).

ERNST HAECKEL first linked phylogeny and ontogeny by what he called the "biogenetic law" saying "ontogeny recapitulates phylogeny". We now have different and molecularly founded reasons to argue that evolution and development are intimately correlated and to advocate an EVO–DEVO–concept. Selection does not act on genes or genomes but on individual organisms or phenotypes. These originate in development from expression of the genetic information. This makes it immediately evident how EVO and DEVO are interwoven (Müller 2007; Gilbert and Epel 2009).

That selection does not act on genes is shown by the observation that several genes often interact to determine a specific given trait. This phenomenon is called epistasis. The organism expressing this trait is then subject to selection. Such epistasis rules out that selection is acting on the single individual genes involved. Development is based on the regulation of the expression of genes. One has used the metaphor that a genome is like a musical instrument such as a grand piano, dead and meaningless unless a musician plays it. A genome per se and in itself cannot be the incarnation. This is readily seen when we compare genome sizes of organisms with vastly different degrees of complexity. For example, the number of genes in:

- man 25,000; about 300 genes different from the chimpanzee (1.3%);
- a little nematode (*Caenorhabditis elegans*) and the fruit fly (*Drosophila melanogaster*) 15–20,000;
- the weed *Arabidopsis thaliana* 27,000.

It cannot be a small number of genes that make up for the differences of complexity between these organisms. Regarding the comparison between humans and chimpanzees, different in about 300 genes, this was already realized quite a while ago by King and Wilson (1975) "... that the genetic distance between humans and the chimpanzee is probably too small to account for their substantial organismal differences ..... that evolutionary changes in anatomy and life are more often based on changes in the mechanisms controlling the expression of genes than on sequence changes .....".

This is currently developed in the vividly emerging field of epigenetics. The basic idea of epigenesis as a principle producing the gestalt of organisms dates back to JOHANN FRIEDRICH BLUMENBACH (1752–1840, see Gierer 1998) and then was picked up with giving it more precision by CONRAD HAL WADDINGTON (1905–1975). Molecular epigenetics is a system of reading the genetic information of DNA. The molecular mechanism of epigenetic regulation is based on the structure and conformation properties of chromatin modulated by acetylation and methylation, respectively, of DNA and nucleosomal histones. Only in the state of acetylation, DNA is accessible for regulator molecules of gene activation or deactivation due to the larger size of the acetyl group as compared to the smaller methyl group. In the state of methylation the genetic information of DNA is silenced. A functional analysis and a high-resolution genome-wide characterization of DNA methylation of *Arabidopsis thaliana* underlines the overarching role gene methylation must exert in the control of biological functions of genes (Zhang et al. 2006). An example showing how this can affect gestalt is given by the ubiquitous ruderal plant *Linaria vulgaris*. This species is normally characterized by bilateral symmetry of its yellow flowers.

There is also a rare form with radial symmetry of the flowers. CHARLES LINNÉ took it for a different genus which he named *Peloria*. We now know that both have identical DNA. The only difference is methylation of the promoter DNA of a single gene (*cycloidea, Lcyc*) in *Peloria* (Cubas et al. 1999; Paulsen 2007; Daxinger and Whitelaw 2010).

Epigenetic variations may be directed by the environment (Jablonka and Lamb 1989). There is increasing evidence that chromatin methylation patterns are strongly modified by environmental stress (Bond and Finnegan 2007; Chinnusamy and Zhu 2009; Adams 2010; Daxinger and Whitelaw 2010; Verhoeven et al. 2010), such as salt stress, nutrient stress, e.g., nitrogen deficiency (Kou et al. 2011), and chemical induction of anti-herbivore and anti-pathogen defenses (Verhoeven et al. 2010).

Epigenetic mechanisms are involved in memory functions of plants. Memory processes in the control of plant growth and morphogenesis (Thellier 2012; Thellier et al. 2012) and also in priming of defense reactions by previous attack (Bruce 2010; Heil 2010; van Hulten et al. 2010) comprise a form of habituation where after exposure to a first stimulus, subsequent responses to a second stimulus of the same type are modified. Most importantly there is a second form of memory which allows storage of information and recall of that information and is therefore termed STO/RCL. Storage of information can occur for various kinds of stress, such as manipulation of plants, drought, wind, cold shock, and even low-intense electromagnetic radiation. At the molecular level proteins are involved in the STO/RCL functions. Possibly small RNAs are participating in the signaling cascades because, as we shall see in a moment, epigenetic modifications where small RNAs are involved (Chinnusamy and Zhu 2009), could well be the major mechanism of STO/RCL. Stress memory appears to be epigenetic (Chinnusami and Zhu 2009; Verhoeven et al. 2010). This requires that stress-induced methylation patterns are not reset to the basal level when the stress is relieved and that therefore methylation is kept as the stress memory (Chinnusamy and Zhu 2009). The physiological experiments show that storage of information is robust, and it can be recalled after many days and weeks (Thellier 2012; Thellier et al. 2012). Studies of epigenetics demonstrate that information of stress received by plants can even be transferred to subsequent generations. Stress-induced methylation changes that are not reset can be transmitted through the germ line and are mostly heritable. They can be transferred through several generations (Jablonka and Lamb 1989; Bird 2002; Molinier et al. 2006; Bond and Finnegan 2007; Saze 2008; Verhoeven et al. 2010).

The inheritance of epigenetically established traits has evolutionary implications. One example, which we have already seen above, is that of the morphologically so different phenotypes of *Linaria vulgaris* and *Peloria*. Phenotypes resulting from heritable epigenetic variation will be subject to evolutionary selection (Verhoeven et al. 2010). Inherited epigenetic variations can also contribute to occupation of different niches with reproductive isolation between populations, and thus become drivers of speciation (Jablonka and Lamb 1989; Verhoeven et al. 2010). As we have seen above and as Richards (2006) focuses it, phenotypic variation as raw material on the playground of evolutionary selection is based on the two components of genetic and environmental variation. Inherited epigenetic variation modulated by environmental inputs blurs the line between EVO and ECO. Moreover, epigenetic modifications constitute a strong link between EVO and ECO (Richards 2006).

2.3 Ecology

2.3.1 The Step from EVO–DEVO to EVO–DEVO–ECO

Why is it so important to add ECO to EVO–DEVO? The term "ecology" was coined by ERNST HAECKEL in 1866 only after CHARLES DARWIN published the "Origin of Species". DARWIN does not talk about ecology although with our current understanding natural selection is eminently ecological, because the selective pressure driving evolution is exerted by cues of the environment. While evolution selects genotypes (Sect. 2.1: EVO) and selection acts on the phenotypes generated from the information of genotypes in development (Sect. 2.2: DEVO), ecology gives a frame for the responses of phenotypes to the conditions of the environment. We may distinguish two types of evolutionists (1) those who are not ecologically biased and (2) the evolutionary ecologists who have strong ecological interests. The former will consider selection of genotypes and evolution independent of the dynamics of environmental conditions. They do not need ecology for building evolutionary or phylogenetic trees from the genomics of molecular genotype comparisons (Sect. 2.3.2). The latter will strongly envisage the dynamics of environmental cues in niches, habitats, and ecosystems as the ecological frame within which evolution becomes manifest. They support adding ECO to the overall concept (Sect. 2.3.3). The distinction between the two may be gradual though.

2.3.2 Selection and Evolution Under "Constant Conditions"

The intriguing question is if selection and evolution can occur without any environmental and ecological dynamics, i.e., under constant conditions. There is now much evidence coming from both in-silico studies with digital organisms and experiments with actual living microorganisms that this is so, evolution does occur without environmental changes (Schuster 2011).

Digital organisms are computer programs that self-replicate and mutate randomly. The mutations then compete and are selected during the process. This then drives their increased fitness and hence evolution (Lenski et al. 1999, 2003). Such history of digital organisms also involves evolution of complex features by mutational modification of existing structures and functions (Lenski et al. 2003). The digital organisms share the properties of self-replication, mutation, competition, and evolution under given and constant conditions with real living microorganisms.

Given their short generation times mostly readily cultivable microorganisms, such as viruses, bacteria, and yeasts, are ideal models for following actual evolution of living organisms experimentally in real time (Elena and Lenski 2003). They can be observed under constant conditions for hundreds, thousands, and even tens of thousands of generations. Mutations that turn out to be better suited for fitness in an unchanged given environment are positively selected and outcompete less fit mutations. Progressive genetic adaptation based on mutations can continue

indefinitely in constant environments. Factors determining this, and thus, driving evolution may be glucose limitation, temperature, fungicides, and the like (Elena and Lenski 2003). Mutants more fit for dealing with this win the race of evolution.

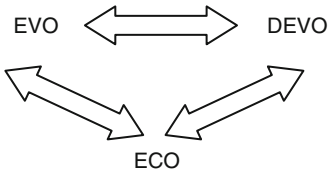
However, we must note two essential points here: The process involves (1) environmental factors albeit constant and (2) competition. Both are eminently ecological categories. Competition is dynamic, and therefore it is not even a "constant" condition. Hence while evolution definitely is possible under a stable and constant pressure of environmental factors, it cannot be said to be independent on ecological dynamics. Where competition is involved there are no constant conditions.

2.3.3 Responses of Phenotypes to Dynamic Networks of Environmental Factors

As we have seen in the previous section, we cannot dismiss ecology as part of EVO–DEVO even if we realize selection and evolution under a constant continuous rule of environmental cues. The essential role of ecology becomes still more evident, of course, when we consider the influence of dynamic changes of environmental conditions, which is the much more normal situation in nature as compared to experiments with microbial cultures.

Selection is on organisms. Irrespective of whether the selective pressure is on individuals (DARWIN) or species (GOULD) (see Sect. 2.1), it is always on the expressed phenotypes. When expression of the genotype information under certain environmental conditions generates a certain phenotype, the individuals carrying this phenotype will have to cope with the conditions given. However, under the environmental input which they receive the phenotypes can exert feedback on the genotype. If there is sufficient plasticity, ontogenetic development can set in, where expression is changed and modification of the phenotype is reached (Lüttge 2005). An example is given by C_3-photosythesis/crassulacean acid metabolism (CAM) intermediate plants, such as *Mesembryanthemum crystallinum*, species of *Clusia,* and several others (Lüttge 2005). Under nonstressful conditions these species perform C_3-photosynthesis. CAM is an ecophysiological biochemical adaptation to stressful conditions mainly affecting water relations under the effect of often network-like interacting environmental factors, such as limited water supply itself, irradiance, temperature, and salinity (Lüttge 2004). Under environmental changes individual plants of these species can switch between the physiological phenotypes (= physiotypes) of C_3 and CAM, respectively. In *M. crystallinum* the switch between C_3-photosynthesis and CAM is accompanied by stress-induced specific cytosine methylation of satellite DNA and therefore most likely under epigenetic control (Dyachenko et al. 2006; Chinnusamy and Zhu 2009).

Looking at these environmental influences, it becomes quite evident that ecology comes into the play of feedback and feedforward interactions between EVO and DEVO to constitute the network of



Epigenetics is a matter of development where stem cells have central functions. We shall consider their basic properties next to lay the ground for our thesis that at the higher scalar level of habitats and ecosystems in analogy, we can consider "stem species" as fundamental elements of hierarchical organizational systems.

3 Stem Cells in Development

As a basis for our aim to introduce a "stem species" concept in this essay, we must undertake here a very brief excursion to the stem cells in mammals and in plants. Some basic definitions and features of stem cells need to be looked at for comparison with what we may call "stem species" at a higher scalar level above organisms. May we use stem cells only as a metaphoric analogy of "stem species" or may we even speak of homologies?

3.1 Mammalian Stem Cells

The term and concept of stem cells comes from animal embryology and development, predominantly from mammals including humans. It plays an increasing role in medicine for therapies and for regenerative medicine.

A lexical definition is:

> Stem cells are biological cells found in all multicellular organisms, that can divide through mitosis and differentiate into diverse specialized cell types and can self renew to produce more stem cells. (Wikipedia 2011)

We might expand that a bit (Alberts et al. 2004). Stem cells are nondifferentiated cells that can divide continuously and indefinitely having an unlimited developmental potential. The daughter cells can either remain nondifferentiated or differentiate to specific kinds of cells. With respect to their occurrence within the organisms, we may distinguish the following types:

- embryonic stem cells,
- adult stem cells
  - somatic stem cells derived from an organ of the organism,
  - germ line stem cells.

Essentially stem cells are self-renewable and have different degrees of potency. The latter criterion allows distinguishing different classes:

- Totipotency or omnipotency: Such stem cells can construct a complete viable organism. Strictly totipotent are only the fertilized egg, i.e., the zygote, and its immediate descendent cells originating from division of the zygote.
- Pluripotency: Such stem cells can differentiate into nearly all cell types of an organism.
- Multipotency: Such stem cells can differentiate into a number of cell types belonging to a closely related family of cells.
- Oligopotency: Such stem cells can differentiate only into a small number of cell types.
- Unipotency: Such cells are still stem cells as they have the property of self-renewal, but they can generate only one single type of cells.

The different degrees of potency allow stem cells to exert various repair functions. Mammal stem cells have mobility. They can replace and renew differentiated cells that have been damaged and died in various organs. Stem cell engineering now aims at using these properties for developing new therapies in regenerative medicine. These particular properties of stem cells issued the idea to search for species as analogies with similar functional properties at the ecological level and to call them "stem species".

3.2 Plant Stem Cells

All plant tissues develop from meristems. Primary meristems are the apical meristems of shoots and roots. They originate directly from cells of the embryos, i.e., they are "primary embryonic meristems". Meristems generate different organs in roots and shoots, therefore in the center of plant meristems there must be and there are in fact stem cells (Weigel and Jürgens 2002). In the shoot leaves, flowers and branches are generated. This can go on almost forever as life of some plants may be very long. For example, *Pinus longaeva* (D.K. Bailey) in northwestern America may get as old as 5,000 years or more. The oldest currently living tree is 4,700 years old and in 1964 a 4,950-year-old tree was felled. The stem cells which are generating leaves, flowers, and branches are immortal over all this time.

Thus, basically mammalian and plant stem cells are similar. There are some conspicuous differences though. First of all there is no critical question regarding totipotency or omnipotency of plant stem cells. By great contrast to the mammalian stem cells, totipotency is not restricted to the zygote and its immediate descendent cells. In contrast to the determinate ontogenies of most animals, there are the indeterminate growth patterns of most plants (Friedman and Diggle 2011). Whole plants can be regenerated from meristems of all kinds, from tissue slices and even from single isolated somatic cells, a process called somatic embryogenesis. Totipotency is known from a variety of experiences and observations, such as

pruning, grafting, development of adventitious plants from leaf cells, e.g., in the genus *Kalanchoë*, and on tillers, etc. Another difference is that in contrast to mammalian stem cells, plant cells have no motility within the organism and its organs due to their cell walls and their position in the center of meristem tissues.

In conclusion, like mammal stem cells plant stems cells have amazing regenerative powers and repair functions (Weigel and Jürgens 2002).

4 Invaders, Nurse Species, and Pioneer Species in Ecosystems

Before we can consider "stem species" as a new category, we must be able to distinguish them from other established categories of functional classes of species, such as invaders, nurse species, and pioneer species.

4.1 Invaders

Invaders are species which newly arrive in existing ecological systems such as niches, habitats, or ecosystems. They get established in their new host systems by outcompeting resident species, and thus, disturbing and modifying the host systems. They display exaptation based on their exaptive pool of dormant traits. The classic work of Elton (1958) was a pioneer study to indicate the impact of invasive species. An invasive species is by definition exotic to the system it invades, which often generates the mistaken notion that all exotic species are invaders (D'Antonio and Meyerson 2002). Sakai et al. (2001) have demonstrated the strong correlation between certain bionomic traits and the invasive potential of species, which strengthens the predictive power of science to determine invaders and therefore to manage them. Thus, even native species can have dormant traits that manifest themselves upon disturbance. Scarano (2009) reviews the interesting case of *Andira legalis* (Vell.) Toledo, a tropical legume shrub that often displays small isolated populations in coastal habitats, which can turn highly abundant in response to fire and subsequently outcompete other species in such a way that local diversity is reduced. Invaders by specific contrast to "stem species" have no potential of repair. On the contrary they often accelerate devastation and change.

4.2 Nurse Plant Species: Facilitators

Nurse plants are species established in the space of a system due to their adaptation. They provide resources to other species (microbes, animals, plants) in facilitation or mutualism and/or by shaping niches in competition, so that the nursed species can also get established in the space of the system. There is natural facilitation by nurse species as well as anthropomorphic facilitation.

4.2.1 Natural Facilitation

The nurse-plant syndrome occurs when plant species shelter seedlings, young and/or adult individuals of other species throughout their ontogeny (Franco and Nobel 1989). Thus, nurse plants promote facilitation enhancing fitness, survival, and/or growth of associated species (Callaway et al. 2002; Bruno et al. 2003; Brooker et al. 2008). It often results in nucleation, i.e., formation of vegetation clumps or islands. Whenever nurse plant effects go beyond the scope of facilitation only and affect the physical space where other species live, and such direct effects last longer than the lifetime of the nurse plant species, they are called ecosystem engineers (Hastings et al. 2006), which is a concept we will come back to in Sect. 5.1.

The nurse plant syndrome and facilitation mechanisms are well known for arid and alpine zones, and fine examples emerge from the papers cited in the above paragraph. For the tropical environments some of our own studies have been reviewed in Dias and Scarano (2007) and Scarano (2002, 2009) and dwell on the examples of *Clusia hilariana* and bromeliads in a coastal sandy plain ecosystem in Brazil, named "restinga". *C. hilariana* is phytosociologically dominant at the so-called *Clusia* scrub, which is the predominant physiognomy in the restingas at the northern coast of the State of Rio de Janeiro (Pimentel et al. 2007). It consists of vegetation islands of various sizes surrounded by white sand. This tree can be as tall as 8 m (Dias et al. 2006) and displays a number of peculiar features, such as (1) dioecy (Faria et al. 2006); (2) seedling occurrence predominantly inside the tanks of terrestrial bromeliads (Scarano 2002), which are nurse plants themselves; (3) CAM metabolism (Lüttge 2006); and (4) an aboveground biomass stock and understory litter comparable to the entire woody component of many neotropical savannas (Dias et al. 2006). Curiously, however, *Clusia* is a genus with many hemi-epiphytic stranglers and/or rupicolous species (Lüttge 2006) that live in the neighboring rainforest habitats. More importantly, *C. hilariana* is the most abundant woody species locally (Pimentel et al. 2007) and it has a positive effect on both understory seedling density and richness, which is partly related to the activity of seed dispersers that use male and female plants indistinctly. Furthermore, Dias et al. (2006) indicated that slow decomposition may play an important role on carbon accumulation and that *C. hilariana* despite its conservative strategy of carbon acquisition via CAM, gives a high contribution to biomass stock in this nutrient-poor coastal vegetation. Therefore, in addition to the positive role played on local biodiversity, this plant might also strongly affect ecosystem processes such as productivity and nutrient cycling that, in turn, are also likely to affect recruitment process and species composition. In Scarano (2009), we proposed that this combination of biotic effects with a long-lasting physical effect on ecosystem processes qualify this species as an ecosystem engineer (see Hastings et al. 2006). In Sect. 5.2, we will show why we now think it is better defined as a stem species.

4.2.2 Anthropomorphic Applied Facilitation

Perhaps the most well-known example of applied facilitation is agro forestry, where woody plants and herbaceous crops or pastures are subject to integrated management. For instance, it is often observed in tropical savannas that a larger diversity of herbal vegetation builds up underneath savanna trees. This is due to protection by the trees, lower water stress due to shading at high solar radiation, and nutrient supply by litter and perching birds. In the case of trees with symbiotic fixation of atmospheric nitrogen, such as species of *Acacia*, it was estimated that about 40 trees per hectare provide sufficient N-fertilization to support pasture or crops in agro-forestry (Lüttge 2008).

Another example is management of degraded pasture and range lands by afforestation where there was no forest before or reforestation restoring previous woodlands. In many geographic regions especially in the tropics and subtropics, exotic trees are much used for this purpose (Lüttge 2008; Feyera et al. 2002; Grams and Lüttge 2010, more references there), e.g., often monocultures of *Eucalyptus*. The advantages and disadvantages have been surveyed elsewhere (Lüttge 2008; Feyera et al. 2002). The disadvantages in places get dominating so that many attempts are started to restore secondary forests which come close to original native forest.

There is more experience of handling exotic trees than native ones. Thus, one uses the exotic trees as facilitators or nurse trees. With the appropriate silvicultural management under the protection of canopies of exotic forest plantations, up to 175 native woody species have been regrown (see Feyera et al. 2002; Grams and Lüttge 2010). In an Ethiopian plantation of *Eucalyptus saligna*, a native forest of *Podocarpus falcatus* is regenerated. The photosynthetic capacity of *E. saligna* and *P. falcatus* is similar, but the *Eucalyptus* is using much more water. With thorough thinning due to regular coppicing of the *Eucalyptus, P. falcatus* becomes competitive and can outcompete the *Eucalyptus* (details in Feyera et al. 2002; Lüttge et al. 2003; Fetene and Beck 2004; Grams and Lüttge 2010). *Eucalyptus* monocultures can destroy water relations of entire landscapes with adverse effects on adjacent agriculture and even the water supply of cities. In the National Park of Mount Entoto at the rim of Addis Ababa at 2,600–3,100 m a.s.l., one is running a reforestation experiment with a diversity of more than half a dozen of native tree species (*Acacia abyssinica, Hagenia abyssinica, Juniperus procera* (syn. *J. excelsa*), *Olea europaea, Podocarpus falcatus, Prunus africanus*)(Ethiopia Heritage Trust, eht@ethionet.et).

It is clear from these examples that an exotic tree such as *Eucalyptus* can exert repair functions and serve as facilitator and nurse tree. However, these examples also clearly underline the difference to “stem species”. In plantations *Eucalyptus* is not sustainable let alone self-renewable, because it exhausts resources, such as water and soil nutrients, and after a few generations cannot be supported longer. This definitely rules out that we call it a “stem species”.

4.3 Pioneer Plant Species

Pioneer plants are species acquiring pure empty space as a resource in a stochastic manner (Grams and Lüttge 2010). They function as foundation species affecting the establishment of new ecosystems at different scalar levels. They can and most likely will develop to nurse species. There is, of course, a plethora of possible examples of pioneer species in the biosphere. Here we briefly touch some outstanding examples.

4.3.1 Biofilms and Soil Crusts

Microorganisms, especially bacteria can get established and start life on almost any imaginable bare surface including surfaces of buildings and other objects around human settlements. Biofilms are mucilaginous excretions of bacteria embedding their colonies as a joint medium that provides protection and enables for metabolic communication. Thus in the biofilms bacteria may act as pioneers for the establishment of other life.

Soil crusts are similar to biofilms but more complex (Belnap and Lange 2001). They are often microscopic and hence often overlooked ecosystems that pioneer on bare surfaces of the sand of dunes, in savannas and deserts and other dry sites where larger vegetation cannot get established or forms gaps. They represent layers of soil particles that adhere to each other *via* contacts with (micro-) organisms and/or their excretions. Such layers have a thickness of a few millimeters up to centimeters but can extend over quite extended surfaces. They constitute a complex community of organisms with cyanobacteria, eukaryotic algae, fungi, lichens and bryophytes, and small animals (nonvertebrates).

The act of pioneering by biofilms and soil crusts is acquisition of new empty space. This does not really imply an act of repair, and therefore, we would not call the species involved "stem species".

4.3.2 Bare Rocks of Tropical Inselbergs

Inselbergs are large rock-outcrops especially from tropical savannas and rainforest (for reviews see Barthlott et al. 1993; Porembski and Barthlott 2000; Lüttge 2008; Porembski 2011). Their often rich and diverse flora originates from pioneer species that have arrived on the bare rock. Again this was not "repair" but acquisition of space. The bare rock is generally covered by biofilms of cyanobacteria and crusts with lichens. There are many different niches for life, including erosion-shaped pot holes and vegetation islands developed out of them. High irradiance and water only from rain with high losses by runoff are the major environmental stresses. The adaptation of desiccation tolerance is relatively frequent among the vascular plants on inselbergs (Porembski 2011).

4.3.3 Maritime Volcanic Islands

A favorite example in the ecological literature is maritime volcanic islands where life was arriving after volcanic outbreak and formation of the new islands. Plants can often travel long distances with their propagation units or diaspores. Thus such pioneer species show mobility like stem cells and of course also the "stem species" as discussed in Sect. 5. It is noteworthy that often the diaspores of some Asteraceae can cover large distances due to their pappus hairs as flight device. Adaptive radiation after separation from their populations of origin can lead to new speciation from the founder populations. Examples are the Asteraceae *Argyroxiphium sandwicense* and *Dubautia menziesii* on the islands of the Hawaii archipelago and the genus *Scalesia* on the Galápagos Islands (McMullen 1999). Of particular interest are the various cacti on the Galápagos Islands. They are all plants performing CAM as an adaptation to stress of water scarcity and high solar radiation (Lüttge 2004). Their success as pioneers on the bare black volcanic rocks was due to this adaptation, i.e., the preadaptation they brought with them. The further development in the cactus genera on the Galapágos Islands shows interesting differences. In the genus *Opuntia* due to evolution by radiative adaptation, there are now 6 species and 8 varieties together forming 14 different lineages (McMullen 1999; Lüttge 2010b). *Jasminocereus thouarsii* is the only species of its genus. It is morphologically variable and has three varieties. Here it might be that it found niches where the environmental pressure has not been tough enough to drive further speciation during the age of only four million years of the Galápagos Islands. By contrast *Brachycereus nesioticus* is growing solitarily on bare black sun exposed lava. It is also the only species of its genus. It may be so perfectly adapted to its niche on the lava that no further selection and speciation was effective (Lüttge 2010b).

There was no "repair" on these geologically recent islands but shaping newly emerging life by preadapted newcomers. Hence, once again the species named above will not be called "stem species".

5 Stem Species

5.1 The Concept

"Stem species" correspond to stem cells in at least two ways:

- "Stem species" have different degrees of ecological potency (see Sect. 3.1), up to pluripotency, due to their exaptive pools and functional-shift capacities for creating different life-forms corresponding to the different cell types at the stem cell level. "Stem species" can provide the basis for building up a complete new viable ecosystem.
- "Stem species" have repair function. They share the property of mobility with mammalian stem cells. They can substitute for previously present adapted species that may have vanished.

Thus, "stem species" are species getting deterministically established in spatio-functional systems. For their establishment they use functional shift of traits for exaptation as explained in Sect. 2.1.2. With their own establishment they establish new niches. In this way they exert powerful repair functions in devastated systems, building functions in new systems, and therefore, collectively, work out as a repair system for the biosphere as a whole. So they constitute the basis for self-organization eventually securing self-sustainability of the systems (Scarano and Garbin 2012).

The "stem species" share some properties and functions with nurse species, pioneer species as well as species that act as ecosystem engineers. As we have seen (Sect. 4.2) nurse species can have repair functions. Pioneer species do not act so much via repair functions but can establish new niches, habitats, and ecosystems in inorganic life-less space which is obviously free of any resources except the space itself (Grams and Lüttge 2010). Pioneer species are adapted to this particular space. When they get established in such space after their diaspores arrived, this is due to their adaptation or preadaptation as it were. Ecosystem engineers "modulate the availabaility of resources to other species, by causing physical state changes in biotic or abiotic materials" (Jones et al. 1997). Stem species share these properties with nurse, pioneer, and ecosystem engineers' species. However, they go beyond that. They display ecological pluripotency. They do not depend on (pre-) adaptation but operate with exaptation.

5.2 Examples

The two examples we describe here originate from tropical vegetation, in Brazil (see also Scarano and Garbin 2012). The plants involved have in common several features (1) they have considerable ecological plasticity that allowed them to colonize novel habitats over varying time scales, (2) they have apparently low-habitat requirements, (3) they are facilitators, and (4) they have long-distance dispersal. They are also insufficiently known in regard to their ecology, physiology, and genetics.

5.2.1 *Clusia hilariana* in the Atlantic Forest Complex

The genus *Clusia* typically has many hemi-epiphytic stranglers and/or rupicolous species (Lüttge 2006) that live in rainforest habitats. In the case of the Brazilian Atlantic rainforest, some such species migrated to sandy plains, known as restingas, which were formed by the coast during the Quaternary (Scarano 2002, 2009). *Clusia hilariana* is one such species. However, the plasticity of the genus and of the species is such that in the restingas of northern Rio de Janeiro (SE-Brazil), this species occurs as an 8 m tall tree (Dias et al. 2006) and that through facilitation processes in the restinga it is largely responsible for diversity in land (Dias and Scarano 2007), soil (Kreuzer

et al. 2007), and possibly even in adjacent water bodies (Pimentel et al. 2007). Thus, *C. hilariana* exerts many positive effects on community diversity. Moreover, it plays a marked functional role: its aboveground biomass stock and understory litter is comparable to the entire woody component of many neo-tropical savannas (Dias et al. 2006). It has enough ecological, physiological, and morphological plasticity to, in time, colonize novel habitats and subsequently facilitate the onset of a diverse community. Therefore, *C. hilariana* fits our concept of stem species.

5.2.2 Nitrogen Fixers in the Flooded Forests of the Amazon

Another conspicuous example of such regeneration power of the planet comes from the Brazilian Amazon. From 1979 to 1989 bauxite washing tailings were continuously discharged into Lake Batata (State of Pará, Central Amazon, Brazil) and the surrounding *igapó* forest (i.e., forest seasonally flooded by low-nutrient waters). When the discharge was halted, circa 30% of the lake area with its marginal *igapó* forest was buried by a 4–5 m bauxite tailings layer (Scarano et al. 1998). Frequent and prolonged exposure to full sunlight during the dry season has led to dehydration and consolidation of the bauxite tailings. The bauxite tailings substrate consists of 75% clay, 21% silt, 3% fine sand, and 1% coarse sand. It differs from nonimpacted *igapó* soil in the proportions of clay (49%), silt (37%), and fine sand (13%) (Dias et al. 2012), and therefore constitutes a new habitat. Perhaps surprisingly, many native *igapó* species began to spontaneously regenerate and grow on the top of this substrate, particularly in areas where water was more still during flooding. Vectors of seed dispersal in these forests are to a large extent water and fish (Mannheimer et al. 2003) and therefore sites with water currents and fast flow during flooding are less prone to establishment. Thus, a large-scale reforestation program was set in place to provide forest cover to this new environment (Bozelli et al. 2000). After over 20 years since the impact happened and 15 years since man-induced forestation started, it is now apparent that nitrogen fixing legumes (*Acosmium nitens* (Vogel) Yakovlev and *Dalbergia inundata* Spruce ex Benth.; Souza et al. 1994) are the most abundant species in the site, both due to spontaneous regeneration and to successful performance of planted seedlings (Scarano et al. 1998; Dias et al. 2012). Nitrogen fixation by these species in such a nutrient-poor substrate can possibly be a factor contributing to the high diversity found in this new habitat, which would also fit them in our stem species concept.

6 Conclusions

Plants we are here calling stem species are insufficiently known in regard to their ecology, physiology, and genetics. Plants known to science under all those angles are usually productive (agriculture or forestry) or model plants (e.g., *Arabidopsis thaliana*). Often rare or threatened species that are in the focus of numerous conservation biology studies are also studied in detail. Conversely we know

astonishingly little about the comportment of the most common plants that are neither economically relevant nor rare. They are largely understudied, at least in the tropics. Some of the traits described above for stem species (high plasticity, long-term dispersal, low-habitat requirements) are often found in common species. Thus, we would argue that plants with high repair potential of Gaia, and thus, the stem species, are likely to be found among common plants and among plants with no known economic importance. These are plants that have not so much attracted scientific studies and we therefore know little about them. In this essay we have explicitly mentioned only two examples and the scientific search for more such stem species now is an important challenge. EVO–DEVO–ECO would provide us with the necessary clues for the search for the stem species. If indeed we can expect exaptative surprise by common species offering repair functions of ecosystems, outlook may be not totally pessimistic.

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Roles of Organic Acid Metabolism in Plant Tolerance to Phosphorus-Deficiency

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Abstract On 30–40% of the world's arable land, crop yield is limited by phosphorus (P) availability. Phosphorus fertilizer use increased fourfold to fivefold between 1960 and 2000 and the demand for P was predicted to increase by 50–100% by 2050 with increased global demand for food and diets. Continually increasing demand for P will deplete existing phosphate (Pi) rock reserves by the end of the century. Improvement of soil P-acquisition and utilization by plants is one approach to alleviate the scarcity of P resources and to reduce environmental pollution. Many plants have evolved different strategies of enhancing P-acquisition from low-P soils, and one of these strategies involves the secretion of organic acid (OA) anions. Although the causes are not fully understood, the P-deficiency-induced secretion of OA anions may be related to several factors, including (a) internal concentrations of OAs in plant tissues; (b) proteoid or cluster root formation; (c) permeability of root membranes; (d) root plasma membrane H^+-ATPase; and (e) anion channels. Besides increased acquisition of soil P, plants respond adaptively to P-deficiency through the induction of alternative glycolytic pathways and tonoplast pumping bypassing Pi- and/or adenylate-dependent reactions. Apart from pyrophosphate (PPi)-dependent tonoplast pyrophosphatase (V-PPiase), several Pi- and adenylate-independent glycolytic bypass enzymes [i.e., UDP-glucose pyrophosphorylase (UGPase), PPi-dependent phosphofructokinase (PPi-PFK), NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (NAD-G3PDH), phospho*enol*pyruvate carboxylase (PEPC), phospho*enol*pyruvate phosphatase (PEPP), pyruvate phosphate dikinase (PPDK), NAD-malic enzyme (NAD-ME)] in plant tissues have been reported to be upregulated by P-deficiency. Genetically modified plants and cells with higher P-deficiency-tolerance by overexpressing genes for the transporter and biosynthesis of OAs, as well as V-PPiase have been obtained. In addition, some aspects needed to be further studied are also discussed.

1 Introduction

Phosphorus (P) is an essential macronutrient element required for the normal growth and development of higher plants and P is considered to be the second-most important nutrient element limiting agricultural production after nitrogen (N). The concentration of P in plants ranges from 0.5 to 5 mg g^{-1} DW (Vance et al. 2003). The element plays an important role in many metabolic processes in plants, including respiration, glycolysis, photosynthesis, energy generation, nucleic acid biosynthesis, enzyme activation/inactivation, carbohydrate metabolism, redox reactions, signaling, N fixation, membrane synthesis, and stability (Plaxton and Tran 2011; Vance et al. 2003). Although the amount of total P is abundant in many soils, the concentration of soluble P in the soil solution is commonly 1–2 μM due to its binding to soil mineral surfaces and fixation into organic forms (Kochian et al. 2004). In acidic soils, free iron (Fe) and aluminum (Al) oxides bind native and fertilizer P into forms unavailable to plants, while in alkaline soils, the amounts of magnesium (Mg) and calcium (Ca) compounds are usually high, binding P into forms unavailable to plants (Kochian et al. 2004;

López-Bucio et al. 2000a; Vance et al. 2003; Yan et al. 2006). Therefore, P is one of the most immobile, unavailable, and inaccessible macronutrients present in soils (Holford 1997; Vance et al. 2003) and is often the most limiting mineral nutrient in almost all soils (Kochian et al. 2004). On 30–40% of the world's arable land, crop yield is limited by P availability (Vance et al. 2003). For this reason, the application of P fertilizers to cropland has become a routine agricultural practice to raise crop productivity and quality. However, the use of P fertilizers is not only expensive and non-sustainable, but also pollutes environments. Phosphorus fertilizer use increased fourfold to fivefold between 1960 and 2000 and the demand for P was predicted to increase by 50–100% by 2050 with increased global demand for food and diets (Cordell et al. 2009; Vance et al. 2003). Continually increasing demand for P will deplete existing phosphate (Pi) rock reserves by the end of the century (Cordell et al. 2009; Vaccari 2009).

Many plants have evolved different strategies of enhancing P-acquisition from low-P soils, and one of these strategies involves the secretion of organic acid (OA) anions (Fukuda et al. 2007; Raghothama 1999; Ryan et al. 2001; Vance et al. 2003). Besides increased acquisition of soil P, plants respond adaptively to P-deficiency through the induction of alternative glycolytic pathways and tonoplast pumping bypassing Pi- and/or adenylate-dependent reactions (Plaxton and Podestá 2006; Theodorou and Plaxton 1993; Vance et al. 2003). In this paper, we summarize the recent progress in our understanding of the roles of OA metabolism in plant tolerance to P-deficiency.

2 Phosphorus-Deficiency-Induced Secretion of Organic Acid Anions from Roots

Phosphorus-deficiency induces secretion of OA anions from roots of many plant species (Table 1). Hernández et al. (2007) and Huang et al. (2008) observed that the amount of OAs was less in P-stressed roots than in P-sufficient roots of bean (*Phaseolus vulgaris*) and barley (*Hordeum vulgare*); the reduced amount of OAs in the P-deficient roots likely reflected the exudation of OA anions. These OA anions can desorb Pi from mineral surfaces and solubilize Pi from Al-, Fe-, and Ca-phosphates by chelating the metals, thus increasing soil solution P concentration (Ryan et al. 2001). Phosphate dissolution rates can be greatly enhanced in soils in the presence of OA anions such as malate, citrate, and oxalate, leading to 10- to 1,000-fold higher concentration of P in soil solution depending on soil type, OA species, and concentration (Jones 1998; Lan et al. 1995; Plaxton and Tran 2011). In solubilizing inorganic Pi compounds in the soil, citrate and oxalate are very effective, whereas tartrate and malate are moderately effective, and acetate, lactate, and succinate are least effective (Nagarajah et al. 1970). Shen et al. (2001) observed that the higher P mobilizing activity in P-deficient root exudates agreed with large secretion of glutarate induced by P-deficiency, concluding that glutarate

Table 1 Plant species with phosphorus (P)-deficiency-induced secretion of organic acid (OA) anions by roots

Plant species	OA anions released	References
Alfalfa (*Medicago sativa*)	Citrate	Lipton et al. (1987)
Arabidopsis thaliana	Citrate, malate	Narang et al. (2000)
Bean (*Phaseolus vulgaris*)	Citrate, tartrate, acetate	Shen et al. (2002)
Cabbage (*Brassica oleracea*)	Citrate	Dechassa and Schenk (2004)
Chickpea (*Cicer arietinum*)	Malate, citrate, malonate	Neumann and Römheld (1999)
Cowpea (*Vigna unguiculata*)	Citrate	Jemo et al. (2007)
Elephantgrass (*Pennisetum purpureum*)	Glutarate	Shen et al. (2001)
Hakea prostrata	Citrate	Shane et al. (2003)
Lupinus luteus	Citrate	Hocking and Jeffery (2004)
Maize (*Zea mays*)	Citrate, malate, *trans*-aconitate	Hinsinger (2001); Li et al. (2008)
Pigeonpea (*Cajanus cajan*)	Citrate, piscidate	Ishikawa et al. (2002)
Potato (*Solanum tuberosum*)	Succinate	Dechassa and Schenk (2004)
Purple lupin (*Lupinus pilosus*)	Citrate, malate	Ligaba et al. (2004b)
Radish (*Raghanus satiuvs*)	Tartrate, malate, succinate	Zhang et al. (1997)
Rape (*Brassica napus*)	Malate, citrate, succinate, acetate, tartrate	Hoffland et al. (1989); Zhang et al. (1997, 2011); Zhou et al. (2012)
Rice (*Oryza sativa*)	Citrate, oxalate	Begum et al. (2005); Hoffland et al. (2006); Kirk et al. (1999)
Soybean (*Glycine max*)	Malate, oxalate	Dong et al. (2004); Liao et al. (2006)
Sudangrass (*Sorghum vulgare*)	Succinate, *cis*-aconitate isocitrate, fumarate, *trans*-aconitate, citrate	Schwab et al. (1983)
Sugar beet (*Beta vulgaris*)	Salicylate, citramalate	Khorassani et al. (2011)
Stylosanthes guianensis	Citrate	Li et al. (2009)
Tea (*Camellia sinensis*)	Malate, citrate	Lin et al. (2011)
White lupin (*Lupinus albus*)	Citrate, malate	Hocking and Jeffery (2004); Johnson et al. (1996a); Kania et al. (2003); Keerthisinghe et al. (1998); Li and Liang (2005); Neumann et al. (1999); Shen et al. (2005)

represented higher P mobilizing activity in P-deficient root exudates, thus enhancing P mobilization. The ability of pigeonpea (*Cajanus cajan*) to utilize Fe-P was attributed to the exudation of piscidate from roots (Ae et al. 1990). Gardner et al. (1983) showed that citrate secreted from P-starved proteoid roots of white lupin (*Lupinus albus*) reacted with Fe-P and then P was released by reduction of Fe^{3+} to Fe^{2+} on the root surface. Khorassani et al. (2011) observed that P-deficiency resulted in an enhanced release of citramalate and salicylate from sugar beet roots and both metabolites released soil P. The release of OA anions also (a) cause organic P to become more susceptible to hydrolysis by secreted acid phosphatases (APases); and (b) stimulate the activity of symbiotic rhizosphere microbes, thus facilitating root P-acquisition (Fang et al. 2009; Plaxton and Tran 2011;

Ryan et al. 2001; Vance et al. 2003). Therefore, the root secretion of OA anions is considered as a principal mechanism in alleviating P-deficiency (Vance et al. 2003). Increasing evidence has shown that many P-efficient plant species or cultivars secrete larger amounts of OA anions than P-inefficient ones when exposed to P-deficiency, including barley (Gahoonia et al. 2000), maize (*Zea mays*; Hinsinger 2001), rape (*Brassica napus*; Zhang et al. 2011; Zhou et al. 2012), *Arabidopsis* (Narang et al. 2000), supporting root secretion of OA anions as a principal adaptation for some P-deficient plants. Dechassa et al. (2003) revealed that cabbage (*Brassica oleracea*) was superior to carrot (*Daucus carota*) and potato (*Solanum tuberosum*) in P-efficiency, because the secretion of citrate by cabbage roots increased largely in response to P-deficiency by which it can additionally increase its P-uptake efficiency, whereas potato and carrot displayed little evidence of possessing such a mechanism (Dechassa and Schenk 2004). However, Ishikawa et al. (2002) showed that the release of citrate and piscidate could not explain the genotypic variation in low-P availability of pigeonpea, although both OA anions released from roots might play a partial role in solubilizing insoluble P in soils. In addition, several reports showed that the release of OA anions was not induced by P-deficiency in some plant species, such as soybean (*Glycine max*; Nian et al. 2003), wheat (*Triticum aestivum*) (Delhaize et al. 1993), carrot (Dechassa and Schenk 2004), rape (Ligaba et al. 2004a), *Citrus sinensis* and *Citrus grandis* (Yang et al. 2011). Wouterlood et al. (2004a, b, 2005) showed that plant P status only had a limited influence on the concentration of P-mobilizing carboxylates in the rhizosphere of chickpea (*Cicer arietinum*). In this section, we will discuss five aspects that have been implicated in the regulation of OA anion secretion.

2.1 Internal Concentrations of Organic Acid Anions in Roots and Leaves

Evidence that the secretion of OA anions from roots could play a key role in mobilizing P was first obtained when researchers found that P-deficiency increased the concentration of OAs in the roots of certain plants (López-Bucio et al. 2000a). An increase in the concentration of OAs has been reported for P-deficient rape (Hoffland et al. 1989), white lupin (Johnson et al. 1996a; Neumann et al. 2000), cabbage (Dechassa and Schenk 2004), and chickpea (Neumann et al. 2000) roots. A study with maize showed that the amounts of citrate in the roots and exudates of low-P-tolerant mutant 99038 were higher than in wild-type Q1-319 under P-sufficient and P-deficient conditions. The increased accumulation and secretion of citrate might be coordinately regulated by increased biosynthesis and decreased degradation of citrate, because the amounts and activities of malate dehydrogenase (MDH, EC 1.1.1.37) and citrate synthase (CS, EC 2.3.3.1) in the roots of 99038 plants increased compared with Qi-319, whereas those of NAD-isocitrate dehydrogenase (NAD-IDH, EC 1.1.1.41) and aconitase (ACO, EC 4.2.1.3) decreased (Li et al. 2008). Yao et al. (2011) reported that in rape roots, the protein expressions

related to glycolysis and tricarboxylic acid cycle, such as fructose-bisphosphate aldolase (EC 4.1.2.13), glyceraldehyde-3-phosphate dehydrogenase (G3PDH), and MDH, were enhanced to meet the requirement of OA secretion. Recently, our work with tea (*Camellia sinensis*) trees showed that P-deficiency-induced release of malate and citrate by roots resulted from the increased accumulation of root malate and citrate because the release of malate increased linearly with increasing root malate concentration and the P-deficiency-induced increase in root citrate release coincided with increased concentration of root citrate (Lin et al. 2011). In the proteoid roots of P-deficient white lupin, the accumulation of citrate and malate coincided with enhanced activities of phospho*enol*pyruvate (PEP) carboxylase (PEPC, EC 4.1.1.31), CS, and MDH, and elevated steady-state amounts of PEPC, MDH, and CS mRNAs (Johnson et al. 1996a, b; Uhde-Stone et al. 2003a). In maize roots, P-deficiency-induced increase in OA concentration was related to increased PEPC activity (Gaume et al. 2001). Ligaba et al. (2004a) observed that P-sufficient rape roots had significantly higher rates of biosynthesis and release of malate. In the roots of P-deficient white lupin, chickpea, and tomato (*Lycopersicon esculentum*), the increased amount of citrate was accompanied by increased PEPC activity and decreased ACO activity (Neumann et al. 1999). In tea roots, malate concentration increased with the increasing PEPC activity and the decreasing NADP-malic enzyme (NADP-ME, EC 1.1.1.40) activity, respectively, and P-deficiency-induced citrate accumulation was accompanied by the increased PEPC and CS activities and the decreased NADP-IDH (EC 1.1.1.42) activity (Lin et al. 2011). These results indicate that the P-deficiency-induced accumulation of malate and citrate in plant roots was associated with increased biosynthesis and/or with decreased degradation (Lin et al. 2011; Neumann et al. 1999). The increase in OA concentrations in P-deficient roots could also be associated with decreased respiration (Johnson et al. 1994; Vance et al. 2003) and dilution due to decreased root growth (Lin et al. 2011). Transcriptomic study with rice (*Oryza sativa*) roots showed that several genes related to glycolysis increased their expression levels in response to P-deficiency, whereas the expression levels of *OsAMT1*, which encodes the ammonia transporter, and glutamine synthase (EC 6.3.1.2) homologues were downregulated by P-deficiency, suggesting that the carbon supply for OA biosynthesis is accelerated, whereas the carbon flow for amino acid biosynthesis is repressed because OA biosynthesis consumes carbon skeleton molecules as a source of amino acids (Wasaki et al. 2003). Proteomic data also supports this conclusion (Fukuda et al. 2007). All these phenomena could result in more OA available for the root release. However, the correlation between the internal citrate concentration and citrate release from white lupin roots was weak (Neumann et al. 1999, 2000). A low concentration of succinate in potato roots in response to P-deficiency was associated with an increased rate of succinate secretion (Dechassa and Schenk 2004). In maize, the increase in OA concentration was associated with an increase in exudation for the low-P tolerant genotype only; while the low-P susceptible genotype was characterized by high OA concentration in roots and low OA exudation (Gaume et al. 2001). Watt and Evans (1999b) reported that neither *in vitro* PEPC nor CS activity correlated with the rate of citrate release from the proteoid roots of low-P white lupin.

Interestingly, tea root malate release and accumulation was induced by both 0 and 40 μM P, while root citrate release and accumulation was induced only by 0 μM P (Lin et al. 2011). In white lupin, young, developing cluster roots mainly excreted malate, whereas mature cluster roots mainly released citrate (Langlade et al. 2002). Studies with P-deficient white lupin showed that the limitation of ATP or Pi at later stages of proteoid root development might decrease the activity of ATP-citrate lyase (EC 2.3.3.8), which catalyzes the cleavage of citrate into acetyl-CoA (Ac-CoA) and oxaloacetate (OAA), thus increasing the accumulation of citrate in mature and senescent proteoid roots, while its activity was upregulated in juvenile proteoid roots of white lupin (Kania et al. 2003; Langlade et al. 2002; Massonneau et al. 2001). In primary roots of lupin and maize, ATP-citrate lyase activity was positively correlated with malate exudation (Langlade et al. 2002). Based on these results, it was concluded that ATP-citrate lyase activity could be responsible for the switch between malate and citrate excretion in the different developmental stages of white lupin cluster roots. Therefore, it is likely that ATP-citrate lyase in tea roots was induced by 40 μM P, but inhibited by 0 μM P. Thus, citrate release and accumulation was induced by 0 μM P. The difference in root accumulation and release of malate and citrate in response to P supply indicates that malate and citrate might function at different conditions of P-deficiency.

Relatively few studies have investigated the effects of P-deficiency on leaf OA metabolism. According to Hoffland et al. (1992), OA anions released from the roots of P-deficient rape and hedge mustard (*Sisymbrium officinale*) mainly resulted from increased PEPC activity in the shoots. Watt and Evans (1999a) observed that the rate of citrate exudation from proteoid roots of white lupin grown with low-P varied diurnally, with maximal rates during the photoperiod. Light also increased exudation of citrate, malate, and succinate from cabbage roots after 14 days of P-starvation (Dechassa and Schenk 2004). This may be associated with photosynthesis, which provides carbohydrates for the biosynthesis of OAs. Phosphorus-deficiency also increased PEPC activity of tomato leaves (Pilbeam et al. 1993). However, P-deficiency decreased PEPC activity of rice (Nanamori et al. 2004) and maize (Usuda and Shimogawara 1992) leaves. Microarray analysis of P-deficient *Arabidopsis* leaves and roots revealed a significant downregulation of both PEPC and MDH and a significant upregulation of pyruvate kinase (PK, EC 2.7.1.40) (Wu et al. 2003). Interestingly, PEPC activity in *Brachiaria* hybrid leaves increased in response to low soil P, but decreased in response to low-P supplied in hydroponics solution, whereas total OA level decreased in the leaves from plants grown in both soil and hydroponics solution (Begum et al. 2006). In tea leaves, the accumulation of malate was slightly induced only in 0 μM P-treated leaves, and no induction was observed in the citrate level. The higher malate concentration in 0 μM P-treated leaves could be caused by less dilution due to decreased shoot growth, rather than by decreased degradation, because NADP-ME activity was not reduced (Lin et al. 2011). Also, more sugar might be utilized to biosynthesize OAs in tea leaves due to the decreased demand for reduced carbon in growing sink tissues, because CO_2 assimilation decreased to a lesser extent than shoot growth (Li et al. 2009). In contrast to tea leaves, P-deficiency increased the concentration of citrate in rape and hedge

mustard shoots, whereas malate concentration was affected little in hedge mustard shoots and even declined in rape shoots (Hoffland et al. 1992). Achituv and Bar-Akiva (1978) showed that citrate concentration in P-deficient lemon (*Citrus limon*) leaves was almost 20 times higher than in controls, while P-deficiency-induced increase in malate concentration was much less. Nanamori et al. (2004) observed that in rice leaves, the marked decrease in oxalate concentration in response to P-deficiency was accompanied by increased malate and citrate concentration. Therefore, it is not well known how P-deficiency affects the accumulation of OAs in plant tissues and their release by roots.

2.2 Proteoid or Cluster Root Formation

Grown under P-deficiency, white lupin proteoid roots release 20- to 40-fold more citrate and malate than P-sufficient roots, and the amount of carbon released in citrate and malate can comprise up to 25% of the total plant dry weight (Dinkelaker et al. 1989; Keerthisinghe et al. 1998; Shane and Lambers 2005; Vance et al. 2003; Watt and Evans 1999b). However, the formation of proteoid roots does not affect plant dry matter yield or shoot to root dry matter ratio (Keerthisinghe et al. 1998). Proteoid root formation is predominantly affected by the P status of the plants, being induced at low-P levels and repressed at high P-levels (Abdolzadeh et al. 2010; Dinkelaker et al. 1995; Keerthisinghe et al. 1998; Shen et al. 2005). In contrast to proteoid root formation, a first visible symptom of P-deficiency (Neumann et al. 1999, 2000), root exudation of OA anions is a symptom of more-severe P-deficiency. The quantity and the composition of OA anions released from proteoid roots vary at different stages of proteoid root development. With the development of proteoid roots, the release of malate decreases, while the release of citrate increases and reaches maximum levels in mature root clusters. In senescent clusters, however, almost no citrate release is detectable despite high internal citrate concentration in the root tissue (Neumann et al. 1999). This may explain the difference in the composition of OA anions in root exudates of white lupin observed in earlier studies, ranging from only malate (Braum 1995), to equal amounts of malate and citrate (Johnson et al. 1996a) to almost exclusively citrate (Gardner et al. 1983). Peñaloza et al. (2002) reported that the rates of malate and citrate release by the whole root system of white lupin plants were similar from seedling emergence to plant senescence due to P-deficiency. Malate was predominantly released from apices of both seedling taproots and proteoid roots, while citrate release was restricted to proteoid root clusters.

2.3 Permeability of Root Membranes

In sudangrass (*Sorghum vulgare*), Graham et al. (1981) reported that P-deficiency-induced increase in root exudation was related to the changes in permeability of root membranes, rather than to the changes in root concentrations of amino acids

and reducing sugars, proposing that increased release of metabolites may be caused by increased root membrane permeability due to P-deficiency-induced decrease in phospholipids level. If this holds true for P-deficiency-induced secretion of OA anions, the greater the degree of P-deficiency, the greater the amount of OA anions secreted from roots. However, Ohwaki and Hirata (1992) reported that the larger amount of carboxylic acids observed in the exudates of chickpea was not directly correlated with the concentration of phospholipids-P in the roots. Phosphorus-deficiency-induced release of OA anions from radish (*Raghanus satiuvs*) roots peaked 6 days after P was deprived, and then decreased over time (Zhang et al. 1997). Similar result has been obtained for P-starved bean plants (Shen et al. 2002). Therefore, it is likely that an active mechanism is involved in P-deficiency-induced release of OA anions.

2.4 Root Plasma Membrane H^+-ATPase

Yan et al. (2002) observed that the plasma membrane derived from active proteoid roots of P-deficient white lupin plants had higher plasma membrane adenosine triphosphatase (H^+-ATPase, EC 3.6.3.6) activity and concentration, H^+-pump activity, pH gradient across the membrane, and passive H^+ permeability than those from other roots. However, plasma membrane H^+-ATPase of active proteoid roots displayed lower vanadate sensitivity and more acidic pH optimum. Therefore, these authors concluded that the release of OAs involves two separate transport processes: an active efflux driven by plasma membrane H^+-ATPase and a passive efflux of OA anions mediated by channel-like transporters. Obviously, modulation of plasma membrane H^+-ATPase is involved in P-deficiency-induced secretion of OA anions. Later, Ligaba et al. (2004b) observed that P-deficiency enhanced plasma membrane H^+-ATPase activity, H^+-pump activity, and secretion of citrate and malate in greater purple lupin (*Lupinus pilosus*) roots, and that 1 mM vanadate inhibited H^+-ATPase activity by 80% in vitro and citrate release by 60% in vivo, but did not suppress malate. These results indicate that P-deficiency-induced secretion of citrate, but not malate, may be attributed to increased H^+-ATPase activity and H^+ efflux. Shen et al. (2006) showed that P-deficiency-induced increase in plasma membrane H^+-ATPase in soybean roots was caused by its transcriptional and translational regulation. However, the release of H^+ did not coincide with increased exudation of carboxylate anions in *Arabidopsis thaliana*, tomato, wheat, and soybean roots (Narang et al. 2000; Neumann and Römheld 1999; Tang et al. 2009). Recently, Tomasi et al. (2009) showed that two different mechanisms of OA secretion existed in cluster roots of P-deficient white lupin. The first component mediated a basal rate of malate and citrate release and was not directly linked to the plasma membrane H^+-ATPase activity. The second one corresponded to the burst of citrate release and was strictly plasma membrane H^+-ATPase activity dependent.

2.5 Anion Channels

Organic acids occur predominately as OA anions at the high cytosolic pH of 7.0–7.5, and the release of these anions from roots may be a passive process resulting from a steep gradient in OA anion concentration and the electrochemical potential across the plasma membrane (Wang et al. 2007). Thus, it is likely that P-deficiency activates an anion channel or a transporter in the plasma membrane to initiate OA anion secretion. Inhibition of OA anion secretion from roots of P-deficient elephantgrass (*Pennisetum purpureum*), white lupin, and purple lupin by exogenous application of anion-channel inhibitors indicates involvement of anion channels (Ligaba et al. 2004b; Neumann et al. 1999; Shen et al. 2001; Wang et al. 2007). The use of patch clamp technique also supports the role of anion channels in secretion of OA anions from roots in response to P-deficiency (Diatloff et al. 2004; Zhang et al. 2004).

3 Alternative Glycolytic Pathways and Tonoplast Proton Pumping Bypassing Phosphorus- and/or Adenylate-Dependent Reactions

The efficient use of internal P pools is considered as another important adaptation for P-deficient plants (Nanamori et al. 2004; Vance et al. 2003). Because the levels of ATP, ADP, and related nucleoside Ps in plant cells were markedly reduced by P-deficiency (Dancer et al. 1990; Duff et al. 1989; Plaxton and Tran 2011), many Pi- and/or adenylate-dependent metabolic processes (classical glycolysis and ATP-dependent H^+-pump) may be impaired by P-deficiency. In response to P-deficiency, many plants show remarkable flexibility in adjusting metabolic rates and utilizing alternative metabolic pathways (Fig. 1). This metabolic flexibility permits plants to bypass Pi- and/or adenylate-dependent reactions. Pyruvate is typically derived from PEP during the glycolytic conversion of ADP to ATP catalyzed by PK and its activity may be limited due to decreased ADP level under P-deficiency. Indeed, PK activity decreased in the roots of 0 μM P-treated tea plants (Lin et al. 2011). Similar results were obtained from the suspension-cultured *Catharanthus roseus* cells (Li and Ashihara 1990). However, P-deficiency did not affect PK activity in bean roots (Juszczuk and Rychter 2002). It has been proposed that in many plants, the kinases of classic glycolysis [i.e., hexokinase (EC 2.7.1.1), ATP-dependent phosphofructokinase (ATP-PFK, EC 2.7.1.11), PK etc.] are maintenance enzymes that are insensitive to environmental stress (Sung et al. 1988). Selective maintenance of these kinases under P-deficiency may ensure that the respiratory machinery of the plant is in place should sufficient P be restored. In contrast to roots, P-deficiency increased PK activity in leaves of tea (Lin et al. 2011), *Brachiaria* hybrid, and rice (Nanamori et al. 2004). Wu et al. (2003) observed that P-deficiency induced PK gene expression of *Arabidopsis* leaves.

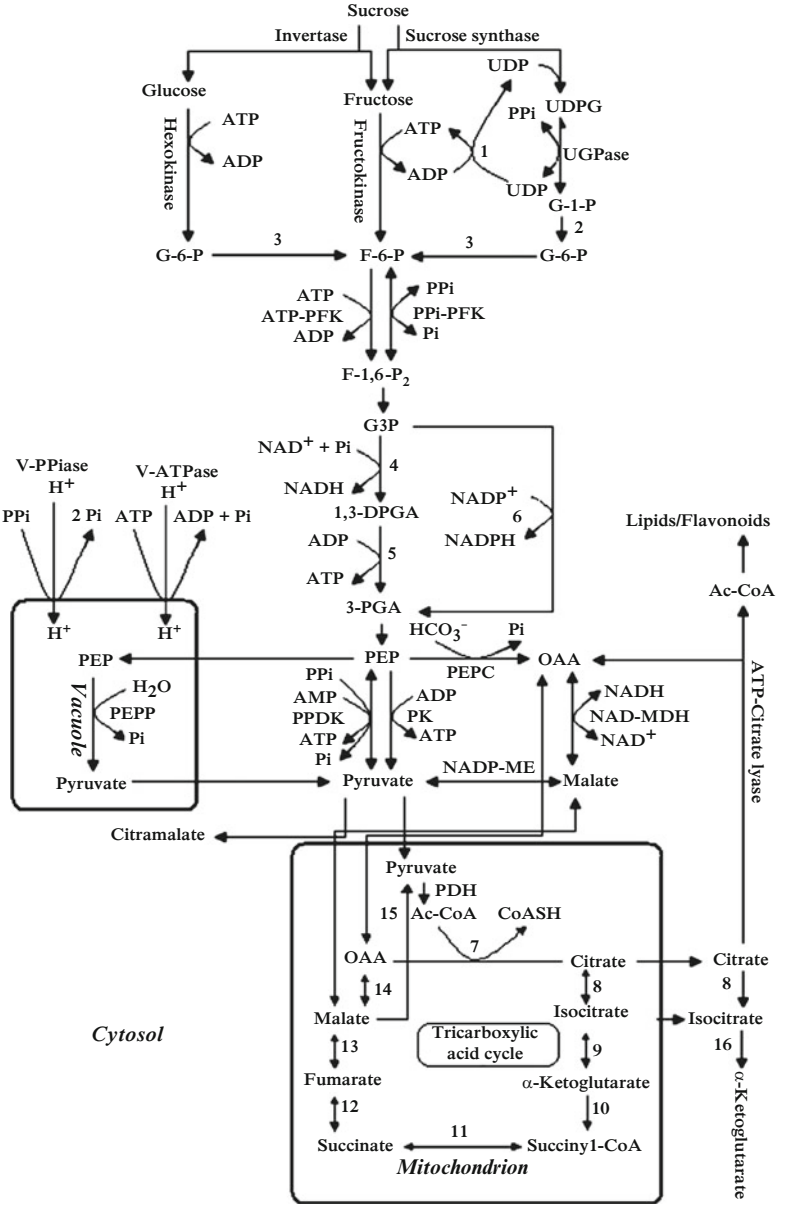

Fig. 1 A diagram showing the reactions involved in the accumulation and secretion of some organic acids (OAs) in phosphorus (P)-deficient plants. *Ac-CoA* acetyl-CoA; *ATP-PFK* ATP-dependent phosphofructokinase; *1,3-DPGA* 1,3-diphosphoglycerate; *F-1,6-P_2* fructose-1,6-bisphosphate; *F-6-P* fructose-1-phosphate; *G-1-P* glucose-1-phosphate; *G3P* glyceraldehyde-3-phosphate; *G-6-P* glucose-6-phosphate; *NAD-MDH* NAD-malate dehydrogenase; *NADP-ME* NADP-malic enzyme; *OAA* oxaloacetate; *PDH* pyruvate dehydrogenase; *PEP* phospho*enol*pyruvate; *PEPC* PEP carboxylase; *PEPP* PEP phosphatase; *3-PGA* 3-phosphoglycerate; *Pi* phosphate; *PK* pyruvate kinase; *PPDK*

Apart from PK, PEP phosphatase (PEPP, EC 3.1.3.60) can also produce pyruvate from PEP in the vacuole, but does not require ATP. The enzyme can bypass the ADP-dependent PK reaction and is thought to play an important role in maintaining pyruvate production under low-P conditions (Duff et al. 1989). Phosphorus-deficiency-induced increase in PEPP activity has been observed in roots of tea (Lin et al. 2011), bean (Juszczuk and Rychter 2002), and *C. sinensis* (Yang et al. 2011) and cells of *Brassica nigra* suspension (Duff et al. 1989). Nanamori et al. (2004) showed that PEPP activity was induced by P-deficiency: 5.6 times in the *Brachiaria* hybrid leaves and 6.0 times in rice leaves and that the ^{14}C distribution ratio to amino acids and OAs slightly increased in the former and decreased in the latter with P-deficiency, suggesting that PEPP might function to facilitate the carbon flow to the tricarboxylic acid cycle in *Brachiaria* hybrid leaves, but not in rice ones. However, P-deficiency did not affect PEPP activity in tea leaves except for a slight increase under 40 μM P (Lin et al. 2011) and in non-Al-treated roots and leaves of *C. grandis* (Chen et al. 2009). In *C. grandis* roots, PEPP activity increased as P supply increased from 0 to 50 μM, then decreased under 200 μM P (Yang et al. 2011). Another alternative route of PEP catabolism and pyruvate production is through combined action of PEPC, cytosolic NAD-MDH, and mitochondrial NAD-ME (EC 1.1.1.39). This pathway can circumvent the PK reaction and thus maintain the carbon flow from glycolysis to the tricarboxylic acid cycle by avoiding the use of ADP but releasing Pi (Theodorou and Plaxton 1993). Gregory et al. (2009) reported that in *A. thaliana* suspension cells and seedlings, P-deprivation resulted in (a) marked increase in the expression of genes encoding PEPC isozyme AtPPC1 and PEP carboxylase kinase (PPCK, EC 2.7.11.1) isozymes AtPPCK1 and AtPPCK2; (b) >2-fold upregulation of PEPC specific activity and the amount of 107-kDa PEPC polypeptide (p107); and (c) in vivo phosphorylation of p107, which could be reversed following P resupply. Uhde-Stone et al. (2003a, b) observed that in the proteoid roots of P-deficient white lupin, genes encoding PEPC and NAD-MDH were induced. A study with tea trees showed that P-deficiency increased the activities of PEPC and NAD-ME in roots and of NAD-ME and NAD-MDH in leaves, but slightly decreased root activity of NAD-MDH (Lin et al. 2011). Juszczuk and Rychter (2002) observed that 16 days after start of P-starvation, bean roots showed increased PEPC and NAD-ME activities, but decreased NAD-MDH activity. At the absence of Al, P-deficient *Citrus* roots had higher PEPC activity and lower NAD-MDH activity

Fig. 1 (continued) pyruvate Pi dikinase; *PPi* pyrophosphate; *PPi-PFK* PPi-dependent phosphofructokinase; *UDPG* UDP-glucose; *UGPase* UDPG pyrophosphorylase; *V-ATPase* tonoplast adenosine triphosphatase; *V-PPiase* tonoplast pyrophosphatase; *1* nucleoside diphosphate kinase; *2* phosphoglucose mutase; *3* phosphoglucose isomerase; *4* NAD-dependent glyceraldehyde-3-P dehydrogenase (NAD-G3PDH); *5* 3-phosphoglycerate kinase; *6* NADP-G3PDH; *7* citrate synthase (CS); *8* aconitase (ACO); *9* NAD-isocitrate dehydrogenase (NAD-IDH); *10* α-ketoglutarate dehydrogenase; *11* succinate thiokinase; *12* succinate dehydrogenase; *13* fumarase; *14* NAD-MDH; *15* NAD-malic enzyme (NAD-ME); *16* NADP-IDH [redrawn from Lin et al. (2011) and Plaxton and Tran (2011)]

(Yang et al. 2011). Considering that NAD-MDH activity was the highest among these enzymes (Chen et al. 2009; Lin et al. 2011; Yang et al. 2011), it was possible that the NAD-catalyzed reaction did not limit the malate production in plant roots.

Pyrophosphate is a by-product of a host of biosynthetic reactions, including the polymerization reactions involved in the final steps of macromolecule biosynthesis (Plaxton and Tran 2011). The large amounts of PPi produced during biosynthesis can be a significant energy donor, replacing ATP under P-deficiency, when ATP level is low (Dancer et al. 1990; Duff et al. 1989). In contrast to ATP, PPi levels appeared to be insensitive to P-deficiency (Dancer et al. 1990; Duff et al. 1989; Rychter and Randall 1994). Plant cytosol usually contains a considerable pool of PPi (Rea and Poole 1993; Weiner et al. 1987), and the theoretical energy released by the cleavage of PPi is estimated to be approx. 50% of that from ATP hydrolysis (Weiner et al. 1987). Therefore, reactions that use PPi instead of ATP provide an obvious bioenergetic advantage to P-deficient plant cells. A third bypass of pyruvate production from PEP can be catalyzed by pyruvate phosphate dikinase (PPDK, EC:2.7.9.1), a reversible enzyme. The enzyme can catalyze AMP, PEP, and PPi to ATP, pyruvate, and Pi. In maize roots, the P-deficiency-induced increase in protein expression for PPDK was greater in the low-P-tolerant mutant 99038 than in wild-type Qi-319, which may be one of the reasons for the higher tolerance to low-P in the mutant compared to the wild-type (Li et al. 2007, 2008).

Typically, the interconversion of fructose-6-phosphate (F-6-P) and fructose-1,6-bisphosphate (F-1,6-P_2) is catalyzed glycolytically by an ATP-PFK and gluconeogenically by a fructose-1,6-bisphosphatase (EC 3.1.3.11). An alternative pathway is catalyzed by a reversible PPi-dependent phosphofructokinase (PPi-PFK; EC 2.7.1.90) that can bypass the ATP-PFK under P-deficiency (Theodorou and Plaxton 1993). Phosphorus-deficiency increased PPi-PFK activity in suspension cells from leaf petioles of *B. nigra*, but did not affect ATP-PFK activity (Duff et al. 1989). Phosphorus-deficiency also increased PPi-PFK activity in *B. napus* suspension cells, *A. thaliana* seedlings, and the roots of *B. napus*, *B. oleracea*, *Brassica carinata*, *Beta vulgaris*, buckwheat (*Fagopyrum esculentum*), *Sinapis alba*, *Sinapis arvensis*, and white lupin (Murley et al. 1998; Plaxton and Carswell 1999). In the proteoid roots of P-deficient white lupin (Uhde-Stone et al. 2003a, b) and in the leaves of ryegrass (*Lolium perenne*; Byrne et al. 2011), genes encoding PPi-PFK were induced. Recently, our work with roots of tea, *C. sinensis,* and *C. grandis* showed that P-deficiency decreased the expression of *ATP-PFK*, and increased the expression of *PPi-PFK* (Lin et al. 2011; Yang et al. 2012). Misson et al. (2005) reported that P-deficiency markedly induced PPi-PFK gene expression in *A. thaliana* leaves. A study with transgenic tobacco (*Nicotiana tabacum*) plants overexpressing 6-phosphofructo-2-kinase (EC 2.7.1.105) showed that the glycolytic contribution of PPi-PFK increased and ATP-PFK decreased in response to P-deficiency (Fernie et al. 2002). The P-deficiency-induced increase in PPi-PFK activity appears to be plant specific. In plants that typically possess sufficient PPi-PFK activity when grown under sufficient P, the activity of PPi-PFK stayed unchanged or declined under P-deficiency (in tomato root cultures, suspension cells of *Nicotiana sylvestris*, tobacco roots, and suspension cells of *C. roseus*; Murley et al. 1998; Plaxton and Carswell 1999).

In addition to PPi-PFK and PPDK reactions, PPi might be used as an energy donor for the active transport of protons into the vacuole by a tonoplast pyrophosphatase (V-PPiase; EC 3.6.1.1). A study using suspension-cultured cells of rape showed that P-deprivation increased the activity and the H^+-pumping action, as well as the amount of V-PPiase, but not of tonoplast ATPase (V-ATPase, EC 3.6.3.14) (Palma et al. 2000). Yang et al. (2007) found that P-deficiency resulted in increased expression of *V-PPiase* in *Arabidopsis*, and that transgenic *Arabidopsis*, tomato, and rice plants overexpressing the gene outperformed controls when exposed to low-P. Lin et al. (2011) observed that in tea roots, P-deficiency decreased the expression of the gene for V-ATPase subunit A (V-ATPase A), and slightly increased the expression of the gene for V-PPiase. In *C. sinensis* and *C. grandis* roots, however, P-deficiency increased *V-ATPase A* expression, but did not significantly affect *V-PPiase* expression (Yang et al. 2012). The expression of gene for V-ATPase A in rice roots and leaves and the expression of protein for V-ATPase A in maize roots was enhanced by P-starvation, and suggested that the upregulation might strengthen the proton transport and provided the required energy to maintain an electrochemical gradient across the tonoplast to facilitate P transport (Guo et al. 2008; Li et al. 2007; Xia et al. 2002). Wasaki et al. (2003) showed that the expression of *V-ATPase* and *V-PPiase* was upregulated by P-deficiency.

Plant cells possess two cytoplasmic pathways [i.e., ATP-dependent invertase (EC 3.2.1.26) pathway and PPi-dependent sucrose synthase (EC 2.4.1.13) pathway] for the conversion of sucrose to hexose-phosphate (hexose-P). In the PPi-dependent sucrose synthase pathway, sucrose synthase catalyses the reversible conversion of sucrose to UDP-glucose (UDPG) and fructose. UDPG may then be used for cellulose biosynthesis or converted to glucose-1-phosphate (G-1-P) and glucose-6-phosphate (G-6-P) *via* the UDPG pyrophosphorylase (UGPase, EC. 2.7.7.9) and phosphoglucose mutase (EC 5.4.2.2) reactions. In the ATP-dependent invertase pathway, invertases hydrolyze sucrose to glucose and fructose. Glucose and fructose are further converted to hexose-P by hexokinase and fructokinase (EC 2.7.1.4), respectively (Alonsoa et al. 2007; Plaxton and Tran 2011). No ATP is needed for the conversion of sucrose to hexose-P *via* the sucrose synthase pathway, whereas two ATP are needed for the invertase pathway. Ciereszko et al. (2001, 2005) showed that P-deficiency increased UGPase protein concentration and activity and its gene expression in the roots, stems/flowers, and leaves of *A. thaliana* except that UGPase gene expression in stems/flowers was not affected by P-deficiency. Li et al. (2008) observed that P-deficiency-induced increase in the amount of UGPase in the roots of low-P-tolerant maize mutant was higher than in that of wild-type Qi-319. Phosphorus-deficiency increased the activity of UGPase in soybean leaves (Fredeen et al. 1989). The activities of sucrose synthase, phosphoglucose mutase, and fructokinase greatly increased in P-deficient juvenile and mature cluster roots of white lupin (Neumann et al. 2000). In bean roots, the activities of fructokinase and hexokinase responsible for phosphorylation of hexoses decreased in response to P-deficiency (Rychter and Randall 1994). In another study with bean root tips, Ciereszko et al. (1998) observed that sucrose

synthase activity increased more than twice and neutral invertase activity about 30%, but did not affect acid invertases, concluding that sucrose synthase might have an increased role in sucrose hydrolysis in the growing parts of root under P-deficiency. These results indicate that during P-deficiency, the conversion of sucrose to hexose-P proceed *via* a PPi-dependent pathway requiring UGPase (Hammond et al. 2004). The PPi released in the biosynthesis of UDPG could then be hydrolyzed to Pi, thereby increasing the availability of Pi for P-deficient plants.

Duff et al. (1989) found that following nutritional Pi-deprivation of *B. nigra* suspension cells, the specific activity of non-phosphorylating NADP-G3PDH (EC 1.2.1.13) was greatly enhanced, whereas that of Pi-dependent NAD-G3PDH (EC 1.2.1.12) was reduced by about 6-fold. It was hypothesized that nonphosphorylating NADP-G3PDH bypassed Pi-dependent NAD-G3PDH and phosphoglycerate kinase (EC 2.7.2.3) (Duff et al. 1989; Theodorou and Plaxton 1993). However, our recent work showed that P-deficient *Citrus* roots displayed higher or similar expression of NAD-G3PDH, but lower or similar expression of NADP-G3PDH, indicating that the NAD-G3PDH is not bypassed by NADP-G3PDH in P-deficient *Citrus* roots. Similar results have been observed in the leaves of P-deficient rice (Shinano et al. 2005), the proteoid roots of P-deficient white lupin (Uhde-Stone et al. 2003a, b), and the roots of P-deficient maize (Li et al. 2007). Therefore, NAD-G3PDH, not NADP-G3PDH may be involved in the root adaptation mechanism of some plants to P-deficiency.

4 Genetic Engineering Technology for the Transporter and Biosynthesis of Organic Acids

The production of transgenic plants with an enhanced ability to biosynthesis and/or secrete OAs appears to be an appealing strategy to produce P-deficiency-tolerant plants. Researchers have manipulated the capacity of OA biosynthesis and secretion in plant cells, in the hope that this will result in enhanced P-deficiency-tolerance of a given plant genotype by increasing secretion of OAs. Table 2 summarizes the attempts to obtain transgenic plants or cells with higher P-deficiency-tolerance by overexpressing genes involved in OA biosynthesis and secretion. Koyama et al. (1999) reported that transgenic carrot cell lines, carrying *A. thaliana* mitochondrial CS gene, displayed a higher CS activity, more citrate secretion, and enhanced growth in an $AlPO_4$-medium in comparison with untransformed wild type. Later, they overexpressed a carrot mitochondrial CS gene in *A. thaliana* and which resulted in improved growth on a P-limited soil due to increased citrate secretion from roots (Koyama et al. 2000). López-Bucio et al. (2000b) reported that overexpression of *Pseudomonas aeruginosa* CS gene driven by the CaMV35S promoter in tobacco resulted in increased levels of biosynthesis and secretion of citrate by roots and enhanced P-acquisition. Citrate-overproducing plants yielded more leaf and fruit biomass when grown under P-limiting conditions and required

Table 2 Transgenic plants or cells with higher phosphorus (P)-deficiency-tolerance overexpressing genes for the transporter and biosynthesis of organic acid (OA)

Genes	Origins	Transgenic plants or cells	Increased secretions of OA anions	P-deficiency-tolerance	References
Citrate synthase (*CS*)	*Arabidopsis thaliana*	Carrot (*Daucus carota*) cell	Citrate	+	Koyama et al. (1999)
CS	Carrot	*A. thaliana*	Citrate	+	Koyama et al. (2000)
CS	*Pseudomonas aeruginosa*	Tobacco (*Nicotiana tabacum*)	Citrate	+	López-Bucio et al. (2000b)
Malate dehydrogenase (*MDH*)	*Penicillium oxalicum*	Tobacco	Malate	+	Lü et al. (2012)
Nodule-enhanced form of MDH (neMDH)	Alfalfa (*Medicago sativa*) nodule	Alfalfa	Citrate, oxalate, malate, succinate, acetate	Enhanced P concentration in shoot and root tissues	Tesfaye et al. (2001)
*Phospho*enol*pyruvate carboxylase PEPC*	Maize (*Zea mays*)	Rice (*Oryza sativa*)	Oxalate	+	Begum et al. (2005)
TaALMT1	Wheat (*Triticum aestivum*)	Barley (*Hordeum vulgare*)	Unknown	+	Delhaize et al. (2009)

less P fertilizer to achieve optimal growth when compared to the controls. However, the result was not repeated for the same transgenic plants by Delhaize et al. (2001), who concluded that expression of the *P. aeruginosa* CS gene in plants is unlikely to be a robust and easily reproducible strategy for enhancing P-nutrition of crop and pasture species. Tesfaye et al. (2001) observed that a 1.6-fold increase in MDH specific activity in root tips of selected transgenic alfalfa (*Medicago sativa*) plants, carrying an alfalfa nodule-enhanced form of MDH (neMDH) led to a 4.2-fold increase in root concentration as well as a 7.2-fold increase in root exudation of citrate, malate, succinate, and acetate compared with untransformed controls, which resulted in increased P accumulation in roots and shoots. Overexpression of a mitochondrial MDH gene from the mycorrhizal fungus *Penicillium oxalicum* in tobacco resulted in improved P-acquisition by enhancing secretion of malate from roots (Lü et al. 2012). Overexpression of a complete maize PEPC gene in rice increased OA biosynthesis and oxalate exudation and enhanced the adaptive capacity of transgenic rice lines to low-P soil conditions (Begum et al. 2005). However, the transgenic alfalfa over-expressing PEPC did not show increased accumulation and secretion of OAs (Tesfaye et al. 2001). In short-term pot trials (26 days), transgenic barley expressing wheat *TaALMT1* was more efficient than a non-transformed sibling line at taking up P on acidic soil. Transgenic plants showed a >2-fold increase in grain production on acidic soil compared with the untransformed controls (Delhaize et al. 2009).

5 Concluding Remark

Plants respond adaptively to P-deficiency through many diverse physiological and biochemical changes (Fig. 1; Lin et al. 2011; Hammond et al. 2004; Plaxton and Tran 2011; Vance et al. 2003). In previous studies, multiple mechanisms of P-deficiency-tolerance in higher plants have been suggested (Fang et al. 2009; Plaxton and Tran 2011; Vance et al. 2003). Phosphorus-deficiency-induced secretion of OA anions from roots is considered as a principal mechanism in alleviating P-deficiency, but the mechanisms which lead to the secretion of OA anions are not fully understood. Alteration in OA metabolism and activation of anion channels have been suggested to be involved in the P-deficiency-induced secretion of OA anions and transgenic plants or cells overexpressing genes for OA biosynthesis and transporter have displayed increased secretion of OA anions and enhanced P-acquisition under P-limiting conditions (Table 2). However, the responsible mechanisms are not well known and more genes controlling these processes need to be cloned in the further. There have been many reports about P-deficiency-induced changes in biosynthesis and secretion of OA anions in the roots of herbaceous plants, but little is known about the effects of P-deficiency on the biosynthesis and secretion of OA anions in tree roots (Yang et al. 2011, 2012). Organic acid anions released from the roots of P-deficient plants have been shown to mainly result from increased PEPC activity in the shoots (Hoffland et al. 1992), but relatively few studies have investigated the effects of

P-deficiency on leaf OA metabolism. It is clear that remarkable progress has been made in our understanding of plant adaptation to P-deficiency through the induction of alternative glycolytic pathways and tonoplast pumping bypassing Pi- and/or adenylate-dependent reactions. These changes in metabolism (Fig. 1), although not always identical in all plants, serve to efficient use of internal P pools by replacing P in metabolites. However, it is still unclear how the carbon flux through alternative glycolytic and tricarboxylic acid cycle enzymes is regulated and that the extent to which the changes in metabolism influence the tolerance of plants to P-deficiency. Thus, a throughout understanding of the P-deficiency-induced metabolic changes requires the effective integration of genomic, transcriptomic, proteomic, metabolomic, and enzymological/biochemiscal approaches. Although some transgenic plants overexpressing PEPC or CS gene did not display increased accumulation and secretion of OAs (Delhaize et al. 2001; Tesfaye et al. 2001), genetically modified plants with higher P-deficiency-tolerance by overexpressing genes for the secretion and biosynthesis of OAs may still be a potentially rewarding area of research in the future. Biochemical and molecular studies have revealed a conserved multigenic P-deficiency-inducible rescue system in higher plants (Abel et al. 2002; Fang et al. 2009; Gaxiola et al. 2011). Coexpression of multiple genes may be needed to produce plants with high levels of P-deficiency-tolerance. Finally, transgenic plants with significantly increased P-deficiency-tolerance will be produced through the collaboration between plant breeders and plant physiologists.

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The Production and Protection of Nectars

María Escalante-Pérez and Martin Heil

Contents

Abstract Nectar secretion serves two important mutualisms. Floral nectar (FN) mediates pollination whereas extrafloral nectar (EFN) serves the indirect defence against herbivores. Research over the last decade has focused on the anti-microbial protection of nectars. The Nectar Redox Cycle consists of several nectar proteins (nectarins) in FN of ornamental tobacco and produces reactive oxygen species that keep the nectar free of microbes. Hydrolytic enzymes such as chitinases, glucanases and other pathogenesis-related (PR) proteins serve the same protective function in FN and EFN of different species, although via different biochemical mechanisms.

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By contrast, little is known about how nectaries are formed, where nectar components are produced and how nectar flow is controlled. Genes with a central role in flower development and nectary formation are *CRABS CLAW* (*CRC*) and *BLADE-ON-PETIOLE* (*BOP*) 1 and 2, but more studies are required to understand the genetic control of nectary formation and the mechanisms by which plants control nectar flow and composition.

1 Introduction

Nectar can be produced on all aerial parts of plants, attracts important animals to plants and forms the basis for honey production. Therefore, nectar has been investigated for centuries. In particular, nectar research has seen multiple important discoveries in the last half of the nineteenth century and the first half of the twentieth century. Since then, nectar research has become the "sleeping beauty" of plant sciences (Heil 2011; Escalante-Pérez and Heil 2012). Research into central topics related to nectar lags dramatically behind other topics in plant sciences. We know almost nothing on the genetic control of the formation of the nectar-secreting structures (the nectaries) or on the synthesis and the release of the components of nectar. Considering that the major function of nectar is the attraction of animals, it appears surprising that the most important discoveries that have been made in nectar research over the last decade concern the protection of nectar from micro-organisms. Nectar basically represents an aqueous solution of mono- and disaccharides and amino acids, and it therefore represents a perfect growing medium for micro-organisms, in particular yeasts, bacteria and fungi. The emerging pattern is that nectars are protected from microbial infection by multiple sets of nectar proteins (nectarins) (Carter and Thornburg 2004a; Thornburg 2007; Heil 2011).

In this chapter, we review the recent discoveries on the protection of nectar via nectarins and the limited information on genes that are involved in the formation of the nectaries and on the physiological and hormonal control of nectar flow. We close our chapter with some concrete suggestions concerning how untargeted "omics" techniques can be applied to investigate crucial questions in nectar research, using non-model plants that appear to be much more promising for this research area than the classical model plants, due to the size of their nectaries and the amounts of nectar produced.

1.1 The Functions of Nectar

Nectar serves two types of plant–animal mutualism in which plants make use of the mobility of animals: pollination and indirect defence (Heil 2008, 2011; Brandenburg et al. 2009). By definition, floral nectar (FN) is secreted within the flower and serves pollination, whereas extrafloral nectar (EFN) is secreted commonly, but not necessarily, outside the inflorescences and serves the indirect defence of the plant via tritrophic interactions (Bentley 1977; Elias 1983).

How important are nectars for the maintenance and efficiency of these mutualisms? Pollination by animals is generally assumed to increase the specificity of the pollen transport among flowers and thereby guarantee adequate fertilisation and outcrossing (de la Barrera and Nobel 2004; Brandenburg et al. 2009). Surprisingly little experimental evidence exists, however, for a positive correlation of nectar quantity with the fitness benefits that result from FN secretion. Although we can assume that the presence of nectar benefits plants by increasing pollination rates (Neiland and Wilcock 1998), we must ask: does more nectar mean more service, and what can plants do to minimise the resulting costs? Only two recent studies have investigated the correlation among FN consumption rates and pollinator efficiency, and they revealed contrasting patterns. The nicotine in the FN of wild tobacco (*Nicotiana attenuata*) reduced the nectar uptake by pollinators during single visits but increased the number of visits by hummingbirds, thereby maximising outcrossing with minimum investment in FN amounts (Kessler and Baldwin 2007; Kessler et al. 2008). By contrast, flowers of *Petunia* plants that combined reduced FN amounts with unchanged morphological traits of the flower paid for this attempt to "cheat" their pollinators with reduced visitation frequency by *Manduca sexta* moths and a concomitant reduction in seed production (A. Brandenburg, personal communication; Brandenburg 2009). Do plants gain or lose fitness when reducing their FN secretion? Unfortunately, no generalisations are possible based on only these two studies.

Surprisingly, the situation is much clearer for EFN, likely because the ecological role of EFN has been controversially discussed for so many decades (Escalante-Pérez and Heil 2012). Hundreds of ant exclusion experiments demonstrated that ants can benefit plants by reducing overall herbivore pressure (Bentley 1977; Davidson and McKey 1993; Heil and McKey 2003). Higher EFN availability can increase the survival rates of ant workers (Lach et al. 2009) and other predators (Limburg and Rosenheim 2001) and also ant activity and aggressiveness (Sobrinho et al. 2002; Ness 2006; Heil et al. 2009). Studies using wild cotton (*Gossypium thurberi*) demonstrated that fewer ants visited plants with experimentally reduced numbers of extrafloral nectaries; leaf damage on these plants was higher and seed number was lower compared to plants with natural levels of EFN (Rudgers 2004). Inducing lima bean (*Phaseolus lunatus*) plants to produce more EFN or supplementing them with artificial EFN had positive effects on the number of ant workers foraging on plants, with positive effects on fitness-relevant traits such as growth rates and numbers of flowers and fruits (Heil 2004; Kost and Heil 2005, 2008). In fact, indirect defence via ants represents one of the few anti-herbivore defence strategies for which a clear effect on net herbivory rates and plant fitness has been shown for different species (Chamberlain and Holland 2009). In summary, a positive correlation of investment with benefit for the plant has been shown for EFN, but the generality of this assumption has yet to be proven for FN.

1.2 Attractive Nectar Components

All the above-mentioned functions depend on the fact that nectar presents an appetising meal to its legitimate consumers. The compounds that mainly contribute

to this function have been reviewed repeatedly (Nicolson et al. 2007; González-Teuber and Heil 2009a; Nepi et al. 2009; Heil 2011; Escalante-Pérez and Heil 2012). Therefore, we present only a very short overview. Carbohydrates and free amino acids in the nectar are most important for its attractive function. Most authors assume the traits of nectar to be adapted to the nutritional preferences of the consumers (Baker and Baker 1982, 1983; Pacini et al. 2003; Johnson and Nicolson 2008; Kromer et al. 2008). The composition and concentration of nectar determines the spectrum of nectar consumers, because animals differ in their nutritive preferences. For example, hummingbirds, butterflies, moths and long-tongued bees usually prefer sucrose-rich floral nectars, as do most ant species that feed on EFN, whereas short-tongued bees and flies prefer FN rich in hexoses (Blüthgen and Fiedler 2004; Nepi and Stpiczyńska 2008; González-Teuber and Heil 2009a; Nepi et al. 2009). However, some nectarivorous birds and ants lack the sucrose-cleaving enzyme invertase, are thus not able to assimilate sucrose and consequently prefer sucrose-free nectars (Martínez del Rio 1990; Heil et al. 2005).

Besides sugars, amino acids can significantly affect the attractiveness of both FNs and EFNs to the respective consumer guilds (Baker and Baker 1973; Baker et al. 1978; Lanza and Krauss 1984; Alm et al. 1990; Lanza 1991; González-Teuber and Heil 2009a,b). Many adult insects feed only on liquids whereas mammals and birds can feed on solid items also. We can therefore assume that insect-pollinated flowers should possess more amino acids in their nectar than vertebrate-pollinated flowers. High amino acid concentrations are indeed commonly found in FNs from flowers that are adapted to butterflies (Baker and Baker 1982), flies (Potter and Bertin 1988) or bees (Petanidou et al. 2006) and in many EFNs (González-Teuber and Heil 2009a,b). Ants as well as many insect pollinators can show strong preferences for specific, usually essential amino acids (Blüthgen and Fiedler 2004; Carter et al. 2006; González-Teuber and Heil 2009b). Besides contributing to the amino acid metabolism of consumers in general, some amino acids might play very specific roles. For example, many insects preferentially utilise proline as an energy source during the initial phases of insect flight and can taste proline, which activates, for example, the salt receptor cells of flies (Shiraishi and Kuwabara 1970; Hansen et al. 1998). Indeed, many FNs contain proline in high concentration and some insects, such as honeybees, prefer nectars rich in proline (Carter et al. 2006; Bertazzini et al. 2010).

Sugars and amino acids are by far the most common nutritious components of nectar, but lipids or free fatty acids can also contribute to the nutritive value of nectars, at least of certain FNs. The FN of *Jacaranda mimosifolia* contains free fatty acids in concentrations that are significantly higher than their respective solubility in aqueous solution (Kram et al. 2008). This phenomenon was explained by the presence in nectar of a lipase, Jacaranda Nectar Protein 1 (JNP1) in this nectar. JNP1 is a 43-kDa protein similar to members of the plant GDSL lipase/esterase gene family, which was suggested to actively cleave off fatty acids of parent lipid particles, which later play a role in pollinator attraction to *Jacaranda* nectar (Kram et al. 2008).

Whereas nectar sugars and amino acids have been investigated since decades, nectar odours were considered as a relevant signal for pollinators only recently (Raguso 2004). Butterflies and moths preferred artificial flowers containing scented nectar over those that contained pure sugar solutions (Weiss 2001), and parasitoid wasps localised cotton (*Gossypium hirsutum*) EFN using only its odours (Röse et al. 2006). The origin of FN scent has been linked to volatiles released from petals that are absorbed and re-released by the nectar (Raguso 2004). However, a wide array of VOCs were found in the nectar of wild tobacco (*Nicotiana attenuata*), and many of these compounds have not been detected in other flower parts, suggesting that in certain species, nectar emits its own scent (Kessler and Baldwin 2007).

2 The Protective Function of Nectar

2.1 The Ecology of Nectar Protection

As we have explained earlier, nectar is a metabolite-rich fluid, which is freely offered to visiting pollinators or defenders to maximise rates of pollen transfer or protection from herbivores. These flower-visiting insects often harbour micro-organisms that have the potential to colonise the nectar itself (Herrera et al. 2010) or even use the nectary as an entrance to infect other plant tissues (Sasu et al. 2010). Thus, the potential for deleterious infections is high. Yeasts are commonly reported in FN (Herrera et al. 2009, 2010), to which they are transmitted by pollinators (Herrera et al. 2010). Similarly, the bacterial pathogen, *Erwinia tracheiphila*, uses flower-visiting beetles as vectors (Sasu et al. 2010). Nectar-infesting yeasts affect multiple nectar traits such as temperature (Herrera and Pozo 2010) and composition, with largely unknown effects on the plant–pollinator interaction. A first study into their ecological relevance revealed that infection of FN of *Helleborus foetidus* with yeasts (*Metschnikowia reukaufii*) reduced several fitness-relevant parameters such as fruit set, seed set and seed mass, although the net outcome was site dependent and could shift to a positive effect, depending on temperature (C.E. Herrera, personal communication). Yeasts are likely to be adapted to the ephemeric and chemically variable environment that is represented by FN. It has been hypothesised that they optimise their growth in different sugar environments via epigenetic processes (Herrera et al. 2012).

Whereas it remains to be studied whether nectar-infesting yeasts usually have positive or negative effects on plant–pollinator interactions, earlier studies considered the possibility that phytopathogens use nectaries as an entry to infect plant tissues (Ivanoff and Keitt 1941; Keitt and Ivanoff 1941). For example, *Erwinia amylovora* is exceptional among bacterial plant pathogens in its ability to infect plants through the nectary stomata (Bubán et al. 2003; Farkas et al. 2007). Similarly, a recent study demonstrated that also the congeneric pathogen, *E. tracheiphila*, can move through the nectary into the xylem of the pedicel (Sasu et al. 2010). Thus, nectar clearly needs some kind of anti-microbial protection, in order to control the flora of micro-

organisms within the nectar and to protect the nectary or adjacent tissues. Besides micro-organisms, non-pollinating animals ("nectar thieves") can represent an important threat against which plants need to protect their nectar (Stephenson 1982; Adler 2000; Adler and Irwin 2005).

In the following, we review the compound classes and mechanisms that have been reported to protect nectar from micro-organisms and nectar thieves. According to the hypothesis on "toxic nectars", compounds that protect nectars against illegitimate consumers comprise mainly alkaloids and non-proteinogenic amino acids (Baker and Baker 1973; Baker et al. 1978; Adler 2000). By contrast, the protection against infections is achieved mainly by proteins. Nectar proteins have been discovered very early (Buxbaum 1927; Lüttge 1961) but became the target of detailed molecular and functional investigations only recently.

2.2 The Nectar Redox Cycle

For many nectars, the lack of infection can be explained by the action of enzymes that exert direct anti-pathogen effects or that produce hydrogen peroxide, H_2O_2. The first case in which the protein-based protection of nectar from micro-organisms has been studied in detail is the nectar of ornamental tobacco (*Nicotiana langsdorffii x Nicotiana sanderae*), in which an array of five proteins were identified, the so-called nectarins (Carter et al. 1999). These nectarins range in size from 29 to 65 kDa and accumulate in nectar to concentrations of 250 mg mL^{-1}. They serve the protection of the nectar through a biochemical pathway called the *Nectar Redox Cycle* (NRC) (Carter and Thornburg 2004a).

Mainly, NEC1, NEC3 and NEC5 are involved in the *Nectar Redox Cycle*: NEC1 and NEC5 together maintain high peroxide levels. NEC1 represents approximately 50 % of the total nectar proteins in *Nicotiana* sp. and was characterised as a manganese-containing superoxide dismutase (Carter and Thornburg 2000), whose enzymatic function is related to the generation of H_2O_2 in nectar. NEC5 is a berberine bridge enzyme-like protein with glucose oxidase activity that functions together with NEC1 in the production of high peroxide levels. Nectar of ornamental tobacco can accumulate up to 4 mM of hydrogen peroxide (Carter and Thornburg 2000), which is 40 times the level produced by human neutrophils in response to microbial attack (Prince and Gunson 1987). Several species of bacteria, including *Pseudomonas syringae*, *Pseudomonas fluorescens* and *Salmonella typhimurium*, were completely inhibited by the H_2O_2 concentrations in nectar (Carter and Thornburg 2000; Carter et al. 2007) and specific destruction of the H_2O_2 using catalase resulted in nectar that no longer inhibited the growth of these species. These findings are consistent with a role for the NRC in microbial growth inhibition. However, this obstacle can be overcome by the floral pathogens *Erwinia amylovora* and *Pantoea agglomerans* that grew well in untreated nectar and were resistant to H_2O_2 at the concentrations found in nectar (Carter et al. 2007).

Because H_2O_2 is highly unstable, it breaks down to form free hydroxyl radicals. These radicals are scavenged by ascorbate. NEC3 is a bifunctional enzyme with monodehydroascorbate reductase activity as well as carbonic anhydrase activity. Carter and Thornburg (2004a) proposed that the monodehydroascorbate reductase activity functions to convert monodehydroascorbate, produced from the detoxification of hydroxyl free radicals, to ascorbate. In addition, also NEC5 can use dehydroascorbate in place of oxygen as a terminal electron acceptor, thereby converting dehydroascorbate back to ascorbate. The carbonic anhydrase activity buffers the pH of nectar with bicarbonate, the same buffer found in blood (Carter and Thornburg 2004b). Finally, NEC2 represents the inactive breakdown product of NEC3, whereas NEC4 is a defence protein that inhibits an endoglucanase that hemicellulose-degrading fungi use to degrade the hemicelluloses in the nectary cell walls (Naqvi et al. 2005; Park and Thornburg 2009).

The NRC pathway is initiated by a developmentally regulated nectary-expressed NADPH oxidase (NOX1) that begins to produce superoxide just prior to anthesis (Carter et al. 2007). Temporal expression patterns demonstrated that the superoxide production (NADPH oxidase activity) is coordinated with nectar secretion, the expression of *NEC1* and the expression of *NOX1*, the putative gene for the nectar NADPH oxidase (Carter et al. 2007). NADPH oxidase was found to be expressed in the early stages of flower development although superoxide was generated later, implicating post-translational regulation of the NADPH oxidase in the nectar (Carter et al. 2007). The superoxide is subsequently cleaved into oxygen and hydrogen peroxide by NEC1 (Carter et al. 1999; Carter and Thornburg 2000).

2.3 PR Proteins and Other Hydrolytic Enzymes

Which other options do plants possess to protect nectar from infestation? The NRC was not found in FN *Petunia hybrida* even though this species belongs to the same family (Solanaceae) as tobacco (Hillwig et al. 2010). By contrast, *Petunia* nectar contains several proteins with ribonuclease activity. Ribonucleases (RNases) are proteins that degrade RNA (D'Alessio and Riordan 1997; Yang 2011). Two nectary-expressed RNases from *Petunia*, RNase Phy3 and RNase Phy4, were found to be expressed in a pattern similar to that found for tobacco nectarins, with RNase Phy4 being expressed in petals, ovaries and nectaries and RNase Phy3 mainly expressed in ovaries, stigma and nectaries (Hillwig et al. 2010). Kram and co-workers also speculated that a second role of the FN lipase of *Jacaranda*, JNP1, might be anti-microbial protection (Kram et al. 2008). JNP1 is similar to members of the plant GDSL lipase/esterase gene family that is generally involved in responses to biotic and abiotic stressors (Oh et al. 2005; Hong et al. 2008). For example, an *Arabidopsis* GDSL lipase, AtGLIP1, has direct anti-microbial activity against the necrotrophic fungus *Alternaria brassicicola*, likely through disruption of spore wall integrity (Oh et al. 2005). Although an anti-microbial activity has not yet been

demonstrated for JNP1, the growth of *Escherichia coli* cells that heterologously expressed *JNP1* was profoundly diminished (Kram et al. 2008).

In fact, hydrolytic enzymes that directly attack micro-organisms are likely to represent a common and taxonomically widespread means by which plants protect nectar. For example, xylosidases in FN of *Cucurbita pepo* might reduce the virulence of pathogens via the degradation of oligosaccharides that are released by the nectary cell walls in response to hydrolytic microbial enzymes (Nepi et al. 2011a). A class of proteins that is emerging as a particularly common defensive mechanism of nectar are pathogenesis-related (PR) (Van Loon 1999; Van Loon et al. 2006). More than 50 proteins with sizes between 20 and 50 kDa were discovered in the EFN of *Acacia* myrmecophytes, plants that constitutively produce high amounts of EFN to nourish symbiotic ant colonies for their indirect defence (González-Teuber et al. 2009). The majority of these proteins were identified as PR proteins such as chitinases, glucanases and thaumatin-like and osmotin-like proteins. No fungal growth was detected in field-derived EFN samples of *Acacia* myrmecophytes although fungi could be cultivated from EFN derived from closely related but non-myrmecophytic species which lack high activities of chitinases and glucanases (González-Teuber et al. 2009). Further studies indicated that EFN of *Acacia* myrmecophytes successfully inhibited *in vitro* growth of important fungal pathogens such as *Phytophthora parasitica*, *Fusarium oxysporum* and *Alternaria alternata* (González-Teuber et al. 2010). Chitinases and peroxidases were also found in the FN of *Allium porrum* (Peumans et al. 1997) and of *Petunia* (Hillwig et al. 2011), and chitinases were found in the FN of *Rhododendron irroratum* (H.-G. Zha, personal communication). Chitinases and other PR proteins, thus, are likely to represent a common and taxonomically widespread type of nectarins.

Finally, nectar proteins have also been discussed to function in the protection from insects. For example, the first defensive enzymes that were detected in FN are an alliin lyase and a mannose-binding lectin in FN of *Allium porrum* (Peumans et al. 1997). The lectin, a 13-kDa protein, was found to be particularly abundant, since it represented about 75 % of the total nectar protein, accumulating to approximately 150 mg mL^{-1} of nectar (Peumans et al. 1997). Taking into consideration that lectins have anti-insect properties (Hilder et al. 1995; Rahbé et al. 1995), and that alliinase is involved in the synthesis of defence-related compounds (Rabimkov et al. 1994), both of these proteins are thought to function in the defence of nectar. In summary, PR proteins and other nectarins play a highly significant role in the protection of both FN and EFN against micro-organisms and, likely, insects.

2.4 Non-Protein Compounds

Secondary metabolites such as alkaloids and phenols are commonly associated with herbivore defence but have been also described as common nectar components (Baker 1977; Adler 2000). They are usually regarded as “toxic compounds” that plants secrete into floral nectar to achieve a protection from nectar robbers (Adler 2000). For example, FN of *Catalpa speciosa* contains iridoid glycosides that fended

off nectar robbers but not legitimate pollinators (Stephenson 1982). Similarly, phenols in FN of *Aloe vryheidensis* lowered its palatability to generalist insects and, thus, the attraction of non-effective pollinators to the flowers of this species (Johnson et al. 2006). By contrast, (Adler and Irwin 2005) manipulated contents of gelsemine, which represents the principal FN alkaloid of *Gelsemium sempervirens,* and found that the supplementation of gelsemine to FN deterred not only nectar robbers but also effective plant pollinators, thus decreasing the number of flowers probed and the time spent per flower by both, pollinators and nectar robbers.

Why should plants fend off legitimate pollinators? As mentioned earlier, short visitation times by pollinators do not necessarily represent a fitness disadvantage for plants (Kessler and Baldwin 2007) and more studies using experimental manipulations of nectar secondary metabolites are needed to understand the detailed functions of "toxic compounds". Besides behavioural assays, identifying the source of secondary metabolite production in plants seems to be crucial to understand their real ecological functions. Secondary metabolites might be synthesised in the nectaries themselves, a situation that would make an adaptive function highly likely, but they can also be derived directly from the phloem and xylem (Adler 2000), thus suggesting a function that is not necessarily related to their appearance in the nectar.

3 Nectary Structure and Development

3.1 Nectary Structure

The anatomy and ultrastructure of nectaries have been reviewed recently (Escalante-Pérez and Heil 2012); here, we therefore present only a very short overview. Nectaries are structures, or simply areas on the plant surface, where nectar is being secreted. Nectaries are structurally highly diverse and no typical or representative type of nectary can be defined (Elias 1983; Pate et al. 1985; Fahn 1988). Floral and extrafloral nectaries are not principally different, although several characteristics might be more common in one than in the other functional group. The anatomically most simple nectaries are "gestaltless" (Frey-Wyssling and Häusermann 1960) (i.e., without any externally visible structure), and in this case can only be identified as areas where nectar appears on the plant surface. Gestaltless nectaries appear to be more frequent among the extrafloral nectaries (Kirchoff and Kennedy 1985), whereas floral nectaries are normally well defined (Bernadello 2007). Most nectaries, however, form anatomically distinct structures with a complex ultrastructure.

The nectariferous tissue usually consists of two main components: an epidermis and a specialised nectary parenchyma that produces or stores the pre-nectar (Fahn 1979). A third component can sometimes be distinguished as subnectary parenchyma by its more loosely packed cells (Stpiczyńska 2003; Kaczorowski et al.

2008). Interestingly, many nectaries are characterised by a continuous cuticle present on the surface of the nectar epidermis (Gaffal et al. 1998). Therefore, most nectaries secrete through modified stomata that remain permanently open or through specialised trichomes (Fahn 1988; Wist and Davis 2006; Gaffal et al. 2007; Vassilyev 2010). The nectary parenchyma is generally composed of several layers of small isodiametric cells with thin walls, dense granular cytoplasm, small vacuoles and relatively large nuclei (see (Heil 2011) for details and references). These cells represent typical secretory cells and share their characteristics (D'Amato 1984) such as, for example, being rich in ribosomes and mitochondria and possessing an elaborate endoplasmatic reticulum, many dictyosomes and—usually—a high number of vesicles. Another well-defined characteristic of this tissue is the high number of plasmodesmata, a trait that is supposed to ensure rapid trafficking of metabolites among the cells (Fahn 1979), perhaps due to a comparably low specificity concerning the molecules that are transported (Terry and Robards 1987). Below the nectary parenchyma, some nectaries present a subnectary parenchyma that can be differentiated by its large cells with bigger vacuoles, less dense cytoplasm and larger intracellular spaces (Durkee 1982; Cawoy et al. 2008). The subnectary parenchyma is generally rich in chloroplasts and might contribute to the production of nectar sugars, although it is commonly believed not to be directly involved in nectar production (Nepi et al. 2007). Its main function appears the communication with the vascular system and perhaps some contribution to the synthesis of nectar sugars.

Nectaries can be connected to the phloem, the xylem, both, or have no direct vascular connection (Fahn 1988; Wist and Davis 2006). In most species studied, floral nectaries are vascularised by phloem only or are not directly vascularised at all (Fahn 1988). When the nectary is vascularised, phloem and xylem bundles are always present in subnectary parenchyma. The xylem vessels in general terminate in this tissue, whereas phloem strands branch into the nectary parenchyma. In short, nectaries usually are equipped for rapid exchange of water and water-soluble compounds with the vascular system. Although the chloroplasts that are localised in the subnectary parenchyma are likely to contribute to the carbohydrate content of the nectar, different studies indicate that carbohydrates are transported from elsewhere in the plant into the nectary. Studying $\delta^{13}C$ signatures in nectars of CAM plants, Luttge et al. (1985) could exclude direct (that is, current) CO_2 fixation as a source of current nectar production. A study using ^{13}C-labelled CO_2 also indicated that young leaves of *Rhizinus communis* secrete EFN when still being net importers of assimilates. In summary, floral and extrafloral nectars depend for nectar secretion on assimilates that are produced outside of the nectary itself and that are likely to accumulate, at least in part, in the nectary parenchyma before nectar secretion begins.

3.2 CRABS-CLAW—*A Central Player in Nectary Development?*

Although nectaries are functionally important structures whose ultrastructure has been investigated in multiple studies, almost nothing is known on how plants control their development. The gene *CRABS CLAW* (*CRC*) is a key gene for nectary development in *Arabidopsis* (Bowman and Smyth 1999; Baum et al. 2001; Lee et al. 2005a). It was identified in the carpel and floral nectaries of *Arabidopsis thaliana*, where it controls the width and elongation of the gynoecium. CRC belongs to the small YABBY family of transcription factors and is a player of the ABC model (Baum et al. 2001), a set of genes which determine the identity of floral organs (Coen and Meyerowitz 1991). The CRABS CLAW protein contains a zinc finger and a helix–loop–helix domain that are thought to mediate DNA binding (Bowman and Smyth 1999). Mutants of *CRABS CLAW* cause the gynoecium to develop into a wide, short structure that totally lacks floral nectaries, making *crc* the only single-gene mutant that lacks floral nectaries (Bowman and Smyth 1999). *CRC* expression in *A. thaliana* is mostly limited to developing carpels and nectaries, where its expression commences before the emergence of nectar glands and continues until after anthesis. Expression of *CRC* persists at high levels in nectaries throughout the secretory process. Recent transcriptomic analyses of *A. thaliana* and *Brassica rapa* nectaries confirmed that the expression of *CRC* (At1g69180) was more than 200-fold higher in nectaries than in the reference tissue (Kram et al. 2009; Hampton et al. 2010). Thus, CRC could potentially play an indirect role in the regulation of nectar synthesis and secretion. However, since *crc* mutants lack nectaries it is not understood what effect, if any, this gene has on de novo nectar production (Bowman and Smyth 1999).

CRABS CLAW is regulated by a number of positive and negative regulators expressed in the nectary Anlagen (Lee et al. 2005a). A phylogenetic footprinting of the *CRABS CLAW* promoter identified a number of regulatory elements in the promoter that included putative binding sites for *LEAFY* and MADS-box transcription factors (Lee et al. 2005b); however, the tissue-specific factor that limits the expression to nectaries and carpels remains unknown. Lee and co-workers proposed that B-class and C-class genes act redundantly with each other and in combination with the *SEPALLATA* genes to activate *CRABS CLAW* in the nectary Anlagen. The transcription factors *SHATTERPROOF1/2* may also participate in the transcriptional regulation of *CRABS CLAW* in appropriate backgrounds (Lee et al. 2005a).

Additional studies examined *CRC* in nectaries from a broad range of eudicot species demonstrating that both Rosids and Asterids (Brassicaceae, Solanaceae and Malvaceae) used *CRABS CLAW* as a general regulator of nectary development (Lee et al. 2005b). CRC expression in extrafloral nectaries of cotton (*Gossypium hirsutum*) implies alterations in spatial and temporal control of gene regulation relative to that of floral nectaries. On the other hand, no expression of *CRC* was found in *Aquilegia* (Ranunculaceae), a basal eudicot. These interesting results indicate that *CRC* expression is conserved in nectaries from several core eudicot species and that it is required for nectary development in several species within core

eudicots, regardless of nectary position and morphology (Lee et al. 2005b). The ancestral function of *CRC* might be the regulation of carpel development, a role probably conserved throughout angiosperms (Fourquin et al. 2005; Lee et al. 2005b). Thus, *CRABS CLAW* is widely expressed among the angiosperms and appears to function early in nectary gland formation.

Like *CRC*, *BLADE-ON-PETIOLE 1* (*BOP1*) and *BOP2* encode redundant zinc finger transcription factors containing a YABBY domain that promote morphological asymmetry during leaf and floral development (McKim et al. 2008). *BOP1* and *BOP2* also were found to promote growth of nectaries. Nectaries are not entirely absent in *bop*1/2 double mutants, but these mutants fail to form a normal nectary structure and instead develop minor projections at the base of the stamens that lack any typical nectary key characteristics such as parenchyma and secretory tissue as well as modified stomata (McKim et al. 2008). Since *BOP1* and *BOP2* are not necessary for *CRC* expression, the significant reduction in nectary formation is not a consequence of *CRC* deregulation, but instead *BOP 1* and *2* might act with *CRC* to promote normal nectary development (McKim et al. 2008).

4 The Control of Nectar Flow

4.1 Ontogenetic and Ecological Patterns in Nectar Flow

Plants possess several internal and environmentally controlled mechanisms to adjust nectar production rates to factors such as flower or leaf developmental stage, consumption rate (reviewed in Heil 2011) and, in the case of EFN, the current level of herbivory. Flowers can even re-absorb nectar that has not been consumed (reviewed in Pacini et al. 2003). In the following paragraphs we list the patterns of FN and EFN secretion as observed at the phenotypic level and then report the few mechanisms that have been suggested as physiological or genetic causes of these patterns.

First, FN secretion in flowers must evidently be synchronised with flower development. We refer to Ren et al. (2007a,b) for detailed studies on the ontogeny of nectar secretion in flowers of ornamental tobacco and the underlying physiological mechanisms. Besides this ontogenetic level, many plants produce their FN or EFN in diurnal rhythms and these rhythms appear, at least in part, to be adapted to consumer activity, rather than being simple consequences of diurnal assimilation patterns (Bentley 1977; Tilman 1978; O'Dowd 1979; Corbet and Delfosse 1984; Heil et al. 2000). Besides these likely endogenous rhythms, the secretion of EFN with few exceptions is induced in response to mechanical damage or herbivory (Heil 2011).

An adjustment of FN net production to consumption rates has been demonstrated in various species (Corbet and Delfosse 1984; Gill 1988; Pyke 1991). *Macaranga tanarius* reduced EFN secretion in the absence of consumers and increased it immediately after consumer access (Heil et al. 2000). Quantitative adjustments of

apparent net production could, however, result either from an inhibited de novo secretion or from a re-absorption of accumulated nectar. Whereas it is not known whether plants really can adjust the de novo secretion, a re-absorption of FN has been shown unambiguously with different methods (Nepi and Stpiczyńska 2008), including labelling studies (Pederson et al. 1958; Ziegler and Lüttge 1959). Re-absorption of non-consumed FN appears common and can occur separately for water and sugars even during the active secretion process (Nepi et al. 2011b), although this phenomenon remains to be demonstrated for EFN (Nepi and Stpiczyńska 2008).

4.2 Hormonal Control of Nectar Flow

As mentioned earlier, EFN secretion in most species functions as an inducible defence mechanism and is enhanced in response to mechanical damage or herbivory. This induction is mediated via the octadecanoid signalling pathway and can be induced by the application of jasmonic acid, JA (Heil et al. 2001, 2004; Wooley et al. 2007; Pulice and Packer 2008; Heil 2011). Floral nectar secretion by *Brassica napa* is under control of the same hormone (Radhika et al. 2010), and recent observations on FN secretion by Lima bean indicate that the induction of FN flow by jasmonic acid also can be observed in taxonomically unrelated species (J. Hernández-Cumplido and M. Heil, unpublished). Thus, FN and EFN secretion might be under control of the same hormone.

4.3 Physiological and Genetic Control of Nectar Flow

How do plants control nectar flow, and where are the nectar components being synthesised? Despite the various reports on nectary-expressed genes, very little is known of the downstream mediators of nectary development, nectar synthesis and secretion. The classical hypothesis was that nectar represents "secreted phloem sap" (Agthe 1951; Zimmermann 1954; Lüttge 1961; Fahn 1988; de la Barrera and Nobel 2004). This hypothesis is anatomically supported by the observation that the companion cells of the nectary phloem commonly possess well-developed wall ingrowths which increase the surface area, thereby facilitating the uploading of pre-nectar component into the adjacent nectary parenchyma (Davis et al. 1988; Wist and Davis 2006). Unfortunately, all classical studies into the quantitative control of nectar flow focused their efforts on the carbohydrates but ignored other nectar components such as amino acids and proteins. Peumans et al. (1997) compared protein contents and the specific activities of alliinase and chitinase of FN of *Allium porrum* to the values found in flower stalk exudates and found strong discrepancies, which indicates that nectar represents more than secreted phloem sap and that significant biochemical conversions and de novo synthesis of nectar

components are likely to take place in the nectary tissue itself. Otherwise, young leaves of *Rhizinus communis* produced EFN when still being net importers of assimilates (Radhika et al. 2008), which indicates that at least some nectar compounds are transported from elsewhere in the plant to the actively secreting nectaries.

Nectar secretion patterns are likely to be genetically determined (Mitchell 2004; Leiss and Klinkhamer 2005a). At the population level, differences in floral nectar production among plants might be genetically determined and interact with environmental conditions (differences in soil moisture, exposure, type of substrate, etc.; see Leiss and Klinkhamer 2005b). Most genetic studies on floral nectar variability concerned production rates, while very little is known about the heritability of other major traits—such as sugar ratios, amino acid composition, taste and scent (Leiss et al. 2004; Mitchell 2004; and references therein). Some studies indicated abundant genetic variation in FN traits (Mitchell 2004), but we are aware of only one study that concerned heritability of extrafloral nectary traits (Wooley et al. 2007). In *Mimulus* (Scrophulariaceae) and *Petunia* (Solanaceae), a minimum of two quantitative trait loci (QTLs) were found to be involved in controlling the amount of nectar produced, whereas the hexose:sucrose ratio in *Petunia* was under the control of one major QTL (Galliot et al. 2006). Stuurman and co-workers analysed phenotypic and genetic differences in colour, shape, nectar reward and scent. Nectar volume was controlled by two QTLs, one affecting volume pleiotropically by altering flower size and another affecting nectary physiology (Stuurman et al. 2004). Their additive effects accounted for almost the entire difference in nectar volume between the two species. No significant QTL was detected for nectar concentration, but a single QTL decreased the proportion of sucrose (Stuurman et al. 2004), which is consistent with the activity of an invertase.

Unfortunately, though, the molecular events that are involved in the synthesis and secretion of nectar are still relatively poorly understood and thus, central questions remained unresolved. For example, no detailed pathway has been presented for the uploading of sugars and other nectar components from the phloem to the nectariferous tissue and for their transport to the secretory cells and subsequent release (but see Heil 2011, and URL http://www.youtube.com/watch?v=Nd8ryN_7BP8 for a recent suggestion). In the following we review the limited evidence that exists concerning the control of nectar flow and the synthesis of important nectar components. Previous to and during secretion, the nectary parenchyma is subject to significant ultrastructural changes that indicate a high metabolic activity. For example, parenchyma cells frequently undergo continued cell division during secretion (Gaffal et al. 1998). While vacuoles in the pre-secretory phase are usually small, their volumes increase at the time of secretion. During this phase, a boost in the energy requirements leads to a more active endoplasmic reticulum and an accumulation of mitochondria and ribosomes in the cytoplasm of parenchymatous cells, while dictyosome numbers are reduced (Zhu and Hu 2002).

Accumulation of starch in amyloplasts is a prominent feature of many, but not all nectaries (Fahn 1979; Nepi et al. 1996; Peng et al. 2004; Stpiczyńska et al. 2005;

Ren et al. 2007b). In these cases, the time frame of nectar production is correlated with that of starch degradation, which is likely to be the source of sugar flow into nectar. The complete canonical sucrose biosynthetic pathway was found to be upregulated in mature lateral nectaries of Arabidopsis when compared with non-nectary reference tissues (Kram et al. 2009). Ren et al. (2007a) identified 26 target genes that participate in starch anabolism and catabolism in nectaries from ornamental tobacco. The anabolic genes include a sucrose synthase, whose expression was observed early in nectary development. Additionally, ADP-glucose pyrophosphorylase and starch synthase 3 were detected at mRNA levels, protein levels and as enzymatic activities during early stages and then declined as the nectary matured. By contrast, the expression of catabolic genes, including isoamylase 1, α-amylase and β-amylase, was detected at later stages (that is during active secretion). Besides rapidly liberating mono- and disaccharides, the hydrolysis of starch will also enhance osmotic pressure within the nectary and thereby drive water flow into the nectary (Nepi et al. 2011b). Finally, genes that were expressed throughout nectary development include a starch branching enzyme 1 and starch phosphorylase, which might participate in both starch synthesis and degradation. The switch from starch anabolism to catabolism seems to be a key step in normal nectary development and function (Ren et al. 2007a,b).

In the most likely scenario, the photoassimilates move from the sieve elements to nectary parenchyma cells by plasmodesmata and multivesicular structures. The simplest regulation for sucrose release is possibly conducted by an extracellular invertase, which hydrolyses the starch into glucose and fructose, thereby creating hexose-rich nectars and controlling the source–sink relationships that prevent reloading into the phloem (Sturm and Tang 1999; Roitsch et al. 2003; Peng et al. 2004). A recently discovered apoplastic invertase of *Arabidopsis thaliana* represents the first gene whose function is required for floral nectar secretion (Ruhlmann et al. 2010). Phloem-derived sucrose appears to be hydrolysed into glucose and fructose by CELL WALL INVERTASE 4 (CWINV4) in both pre- and post-anthesis flowers, thus allowing nectaries to maintain a constant sink status (Ruhlmann et al. 2010). Whereas wild-type flowers accumulated high levels of starch within the secretory stomata, *cwinv4* mutant flowers accumulate starch within the receptacle and pedicel and not within the nectaries themselves. It is likely that CWINV4 also is involved in the cleavage of sucrose into glucose and fructose in actively secreting cells, because *cwinv4* mutants failed to secrete nectar. Furthermore, an orthologue to *AtCWINV4* in *B. rapa*, *BrCWINV4*, also displays a nectary-specific expression profile. This suggests a conserved role for cell wall invertases in nectar secretion, at least within the Brassicaceae (Ruhlmann et al. 2010).

Whereas research has advanced in the context of sugar metabolism, it is still not known where non-carbohydrate nectar components are synthesised and how these compounds enter the nectar. Preliminary evidence indicates that at least the nectarins of ornamental tobacco are likely to be synthesised in the nectary tissue itself. The promoter responsible for *NEC1* and *NEC5* expression has been characterised (Liu et al. 2009). *MYB305* is expressed exclusively in flowers and

represents a transcription factor that directly binds to and activates transcription of both the *NEC1* and *NEC5* promoters. Temporally, *MYB305* expression precedes that of *NEC1* and *NEC5*, as would be expected if the *MYB305* factor regulates expression of *NEC1* and *NEC5*. Deletions of the promoter binding sites from the *NEC1* promoter significantly reduced its expression in nectary tissues. Ectopic expression of *MYB305* in foliage was able to induce expression of *NEC1* and *NEC5*, as well as two flavonoid biosynthetic genes in the foliage. Finally, RNA interference knockdown of *MYB305* resulted in reduced expression of both nectarins and flavonoid biosynthetic genes (Liu et al. 2009). Recently, these results have received further support from studies on *myb305* RNAi knockdown tobacco plants, which accumulated lower levels of starch in their nectaries and exhibited lower expression levels of α-amylase, ß-amylase and isoamylase 3 than wild-type plants (Liu and Thornburg 2012).

5 Conclusions and Outlook

The ecological roles of nectars are well defined and multiple studies exist on the nectar components that mediate the attraction of mutualistic animals. An emerging new general pattern is that nectars contain nectar proteins (nectarins) as a means of anti-microbial protection. Physiological and genetic studies demonstrated that the synthesis and accumulation of starch and its subsequent degradation form key steps in the secretion of FN of ornamental tobacco, although the relevance of these processes remains to be proven for other plant species and types of nectaries. Extrafloral nectaries in particular often lack a detectable accumulation of starch and secrete EFN in response to herbivore-inflicted damage rather than in an ontogenetically determined pattern; they, therefore, require more flexible mechanisms to control nectar secretion patterns.

Molecular research into the formation and function of nectaries has likely been impaired by the general focus of the plant sciences on few model plants that either lack nectaries (rice, wheat, maize) or that have very small nectaries of limited relevance in the biology of the plant (mostly the selfing *Arabidopsis*). The knowledge on genes that are involved in the formation of nectaries or in the synthesis of nectar components other than sugars remains therefore highly scattered. We suggest the use of untargeted techniques to study the transcriptomes and the proteomes of nectaries in the non-secreting and the secreting stage and to combine biochemical analyses of the nectar with the respective analyses of the contents of phloem and xylem, in order to elucidate the sites where important nectar components are produced and how nectar secretion is regulated at the quantitative level. Any given compound that is synthesised in the nectary parenchyma itself and for which active transportation or secretion mechanism exists is highly likely to have an ecologically relevant function, whereas compounds that are present in the phloem at concentrations similar to—or higher than—in the nectar, and for which no secretion mechanism can be discovered, are likely to passively leak into the nectar. However, the transcription in the nectar

parenchyma of genes that are directly involved in the synthesis of nectar components has so far only been reported for the starch metabolic genes and nectarins of ornamental tobacco (Carter and Thornburg 2004a; Carter et al. 2007; Ren et al. 2007a,b; Liu et al. 2009). Finally, nectarins—besides their protective function (Carter and Thornburg 2004a; González-Teuber and Heil 2009a)—also play important roles in the chemical conversion of the secreted nectar (Heil et al. 2005; Kram et al. 2008), and specific nectarins might even affect the early development of the fruit (Zha et al. 2012). These findings, together with the role of yeasts in the chemical conversion of the nectar, indicate that the control of the biochemistry of nectar and its effects on biologically relevant traits do not stop with nectar secretion. Processes that are ongoing in the nectar need to be considered as well, in order to understand what finally mediates pollination and indirect defence: the liquid nectar as it is found in flowers and on extrafloral nectaries by the respective consumers, including mutualists, micro-organisms and nectar thieves.

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Part IV
Ecology

Identifying Geographically Based Metapopulations for Development of Plant Materials Indigenous to Rangeland Ecosystems of the Western USA

Douglas A. Johnson, B. Shaun Bushman, Thomas A. Jones, and Kishor Bhattarai

Contents

Abstract Rangeland ecosystems account for about half of the earth's land surface. They play an important role in providing forage for livestock and wildlife, and they serve as critical watershed areas. Many of the world's rangelands have been degraded by overgrazing, marginal crop production, mineral and energy extraction, recreation, and other human-caused disturbances. This degradation has led to invasion by exotic weeds and subsequent increases in fire frequency. This, in combination with uncertainties associated with global climatic change, has resulted in a critical need for plant materials to restore and revegetate rangeland ecosystems. The assessment of genetic variation and its phenotypic expression in important rangeland plant species (especially forbs) is critical in defining population structures (genetically

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differentiated groups) that could be used in rangeland restoration/revegetation efforts. We used common-garden studies and DNA-based analysis of genetic variation to assess genetic diversity in three rangeland legume species indigenous to rangeland ecosystems of the Great Basin Region of the western USA. Results of these studies are presented as three case studies that describe data collection procedures, analysis, and interpretation used to identify population structures in each species. These data formed the basis for combining plant collections into geographically based metapopulations for these three legume species that are being used to develop plant materials for commercial seed production and subsequent use on rangelands of the Great Basin.

1 Introduction

Rangeland ecosystems have been defined by Allen et al. (2011) as "land on which the indigenous vegetation (climax or sub-climax) is predominantly grasses, grass-like plants, forbs or shrubs that are grazed or have the potential to be grazed, and which is used as a natural ecosystem for the production of grazing livestock and wildlife. Rangelands may include natural grasslands, savannas, shrublands, many deserts, steppes, tundras, alpine communities and marshes." Rangeland ecosystems cover an estimated 50 % of the world's land area (Holechek et al. 2010) and play an important role in providing meat, milk, wool, leather, and other animal products to human populations. In addition, rangelands (especially those at higher elevations) serve as critical sources of water for both urban and agricultural use.

Rangeland ecosystems in the western USA are increasingly vulnerable to mismanagement, weed invasion, human-caused disturbances, and soil erosion (Pierson et al. 2011). Although some of these problems can be solved by improved management practices that result in natural recovery, many rangeland ecosystems may suffer from long-term reductions in biodiversity, altered nutrient and water cycling, diminished livestock and wildlife forage production, increased wildfire frequency, and increased soil erosion and stream sedimentation (Sheley et al. 2008). These degraded rangelands may not recover for decades (or longer) without human intervention, so restoration/revegetation may be required to improve degraded conditions, speed recovery, and prevent further erosion and degradation. Because plant materials are critical to optimize the success of restoration/revegetation efforts, rangeland managers, restoration/revegetation specialists, and the commercial seed trade have considerable interest in the development, planting, and management of rangeland plant materials.

The development of plant materials for use in rangeland ecosystems is a long-term process. A framework for the development of plant materials for semiarid rangelands was discussed by Johnson et al. (1981). The initial stage of plant material development involves the identification of candidate plant species, assembling diverse collections of these species, and developing populations for release to the commercial seed trade. In the population development stage prior to release, determining and applying appropriate selection procedures is an important consideration. It is critical to keep genetic considerations in mind when planning rangeland restoration/revegetation

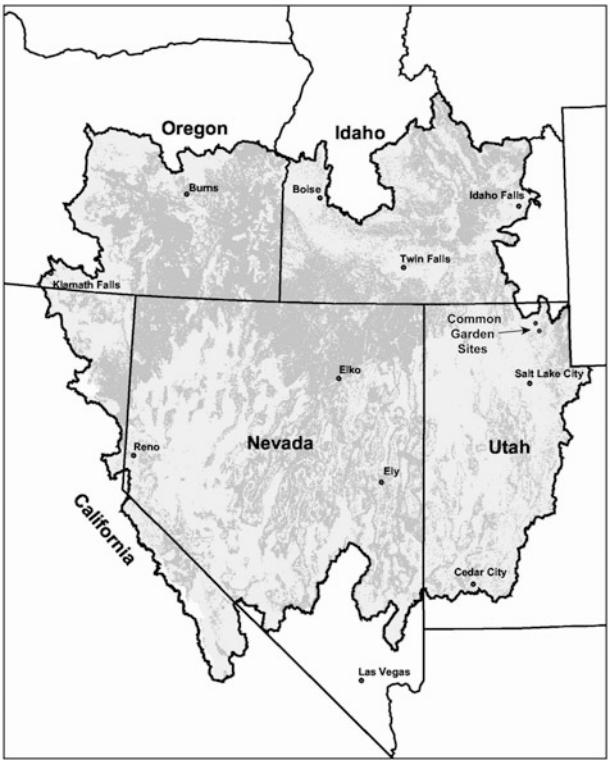

Fig. 1 Map of the floristic Great Basin showing sagebrush (*Artemisia)*–steppe rangelands (*shaded*) and location of the two common-garden sites in northern Utah (public domain figure provided by S.E. Hanser)

projects in relation to site potential, seeding objectives, desired plant community, community seral status, weed invasion, economic limitations, and ecological adaptation (Jones and Johnson 1998). Coupled with these considerations, rangeland managers should be cognizant of the potential effects of global change on their restoration/revegetation efforts (Rice and Emery 2003; Chambers 2008a). Thus, information is critically needed concerning the application of practical procedures to assess genetic variation and its phenotypic expression in important rangeland plant species for use in defining population structures that could be used in the development of plant materials for restoration/revegetation of degraded rangelands.

The Great Basin Region covers an area of approximately 293,042 km^2 in the western USA (U.S. Geological Survey 2011) (Fig. 1) and predominantly comprises rangeland ecosystems. Nearly the entire state of Nevada is included in the Great Basin, which also extends into California, Oregon, Idaho, and Utah. It is bordered by the Sierra Nevada Mountains to the west, the Rocky Mountains and the Colorado Plateau to the east and south, the Mojave Desert to the south, and the Columbia Plateau to the north. Elevations in the Great Basin commonly range from 1,200

to 2,000 m, with the lowest elevation occurring in Death Valley (−282 m) in Nevada and the highest elevation on Mt. Whitney (4,421 m) in California. Because the Great Basin lies in the rain shadow of the Sierra Nevada Mountains, much of the Great Basin has a semiarid to arid climate that ranges in average annual precipitation from about 100 to 300 mm, reaching more than 500 mm in some montane zones. The Great Basin contains numerous mountain ranges with 33 peaks reaching elevations greater than 3,048 m. Temperature varies considerably from site to site, often according to elevation. The Great Basin receives a large portion of its precipitation as snow during the winter. As a result, the Great Basin is referred to as a cold desert, which has resulted in unique plant adaptations in plants that grow there (Comstock and Ehleringer 1992).

Because of the spread of invasive weeds, in particular cheatgrass (*Bromus tectorum* L.), and the associated increased frequency of fires (Pellant et al. 2004; Chambers 2008b), the Great Basin has been classified as one of the most ecologically endangered regions in the USA (Stein et al. 2000). Each year in the Great Basin, an estimated 3,850 km^2 burns (mean from 1990 to 2010) due to wildfires (Don Major, personal communication 2011). If these areas are left untreated, weed invasion accelerates and results in further degradation (Jessop and Anderson 2007). Postfire restoration/revegetation efforts in the Great Basin require considerable amounts of seed. For example, in 2007, the US Government's Bureau of Land Management (BLM) purchased more than 1.9×10^6 kg of seed for rangeland restoration/revegetation (USDI Bureau of Land Management 2009). This large amount of seeds included a wide variety of both indigenous and introduced plant materials used to meet the specific seeding objectives of the BLM land managers (Table 1). A mix of species is preferred for restoration/revegetation projects because they provide a functionally diverse plant community that is more likely to minimize the risk of weed invasion and improve ecosystem function (Pokorny et al. 2005; Sheley and Carpinelli 2005; Walker and Shaw 2005).

In this chapter, we discuss the challenges that rangeland managers face in meeting the specific objectives of their restoration/revegetation projects and how managers must make their decisions in the context of a suite of ecological and genetic considerations. We present our research with three leguminous [Fabaceae] species indigenous to the Great Basin as three case studies to demonstrate how common-garden- and molecular-marker-based evaluations can be used to identify populations that exhibit similar genetic characteristics (defined here as geographically based metapopulations). These metapopulations are serving as a basis for the release of plant materials of these species to the commercial seed trade. Although this information specifically relates to rangeland ecosystems of the Great Basin and neighboring ecosystems in the western USA, these general procedures may be applicable to plant material development for rangeland ecosystems in other semiarid regions of the world.

Table 1 Plant materials exceeding 5,000 kg on a pure live seed (PLS) basis purchased by BLM in the Fall 2007 Consolidated Seed Buy for rangeland restoration/revegetation in the western USA

Species	Plant material	PLS (kg)
Agropyron cristatum/A. desertorum	Hycrest	174,962
*Elymus wawawaiensis** [a]	Secar	149,631
*Pascopyrum smithii**	Arriba	93,128
Agropyron fragile	Vavilov	85,421
Thinopyrum intermedium	Luna	80,157
Psathyrostachys juncea	Bozoisky	47,920
Astragalus cicer	Eski	45,282
Thinopyrum intermedium	Rush	43,765
Medicago sativa	Ladak	39,761
*Achnatherum hymenoides**	Rimrock	38,239
Sanguisorba minor	Delar	37,476
*Leymus cinereus**	Magnar	33,735
*Poa secunda**	Sherman	33,298
Agropyron fragile	P27	32,027
*Poa secunda**	SI [b]	26,210
Agropyron desertorum	Nordan	25,217
*Elymus lanceolatus**	Critana	24,839
Psathyrostachys juncea	Swift	24,760
Agropyron cristatum	Kirk	24,429
Thinopyrum intermedium	Oahe	21,062
*Elymus lanceolatus**	Bannock	20,209
*Elymus elymoides**	SI	19,944
*Pseudoroegneria spicata**	Anatone	17,359
Thinopyrum ponticum	Alkar	17,165
*Achnatherum hymenoides**	Nezpar	14,488
*Psuedoroegneria spicata**	P-7	14,054
Linum perenne	Appar	13,341
Bromus inermis	Manchar	12,887
Agropyron cristatum	Fairway	12,464
*Elymus lanceolatus**	Sodar	12,107
Kochia prostrata	Immigrant	11,704
*Bromus marginatus**	Garnet	8,196
*Festuca idahoensis**	Joseph	7,637
*Pseudoroegneria spicata**	Goldar	7,243
*Artemisia tridentata**	SI	6,769
*Achnatherum hymenoides**	Paloma	5,663

[a] *indicates a species indigenous to rangeland ecosystems of western USA
[b] SI refers to source-identified plant material collected from a specific site in western USA

2 Restoration/Revegetation Objectives

Opinions vary considerably concerning the best approaches for rangeland restoration/revegetation in the USA (Johnson et al. 2010a). One approach advocates "local-only" plant materials (Rogers and Montalvo 2004), which emphasizes trying to reestablish

population biodiversity patterns (i.e., matching species diversity and richness) present on the site prior to disturbance (Murphy 1989). This approach has the benefit of reestablishing plant materials that were present at the site prior to disturbance, which may fulfill their same ecological role prior to disturbance. However, defining past biodiversity patterns at some sites can be problematic, and disturbances may markedly alter conditions at the site such that some plants originally found on the site may no longer be adapted to the site. In addition, because this approach often necessitates the use of local ecotypes that must be specifically grown for restoration/revegetation of the particular site, seed costs can be relatively high. A second approach involves "assisted evolution" (Jones and Monaco 2009), which may encompass augmentation of genetic variation, enhancement of fitness by artificial selection, or the hybridization of nonindigenous material with indigenous material to increase stress tolerance. The overall goal of artificial selection is to use these techniques to develop plant materials that reflect general historical evolutionary patterns, are adapted to modified environments, are capable of adaptation to contemporary selection pressures, and contribute to restoration of ecosystem structure and function. Recognition of differences in scale and approach have important implications concerning which land management practices should be used to best achieve the specific objectives for the targeted restoration/revegetation site (Wiens 1989; Levin 1992).

Private ranchers, who own a relatively small proportion of total rangeland area in the western USA, generally have considerable latitude to choose the plant materials that meet their specific restoration/revegetation objectives and needs. Many private landowners are interested in meeting objectives related to optimizing livestock production, which is a common use of private rangelands in the western USA. In these cases, fast effective establishment of grazing-tolerant species at an affordable cost is of primary concern, and a wide variety of species are available for this purpose (Alderson and Sharp 1994; Barnes et al. 2007). Frequently, many of these grazing-tolerant plant materials for the Great Basin are introduced species that are commercially available at a low cost and have been selected for improved establishment, persistence, and forage production and quality characteristics over many years (Caldwell et al. 1981; Robins et al. 2012).

The BLM and USDA Forest Service (USFS), the federal agencies responsible for managing most publically owned rangelands in the western USA, are mandated to manage their lands for multiple uses and ensure long-term diversity and sustainability (Richards et al. 1998; Collins and Stritch 2008). Long-term goals for these rangelands are typically to stabilize soil, increase biotic diversity, provide the necessary food and habitat to support a diverse fauna, and confer resilience to new or unpredictable biotic and abiotic challenges (Johnson et al. 2010a). Thus, plant materials sought by BLM and USFS land managers generally focus on a mix of species that are able to support a diversity of products and ecosystem services that society demands from public rangelands. There has been a gradual shift towards use of indigenous North American species for restoration/revegetation. For example, 32 % of the seed purchased by BLM for rangeland restoration/revegetation in 1999 was for species indigenous to rangelands of the western USA compared to 56 % in 2007 (USDI Bureau of Land Management 2009).

BLM and USFS land managers face challenges to identify species that will be appropriate for the given land type, end use of the site, and seeding objectives and will match the amounts and seasonal distribution of annual precipitation at the site. Land managers also must balance their purchase decisions in relation to seed availability of the desired species, seed cost, and past successes or failures of specific plant materials.

In some cases, land managers may be interested in restoring degraded sites with plant materials specifically indigenous to the site or very similar ecological sites in hopes that the site eventually can be reverted to some pre-disturbance state. In such cases, questions concerning how indigenous plant materials interact with the biotic and abiotic characteristics of the disturbed ecosystem are relevant. For example, do the indigenous plants interact with site characteristics in a similar manner as they did prior to disturbance? What are the tolerance limits of the pre-disturbance plant materials to the various disturbances and changed conditions on the site (Millar and Libby 1989; Roundy et al. 1997; Brown and Amacher 1999)? When degraded rangelands have deteriorated to the point where they are no longer functioning ecosystems, it may be not be economically or ecologically feasible to return them to a pre-disturbance state (Jones and Monaco 2009). Plant species present at the site prior to disturbance may no longer be adapted to the site because ecosystems have been drastically (and possibly irreversibly) altered (Jones and Monaco 2009). For example, cheatgrass dominance in the Great Basin can alter key ecosystem processes such as nutrient dynamics (Saetre and Stark 2005) and soil structure (Norton et al. 2007), which may critically affect which species can establish and survive on degraded sites. In such cases, assisted evolution may represent an alternative approach, with the land manager's primary objective being to reinitiate "natural succession that will lead to the reestablishment of ecosystem form and function" (Brown and Amacher 1999). In such cases, whether or not plant materials are indigenous to the site may be of secondary importance to their ability to repair ecosystem form and function.

Jones (2003) and Jones and Monaco (2007) developed the Restoration Gene Pool (RGP) as a framework for organizing plant material selection decisions and clarifying the various plant material options for rangeland restoration/revegetation. They defined four RGPs (primary, secondary, tertiary, and quaternary) in descending order of genetic similarity to the indigenous population of the target species. The primary RGP involves the use of indigenous plant populations from the restoration site. The secondary RGP is seed of the target species not from the particular restoration site, whereas the tertiary RGP involves use of hybridized material between the target species and a closely related taxon. The quaternary RGP consists of a native species different from the target species or a noninvasive introduced species that functions in a similar way as the target species. In recent years (at least for BLM), most restoration/revegetation projects have used secondary and quaternary rather than primary RGP materials because seeds of indigenous populations are often unavailable due to project timing, seed availability, and funding constraints. The primary RGP, however, is now receiving a greater emphasis than in the past (Johnson et al. 2010a). Further research is needed to document the successful implementation of the primary RGP.

Opinions differ widely concerning what plant materials should be used in the restoration/revegetation of degraded rangeland ecosystems in the western USA. Rangeland managers of both privately and publically owned rangelands need to clearly define the objectives of the particular restoration/revegetation project and choose the plant materials that best meet these defined objectives.

3 A Strategy for Plant Material Development

Short-term plant response refers to a plant's ability to germinate, establish, persist, and produce seed (i.e., ecological fitness). The presence of local adaptation in timber species has been estimated by growing collections in common gardens and identifying plant traits that are correlated to environmental variables at the original collection sites, such as mean annual precipitation and mean annual minimum or maximum temperature (St. Clair et al. 2005). In this type of situation, a significant and high correlation coefficient may infer that an environmental variable is associated with genetic variation for a particular trait or traits. Long-term evolutionary potential may be inferred by a population's long-term persistence and seedling recruitment under the site's range of natural variability (Landres et al. 1999). Long-term evolutionary potential can involve estimating the historical gene-flow pattern and the magnitude of heterosis or outbreeding depression when newly seeded plant materials hybridize with local populations of the same species (McKay and Latta 2002).

Several authors have reviewed various aspects of the genetics of plant materials for restoration/revegetation, their evolutionary potential in new sites, and their interaction with adjacent populations of the same or similar species (e.g., Endler 1986; Merila and Crnokrak 2001; Mckay and Latta 2002; Hufford and Mazer 2003; Rice and Emery 2003; Ouborg et al. 2006; Broadhurst et al. 2008; Weeks et al. 2011). The genetic identity of a population may reflect directional evolutionary forces such as natural selection, as well as stochastic forces such as genetic drift (Enjalbert et al. 1999). Geographical gene-flow barriers can enable genetic differentiation between neighboring populations (McKay et al. 2005). In some cases, genetic differentiation has been correlated with phenotypic differentiation among populations for quantitatively inherited traits (Merila and Crnokrak 2001). Isolation by distance has also been reported for some plant species (Raspe and Jacquemart 1998; Stöcklin et al. 2009; Bhattarai et al. 2010; Bushman et al. 2010), but not for others (Franceschinelli and Kesseli 1999; Albaladejo et al. 2009). Strong isolation by distance usually indicates that populations are at equilibrium with respect to gene flow and genetic drift (Slatkin 1993). However, other factors, such as precipitation, temperature, elevation, or disturbance, can influence isolation by distance (Kittlein and Gaggiotti 2008). Isolation by distance can be statistically detected by a positive correlation between genetic and geographic distances among individuals with the Mantel test (Mantel 1967).

Local germplasm has been recommended for use in revegetation because of uncertainties that nonlocal material may be maladapted (Lesica and Allendorf 1999; McKay et al. 2005). A challenge with use of local plant materials for widely distributed indigenous plant species is that maintaining a large number of seed sources can become problematic for seed growers (McKay et al. 2005; Smith et al. 2007; Broadhurst et al. 2008). This is particularly true for sites in the geographically extensive Great Basin where demand for specific local materials is sporadic given the unpredictability of fire occurrences at specific sites. In addition, human-caused disturbances, invasive species, and global change may alter ecosystems to an extent that they become new or "novel" (Hobbs et al. 2006) where local germplasm may no longer be optimally adapted. If selection pressures change due to altered environmental characteristics, fitness of local populations could decline (Sgrò et al. 2011). Nonlocal genotypes have the possibility of hybridization with remnant local material and consequential increases or decreases in progeny fitness (Hufford and Mazer 2003). The introduction of nonindigenous alleles, however, may be advantageous if they confer enhanced adaptation to a planting, particularly in a drastically changed environment (Sgrò et al. 2011). In particular, poor seedling establishment and survival are typically the major constraints that impact indigenous rangeland plant materials in the difficult restoration environments of the Great Basin (Jones and Monaco 2009). As a result, generalizations cannot be made concerning the consequences when nonindigenous alleles are introduced into indigenous rangeland species.

One approach to balance concerns of genetic identity, ecological adaptation, and economical seed production is to combine plant collections (populations) into geographically based metapopulations. Boundaries of natural metapopulations have been identified for several indigenous rangeland plant species from the western USA (Larson et al. 2004; Bushman et al. 2007; Jones et al. 2008; Phillips et al. 2008). Common-garden studies conducted in target environments that evaluate important adaptive and economic traits could be used to compare collections for the genetic component of phenotypic trait variation and for the potential of local adaptation. Molecular genetic markers can be used to estimate variation within collections, infer gene-flow barriers by identifying genetically differentiated groups, test for isolation by distance, and search for correlations with phenotypic variation. Grouping collections into metapopulations of closely related local collections would allow planting of individual metapopulations across a larger geographical area compared to the use of only local populations. These metapopulations could potentially reduce the number of local seed sources required for use in restoration/revegetation programs, thus lowering seed costs. If desired, the metapopulation or the most promising local collections within it could be used as a base population to select for characteristics such as enhanced seedling establishment, high seed yield, reduced seed shattering, less indeterminate growth, and other important traits. Although seed growers may inadvertently select for uniformity in heterogeneous metapopulations during the process of seed production, this situation is typical for seed production in most rangeland species.

The following three case studies provide results from the combined use of common-garden evaluations and DNA genotyping techniques that are guiding the development of geographically based metapopulations for three outcrossing legume species indigenous to the Great Basin Region of the western USA.

4 Case Studies of Three Leguminous Species Indigenous to the Great Basin

Here we present the general procedures used in the development of geographically based metapopulations of three leguminous (Fabaceae) species indigenous to the Great Basin Region of the western USA: basalt milkvetch (*Astragalus filipes* Torr. ex A. Gray), western prairie clover [*Dalea ornata* (Douglas ex Hook.) Eaton & J. Wright], and Searls' prairie clover [*Dalea searlsiae* (A. Gray) Barneby]. The first two letters of the genus and species name will be used to refer to each species: *Astragalus filipes* (ASFI), *Dalea ornata* (DAOR), and *D. searlsiae* (DASE). Although these three legume species are indigenous to rangeland ecosystems of the western USA, some of the general results and principles found for these species may be applicable to other insect-pollinated plant species in other rangeland ecosystems, especially temperate regions with low annual precipitation.

The vast majority of species used for restoration/revegetation of rangeland ecosystems in the western USA are grasses, with very few indigenous legume plant materials currently available. Legumes are of particular interest because they provide biologically fixed nitrogen to associated species, increase plant productivity, enhance forage quality, and provide food sources for herbivores and pollinators (Cherney and Allen 1995; Madison and Robel 2001; Aydin and Uzun 2005; Walker and Shaw 2005). In addition, nitrogen-fixing plants can enhance nitrogen pools, carbon pools, and various fluxes (Liao et al. 2008) that may be important in maintaining and restoring natural succession and inhibiting site colonization by weeds following disturbance. Some rangeland legumes (particularly species of *Astragalus, Lupinus,* and *Oxytropis*), however, can be toxic to livestock because of high levels of nitrotoxins, selenium, and indolizidine alkaloids present in the plant (Williams and James 1975; Williams 1981; Rumbaugh 1983). This is especially problematic in rangelands of the western USA, which are frequently used for livestock grazing. However, results we obtained for the three species in our study (ASFI, DAOR, and DASE) showed that collections of these species had extremely low or non-detectable levels of nitrotoxins, selenium, and indolizidine alkaloids (Bhattarai et al. 2008; unpublished data). For these reasons, we focused our common-garden and DNA genotyping research on ASFI, DAOR, and DASE.

4.1 Procedures Used in Case Studies

Details concerning the procedures, results, and implications of phenotypic and genetic characterizations of the three species are contained in the following publications for each species: ASFI (Bhattarai et al. 2008; Bushman et al. 2010), DAOR (Bhattarai et al. 2010), and DASE (Bhattarai et al. 2011). Only the general procedures are presented here.

Seeds were collected and bulked from at least 100 plants for individual wildland sites across the distribution of each of the three legume species (67 sites for ASFI, 22 sites for DAOR, and 20 sites for DASE) with specific sites identified from specimen location information obtained from regional herbaria. Seed collections from individual sites were kept separate, seeds from each collection site were germinated in a greenhouse, and seedlings were transplanted to two common-garden sites in northern Utah. After an establishment year, field data were collected for morphological and other phenotypic traits (biomass, plant height, number of stems, plant diameter, number of inflorescences, flowering date, plant vigor scores, seed yield, and forage quality characteristics) for 2 years. Analyses of variance (ANOVA) (SAS Institute 2004) and principal component analysis (Horn 1965; Dinno 2010; Oksanen et al. 2010) were used to identify significant differences among collections for phenotypic traits.

The basic use of common gardens to estimate genetic components and phenotypic plasticity was reported by Clausen et al. (1940, 1941). Their studies covered a broad range of elevations (from 17 to 3,050 m above sea level) and associated climatic extremes, and included examination of ecotypes and subspecies. Our case studies report comparisons among and within populations across a narrower altitudinal range (202–2,545 m for ASFI, 110–1,163 m for DAOR, and 1,326–2,036 m for DASE). Several common-garden sites carefully located at multiple strategic sites across the range of species distribution would be ideal. However, the number of sites and their location usually involves a compromise because funding is usually limited, travel to the sites may be expensive and/or time consuming, labor may not be readily available at the site, and appropriate land may not be available at ideal locations. In our common-garden studies, because of these constraints, two common-garden sites were located near Logan in northern Utah, the periphery of distribution for our species (Fig. 1). This may limit the predictive capability of the data we collected; however, the test sites we used were within the temperature and elevation ranges of the species and were representative of sites where seed production of these species would typically occur. It would be ideal to evaluate collections both at multiple strategic locations throughout the range of distribution of the species and locations representative of seed production environments.

A genetic fingerprinting technique that used AFLP markers was used to determine genetic diversity and population structure in the three species (Meudt and Clarke 2007). AFLP markers do not require prior genetic information about the target species, and when generated, are largely considered neutral (implying no adaptive information) and robust in their ability to differentiate populations

(Schlötterer 2004). Modified procedures of Vos et al. (1995) were used to estimate population structures from 474 to 1,194 AFLP marker bands for each species. The Dice similarity coefficient was used to estimate average within-collection diversity (Dice 1945; Leonard et al. 1999), and Dice's distance matrix was then used as input for hierarchical analysis of molecular variance (AMOVA) (Schneider et al. 2000; Vekemans et al. 2002; Excoffier et al. 2005). Analysis of variance (ANOVA) procedures (SAS Institute 2004), neighbor joining cluster dendrograms (Felsenstein 2009), model-based Bayesian structure analysis (Falush et al. 2007), and the Mantel and partial Mantel tests (Mantel 1967; Rohlf 1998; Bohonak 2002; Jensen et al. 2005; Ersts 2009) were used to detect relationships among genetic, phenotypic, and climatic characteristics (mean annual precipitation, mean annual minimum temperature, and mean annual maximum temperature) at the collection sites.

The results of the common-garden and AFLP marker studies were used to identify geographically based metapopulations for each of the three species. In turn, we used these metapopulations as a basis for release of pre-variety germplasms to the commercial seed trade for use in restoration/revegetation projects for rangeland ecosystems in the western USA.

4.2 Case Study 1: Astragalus filipes

Astragalus filipes (ASFI) is a member of the Fabaceae family (Wojciechowski et al. 1999). This North American legume is indigenous to California, Idaho, Nevada, Oregon, Utah, Washington, northern Mexico, and British Columbia, Canada (Barneby 1989; Isely 1998). Plants are perennial, form large clumps of stems, and are upright in growth habit from 20 to 90 cm in height (Welsh et al. 1993). ASFI has high seed production potential and is prevalent in rangeland areas that have recently burned (Bhattarai et al. 2008), which may be especially significant considering the increasing fire frequency on western rangelands (Whisenant 1990) and the importance of fire as an ecosystem management tool in restoring rangeland function. Details of the field common-garden studies are contained in Bhattarai et al. (2008), whereas data and results of the population–structure analysis are presented in Bushman et al. (2010).

Nine phenotypic traits were measured on ASFI during 2 years at two common-garden sites (Providence and Millville) in northern Utah. Significant variation was detected among the collections for all phenotypic traits measured. Biomass yield is a key trait that integrates growth responses to the suite of biotic and abiotic characteristics at the particular common-garden site. High seed yield is critical in the commercial seed trade. High positive correlations were found between August biomass and seed yield at Millville for the 2 years of study ($r = 0.75$, $P < 0.01$; $r = 0.71$, $P < 0.01$). The number of stems was related to August biomass ($r = 0.68$, $P < 0.01$; $r = 0.73$, $P < 0.01$) and seed yield ($r = 0.67$, $P < 0.01$; $r = 0.46$, $P < 0.01$) such that assessment of the number of stems could be used as a surrogate to replace more costly and time-consuming biomass and seed-yield measurements in a selection program.

Principal component (PC) analysis identified two significant components, with the first principal component (PC1) accounting for 60.5 % of the total variation among collections, and the second principal component (PC2) describing an additional 15.4 % of the total variation. The PC1 loadings were high for biomass (0.95), seed yield (0.87), combined plant height and vigor score (0.80), and combined number of stems and inflorescences (0.93). Loadings for PC2 were high for seed mass (g per 100 seeds) (0.71). To estimate if trait variation was related to collection-site environments, correlations were calculated between trait values and collection-site environmental factors. PC1 was negatively correlated with collection-site elevation ($r = -0.71$, $P < 0.0001$) and positively correlated with precipitation ($r = 0.28$, $P < 0.05$) and mean annual minimum temperature ($r = 0.25$, $P < 0.05$). Collections from lower elevations tended to have greater biomass, seed yield, plant height, number of stems and inflorescences, and plant vigor scores, but lower winter mortality than collections from higher elevations. PC2 was positively correlated with elevation ($r = 0.36$, $P < 0.005$) and mean annual precipitation ($r = 0.26$, $P < 0.05$), and negatively correlated with mean annual minimum temperature ($r = -0.35$, $P < 0.005$). The positive correlation between PC2 and elevation suggested that collections from higher elevations tended to produce seeds with greater seed mass (g per 100 seeds) than those from lower elevations. PC2 analysis showed that collections from north-central Oregon generally had greater biomass and seed yield, number of stems and inflorescences, and greater plant height than collections from other locations. Plants of ASFI from collections in close proximity were somewhat more similar morphologically than those from more distant collections, as indicated by a significant positive correlation between geographic distance and morphometric distance ($r = 0.34$, $P < 0.005$). However, a PC clustering of phenotypic variables showed no strong tendencies for grouping of collections.

AFLP marker data showed that similarity index values (estimates of within-population genetic similarity or diversity) ranged from 0.622 to 0.789, which are similar values to those for other insect-pollinated outcrossing species (Bushman et al. 2007; Bhattarai et al. 2011). The similarity index values differed among collections with a geographical trend toward increasing diversity in central Oregon, suggesting again that Oregon may be a center of diversity for ASFI. The lowest within-population diversity was found in the southern periphery of the ASFI distribution in central Nevada and the northern periphery in British Columbia. Bayesian structure analysis of the AFLP marker data showed that collections from Oregon had high levels of admixture, indicating that collections had considerable shared ancestry. Collections from central Nevada and British Columbia were identified as distinctly separate population structures. This distribution pattern might possibly be explained by the expansion of populations from central Oregon to the north after glaciers receded during the last deglaciation (Licciardi et al. 2004). Alternatively, this pattern may simply represent geographical isolation with the bottleneck event limiting the number of alleles in the British Columbia founder population.

Structured groups were also detected for one group of 18 collections from Idaho, eastern Oregon, and eastern Nevada; a second group of seven collections from southern Oregon; and a third group of 30 collections from across California, Oregon, and Washington. Each of these three groups, however, exhibited considerable shared ancestry with few homogeneous collections, suggesting little genetic isolation among these groups. As a result, we recommended combining these three latter groups into one genetically based metapopulation to serve a specific geographical region (Bushman et al. 2010).

The relationship between genetic and geographic distances was investigated using the Mantel test. The correlation between genetic and geographic distance was significant, suggesting isolation by distance. Matrix correlations for elevation, mean maximum and minimum temperatures, and mean annual precipitation were either not significant or only weakly significant when corrected for geographic distance.

To summarize the results, our data from 67 collections suggested a center of diversity for ASFI in central Oregon with population movement north to the dry regions of British Columbia, south to intermittent montane areas in central Nevada, and east to the Snake River Plain in Idaho. Although some population phenotypes varied from high-elevation sites in peripheral areas of distribution, strong gene-flow barriers were only suggested in the extreme northern and southern groups of collections. However, crosses would need to be made between these extreme groups to confirm the existence of possible gene-flow barriers. We decided that one metapopulation of ASFI should be delineated in British Columbia (which is outside the Great Basin), a second in central Nevada, and a third that covers portions of Oregon, Idaho, northern Nevada, California, and Washington (Fig. 2). Because of the observed phenotypic plasticity and lack of strong gene-flow barriers in this third group (and yet the presence of a significant correlation between elevation and quantitative trait variation), we decided to bulk 12 collections across elevations within this group to reflect its broad diversity. These three metapopulations would be more tractable for seed companies to produce and maintain than many highly localized populations. A pre-variety germplasm (NBR-1 Germplasm) was released to the commercial seed trade to represent the third (and largest) metapopulation (Johnson et al. 2008). An additional release of ASFI is in the process of being developed for central Nevada.

4.3 Case Study 2: Dalea ornata

Dalea L. is a widespread genus of the legume family (Fabaceae) comprising 62 species of prairie clovers in North America (USDA Natural Resources Conservation Service 2009). *Dalea* is distinguished from other genera in the Amorphae tribe of Fabaceae by its base chromosome number (x) of 7 (rarely 8) and two collateral ovules (Barneby 1977). DAOR is an insect-pollinated species (Jim Cane, personal communication 2011) that is distributed throughout the northern Great Basin, southern Columbia

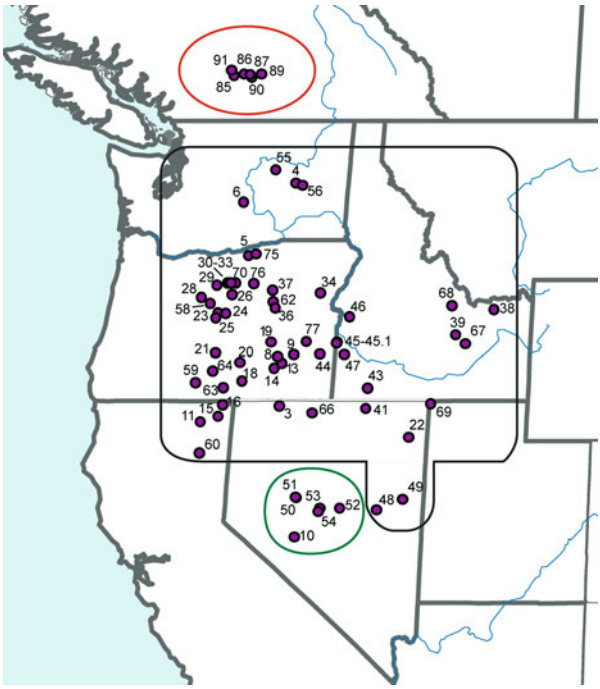

Fig. 2 Map depicting the three geographically based metapopulations for *Astragalus filipes*: (1) one source for British Columbia, (2) a second source for Washington, Oregon, northeastern California, Idaho, northern Nevada, and northwestern Utah, and (3) a third source for south-central Nevada

River Plateau, and Snake River Plain (USDA Natural Resources Conservation Service 2009). Its distribution, lack of toxicity to herbivores, and relatively upright growth habit make it a candidate for commercial seed production. The specifics of the field common-garden and AFLP studies for DAOR are contained in Bhattarai et al. (2010).

Eleven traits were measured for DAOR during two years at two common-garden sites (Millville and Hyde Park) in northern Utah. Significant variation was detected among the collections for all traits measured. Dry-matter yield of several collections of DAOR was equal to or greater than that of *Dalea purpurea* Vent. (seed obtained from Oak Prairie Farm, Pardeeville, WI), a commercially available species used in restoring prairie ecosystems in the midwestern USA. For the two common-garden sites, strong positive correlations were found between dry-matter yield and inflorescence weight ($r = 0.85$, $P < 0.001$; $r = 0.71$, $P < 0.001$) and number of inflorescences ($r = 0.61$, $P < 0.01$; $r = 0.71$, $P < 0.001$), suggesting that selection for high dry-matter yield would likely lead to greater seed yields, similar to our findings with ASFI (Bhattarai et al. 2008). In addition, selection for number of inflorescences could be used to indirectly select for seed yield in DAOR.

Principal component (PC) analysis identified three significant components that accounted for 89 % of the total variation among collections, with PC1, PC2, and PC3 each accounting for 57, 19, and 13 % of the total variation, respectively. The PC1 loadings were high for inflorescence weight (0.95), number of inflorescences at the two sites (0.80, 0.94), dry-matter yield at the two sites (0.91, 0.76), foliage diameter (0.63), and flowering date (−0.57). PC2 loadings were high for plant

height (0.74), flowering date (0.69), and dry-matter yield at Millville (0.56), whereas PC3 loadings were high for foliage diameter (0.64) and plant height (−0.53). A scatter plot of the collections based on PC1 and PC2 scores showed a clustering of collections originating from the Deschutes River and John Day River watersheds in central Oregon, with the remaining collections from southeastern Washington, eastern Oregon, and Idaho forming a large heterogeneous group.

The PC score correlations between trait values and collection-site environmental and topographic characteristics were calculated to identify traits with possible local adaptive significance, similar to the procedures used by Johnson et al. (2010b) in mapping genetic variation and seed zones for *Bromus carinatus* Hook. & Arn. in eastern Oregon. PC1 was not correlated with site elevation, mean annual precipitation, or mean annual temperature. PC2 was correlated significantly and positively with mean annual temperature ($r = 0.71$, $P < 0.0001$) and negatively correlated with mean annual precipitation ($r = -0.47$, $P < 0.05$) and site elevation ($r = -0.43$, $P < 0.05$). PC3 showed significant and negative correlation with site elevation ($r = -0.44$, $P < 0.05$). As a result, plant height, flowering date, dry-matter yield, and foliage diameter have possible adaptive significance in DAOR.

For the AFLP marker data, expected heterozygosity was estimated assuming Hardy–Weinberg equilibrium (as compared to the similarity index which was used for the ASFI data), and the expected heterozygosity in DAOR ranged from 0.09 to 0.17 with a mean of 0.13. Neighbor-joining dendrogram and Bayesian clustering analyses suggested that the five collections from the Deschutes River watershed were a relatively homogeneous genetically differentiated group from the other collections. In addition, 14 collections from Idaho, Washington, and eastern Oregon formed a second group. Three collections from the John Day River watershed were identified as a possible third group; however, the John Day River collections exhibited shared coancestry (genetic admixture) with collections from Idaho, Washington, and eastern Oregon. Further Bayesian clustering tests showed that the Deschutes River watershed group was strongly supported and that the admixture between the John Day group and the remaining collections was consistent, indicating that the John Day River watershed collections were genetically more similar to collections from distant sites than to those from the nearby Deschutes River watershed (Fig. 3).

Although collections from the Deschutes and John Day River watersheds in Oregon were located in relatively close proximity and no apparent gene-flow barrier exists between the watersheds, these collections did show molecular-based genetic differentiation. In addition, no major differences in climate or soils were apparent between the two watersheds. Furthermore, PC analysis of the measured phenotypic traits did not separate the two groups. One possible explanation for the molecular-based genetic differences between the John Day River and Deschutes River collections may be that differential historical disturbances between the two watersheds may have reduced population size in the Deschutes River watershed. The relatively low heterozygosity in collections from the Deschutes River watershed (mean < 11 %) compared to collections from the John Day River watershed (mean $= 16$ %) supports such a differential disturbance. In addition, the homogeneous

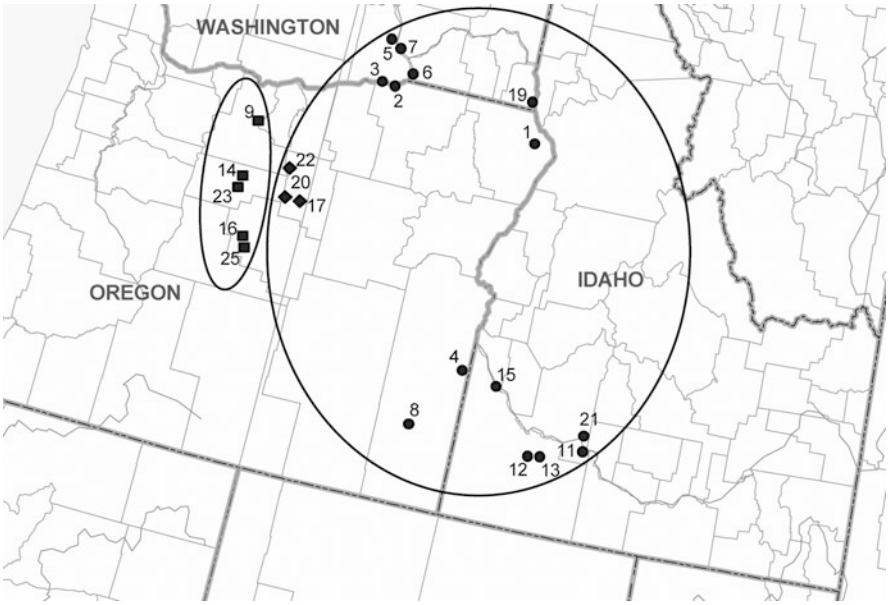

Fig. 3 Map depicting the two geographically based metapopulations for *Dalea ornata*: (1) one source for the Deschutes River watershed in north-central Oregon and (2) a second source for southeastern Washington, central and eastern Oregon, and southwestern Idaho. *Solid rectangles* represent collections from the Deschutes River watershed, *solid diamonds* represent collections from the John Day River, and *solid circles* represent all other collections

grouping of individuals and their north/south distribution along the Deschutes River suggested that these collections may have originated from the same ancestral population. However, additional research is needed to determine the exact placement of John Day River watershed collections within a specific population structure.

The Mantel test was used to determine if the marker-based genetic distances were correlated to the phenotypic, environmental, and geographic distances. A significant positive correlation was detected between the phenotypic and genetic-distance matrices ($r = 0.33$, $P < 0.005$), suggesting that in this case similar evolutionary processes may have operated in the diversification of the measured phenotypic traits and neutral genetic markers. Significant positive correlations also were found between the phenotypic and geographic distance matrices ($r = 0.35$, $P < 0.005$), and the genetic and geographic distance matrices ($r = 0.31$, $P < 0.01$). The genetic-distance matrix was not correlated with differences in precipitation or elevation; however, it was correlated with differences in mean annual temperature ($r = 0.28$, $P < 0.005$). Interestingly, flowering date was the sole discriminatory variable for the Deschutes River watershed group and the remaining collections from Idaho, Washington, and eastern Oregon (including the John Day River group). Flowering date has been considered a life-history trait (Crnokrak and Roff 1995), shown to generally reflect neutral genetic diversity (Merila and Crnokrak 2001). Our results for DAOR indicated that flowering date

was the phenotypic character most highly correlated with environmental characteristics of the collection sites. As a result, flowering date was a critical trait in defining both local adaptation and population structure in DAOR.

In summary, for restoration/revegetation purposes, the presence of genetic admixture and relatively small differences in molecular variance may not necessitate separating the John Day collections as a distinct group from the collections in Idaho, Washington, and eastern Oregon. As a result, two geographically based metapopulations were recommended for DAOR (Fig. 3): one for the Deschutes River watershed and the other for the remainder of the distribution. Accordingly, one pre-variety germplasm was released for the Deschutes River watershed (Majestic Germplasm) and another for sites across Washington, Oregon, and Idaho (Spectrum Germplasm) (Johnson et al. 2011).

4.4 Case Study 3: Dalea searlsiae

Dalea searlsiae (DASE) is a perennial legume in the Fabaceae family that is indigenous to the arid and semiarid southern Great Basin, southwestern Colorado Plateau, and northern Arizona in the western USA (USDA Natural Resources Conservation Service 2009). It is diploid ($2n = 14$, or rarely 16) (Barneby 1977) and primarily insect pollinated (Jim Cane, personal communication 2011). Its relatively upright growth habit makes it a potential candidate for commercial seed production. Specific details of the field common-garden studies and the population–structure analysis for DASE are discussed in Bhattarai et al. (2011).

Twelve traits were measured during 2 years at two common-garden sites (Millville and Hyde Park) in northern Utah. Significant variation was detected among the collections for all traits measured. Several collections exhibited agronomic characteristics comparable to commercially available *Dalea purpurea* Vent. (Oak Prairie Farm, Pardeeville, WI), which is used for restoration/revegetation of grasslands in the midwestern USA. Flowering dates ranged from 8 to 20 June. Dry-matter yield at both common-garden sites was significantly correlated with almost all other phenotypic traits, except for flowering and some forage quality traits. The number of inflorescences at the two common-garden sites showed a high positive correlation with dry-matter yield ($r = 0.88$, $P < 0.001$; $r = 0.64$, $P < 0.01$), inflorescence weight ($r = 0.86, P < 0.001$; $r = 0.76, P < 0.001$), and foliage diameter ($r = 0.80$, $P < 0.001$; $r = 0.86$, $P < 0.001$), and a negative correlation with flowering date ($r = -0.67$, $P < 0.001$; $r = -0.58$, $P < 0.01$). Plant height was positively correlated with the number of stems ($r = 0.71$, $P < 0.001$) and foliage diameter ($r = 0.60$, $P < 0.01$). Flowering date was negatively correlated with collection-site elevation ($r = -0.50, P < 0.05$) and positively correlated with mean annual temperature ($r = 0.50, P = 0.05$), suggesting that earlier flowering occurred in collections from high-elevation sites with a low mean annual temperature.

Principal component (PC) analysis identified three significant components that accounted for 87 % of the total variation among collections, with PC1, PC2, and

PC3 each accounting for 63 %, 13 %, and 11 % of the total variation, respectively. The PC1 loadings were high for inflorescence weight (0.95), dry-matter yield at the two sites (0.86, 0.78), number of inflorescences at the two sites (0.76, 0.61), foliage diameter (0.69), and plant height (0.48). Loadings for PC2 were high for forage quality traits (acid-detergent fiber, 0.75; neutral-detergent fiber, 0.94; crude protein, −0.72) and plant height (0.45), whereas for PC3 loadings were high for flowering date (0.92) and number of inflorescences at the two sites (−0.57, −0.64). Collections with high PC1 scores were considered useful for germplasm release or selection and breeding when the goal is to increase seed and forage for livestock and wildlife. Number of inflorescences, inflorescence weight, number of stems, and foliage diameter were also highly correlated with each other. In addition, the high positive correlations of foliage diameter or number of stems with dry-matter yield and inflorescence weight suggest that foliage diameter or number of stems could be used as surrogate traits to select for dry-matter yield and seed-yield potential.

AFLP marker data showed that the mean similarity index between individuals within each collection ranged from 0.64 to 0.77, with collections from northwestern Utah exhibiting the highest within-collection similarity (lowest genetic diversity). Collections from northwestern Utah represented the most northerly distribution of DASE, and the lower genetic diversity among these collections suggested a possible founder effect (Ray 2001). Both the neighbor-joining dendrogram and the Bayesian clustering results suggested that geographic distances tended to separate population structures. Consequently, isolation by distance was examined using genetic and linear geographic distances using the Mantel test. The genetic-distance matrix was highly and positively correlated with the geographic-distance matrix ($r = 0.77, P < 0.001$), even after controlling for the effects of elevation, precipitation, or temperature. Conversely, the genetic-distance matrix had only a low positive correlation with collection-site elevation ($r = 0.30$, $P < 0.011$) and with the phenotypic distance matrix ($r = 0.37, P < 0.005$).

The four collections from northwestern Utah exhibited the least genetic diversity of the collections, and also generally had lower dry-matter yield and number of inflorescences than the other collections. These collections were from a site located within the area of prehistoric Lake Bonneville (Oviatt 1997), and their differentiation may be due to migration when Lake Bonneville receded to the much smaller area covered by the present-day Great Salt Lake. Collections from across Nevada and southern Utah were the most diverse (lowest within-collection similarity). Two groups within these collections (located in western Nevada and southern Utah on the east–west periphery) showed a trend toward isolation, as indicated by their significant isolation-by-distance test and separation in the neighbor-joining tree, yet they were not separate enough to show significance in Bayesian structuring. Because of the genetically distinct differentiated group in northwestern Utah and the significant isolation by distance for east–west peripheral collections, it is hypothesized that gene flow may be playing a role in shaping the population structure of DASE.

One challenge with DASE was the location of the common-garden sites. Mean annual precipitation at collection sites varied from 152 to 372 mm, whereas precipitation at the common-garden sites was approximately 430 mm. Even so,

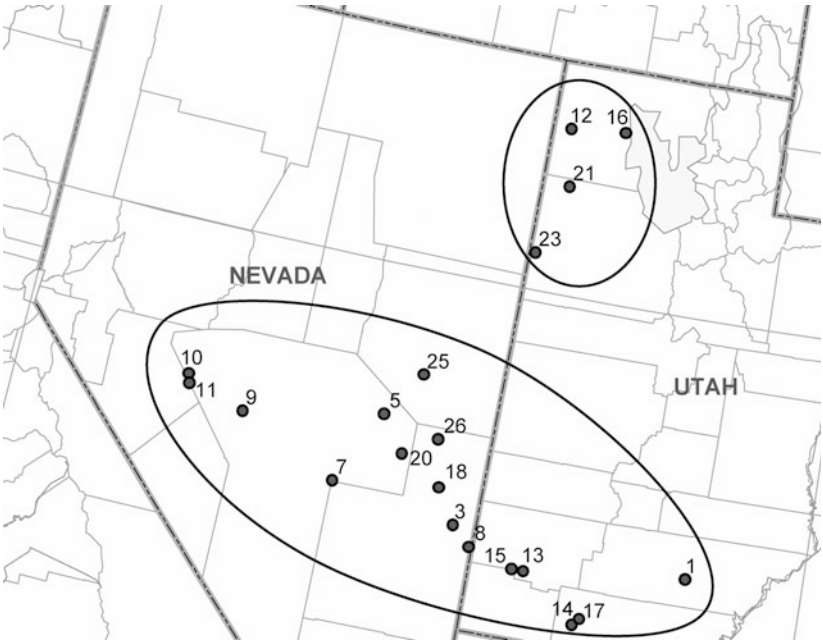

Fig. 4 Map depicting the two geographically based metapopulations for *Dalea searlsiae*: (1) one source for northwestern Utah and (2) a second source for southern Nevada and southwestern Utah

the four collections from northwestern Utah represented the strongest genetically differentiated group in our study and originated in an area with relatively low precipitation and probably high salinity. Consequently, we recommended establishing a geographically based metapopulation for this group. The larger group of collections from Nevada and southern Utah were from areas with a broader range of precipitation. Consequently, additional testing is needed to determine if gene flow within this latter group of collections is sufficient to counteract the possibility of local adaptive forces in areas with extremely low precipitation (McKay and Latta 2002).

In summary, collections of DASE from northwestern Utah and western Nevada exhibited low phenotypic values, whereas collections from southern Utah and eastern Nevada had high phenotypic values. AFLP marker data showed that collections from northwestern Utah were genetically differentiated from those in southern Utah and Nevada. Consequently, we recommend that one population be developed for northwestern Utah and another for southern Nevada and Utah (Fig. 4). Additional reciprocal-transplant studies are needed to clarify if collections from the low-precipitation areas of southeastern Nevada warrant the development of a third population for this area (Fig. 4).

5 Other Tools to Delineate Adaptation Zones for Rangeland Plants

Numerous plant species occur in various rangeland ecosystems in the Great Basin Region of the western USA. Ideally, it would be beneficial to have the two sources of genetic data (similar to those described earlier) for the major species used for rangeland restoration/revegetation projects. Common-garden and DNA marker techniques are being applied to other species through the Great Basin Native Plant Selection and Increase Project (USDA Forest Service 2010). Until these evaluations are completed, other tools are needed to identify which populations of species can be used for specific rangeland areas of the Great Basin.

One option for delineating where various seed sources can be used involves the use of ecoregions (Ward et al. 2008; Miller et al. 2011), which are geographical areas within an ecosystem that denote similar types, qualities, and quantities of environmental resources (Omernik et al. 2000). The approach used to identify specific ecoregions is based on the premise that ecological regions can be identified through the analysis of patterns and composition of biotic and abiotic characteristics that reflect differences in ecosystems (Wiken 1986; Omernik 1987, 1995). These characteristics include geology, physiography, vegetation, climate, soils, land use, wildlife, and hydrology. By recognizing spatial differences in the capacities and potentials of ecosystems, ecoregions can be used to stratify the environment by its probable response to disturbance (Bryce et al. 1999). These general-purpose regions are being used in the USA to structure and implement ecosystem-management strategies across various agencies and nongovernmental organizations responsible for managing different types of resources within the same geographical area (Omernik et al. 2000; McMahon et al. 2001). Ecoregion maps serve as a spatial framework for the assessment and monitoring of ecosystems and ecosystem components (U.S. Environmental Protection Agency 2011).

Another proposed option for identifying where seed sources of rangeland species can be planted involves the development of provisional seed zones, which are defined as areas "within which plant materials can be transferred with little risk of being poorly adapted to their new location" (Bower et al. 2010; USDA Forest Service 2011). Provisional seed zones are intended for species without specific genetic information and were developed using overlays of temperature and precipitation data (1971–2000) in combination with Level III ecoregions (Omernik 1987, 1995). Provisional seed zones follow similar efforts of Vogel et al. (2005) who developed "plant adaptation regions" that involved the overlay of ecoregion maps on the USDA Plant Hardiness Zone map. Bower et al. (2010) proposed using provisional seed zones based on minimum winter temperature and annual precipitation overlaid on Level III ecoregions for trees, shrubs, and woody vegetation. For grasses and herbaceous plants, provisional seed zones are based on average maximum temperature and annual precipitation overlaid on Level III ecoregions. Early work suggests that 35 seed zones would be needed for grasses and herbaceous species in the Great Basin, while 30 zones would be needed for trees, shrubs, and woody vegetation (Andrew Bower, personal communication).

6 Conclusions

Rangeland ecosystems of the Great Basin Region of the western USA and throughout the world are facing unprecedented challenges related to natural and human-caused disturbances. A wide diversity of opinion exists concerning the best approaches and plant materials to restore/revegetate rangelands to a more desirable ecological state. Identification, development, and availability of effective and affordable plant materials are critical to restoration/revegetation efforts. Case studies of three leguminous species indigenous to the Great Basin presented herein used common-garden evaluations and DNA marker analysis to identify geographically based metapopulations for release as pre-variety germplasms to the commercial seed trade. Further field testing and evaluation of these metapopulations are needed to compare these with other plant materials. The procedures used in our three case studies and other approaches reviewed earlier may be useful in developing plant materials of other insect-pollinated, herbaceous plant species for use in the restoration/revegetation of degraded rangeland ecosystems in other parts of the world. Close working relationships between user agencies, plant material developers, and the commercial seed trade are essential for plant materials to be effective, affordable, and available for rangeland restoration/revegetation projects.

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Invasive Alien Plants and Their Effects on Native Microbial Soil Communities

T. Steinlein

Contents

Abstract Invasion ecology has become a significant issue in contemporary ecological research. Detecting the success of alien plant species in invading native plant communities (invasiveness) and observing the plant community's ability to repel this invasion (invasibility) are central topics. In this chapter, the interaction of the native soil community with the alien invader is discussed. The first section describes plant traits of successful invaders that may dramatically alter soil community's composition. Then the effects of exotics on soil structure, physical properties of soils, and changes in nutrient cycling are analyzed. In addition to this biogeographical aspects, the reactions

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of soil communities in the native and nonnative range of alien invaders are compared and assessed. The second part describes the interaction of invaders with soil pathogens, mutualistic fungi and bacteria, and decomposers.

1 Introduction

Since the inspiring work of Charles S. Elton "The ecology of invasions by animals and plants" in 1958, a multitude of case studies, reviews, and meta-analyses have been published on invasive alien plant species and their effects on native species or native plant communities. As a result invasion ecology has become a central topic of contemporary ecological research in the last decades all over the world.

But what is an invasive plant? Cronk and Fuller's (1995) definition underlines the impact on the ecosystem that is invaded: "a plant invader is an alien plant spreading naturally (without direct assistance of people) in natural or semi-natural habitats, to produce a significant change in terms of composition, structure or ecosystem process." In this review, I will use the more neutral and unbiased definition given by Daehler (2001), who argued that the primary criterion for a species to be considered an "invader" (other than being new to a certain region) should be that the new species is spreading in a new environment.

There are several reasons why ecologists are captivated by the field of biological invasions. First of all biological invasions tend to homogenize the earth biota and are—together with the current tremendous changes in land use and the effects of global warming—recognized as one of the most important threats to biodiversity (Lodge 1993; Walker and Steffen 1997; Vitousek et al. 1997; Mooney 1999; Mack et al. 2000). But overall, only a small number of these nonnative plants, animals, and microorganisms have been implicated in the cases of native species extinctions (mainly invasive aliens on islands). Additionally, research on invasive plant species is increasingly focusing on the problems related to global change, as species invasions do not only respond to but are also an integral part of global change (Vitousek et al. 1996).

Second, invasive alien plant species can cause severe economic, environmental, and sometimes even health threats (Mack et al. 2000; Pimentel et al. 2005; Pejchar and Mooney 2009). Already in 1988 Usher stated that there are virtually no natural areas left on our globe that have not felt the impact of nonnative invaders (Usher 1988). Invasive plants can severely alter ecosystem functions and processes (e.g., soil structure and function, nutrient or hydrological cycles, fire frequency and/or intensity, plant–animal interactions), and thus they impair native species and ecosystems by competing directly for the resources (nutrients, space, species relationships, and interactions) that native species require (Levine et al. 2003).

A third and perhaps more positive reason is that biological invasions of specific species provide great natural online experiments for ecologists and evolutionary biologists and their analyses are extremely valuable for understanding population spread, maintenance, and survival (Sakai et al. 2001). In these "experiments" we can

investigate on large spatial and temporal scales (scales which are hardly accessible to experimental ecologists under normal circumstances) how community- and landscape-level processes affect the patterns and abundance of species. The outcome of such analyses may be valid for native as well as for alien plant species.

The problems described earlier must, although they are of global concern, always be evaluated on a local or regional scale. There is evidence that most of the naturalized alien plant species may act ecologically similar to the indigenous (resident) species and occur in new plant communities only at low or mid frequencies (Huston 1997; Davis et al. 2000; Reinhart and Callaway 2006). They may move into previously empty niches or partly displace indigenous species. As Levine et al. (2003) and Hierro et al. (2005) have pointed out, only a small proportion of introduced alien (nonnative) species become dominant on a local (or sometimes regional) scale and transform diverse plant communities into communities dominated by only one species (see also the tens rule according to Williamson 1997).

But invasion ecology has gone through changes since the publication of Elton's book in 1958. Early studies on the invasive potential of alien plant species focused on weedy traits (Baker 1974) and/or on the patterns, mechanisms, and evolution of species invasiveness (e.g., large and few vs., numerous and small seeds, clonal vs. sexual reproduction, high vs. low competitive strength, tolerance to harsh environmental conditions, initial growth rates of seedlings, resource allocation patterns). Thereafter studies have looked at the invasibility of certain native plant communities (Stohlgren et al. 1999). But habitat invasibility, i.e., the susceptibility of a specific habitat to invasions by exotic plants is a very complex attribute. Many factors can contribute in total to differences in habitat invasibility such as disturbance, resource availability, habitat fragmentation and accessibility, evolutionary history, propagule pressure, predation, mutualism, and competition (Richardson et al. 2000a, b). At the moment it is widely recognized that man-made disturbance (e.g., overgrazing, fires, soil disturbance)—even in the attempt to exclude or to "combat" alien plants—is a major reason for the successful establishment and spread of alien plants (Hobbs and Huenneke 1992; Dietz and Steinlein 2003).

In his early work, Elton (1958) suggested that with increasing diversity of a community resistance to invasion may also increase as a consequence of more tightly connected food webs. But even this high biodiversity of the invaded community, which is often thought by many authors to be a safeguard against alien invasion (e.g., Prieur-Richard et al. 2000; Dukes 2001; Kennedy et al. 2002), may not always protect communities from being invaded (Lonsdale 1999; Pyšek et al. 2002). In highly diverse communities, alien plant species may be just as supported by greater habitat diversity as the natives are. In more diverse communities, invasions may be facilitated by a more diverse array of pollinators, dispersers, fungi, and bacteria (Richardson et al. 2000b). Many plant invaders can even profit from enemy release in the introduced range. But plant invasions are very complex phenomena that defy simple causes and explanations in most cases. Generalizations on success of invasive alien plants are rather coarse level and tend to have low predictive power.

In the process of searching in the last two or three decades for causes that promote successful invasions, researchers have drawn attention to a formerly neglected field: the interaction of soil biota and invasive plants. Wardle et al. (2004) showed in a detailed review in what manner and how closely the aboveground and the belowground subsystems are interlinked.

It is hypothesized that soil properties, soil conditions, and the respective soil communities may (or may not) prevent an alien plant species from invading into an indigenous plant community. In general, many authors argue that the invasive potential of an alien plant species depends more on the efficient interaction with the native soil community at a newly invaded site than on above- or belowground competition with the native plants in the community. Charles S. Elton (1958) did not address the effect of soil biota on alien plants (and animals), but many subsequent publications have shown this, especially how pathogenic soil organisms like bacteria and fungi affect the establishment of alien plant species. It should be noted that the study of the effects of soil biota on alien invasion has caused a marked increase in our understanding of the role soil organisms in community ecology, in general. Mordecai (2011) discovered that soil pathogens shape natural communities and developed the feedback concept. Klironomos (2002) stated that there may be no plant/soil feedbacks for natives, but negative feedbacks for invaders. Liao et al. (2008) discussed the relationship of soil biota and the alteration of ecosystem functions in the process of invasion. Rout and Chrzanowski (2009) discovered that invaders may bind local soil mutualists as endophytes.

But an absolutely essential precondition remains: do these soil–plant interactions benefit nonnative species more than they do indigenous plants? There are investigations that affirm this hypothesis: In many plant communities (nonexperimental field studies) soil microbial community of sites dominated by invasive alien plants had negative effects on natives by reducing their growth (Allen et al. 2003; Yu et al. 2005). The invaders were either unaffected or positively affected. However, native species were not negatively affected, if the plants were grown in "invader-free" soil. These neutral or positive effects (nonnative) and negative effects (native) of soil biota were confirmed by controlled experimental studies (Callaway et al. 2003; Reinhart and Callaway 2006).

In this review, I will discuss the following aspects of the interdependence of soil biota and invasive alien plant species: I will start with the plant invaders view showing (1) plant traits, which will drastically alter soil functioning, and then give (2) some information as how alien invaders change plant litter and affect decomposing processes. After this I will discuss (3) biogeographical aspects and (4) changes in soil properties and structure. In the second part, I will focus on the other side of the story: the role of specific groups of microorganisms: I will examine (5) the role of microbial pathogens and parasites in the invasion process, (6) the role of symbiotic relationships (mainly mycorrhizal) of microorganisms and invasive plants, and (7) the effect of alien plants on soil decomposers.

I will not review the effects of nonnative, exotic soil microorganisms (bacteria, fungi) that invade plant communities. Up to now there are very few studies that have investigated these problems. Currently there are no reports on invasive soil-borne bacterial pathogens (Van der Putten et al. 2007).

2 The Plant Invaders View

Invasive species may alter the above- or the belowground community structure through exploitation competition (indirect interactions such as alterations or changes in resource use) and interference competition (direct interactions such as allelopathy in plants via roots or leaves or leaf litter decomposition) (Callaway and Ridenour 2004).

In general, the extent or the quality of changes in soil properties by alien plant species depends on initial soil conditions like climate, topography, soil texture, soil type, nutrient budgets and cycles, water budget, and biotic relations in the specific soil (Dassonville et al. 2008). Alien plants have the potential to alter components of nutrient, and water cycles, and may increase carbon stock by higher net primary productivity (Liao et al. 2008). Liao et al. (2008) detected in a meta-analysis of 94 experimental studies an increase of 5% in root carbon stock compared to an increase of 133% in shoot carbon stock. Compared to ecosystems with only native plants, the aboveground net primary production and the litter decomposition increased by 50–120% in invaded ecosystems. Changes in nutrient cycling processes may reflect alterations in the soil microbial community and invasions by alien plant species may result in dramatic changes in the structure of above-ground (plant community) and belowground (soil community) species abundance and dominance. In addition, invasive plant species can massively alter ecosystem soil functions (Weidenhamer and Callaway 2010). Whether these changes in soil–plant feedbacks are positive or negative depends on the balance of the aggregation of soil-borne pathogens, parasites, and herbivores (negative, Van der Putten et al. 2001) and on the accumulation of mycorrhizal fungi, nitrogen-fixing bacteria, and other plant-stimulating bacteria (positive, Garbaye 1994). These direct effects will in turn affect the belowground plant–plant interactions (Bever 2003).

Overall, the constant "revolution" or the exhaustive change in soil community structure, physical soil properties, nutrients, and water can contribute to and initiate an "invasional meltdown" process (Simberloff 2006). This may favor the invasion or spread of other invasive plants into this already transformed or altered habitat/plant community.

2.1 Traits of Alien Plant Species that Alter Soil Functioning

There are key traits of invasive alien plant species (Fig. 1), some of which are new to specific ecosystems, that may influence ecosystem processes and functions and can dramatically influence other species interactions (predation, herbivory, parasitism, and mutualisms) leading to changes in the abundance of native species (Chapin et al. 2000).

One of the simplest traits is a relative difference in size of individual plants of native and nonnative plant species: a taller size (or higher biomass) and/or a higher leaf area of the alien can help the plant to overtop the native plants. Higher biomass

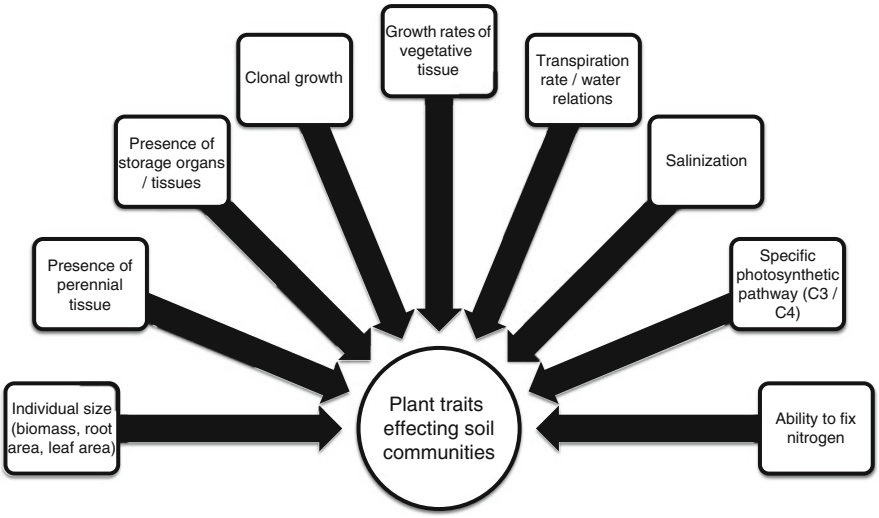

Fig. 1 Specific invader traits that may alter soil functioning in nonnative habitats

or leaf area is direct competitive advantages. These size effects can also lead to a higher shading of the soil, thus influencing the heat budget of the soil. It can also cause a lower interception or precipitation together with higher transpiration and thereby influence the water balance of the soil. In Europe the herbaceous fast growers *Fallopia spec*. or *Heracleum mantegazzianum*, one of the tallest herbs in Europe, are good examples (Tiley et al. 1996; Siemens and Blossey 2007). Steinlein et al. (1996) demonstrated the competitive advantage of overtopping or overshading of native neighbors for the nonnative herb *Bunias orientalis*. Twice as tall as the native ruderals, *Bunias* plants exhibited a threefold higher biomass. The higher standing crop resulted in lower soil water status and increased mineralization rates compared with invader-free sites. But there are also antithetic reports: for example, in the Himalayan region small shrubs (*Lantana camara*) substitute tall oak trees (Bhatt et al. 1994).

Another important trait is the presence of perennial tissue. This may not only alter nutrient cycling processes in the system by storing of nutrients in this tissue (e.g., annual vs. biennial or perennial), but also alter the retranslocation of nutrients from plants to soil or vice versa. Perennial tissue may also result in a high amount of carbon stored in this tissue (with a high C:N ratio) and in the case of woody species results in lignin-rich litter. This may affect the timing and quality of litter production and decomposition rates. Also, the presence of particular storage organs (roots, or in most cases, rhizomes) may accumulate and increase carbon (and nitrogen) pools. An example is the alien giant reed (*Arundo donax*). Stored carbon helps the plant to develop stems that can reach heights of nearly 10 m (ten times higher than natives) causing massive overshading of the natives and resulting in a much higher biomass in the respective community (Dudley 2000). Clonal growth and thereby storage of carbon may also change carbon soil budgets (e.g.,

Phragmites australis in Windham 2001; *Gunnera tinctoria* in Hickey and Osborne 1998).

Besides nutrients one of the most important (and most widely studied) ecosystem processes is the change and modification of the water flow and budget. The exotic *Imperata cylindrica* exhibited a very high evapotranspiration rate, thus drying out the topsoil in the humid lowlands of Papua New Guinea (Hartemink and O'Sullivan 2001). Changes in soil moisture, e.g., by increases in evapotranspiration, may strongly influence microorganisms and processes in the topsoil. But which morphological and physiological properties of alien species have an effect on water relations in the soil? A larger plant size, a higher leaf area, the spatial extent of the above- or belowground parts, differences in rooting depths, or higher transpiration rates may account for these changes. A very prominent example is *Tamarix ramosissima,* which alters water budgets by having higher leaf areas and higher transpiration rates than natives (Sala et al. 1996). The invasive *Halogeton glomeratus* salinizes the soil with sodium by transporting it to the surface and depositing there, thus increasing sodium and nutrients (nitrogen, phosphorus, potassium) content compared to the native ecotone (Duda et al. 2003). For the most part these effects are not caused by physiological alterations (e.g., higher transpiration rates per unit leaf area), but rather as a result of changes in higher leaf area, overall higher growth rates, or a higher total biomass. McCarron and Knapp (2001), for example, did not find differences in soil moisture content between a C_4 grass and several invasive species of C_3 shrubs, although they have different photosynthetic pathways.

Williamson and Fitter (1996) have pointed out that there is a clear correlation between size, leaf area, and invasiveness in the British Flora. The bigger the root and the larger the root area of an (invasive) plant, the greater its influence and zone of control over the soil community.

In 1987 Vitousek et al. showed that an introduction of a new functional plant trait, namely, the ability to fix nitrogen via bacteria, had an enormous effect on plant communities. This classic example of *Myrica faya* altering Hawaiian ecosystems has been extensively cited and summarized by many authors (e.g., Vitousek and Walker 1989; Hobbie 1992; Burleigh and Dawson 1994; Cox 1999; Vogelsang and Bever 2009). *Myrica faya* and its nitrogen-fixing root symbionts (*Frankia* spp.) invaded and changed plant communities that were previously nitrogen limited. Hence, nitrogen-rich habitats were generated. As demonstrated for *Myrica faya,* the ability to fix nitrogen is one of the most "effective" and differentiating traits. Another example of how this trait can dramatically alter plant communities is *Acacia longifolia*, which in Mediterranean coastal dune systems alters native plant communities by increased nitrogen input (Hellmann et al. 2011; Grieve Rascher et al. 2011). Similarly in a riparian region a large input of nitrogen into the soil was reported, caused by the invasive alien tree *Robinia pseudoaccacia*, which may possibly lead to changes in the natural plant community structure (Akamatsu et al. 2011).

But there are also other plant traits that may tremendously influence ecosystem processes and functioning and thereby change the plant species composition of these systems (Fig. 1). The greater the difference in the traits (native/alien species)

or the "newer" the traits that are introduced into an existing plant community, the bigger the effects they will generate.

Each invasion process has its individual rules and depends in most cases on the initial site or soil conditions. Although these new and specific traits described earlier may terrifically alter soil conditions, Daehler (2003) analyzed 79 independent native–nonnative invasive plant comparisons and found out that statistically aliens did not exhibit higher competitive abilities than natives. In 94% of 55 comparisons involving more than one growing condition, the performance of the individuals of the native plant was equal or even superior to that of the invader. He even found some "super invaders" with universal performance advantages over co-occurring natives. In most cases it must therefore be a combination of different plant attributes that in sum causes the advantages of invasive species.

2.2 *How Alien Plant Species Increase Soil Nutrient Concentrations Through Increased Living Biomass and Plant Litter*

We can find copious evidence of the self-facilitating effects of invasive aliens on soil communities thus altering water relations or nutrient concentrations mediated by substantial positive feedbacks between plant roots and soil biota (e.g., Duda et al. 2003; Hawkes et al. 2006; Jordan et al. 2008). Comparing invaded sites with nearby uninvaded sites, most published studies detect increases in soil nutrient stock and/or an increase in resource availability below invasive plant species, even if the plant species are not nitrogen fixers (Duda et al. 2003; Vanderhoeven et al. 2005; Chapuis-Lardy et al. 2006; Liao et al. 2008). There are only a few reports that show the opposite (e.g., Leary et al. 2006).

But what is the mechanism underlying this phenomenon that the presence of invasive aliens leads to higher nutrient levels in the soil? In many cases plant invaders can be described as species with high growth rates. If these plants become dominant in certain sites, the standing crop biomass will be enormously enlarged. In our studies (Steinlein et al. 1996; Dietz and Steinlein 1998; Dietz et al. 1999) the alien invader *Bunias orientalis* caused a 2.6–3.1-fold increase in standing crop, compared to a site with only native ruderal fast growing herbs (*Urtica dioica, Filipendula ulmaria, Artemisia vulgaris, Aegopodium podagraria*). *Heracleum mantegazzianum* (2.1–3.3-fold increase) and *Fallopia japonica* (1.8–2.3) had similar effects on standing crop (Steinlein, unpubl. data). This greater net primary production and higher plant biomass causes a much greater amount of leaf litter, which stimulates the activity of decomposers and may lead to a higher carbon content in the top soil (see Fig. 2). In a screening of Ehrenfeld (2003), nonnative plants exhibited in 14 out of 18 cases an increase in standing crop and in 10 out of 12 cases plants had a higher net primary productivity. Increased carbon means higher activity of the soil microbial community, including nitrogen-fixing bacteria and this elevates nitrogen concentration and perhaps pool size in the topsoil.

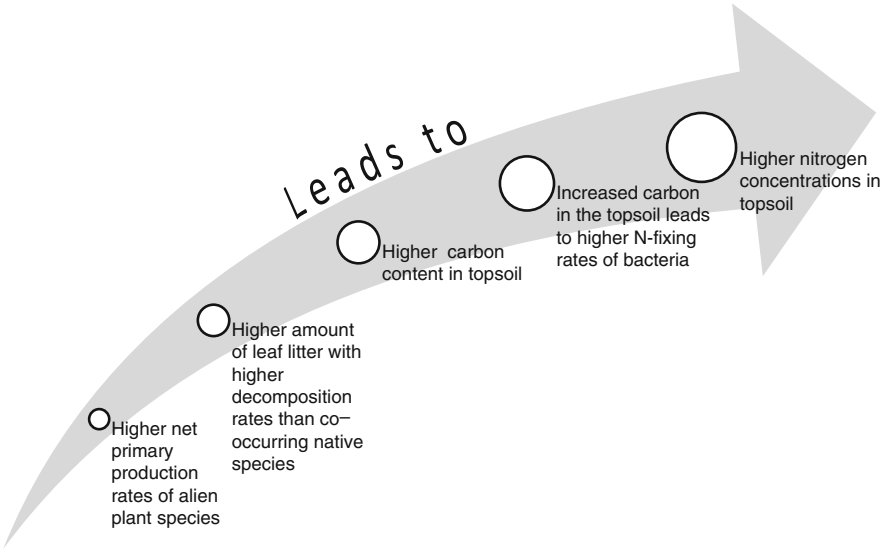

Fig. 2 Model how an increase in biomass of alien invaders may change nitrogen contents and dynamics on invaded sites

However, the increase of cations and phosphorus in the topsoil can be explained by nutrient uplift phenomena. Deep-rooting plant invaders provide access to deeper soil layers and transport these resources to the soil surface (Jobbagy and Jackson 2004). Certainly it is clear that, excepting the invasion of sites poor in nitrogen with nitrogen fixers, invasive plants cannot change nutrient-poor soils into soils with an overabundance in soil resources and infinite availabilities. In a field study with seven invasive plant species invading sites in Belgium, Dassonville et al. (2008) found out that these invaders had a high impact on the increase of resources, but this was always correlated with initial soil site conditions. But Dassonville et al. (2008) argued that "the particular site-specific pattern in the impact that we observed provides the first evidence that alien invasive species may contribute to a homogenization of soil conditions in invaded landscapes." In their study on sites with small pool sizes of the particular nutrients, such as potassium, magnesium, phosphorus, manganese, and nitrogen in the upper soil layers, aliens had large positive effects on soil resources, whereas on sites with higher resource availabilities effects of invasive alien decreased. But in all of their analyses—as described earlier—the invaded plots always had higher aboveground biomass (achieved by higher growth rates or increased ground cover with leaves) and nutrient standing stock (see examples earlier). There may be a kind of nutrient/nitrogen loss or leakage especially on nutrient-rich soils in winter, because the above-mentioned species (*Heracleum mantegazzianum*, *Fallopia spec*., or other perennial herbs) die back in winter and leaves do not cover the soil in winter: leaf material is mineralized and eventually nutrients are washed out.

In many cases increasing nutrient levels are very site specific (Dassonville et al. 2008), depending primarily on the initial soil conditions before the invasion process started. Again this stresses the importance of the specific soil community initially present on an invaded site. Surely environmental factors like soil type or climate may also play an important role. In most cases, however, if there is a change in nutrient availabilities and concentrations, the pool sizes of the respective nutrients are not changed (Ehrenfeld 2003). It must also be mentioned that one has to distinguish clearly between effects that were caused by the invader‘s influence on the soil community and effects that were caused by the soil itself. In many incidents of successful invasions, the invasions occur as a consequence of man-made soil disturbances. It is known that these perturbations of soil layers often lead to nutrient flushes in the topsoil or changes in nutrient dynamics of pools and fluxes (e.g., Guo et al. 2004; Jentsch et al. 2009).

2.3 *Biogeographical Aspects: Native Range Versus Nonnative Range*

In most cases the coevolution or coadaptation with soil biota controls the spread and the relative abundance of invasive species in their home range. But what happens when these plants invade new habitats thus escaping their natural enemies, like competitors, pathogens, and/or predators (ERH, enemy release hypothesis, Elton 1958)? This often leads to an enhanced spread in the new region (Wolfe 2002). The greater the intensities of mutualistic positive relationships (mainly mycorrhizal) and the greater the host specificity (positive feedback of plant species with soil microorganisms), the lesser will be the invasion success in a new nonnative range.

On the other hand, the greater the control by pathogens or parasites in the native range (negative feedback) the higher will be the risk of enhanced spread and the building of monocultures of this species in the nonnative range. In contrast to low host-specific arbuscular mycorrhizal fungi (AM-fungi), we find reports on high host specificity of pathogens and plants (see later). A prominent example is the tree *Prunus serotina*, which has shown a negative feedback with *Pythium* spp., a fungal-like organism. In its home range, the spread, abundance, and performance of *P. serotina* are controlled by *Pythium* (Packer and Clay 2003). But without this negative interaction in the new nonnative range in Europe *P. serotina* expands into forest and linear woody habitats forming dense dominance stands (Starfinger et al. 2003). There is a specific experimental setup to test these home range/new range effects. Plants were grown in soil from their home range and in soils from the new invaded sites. Many of the species investigated were inhibited in growth and reproductive parameters when grown in their “home soil”: e.g., *Centaurea maculosa* (Callaway et al. 2004), *Ammophila arenaria* (Knevel et al. 2004), *Carpobrotus edulis* and *Carpobrotus* × *cf. acinaciformis* (Van Grunsven et al. 2009), and *Tragopogon dubius* (Van Grunsven et al. 2010). These effects may be remarkable: Callaway et al. (2004) used the fungicide Benomyl to sterilize soil from the native range of *Centaurea*

maculosa and compared it to soils from the nonnative range. Growth performance of *C. maculosa* was increased on average by 166% compared to the unsterilized native soil but only increased on average by 24% when compared to the unsterilized nonnative soil.

If there is a high host specificity (e.g., ectomycorrhizal symbiosis) exotic plants like *Pinus* species can also be limited in their spread and establishment if suitable symbionts are not available (Pinaceae species, Nuñez et al. 2009). Generally the number of aboveground (and belowground) pathogens and/or viruses decreased in the introduced regions. There are only very few examples where there are no biogeographical effects, when comparing home ranges with nonnative ranges (see Table 1). Besides the soil community interaction effects described earlier, there are some other hypotheses that try to explain the varying success of alien invaders: Alien invasives may introduce "novel" weapons of biochemical interactions to recipient communities (novel weapons hypothesis, Callaway and Ridenour 2004). Callaway and Aschehoug (2000) showed for the European *Centaurea diffusa* (an European knapweed that has invaded into American grassland) that the produced allelochemicals suppressed both the Eurasian grasses of the home range and the North American grasses on the nonnative sites. But the effect was much higher on the American grasses. *Centaurea maculosa*, native to Europe, produces a (−)-catechin with phytotoxic properties that can be found in soils *C. maculosa* is growing on. Again the "native" European grasses were more resistant to this substance than their American counterparts (Bais et al. 2003). It is interesting because (+)-Catechin can also play an important role in the complexation of Fe, Al, and Ca in the soils (Kidd et al. 2001).

In a study with *Mikania micrantha*, an invasive plant in forests in South China, Chen et al. (2009) found significant effects of the plant on soil carbon, nitrogen, ammonia, and net nitrification rate. Aqueous plant extracts of *M. micrantha* decreased soil pH and increased carbon and nitrogen. Therefore the authors argue that the water-soluble allelochemicals change soil community structure.

Prati and Bossdorf (2004) tested the allelopathic effects of the invasive alien *Alliaria petiolata* (a Brassicaceae species originating in Europe) on the two co-occurring species *Geum laciniatum* (native to America) and *Geum urbanum* (native to Europe). They found a clear reduction in seed germination in *G. laciniatum* but not in *G. urbanum* by the North American *A. petiolata* plants, but surprisingly similar effects on both species by *A. petiolata* plants from Europe.

All of these studies indicate that when these allelochemicals are released into the nonnative soil, they will scramble the soil community and disrupt processes that have been occurring in the communities before the invasion process. But experiments on allelochemicals have to be assessed very carefully: often experimental setups in the laboratory and the respective results differ exceptionally from field results. Especially clay minerals or other soil colloids may fix and inactivate these substances and temperature changes may reduce their activity. It is also uncertain whether these allelopathic substances occur in sufficient concentrations in the soil to impede soil microorganisms (see Müller 2009).

Table 1 Plant–soil biota interactions in native and nonnative distribution ranges of invasive plants. Redrawn from Reinhart and Callaway (2006)

	Soil community effects		
Species	In native range	In nonnative range	Reference
Acer negundo	⊖	⊖○⊕	Reinhart and Callaway (2004)
Acer *platanoides*	⊖	⊖○⊕	Reinhart and Callaway (2004)
Ammophila arenaria	⊖	⊖	Beckstead and Parker (2003)
Ammophila arenaria	⊖	⊖○⊕	Knevel et al. (2004)
Centaurea maculosa	⊖	⊖	Callaway et al. (2004)
Prunus serotina	⊖	○⊕	Reinhart et al. (2003), Starfinger et al. (2003)

+/- and empty circles indicate the effect on growth and performance on the respective plant in its home or nonnative range.

⊖ Strong negative effects ⊖ Mild negative effects

○ No or neutral effect ⊕ Positive effect

Rapid evolutionary changes by genetic differentiation as adaptation to new environments may lead to alterations of native and introduced populations and thus will favor exotics (Bossdorf et al. 2005). Maron et al. (2004) compared *Hypericum perforatum* plants of different genotypes from native and nonnative ranges and found differences in lower pathogen resistance and defense chemistry in the nonnative plant communities. In spite of having higher amounts of carbon (that do not have to be invested into defense chemistry or structure), the authors did not observe higher growth rates in the nonnative plants. A very old hypothesis is the "empty niche" hypothesis (Elton 1958), with exotics using soil resources unused by the local species (in space or time). *Centaurea solstitialis*, a successful invader building dominance stands in grasslands in California, uses "unused" water 60 cm below the soil surface by an extensive and deep-rooting system. The shallow root systems of the native grasses cannot reach such soil depths in their home range (Dyer and Rice 1999).

2.4 Changes in Soil Properties and Structures

The number of publications describing differences found in soil properties of sites dominated by nonnative invasive plants and adjacent sites covered by native plants has increased in the last 20 years. But finding differences is one thing, explaining the mechanistic background of these changes is another. Some of the factors that may change soil inputs are summarized in Fig. 3.

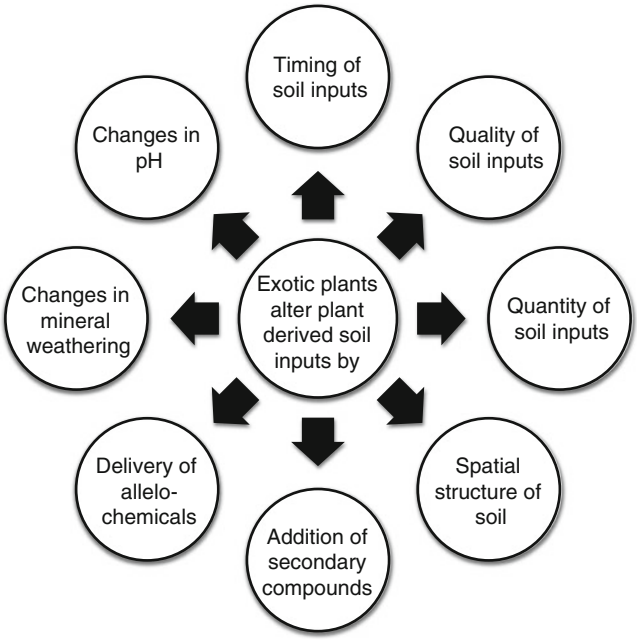

Fig. 3 Possible effects on nonnative soils of alien invasive plants by specific plant-derived soil inputs

There are several reports of changes in the physical properties of soil after the arrival of nonnative species. Kelly et al. (1998) reported that new plant species changed the amount of mineral weathering, thus leading to changes in soil structure. Finzi et al. (1998) reported changes in soil pH caused by trees in temperate forests, leading to higher nutrient availabilities in the soil water phase. Changes in pH may be caused by a higher degree of nitrification and uptake of NH_4^+ (decrease in pH) or a higher uptake of nitrate as the preferential nitrogen source (increase in pH). Sometimes also changes in litter quality (more acidic) may cause a decrease in pH. Early goldenrod (*Solidago gigantea*) was reported to cause 20–30% higher labile phosphorus fractions in the soil and a lower pH compared to invader-free habitats (Herr et al. 2007). The authors ascribe this phenomenon to higher phosphorus turnover rates and mobilization of sparingly soluble phosphorus through rhizosphere acidification in *S. gigantea*. Already in 1999 Saggar et al. found changes in organic matter and changes in soil aggregation under the invasive *Hieracium pilosella*. But also decreases in soil carbon and nitrogen levels occur: due to its low root–shoot allocation the invader *Agropyron cristatum* degrades prairie soils in America (Christian and Wilson 1999).

In brief, nonnative invasive plants will by a higher amount of root exudates, by different root architecture (e.g., higher rooting depth compared to the native plants), or by alterations in the rhizosphere pH modify the nutrient uplift, change the turnover of the microbial community, or cause a shift in the geochemical equilibria

controlling especially the availability of phosphorus. Nonnative plants alter the timing, the quantity and the quality of soil inputs, and the addition of secondary compounds. They may also change the spatial structure of the soil.

By increasing the amount of litter and as a result of increased carbon, invasive plants can "heat up" the cycling process of nutrients through higher turnover rates of the specific nutrients (especially nitrogen and phosphorus). This causes higher mineralization rates and a greater additional supply from deeper soil layers. If the invader species has a rooting system that exceeds that of the native plant species immobilized and stored nutrients (e.g., phosphorus) from deeper soil layers may be transported to the soil surface and integrated into the nutrient cycle. In a field study, Thorpe et al. (2006) exhibited twice as high phosphorus concentrations in the tissues of *Centaurea maculosa* compared to native plants. Soil phosphorus levels in the soil were elevated in sites invaded by *C. maculosa*. This may be due to a better "cooperation" with mycorrhizal fungi as well as a deep, extensive root development that harvests phosphorus from deeper soil layers. Chacón et al. (2009) even demonstrated an increase in organic carbon content under the invader *Kalanchoe daigremontiana* in tropical arid lands. This increase was again due to higher input of plant residues and rhizodeposition and led to higher activities of enzymes in the soil (in this case urease activity was measured that was linked to an enhanced SOM (soil organic matter) and SOC (soil organic carbon)).

But we can also find the opposite: invasive plants like *Berberis thunbergii* (Japanese barberry) one of the most widely known and planted exotic shrubs in the United States had much thinner litter layers than the native shrubs (Ehrenfeld et al. 2001) and *Pinus cortata* (exotic) forests exhibit lower litter mass than the native *Pinus sylvestris* (Agren and Knecht 2001).

Often these negative leaf litter effects can be caused by differences in litter quality, i.e., the invader's litter totally differs from that of the natives possessing "novel compounds." Large oil concentrations (monoterpenes) in the leaves of *Melaleuca alternifolia* together with large amounts of litter resulted in very slow litter decomposition on *M. alternifolia* dominated sites (Boon and Johnstone 1997). "New" secondary compounds of alien invaders may upset regular decomposing processes in soil communities. Some invaders have been introduced to novel ecosystems especially for medicinal purposes and their special and new secondary compounds.

3 The Native Soil Community View

In the soil there are three important functional groups of microorganisms (1) soil microbial parasites and pathogens, (2) mutualistic symbionts, and (3) decomposers. Several studies (e.g., Van der Heijden et al. 1998) have shown that mycorrhizal fungi and bacteria can dramatically alter aboveground biodiversity, productivity, and nutrient dynamics. Some authors postulate—according to the negative density dependence hypothesis of Janzen (1970) and Connell (1971)—that predominately

the negative plant–soil biota feedbacks via plant fungal pathogens, parasites, mycorrhizas, and bacteria "regulate" plant populations densities. Thus the aboveground plant species diversity is merely an upshot of the processes going on belowground (Mills and Bever 1998; Packer and Clay 2000; Klironomos 2002; Bever 2003). While this is the case for many native plants, invasive plants "new" to an invaded site often show the opposite: positive plant–soil feedbacks. It is not clear why these species (e.g., *Centaurea maculosa*, Callaway et al. (2004), more examples see above) have negative soil feedbacks in their home ranges and develop positive effects with soil microorganisms on the invaded sites, leading to dominance stands of populations of these plants. Agrawal et al. (2005) discovered that soil microbial feedbacks (tested for ten congeneric pairs of native/alien species in Canada) were twice as high in native herbs compared to alien invasive herbs. But negative soil feedback effects do not just occur with native plant species. Scheffer (2003) reported that agronomists who tried to plant fruit trees (pear, apple, cherry) and foresters who tried to establish "new" forest trees (Douglas fir and pines) in new regions of the world failed because of direct negative effects of indigenous soil pathogens.

In the following, I will concentrate on the above-mentioned three groups of microorganisms mentioned earlier, although there are certainly a few reports on other groups of organisms and their interference with invasive plants (e.g., nematodes: Van der Putten et al. 2005, microarthropods: Belnap et al. 2005, isopods: Bastow et al. 2008, ground beetles: Dávalos and Blossey 2004).

Soil organisms and invasive plants always act on a local scale (on larger scales soil type and/or climate are important), but local feedbacks between microorganisms and plants will strongly influence both "partners" (Bever 2003). Still there are problems in detecting structural diversity of these microorganisms (Wolfe and Klironomos 2005). Contemporary tools include culture-dependent techniques (incubating soil extracts on various sterile media that favor microbial growth), fatty acid analysis (microbes have signature fatty acids), and nucleic acid analysis (DGGE, t-RFLP). The culture technique approach is a rather old one (but not obsolete) and only a small fraction of soil diversity can be identified by it. Also fatty acid analysis, even though it is new, can only detect broader differences in soil organisms groups. Nucleic acid analysis is becoming one of the most widely used methods in microbial soil ecology. Functional diversity is assessed by measurements of nutrient cycling and soil enzyme activity, carbon substrate utilization techniques (substrate-induced respiration, CLPP), or by measurements of multitrophic functions (Wolfe and Klironomos 2005).

3.1 How Soil Microbial Pathogens and Parasites Are Affected by Alien Invasive Plants

In the last decades, researchers have reconsidered the idea that pathogens do not play a role in natural ecosystems. There is substantial evidence that soil pathogens

play a role (or even a key role) in shaping and structuring plant communities (Packer and Clay 2000; Klironomos 2002; Lee and Klasting 2004; Prenter et al. 2004; Kardol et al. 2006). Host genotype specificity and selective stimulation of soil pathogens by host plants must be assumed and there are some case studies finding pathogenic soil microbes in the root zones of wild plant species (Mills and Bever 1998; Packer and Clay 2000). If there is a high specificity in pathogens alien invaders will have a competitive advantage in getting rid of the pathogens of their native regions. Escaping harmful soil biota of the home range could cause an increase in their spread, distribution, and abundance in the nonnative range (Enemy release hypothesis, ERH). This means that these species get away from an intense density control by host-specific herbivores and pathogens in the root zone. However, Van der Putten et al. (2007) argued that there is an indirect defense against soil-borne pathogens: "when plants escape from their natural enemies, they may also lack those microbes that indirectly defend plants against their natural enemies." Eppinga et al. (2006) also found that pathogens in the newly invaded regions, which accumulate in the root zone of the alien invader, might spread to the roots of native species. If the invader is more tolerant this could be a disadvantage to the native plant. In a meta-analysis, Mitchell and Power (2003) found that invasive plants have less pathogen and virus species than similar native plant species have, indicating that the enemy release (ERH) from pathogens may apply for certain invasive plant species. As mentioned earlier the abundance of a species (i.e., dominance and rarity) can be the result of an effective control by pathogens in the soil. Perhaps on an extended timescale in natural plant communities dominant plants are more tolerant to native pathogens than rare plants. Up to now our knowledge of the specific plant traits (of native and alien species) that are related to an effective control of soil-borne pathogens is marginal.

On the other hand, infectious diseases via pathogens may cause a sudden collapse in biological invasions. Some authors (Simberloff and Gibbons 2004; Hilker et al. 2005) argue that presence or absence of specific pathogens can not only favor the successful spread of invaders but can also stop the spread of invaders or lead to their disappearance. Nevertheless, Hilker et al. (2005) emphasize the fact that the population crash of the invader cannot be caused by native pathogens (otherwise the invaders would not have invaded successfully) but must be the effect of a "subsequently released" or later invaded pathogen. In general, there are very few published studies on such invasive soil pathogens.

3.2 Mutualistic Relationships Between Soil Microbial Symbionts and Alien Invasive Plants

Most of our terrestrial plant species live in symbiosis with soil-borne micro-organisms: we find mycorrhizal fungi, nitrogen-fixing bacteria, and some other plant-growth-promoting rhizobacteria. A total of 80–90% of all plant species have mutualistic relationships with mycorrhizal fungi, a type of efficacious symbiosis

which has existed already for more than 400 million years (Smith and Read 2008). Only a few plant families do not show this type of interaction: Brassicaceae, Chenopodiaceae, Polygonaceae, and Amaranthaceae. These mutualistic relationships stimulate plant growth and reproduction, provide the plant with limited resources (nitrogen, phosphorus), and protect the plant rhizosphere from soil-borne pathogens. Symbiosis with mycorrhiza (and adjacent mycorrhiza helper bacteria) may be responsible, for instance, for up to 80–90% of the phosphorus and 80% of the nitrogen acquired by plants (Van der Heijden et al. 2008). In return for these effects on the plant, the microorganisms receive carbon. About 10–20% of the net photosynthetic rate of the plant is delivered to the fungus and in some cases, if the relationship gets parasitic up to 85% is provided to the fungus (Simard et al. 2002; Allen et al. 2003). Many studies have demonstrated that the intensity of colonization is influenced by diverse environmental factors like light and nitrogen availability, soil pH, temperature, or soil moisture.

Nitrogen-fixing bacteria and mycorrhizal fungi are the two most effective mutualistic relationships we find in the soil. Both can improve the nitrogen status of their host plants, but both can—by their interactions with their (nonnative) host plants—dramatically alter soil of the recipient plant communities (Richardson et al. 2000a).

The type, quality, and magnitude of these mutualistic relationships may vary significantly. Many ectomycorrhizal fungi (e.g., symbionts of Pinaceae) are facultatively dependent, but in only some cases of arbuscular mycorrhiza (AM) is there a strong dependency (e.g., *Citrus* species or *Coffea arabica*). But for most fungi and some nitrogen-fixing bacteria/cyanobacteria there is a more obligate dependency. The host specificity of many *Pinus* species hindered them from invading into new ecosystems in many regions of the world (Poynton 1979). But besides these very specific cases there is up to date relatively little general information about host specificity of mycorrhizal fungi. Already in a root segment of only 10 cm length you can find up to ten different arbuscular mycorrhizal-fungi (Aldrich-Wolfe 2007). Hence we can only speculate about the functional diversity or the strength of interactions of AM-fungi.

From the perspective of a successful invasive alien plant species the various ways the plant reacts to specific fungi/bacteria and the intensity of the plant's response to the fungi/bacteria are important. The strongest responses could be investigated under nutrient limiting conditions and in late successional stages of plant communities, because in these late stages of succession nutrient cycles are tightly closed and nutrients are located in plant individuals (Van der Putten et al. 2007). Štajerová et al. (2009) tested alien invaders in Europe and found 70% of these species having mycorrhiza. Their results supported the positive correlation between the degree of mycorrhizal colonization and the extent to which a species dominates an invaded plant community. However, there are also invasive species (e.g., *Fallopia japonica* and all species of the Polygonaceae) that form dense dominance stands without having mycorrhizal symbionts.

But does the lack of AM-fungi hamper the successful spread of aliens as in the case of the ectomycorrhizal *Pinus* species? No—contrary to ectomycorrhizal and

ericoid mycorrhizas AM-fungi are less host specific and they have a cosmopolitan distribution (currently only around 5% of these AM-fungi have been described). Plants interact with a very large number of the less host-specific AM-species (Eom et al. 2000; Streitwolf-Engel et al. 2001) and it is very implausible that a new alien-fungus alliance will produce a "super-invader." Van der Heijden et al. (1998) and Klironomos (2003) found out that there may be "unusual relationships" between native fungi and exotic plants that may create strange (i.e., unusual) and highly variable ecological effects. Invasive success may be linked to the degree of successful establishment of mutualistic relationships. AM-fungi that tend to have highly parasitic relationships with invasive aliens may repel invasions whereas highly mutualistic relationships will facilitate invasions of aliens (Reinhart and Callaway 2006). It is often the case that the new association of an invader with a native fungus may be strong parasitic. We know little about why and how enhanced mutualistic effects can boost invasions. Up to now it is also unclear why the accidental finding of new fungi/bacteria as a (maybe strong) partner should help the plant to dominate specific plant communities. Since mycorrhiza–plant interrelationships are organized in so-called common mycelial networks (CMN), providing an exchange service of nutrients allocated between plant individuals even of different species growing together on the same site (Wilson et al. 2006), it may be the case that newly invading species use these networks in a parasitic way, without paying the mutualistic costs (Reinhart and Callaway 2006).

Many studies emphasize the role of AM-fungi in competitive interactions (e.g., Carey et al. 2004). The old argument that successful invaders have higher competitive abilities than native plants (Baker 1974) may in fact be caused by the efficient mutualistic relationship of the invader in the new surroundings. Shah et al. (2008) demonstrated significant AM-fungi colonization of the alien invader *Anthemis cotula* across different spatially separated populations, which pointed to the contributory role of AM-fungi in promoting invasions to different habitats in Kashmir Himalaya, India. They also suggested that not the sheer presence/absence of AM-fungi gives an advantage to the nonnative host, but rather that the source of the AM-fungi determines the extent of a specific benefit. Local AM-fungi had greater positive effects on specific traits of the alien invader *A. cotula* than nonlocal AM-fungi. This is a very important finding: relationships of plants with AM-fungi are influenced by the identity of the plant and also their geographical origin. Mummey et al. (2005) found out that the invasive forb *Centaurea maculosa* strongly controlled the AM community of the native grass *Dactylis glomerata* through its root system. Niu et al. (2007) found that the aster *Ageratina adenophora* (originating in Mexico and Costa Rica) invading into native forest communities in China altered soil microbial community structure by strongly increasing abundance of AM-fungi and the changing fungi/bacteria ratio. This led to higher nitrate, ammonia, P, and K contents in the invaded sites compared to adjacent uninvaded sites. In addition, this "invaded" soil had a high inhibitory effect on native plant species but not on the invader. In many studies mycorrhizal fungi are described as being "low" host specific. The results of Niu et al. (2007) hint at a species specificity of mycorrhizal fungi (different effects on *A. adenophora* and natives).

Although mutualistic relationships are in many or even most cases very helpful for the plant (see earlier) there is a large proportion of invasive plant species that are not mycorrhizal (Vogelsang et al. 2004). Ruderal sites or garbage dumps with high disturbances and low densities of mycorrhizal hyphae are better colonized by nonmycorrhizal invaders. Also sites with extremely poor soils may be unsuitable for most invaders that depend on AM mycorrhizas (see Allsopp and Holmes 2001).

Successful invaders like *Alliaria petiolata* (a weedy pest in the USA, not mycorrhizal) use this important interrelationship of mutualistic symbionts as a tool to suppress native plant species by allelochemicals and to reduce AM-fungi abundance in the surroundings of their own root zone as well as the adjacent soil (Roberts and Anderson 2001; Stinson et al. 2006). This led to the "Mycorrhizal Degradation Hypothesis" (Vogelsang et al. 2004).

There are only few studies that have investigated the degree of mycorrhizal dependency of the plants species. Most of these studies were conducted in the laboratory, but it is reasonable to suppose that interrelationships that occur in the field may be restricted to fewer fungi taxa than in the lab because other abiotic or extrinsic factors may play an important role in the field (Van der Putten et al. 2007). But one thing is clear: the lower the degree of specialists and the higher the degree of generalists in mutualistic relations, the easier the entry of alien plants into native communities.

Considerable progress has been made in the last decades in understanding how mutualistic relationships affect the degree of invasiveness of alien plants (Reinhart and Callaway 2006), but up to now the underlying mechanisms that govern these novel interactions are not fully clear and further research in controlled laboratory, greenhouse, or field studies has to be conducted.

3.3 Invasive Plants and Soil Decomposers

Decomposition of litter is one of the most fundamental ecosystem processes as it is responsible for the recycling of nutrients in dead organic biomass. Invasive plants can have strong effects (see also earlier) on this process and also on the detritus-based food webs in the soil in a variety of reactions in terrestrial systems (Ehrenfeld 2003; Lindsay and French 2006).

Whereas plant-pathogenic or plant-mutualistic relationships with soil microorganisms can be of high specificity, saprophytic microorganisms exhibit lower levels of species-specific associations (Wardle et al. 2004). This is because plant species primarily interact via their litterfall with the saprophytic microbial community. As mentioned earlier it is primarily the quality, quantity, and the timing of the delivery of organic material (litter) that the alien invader produces that affect the saprophytic microorganisms. Most important functional groups of saprophytic microorganisms are present in nearly every soil (Beijerink's law: "all microbes are everywhere" Chung and Ferris 1996). Therefore, the dependency of specific saprophytic microorganisms on specific alien plant invaders is low. As saprophytic

species with particular functional capacities are everywhere (see earlier) there is no specific advantage or disadvantage in the lack or existence of a specific microbe for a newly invading species.

But how do exotics stimulate the decomposers? Many invader plants exhibit high amounts of "good quality" litter due to their growth patterns (fast growers) and to the ingredients of their litter (high tissue nutrient concentrations, low secondary compounds for defense, low structural material). These "better" resources for microorganisms may cause shifts in the decomposer subsystems. The effects are therefore the greatest if native and alien invader plants differ most in these traits. A "test" for these changes is the change in the decomposition rates of added material (e.g., Hughes and Uowolo 2006). Koutika et al. (2007) reported clear effects (increases) on soil organic matter (SOM) by different alien species (*Solidago gigantea* and *Prunus serotina*). These species enhance SOM dynamics by increasing carbon mineralization thus stimulating the decomposing microorganisms. However the stimulating effects described earlier cannot be regarded as universally valid; there are also reports of negative (or neutral) effects of exotic plant species on soil nutrient cycling processes (Ehrenfeld 2003). In the decomposing systems there are also microbes that feed on root exudates. These species seem to be more plant species specific than those microbes just decomposing litter in the bulk soil and since the plant–microbe interaction is just more direct these interactions will be stronger for both native and exotic plant species (Van der Putten et al. 2007).

4 Conclusions

Invasive species can modify the soil and its community on sites that they occupy. They have found ways to increase their own fitness relative to that of native species in the particular soils. They can change soil conditions that may downsize the growth or fitness of co-occurring native species. Thus the positive or negative interaction with soil microbes is a critical factor in the successful spread and/or establishment of alien plant species in nonnative plant communities. Many direct and indirect mechanisms have been demonstrated how plants communicate with the soil microbe community. In order to acquire a more detailed understanding of this, many biogeographical studies combining experimental and field studies should be performed in the future. But a major drawback in this investigation will be the analysis of mycorrhizal symbiosis. How does one compare native with nonnative interrelationships if only about 5% of the interacting fungal taxa are known? Analyzing fungal taxa that interact with nonnative aliens and thus investigating possible host specificities of these relations need more attention in future research.

On the other hand, given the continuum of plant parasites feeding on the roots of invaders and herbivores feeding on the leaves, there should be more studies which combine aboveground with belowground investigations in a multitrophic research approach. Even though each invasive alien plant species and every invasion process will still have its own unique history it will be crucial for developing reliable

generalizations about the role of soil biota in promoting or repelling invasive alien plants to test more examples of exotic invaders especially in their biogeographical contexts of home and nonnative ranges.

But despite the intense literature cited above and below soil, Lambrinos's (2002) assertion is still correct: every invasion seems to be a (special) particular case and the need to investigate the specific ecological aspects of these particular cases will remain in the future.

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Biotic Interactions in the Face of Climate Change

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Contents

Abstract Recent evidence suggests shifts in biotic interactions due to climate change. Accordingly, there is a growing body of scientific literature available which includes review articles on specific types of biotic interactions. However, up to date there is no review, which summarizes insights on biotic interactions on a global scale and across ecosystems and species.

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We derived major findings concerning climate change effects on biotic interactions from a formalized literature search in the ISI Web of Science. Increased carbon dioxide levels along with global warming appear to be the most influential factors for the alteration of biotic interactions including, e.g., competition and facilitation, herbivory and pollination, mycorrhiza, parasitism, mutualism, as well as predator–prey interaction. Ecosystems holding large trophic networks and high biodiversity prove to be more resistant towards the effects of climate change than ecosystems disturbed by human influences. Regarding study location, most research up to date is carried out in terrestrial temperate regions, thus proving a strong bias in knowledge sources. The most investigated interaction partners are herbivore insects interacting with grass species. It is often found that indirect effects of altered biotic interactions due to environmental changes on the performance of members of biocoenoses are more pronounced than direct effects of abiotic drivers. With respect to abiotic factors the focus of most investigation is an increase in temperature. Far less data are available on effects of extreme weather events such as drought, heavy rainfall, late frost, and warm spells. Based on our survey, we propose a shift in research towards more complex types of interactions with multiple interaction partners as well as multifactor climate change scenarios and events.

1 Importance of Biotic Interactions in a Global Scope

Biological interactions are essentially omnipresent in all ecosystems. As interactions are a dynamic process, it is of great interest to know, how the dynamics will change with alterations of environmental variables, such as abiotic factors (Jiang and Kulczycki 2004; Hillyer and Silman 2010; Van der Putten et al. 2010; Yang et al. 2011; Andrewartha and Birch 1960). Here, we attribute alterations of abiotic factors to effects of climate change.

Hence, we are interested in the effects of rising temperature, changing precipitation regimes, and elevated concentrations of carbon dioxide. Hand in hand with these alterations comes an increased frequency of extreme weather events. Prominent candidates of these events are drought, heavy rain, winter warm spells, late frost events, and summer heat waves (IPCC 2007).

The influence of climatic changes on biotic interactions has evolved to be an important research frontier (e.g., Easterling et al. 2000; Parmesan et al. 2000; Jentsch et al. 2007; Hegerl et al. 2011). Many researchers in this field agree that knowledge on the reaction of single species towards effects of climate change alone is not sufficient to derive a conclusion on climate change-induced alterations in plant communities and their ecosystem services as well as in the biogeography of vegetation zones (e.g., Walther 2010). Biotic interactions crucially need to be considered, since each species exists in a system alongside with other organisms of various trophic levels (Harrington et al. 1999; Lavergne et al. 2010). Some authors go as far as to state that species interactions can even overrule direct climatic effects (e.g., Suttle et al. 2007). With respect to climate parameters, only multifactor studies can lead to a thorough understanding of the complex phenomena

of biotic interaction dynamics (Edwards and Richardson 2004; Costello et al. 2006; Vittoz et al. 2008; Schlueter et al. 2010; Yang et al. 2011).

A well-defined search (Table 1) in the ISI Web of Science yielded 2,529 articles (published as recently as December 2011) on the topic of biotic interactions in the face of climate change effects. There were about 450 review papers available. However, the majority of these review articles were restricted to certain criteria, such as terrestrial habitats (Caldwell et al. 2003, 2007; Tylianakis et al. 2008), marine habitats (Clarke et al. 2007; Occhipinti-Ambrogi 2007; Hallegraeff 2010; Perry et al. 2010), to specific regions, e.g., Arctica (Dormann and Woodin 2002; Klein et al. 2008) or Australia (Hughes 2003; Mcalpine et al. 2009), to specific ecotypes, such as forests (Ayres and Lombardero 2000; Rennenberg et al. 2006; Rouault et al. 2006; Battisti 2008; Anderson et al. 2011), only selected classes such as insects (Hassall and Thompson 2008) or only certain types of biotic interactions such as herbivory (Bale et al. 2002; Bidart-Bouzat and Imeh-Nathaniel 2008; Traill et al. 2009).

Hence, we present a survey on the research on biotic interactions in the face of climate change. Our results are structured the following way: The first part deals with findings about climatic drivers and biotic interaction types. The second part concerns ecological domains and study locations. The third part follows with a close look on investigated interaction partners. Further we analyze observation period, climatic drivers, research method, and trend versus event.

1.1 Modeling Biotic Interactions

Current modeling of ecological climate change impacts is focused on projections of the availability of potential habitats for species occurrence at the end of this century. Attempts to forecast global warming impacts often rely on bioclimatic envelope models, which combine species with environmental variables to enable projections of species distributions under future climatic conditions (Berry et al. 2002, Peterson et al. 2002, Thuiller et al. 2005). The validity of this approach has been criticized since there are frequent cases where important biotic interactions are not taken into account (Leathwick 2002).

The consideration of biotic interactions in modeling processes, even on macro-ecological scales, is necessary since purely climate-based models are insufficient to quantify potential changes in species distributions due to climate change effects (Araujo and Luoto 2007). Interactions between species can modify or disrupt links between warming and the local distribution of species. Thus, accounting for interactions in modeling can distort expectations of range shifts under global warming.

1.2 Experiment and Observation

Modeling requires reliable biotic data in order to produce realistic forecasts. Such data are provided by lab or field experiments or by field observation. There are only few experiments, in which climate factors as well as community compositions are manipulated, a fact which has already been pointed out by several authors (e.g., Edwards and Richardson 2004; Costello et al. 2006; Jentsch et al. 2007; Vittoz et al. 2008; Schlueter et al. 2010; Beierkuhnlein et al. 2011). Among those few studies, there are multifactor studies, where several climate factors are studied simultaneously on behalf of their combined effect on a single species and studies where the effects of multiple factors on several species are studied individually and in a parallel fashion (e.g., Davis et al. 2010; Dukes et al. 2011; Keuper et al. 2011). Eventually, in the context of experiments and observations, it is worth to mention long-term studies. Some long-term studies on biotic interaction have been running for 20 (Brown et al. 1997) or more years (Edwards and Richardson 2004; Costello et al. 2006). Most long-term studies are field observations that document changing community structures over a period of several decades. An example for a long-term field observation is the study of marine pelagic communities. Over a period of several decades a change in plankton life histories and flower phenology has been observed. A development which led to a mismatch between trophic levels on the one hand and to trophic dead ends and new community dynamics on the other (Edwards and Richardson 2004; Costello et al. 2006; Vittoz et al. 2008; Schlueter et al. 2010).

2 Literature Survey

A literature survey (surveyed period: January 1991 to December 2011) on key topics of biotic interactions and climate change was conducted within publications listed in the ISI Web of Science. The search string (Table 1) consisted of a combination of key terms from the two research areas “climate change” and “biotic interaction.” The search string included specific aspects of interaction such as herbivory and pollination. The search string excluded historical climate changes from the Paleozoic, Paleocene, Holocene, Bronze Age, as well as research in archeology and related fields such as archaeozoology. Only original research articles were considered.

2.1 Criteria for Paper Selection

After the search in the ISI Web of Science relevant papers were extracted, particularly those, whose study objectives were focused on biotic interaction in the face of climate change. Subsequently, the following topics were excluded: viruses and human diseases, planning of family, overfishing and genetic modification within a

Table 1 Search string for the ISI Web of Science

Category	Keywords/search terms in the "ISI Web of Science"
"topic"	"climat* chang*" or "changing climat*" or "global chang*" or "global warming"
AND in "topic"	interact*
AND in "topic"	"plant interact*"or interspecific or "species interact*" or community or symbio* or parasit* or mutual* or mycorrhiz* or herbivor* or pollin* or trophic* or coex* or "bio* interact*" or compet* or facilit* or "predator*prey*"
NOT in "topic"	palaeo* or holocene or "bronze age" or archaeo*

The column "category" indicates the logic operators that we chose from the search options in the ISI Web of Science. The other column shows the search term we used as input to the logic operators

species, and further related topics. Finally, the remaining selection of publications was narrowed down to publications that established a clear link between the analyzed climatic driver and climate change. The initial filtering was based upon the abstract alone, while the last filtering was based upon the full text article.

2.2 *Findings from Relevant Publications on Climatic Drivers and Biotic Interaction Types*

A total number of 2,529 papers were found within the selected time period from January 1991 to December 2011. We extracted 528 publications within the filtering process described earlier.

Publications identified as relevant were sorted into categories, such as interaction partners (plant–plant, plant–pollinator, plant–herbivore, plant–microorganism, mycorrhiza, and animal–animal), climatic drivers (e.g., temperature, CO_2), and kind of influence of climate change effects on biotic interactions.

Our analysis of the final set of publications confirmed that there is an ongoing debate on the effects of climate change on biotic interactions with regard to the following main topics: (1) community structure (interaction strength, diversity, competition and facilitation, top predator stabilization versus bottom-up regulation), (2) timing (de-synchronization, decoupling of flowering and growth), and (3) future development (divergent and convergent evolution). A detailed additional overview and extracted representative statements from the considered publications can be found in Table 2. With regard to these three major issues our literature search revealed the following findings.

2.2.1 Community Structure

There was a common expectation that interaction strength will change, e.g., for the predator–prey interaction (e.g., Helland et al. 2007; Hart and Gotelli 2011) and for

Table 2 Main findings of the reviewed publications with the most expressive results

Interaction type	Climatic driver	Change in BI	Main findings	References
Plant–plant	CO_2	Yes	Biomass decreased when invasions occurred Competition increased	Bradford et al. (2007), Lazzarotto et al. (2010)
	CO_2	No	Small direct response Competition remained unaffected Plant community, as a whole, did not differ between ambient and elevated CO_2	Brosi et al. (2011), Manea and Leishman (2011), Wilsey et al. (1994)
	Precipitation	Yes	Heavy rain: productivity decreased Longevity shortened; solitaire plants accumulate more biomass than plants with neighbors Changes in species composition modified phenological shift Drought: competition shifted to facilitation Resistance of plant communities decreased due to increased climate variability and biodiversity loss Interaction between below ground plant parameters and microbial turnover was changed An intact plant–soil system buffer against drought Phenological response of individual species is modified by community composition First visible changes occurred after 4th and 7th year of drought Competitive advantage of C4 plants decreased	Kikvidze et al. (2006), Jentsch et al. (2007), Kreyling et al. (2008a), Kreyling et al. (2008b), Weber et al. (2008), Levine et al. (2010), Linares et al. (2010a), Volder et al. (2010), Evans et al. (2011), Jentsch et al. (2011)
	Temperature	Yes	Facilitative neighbor effects on survival, phenology, growth, and reproduction Earlier developing Competitive advantage of C4 plants decreased	Nadkarni and Solano (2002), Wipf et al. (2006), Volder et al. (2010)

	Temperature	No	Effects of competition exceeded effects of increased temperature Ecological role remained unaffected	Gedan and Bertness (2010), Clark et al. (2011)
	CO_2 and temperature	No	Interactive effects among treatments rarely significant	Dukes et al. (2011)
	Temperature and precipitation	No	Plant and species abundances, diversity, and evenness did not change	Keuper et al. (2011)
	Temperature and precipitation	Yes	Sphagnum modified effects of climate change at the community level.	Keuper et al. (2011)
Plant–pollinator	Temperature	Yes	Allocation: phenological shifts reduced floral resources Reduced overlap decreased diet breadth of pollinators	Memmott et al. (2007)
Plant–herbivore	CO_2	Yes	Relative growth rates of larvae depended on amount of CO_2 exposition of feeding plants Changes in host plant quality alter interspecific competition among insect herbivores Alterations in leaf chemistry were more important than direct short-term effects of temperature on insect performance Changes in plant quality had effects on the herbivore populations Reduced abundance of phloem-feeding herbivores Increased numbers of parasitoids	Williams et al. (2000), Stacey and Fellowes (2002), Newman et al. (2003), Hättenschwiler and Schafellner (2004), Hillstrom and Lindroth (2008), Strengbom et al. (2008)
	CO_2	No	Consumption and species preference of herbivores were not altered Abundance of chewing herbivores was not altered Plants did not increase the production of cardenolides in response to herbivory	Peters et al. (2000), Strengbom et al. (2008), Vannette and Hunter (2011)
	Precipitation	Yes	Increased precipitation: led to higher winter mortality Changing precipitation: responses of small mammals depended on changes in vegetation	Wang and Zhong (2006), Frank (2007), Martin (2007), Ponti et al. (2009), Gutbrodt et al. (2011)

(continued)

Table 2 (continued)

Interaction type	Climatic driver	Change in BI	Main findings	References
			Belowground productivity underneath grassland was influenced in dependence of grazing Bird species were more indirectly affected by trophic levels below (plants) and above (predators) than by direct effects of increased precipitation Drought: lower concentration of secondary defense compounds Feeding preference changed (palatability)	
	Precipitation	No	Drought: feeding guilt level for leaf mining insects did not indicate drought stress impact	Staley et al. (2006)
	Temperature	Yes	Decreased interannual variation in flowering, advance of flower phenology and phonological asynchrony Seed predator populations increased Alterations in leaf chemistry due to CO_2 were more important than direct short-term effects of warming on insect performance Increased grazing rates Prevention of re-vegetation of bare peat No direct effect on plant production, but increased strength of top-down (indirect) effects and alteration in cascading responses Changed grazing pressure Delay of larvae emergence	McKone et al. (1998), Williams et al. (2000), Schiel et al. (2004), Barton et al. (2009), Chapman et al. (2009), O'Connor (2009), Gaedke et al. (2010), Williamson et al. (2010), Liu et al. (2011), Warren et al. (2011)
	Temperature	No	No effect on synchronization Induced alterations in leaf had no effect on insect performance Timing of peak was unrelated, which indicated no trophic mismatch No change of feeding preference (palatability) occurred	Buse et al. (1999), Williams et al. (2003), O'Connor (2009), Berger et al. (2010)

Plant–micro-organism	CO_2	Yes	Response from selected plant species and changes in community structure, however, effect of increased temperature more pronounced Litter quality decreased	Wolf et al. (2003), Ferreira and Chauvet (2011)
	Precipitation	Yes	Decreased resistance towards infections	Linares et al. (2010b)
	Temperature and precipitation	Yes	Microbial-nitrogen pool is higher under warming and warming plus snow addition	Keuper et al. (2011)
	Temperature	Yes	Overall litter quality decreased, however, direct warming effects are more pronounced than the indirect effects related to altered litter quality on decomposition Saprophagous macrofauna increased Change in community structure	David and Gillon (2009), Ferreira et al. (2010), Ferreira and Chauvet (2011)
Plant–fungi	CO_2	No	No response in plant polycultures	Wolf et al. (2003)
	Temperature	Yes	Altered structure and allocation of arbuscular mycorrhizal hyphal network More carbon losses to the atmosphere from fungal respiration	Hawkes et al. (2008)
	Temperature and precipitation	Yes	Decrease in Tanoak mortality correlated with mean annual precipitation	Davis et al. (2010)
Animal–animal	CO_2	Yes	Diminished escape responses Divergent pheromone-mediated behaviors could alter predator–prey interactions	Mondor et al. (2004)
	Precipitation	Yes	Precipitation change: bird species were more indirectly affected by trophic levels below (plants) and above (predators) than by direct effects of increased precipitation Species abundance altered by shifts in strength of density dependence and in growth rate Drought: decline of herbivores population supported (indirect) predators	Owen-Smith and Mills (2006), Martin (2007), Hart and Gotelli (2011)

(continued)

Table 2 (continued)

Interaction type	Climatic driver	Change in BI	Main findings	References
	Temperature	Yes	Ecosystems with intact top predators are more resistant Strengthened top-down effect by different warming effects on predator and prey Competition altered response to temperature increase Increase in predator attacks on mobile prey Community composition changed and food chain lengthened	Jiang and Kulczycki (2004), Wilmers and Post (2006), Cresswell et al. (2009), Barton (2010), Woodward et al. (2010), Hoekman (2010), Harley (2011), Vucic-Pestic et al. (2011)
	Temperature	No	No variation of bottom-up effects Predation did not alter response to temperature increase No changes in predator attacks on resident prey No breakdown of host–parasitoid synchrony	Jiang and Kulczycki (2004), Hoekman (2010), Klapwijk et al. (2010), Vucic-Pestic et al. (2011)

These publications constituted around 10 % of the total number of analyzed publications. Publications are ordered according to interaction type and climatic driver. The categories CO_2 and temperature represent an increase. *BI* biotic interaction. Information about "change" corresponds to statements by the respective authors regarding statistically significant results

plant–plant interactions (e.g., Kardol et al. 2010, Yang et al. 2011). However, the influence of the two types of effects, direct (e.g., increased temperature, precipitation change) and indirect (e.g., palatability), was highly debated. On the one hand, there was evidence that climate change alters community structure (especially for herbivorous insects, e.g., Lavergne et al. 2010; Schweiger et al. 2010; Walther 2010 and other communities, e.g., Forchhammer et al. 2008; Ims et al. 2008; Villalpando et al. 2009; Harley 2011). On the other hand, it was found that climate change does not alter palatability of plant tissue to herbivores (O'Connor 2009; in contrast to Gutbrodt et al. 2011)—a finding that opposed the existence of any influence from direct or indirect effects.

In the case of plant–plant interactions, evidence suggested that abiotic stress changes competition between plants (Sthultz et al. 2006; Hillebrand et al. 2008). Kidvidze et al (2006) found that competition shifted to facilitation under drought. However, in contrast to these findings, some factors, such as alien species, also acted stabilizing on biotic interactions (Schweiger et al. 2010; Sparks et al. 2010b). Plant–plant interactions could remain robust in the face of climate change effects due to local diversity (Maron and Marler 2007). In the case of animal–animal interactions, Previtali et al. (2009) presented the view that a strong bottom-up regulation is predominant in arid environments. However, ecosystems with intact top predators were likely to exhibit stronger biotic regulation and were more resistant to climate change effects than ecosystems lacking top predators (Wilmers and Post 2006).

2.2.2 Timing

Quance and Travisano (2009) confirmed the importance of temperature for timing. Higher temperature, accompanied by dust deposition and faster snow melt, will lead "to synchronized growth and flowering across the landscape" always including the possibility of modified biotic interactions (Steltzer et al. 2009). However, effects on biotic interactions are not necessarily equal to de-synchronization of species interaction (Ponti et al. 2009; Steltzer et al. 2009). Timing is expected to change for predator–prey interactions, for example, as different species are affected differently by increases in temperature, the interaction strength between predator and their prey is altered (Helland et al. 2007; Berger et al. 2010; Sparks et al. 2010a, b). Based upon the surveyed literature, we found that timing had different effects on different biotic interactions. Timing had an influence on biotic interactions, when climate change effects affected interaction partners with a different magnitude.

2.2.3 Future Development

There is the possibility of divergent evolution (Bidart-Bouzat et al. 2004). However, the number of publications that dealt with the question and consequences of divergent and convergent evolution is very small.

In cases where authors of the reviewed literature made a forecast of the development and outcome of altered biotic interactions the predictions tended to be very specific. Such a case was Ponti et al. (2009), who remarked that from the point of view of agriculture various effects of climate change are rather anticipated. They provided the example of the decreased risk of fly damage for *Olea europaea* due to climate change effects (Ponti et al. 2009).

Various authors found multiple drivers that were independent from climate change effects, but also influenced biotic interactions. Examples for this type of drivers were land use (West and Yorks 2006; Moen 2008; Pompe et al. 2010) and topography (Pettorelli et al. 2005).

2.2.4 Concluding Remarks on Findings of the Authors

In summary, most researchers expected changes in biotic interactions at least for parts of ecosystems. Hawkes et al. (2008) even expected an increasing carbon loss into the atmosphere due to the respiration of arbuscular mycorrhizal fungi. The loss was not balanced by increased growth of these fungi.

Based on the total of our reviewed literature, we found evidence for shifts in biotic interactions. The amplitude of such shifts was variable. Shifts in biotic interactions could favor interaction partners which currently only play a minor role. However, despite these expected shifts in biotic interactions, we did not see any evidence for de-synchronization of biotic interaction partners within near future, when we considered CO_2 increase, rising temperature, and precipitation changes in the frame of climate scenarios found in the reviewed publications.

2.3 Ecological Domains and Study Locations

Independently from our previous categorization of the final set of literature we sorted the individual publications under the aspect of the respective study location. The geographic coordinates of locations or regions where a study took place were visualized in a map of global ecological domains (Bailey 1989). For simplicity we distinguished only between five broad categories of ecosystems: aquatic, forest, grassland, desert, and others. These categories were marked with different symbols (Fig. 1). Research of climate change effects on biotic interactions in terrestrial habitats dominated the field (56% of all relevant papers) compared to research in aquatic ecosystems (39%), regardless of whether in freshwater, saltwater, flowing water, or standing waters (Fig. 1).

The terrestrial habitats were subdivided into forest (18% of all studies excluding aquatic), grassland, savannah, and moor (23%) and desert (2%). Twenty-three percent of the terrestrial studies were not specified by the authors of the respective publications.

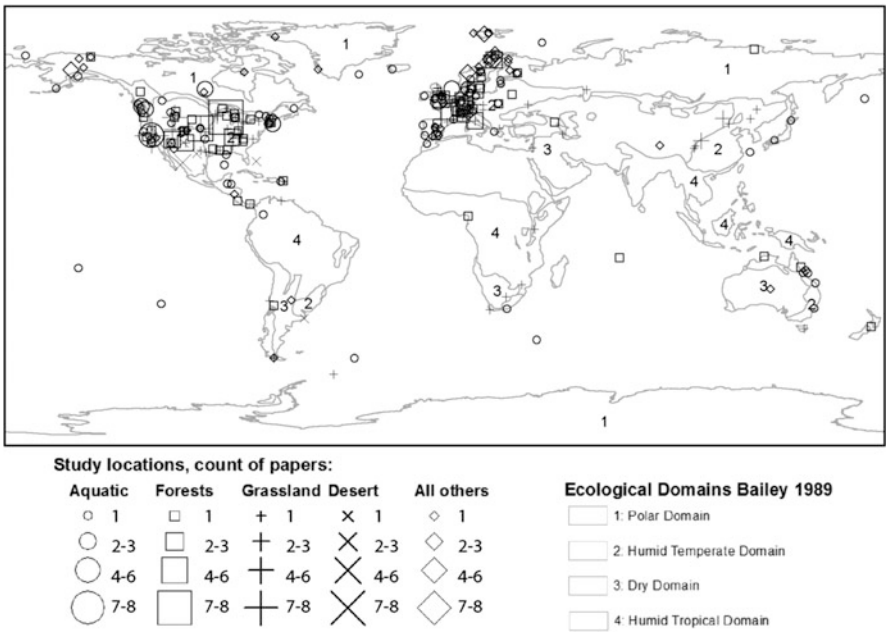

Fig. 1 Locations of research on biotic interactions in the context of climate change. Map from Bailey (1989). Research focused on temperate forest and grasslands. The size of a symbol is related to the number of the publications

In terms of climatic zones, 32% of the research is located in temperate climate zones. Nineteen percent are located in arctic and alpine climate zones. Limnic systems were rarely investigated (9%).

One hundred and sixty-eight publications were not depicted in the map, because the available amount of information did not allow us to match the study location with the ecological domain.

We found a strong focus of research on the northern hemisphere with maquis, deciduous forest, and grassland of temperate latitudes, as well as in smaller proportion on high mountains and boreal forests. Furthermore, marine ecosystems were examined frequently. Based on these observations it becomes clear that the choice of study location is not representative for the manifold biotic interactions occurring in a global scope.

2.4 Interaction Partners

When we looked at the question of interaction partners in the filtered literature, we considered classes in the case of animals and orders in the case of plants. Additionally, bacteria and fungi were specified, but not further resolved. The interactions were

summarized in categories analogue to those mentioned in Sect. 2.2, such as plant–plant, plant–animal, or animal–animal. We paid close attention to details of the interaction types for our examination of the filtered publications, e.g., trophic interaction, competition versus mutualism.

The majority of findings indicated an influence of climate change on biotic interactions independent from the climatic driver (e.g., temperature, CO_2, precipitation) and independent from the kind of biotic interaction (e.g., plant–plant, plant–animal). In plant–plant interactions there is evidence for shifts from competition to facilitation, for decreased resistance of plant communities and modified phenological response of plant communities.

Concerning animal–animal interactions, the focus is on predator–prey interactions. Here, there is evidence that an increased effect of the predator can be induced by changing the overlap of the seasonal peaks or by exploiting the influence of warming on the feeding rate of the predator.

The focus of plant–animal interactions is on herbivory; however, the literature is contradictory concerning the existence of effects of climatic change drivers here.

2.4.1 Plant–Animal

Plant–animal interaction was by far the most commonly analyzed type of interaction (42% of the final set of publications) (see Fig. 2). In 73% of these studies at least one animal interaction partner was an invertebrate, while in 50% of the studies at least one insect species was involved.

In the context of plant–animal interactions (224 interactions) the most examined plant orders were Poales (25%) and Fagales (12%) (Fig. 2). The most prominent animal classes in this context were insects (50%), mammals (13%), and gastropods (6%). While a strong focus was on herbivory (e.g., Wolfe and Ralph 2009), several authors also gave examples for predatory birds and their impact on herbivorous insects or other animals (e.g., Buse et al. 1998; Primack et al. 2009).

In the context of insect–plant interactions only a few publications dealt with *pollination* (e.g., Harrison 2000; Memmott et al. 2007; Dupont et al. 2009; Alba-Sanchez et al. 2010; Fabina et al. 2010; Forrest et al. 2010; Memmott et al. 2010; Sparks et al. 2010a, b; Aldridge et al. 2011). However, these studies did not provide consistent results. Memmott et al. (2007) stated that an overlap between plants and pollinators and a decreased diet breadth of the pollinators could be expected to occur. They expected the extinction of pollinators, plants, and their interaction. Dupont et al. (2009) considered an annual turnover in composition of both plant and animal communities and particularly in their interactions, while the descriptors of the overall network structure remained relatively stable. This result indicated that the regional pool of plants and their pollinators contained many functionally equivalent species. Within the body of analyzed publications insects were the only class that was considered as a pollinator.

Herbivory was a popular research topic in the category of publications on *insect–plant* interactions. The feeding behavior of insects on plants was examined

	Anthozoa	Aves	Bivalvia	Branchiopoda	Gastropoda	Insecta	Mammalia	Maxillopoda	Osteichthyes	Other animal	algae	Asterales	Brassicales	Caryophyllales	Coniferales	Ericales	Fabales	Fagales	Lamiales	Magnoliales	Malpighiales	Malvales	Other plants	Poales	Rosales	Saphindales	bacteria	fungi
A	1																											
A		14																										
B			2																									
B	1			3																								
G			2	1	1																							
I		4			2	32																						
M		5				1	16																					
M		2	2	1	1		1	4																				
O		3		1		3	3	2	12																			
o		3	1	2	1	3	1		4	6																		
a	8		2	6	3		1	2	3	7	12																	
A						10	1					11																
B					1	11				1		3	1															
C						1						5	1	1														
C						8						1			13													
E						3						4	1	1	1	7												
F					1	11	1					14	1	4	3	4	11											
F		2				15	4			2		3			17	10		9										
L				1		8	1					13	1	3	2	2	12	2	1									
M						9	2			2			1		2	1	1	7	1	3								
M						1				1				1	2	1		4		1								
M						8				1		1		1	1		2	2	1	2	1							
o		5			1	17	8		1	9					5			3	2				14					
P					3	12	7			3		20	2	7	6	12	19	11	20	5	3	3	12	40				
R					1	6				1		3			6	2	1	10	2	5	2	4		7	4			
S						8	1								5		2	8	2	6	2	2			3	1		
b	1	1				6	1		1	1	1		1		1	1	1	1				3	4	2			2	
f				1		3	1			2		1			7	3	2	7	2				7	5	2		4	1
Σ	***11***	***39***	***9***	***17***	***18***	***182***	***55***	***15***	***33***	***51***	***45***	***90***	***25***	***25***	***80***	***53***	***90***	***117***	***76***	***48***	***19***	***32***	***88***	***199***	***59***	***40***	***32***	***48***

Fig. 2 Interaction partners: numbers of publications, which examined biotic interaction under climate change effects; animals (class), plants (order), bacteria, and fungi. The category "others" summarizes all further groups of belowground organisms, (soil) microbes, plankton, decomposers, chytridiomycetes, and parasites. The scheme neglects any orders or classes with less than ten references. The total is the sum of all values in the row added to the values in the corresponding column. The abbreviations in the far-left column correspond to the categories at the top of the table

frequently. One example was Davis et al. (1998), who claimed that temperature determines the outcome of competition. However, findings were contradictory concerning the existence of effects of climatic change drivers also in case of insect herbivory. Peters et al. (2000) found no alteration in consumption or preference of herbivores due to increasing CO_2. Other studies showed that changes due to CO_2

existed and that alterations in leaf chemistry were more important than direct short-term effects of warming on insect performance (Williams et al. 2000).

The strength of top-down effects also increased with rising temperature (McKone et al. 1998; Barton et al. 2009; Chapman et al. 2009). However, there was also indication that warming has no effect on herbivores (Buse et al. 1999; Williams et al. 2003).

The discussion of herbivory and plant–animal interactions in the context of precipitation changes remained open. Far-reaching consequences such as changes in vegetation and in belowground activity were expected. In the case of vegetation and belowground activity, grazed and un-grazed grasslands were differentiated (Wang and Zhong 2006; Frank 2007; Martin 2007; Ponti et al. 2009). Opposing to this, there was also evidence that drought, like warming, has no effect on herbivore insects (Staley et al. 2006).

2.4.2 Plant–Plant

Regarding plant–plant interaction publications researchers mainly referred to competition; however, in the most cases there was no differentiation between competition and facilitation. The four mainly examined orders were Poales (26%), Fagales (19%), Lamiales (12%), and Coniferales (11%). Only few papers dealt with plant–plant interaction in water (9%) or wetlands (3%) (Fig. 2).

Most authors agreed that warming, precipitation change, and CO_2 increase would lead to changes in plant–plant interaction (Table 2). Evidence to support this statement was shifts from competition to facilitation, a decreased resistance of plant communities, more biomass, or modified phenological response by community composition (Kikvidze et al. 2006; Weber et al. 2008; Jentsch et al. 2009).

The results from our analysis of plant–plant interactions in the final set of publications can be roughly divided up into three parts "competition of native plants," "invasion," and "community composition," although these parts cannot be clearly separated from one another.

We could not extract a general agreement on the effect of climatic drivers on competition among plants (e.g., Linares et al. 2010a; Dijkstra et al. 2010; Huebler et al. 2011; Lau et al. 2010; Levine et al. 2010; Reyer et al. 2010). On the one hand, authors observed an increased *competition* due to climate stressors (Huelber et al. 2011). Further, a decline in growth and rhizosphere effects on carbon and nitrogen cycling was observed and also attributed to the interaction of climate stressors and competitors (Linares et al. 2010a; Dijkstra et al. 2010). On the other hand, we found studies where experimentally simulated climate change did not alter interactions among plants. Further it was observed that precipitation response of competing plants was negligible (Levine et al. 2010; Reyer et al. 2010). We stress the observation made by Levine et al. (2010) who found that dominating competitors are more sensitive to climate stressor and, thus, climate change can shift dominances in biotic interactions.

Unlike the previous issue, we found no objection to studies that revealed a change in *community composition* due to climate change effects, which is strongly linked to the availability of water (e.g., Kardol et al. 2010; Yang et al. 2011).

We found that the aspect of *invasion* in plant communities in the face of climate change is again discussed controversially. It remains unclear, if invasive species profit from altered growth conditions triggered by climate change effects. Manea and Leishman (2011) observed a decrease in productivity in native plants, but not in related invasive species within the same communities due to warmer climate (Verlinden and Nijs 2010). Furthermore, Dukes et al. (2011) found that elevated CO_2 fostered the growth of invasive plants while native plants responded much weaker to climate stressors. However, this influence of CO_2 is disputed since Manea and Leishman (2011) observed improved competitivity in the majority of invasive plants independent from the concentration of CO_2.

The most commonly observed extreme event in the context of plant–plant interactions was *drought*. Here, we found agreement among several authors that a drought event has severe consequences for single species, native and invasive, as well as for plant communities and the competition of its constituents (e.g., Linares et al. 2010b; Evans et al. 2011; Jentsch et al. 2011; Jimenez et al. 2011; Kane et al. 2011; Mikkelsen et al. 2008; Volder et al. 2010).

A further precipitation related abiotic factor, which was only studied in the context of plant–plant interactions, was *snow coverage* (e.g., DeMarco et al. 2011; Huelber et al. 2011). DeMarco et al. (2011) found an influence of the height of snow coverage on the availability of nitrogen and, hence, on the juvenile growth of plants. The authors reported that the higher the snow coverage was, the more nitrogen was for plants. Thus, plants with a high demand of nitrogen profited from increased height of snow layers. Additionally, Huelber et al. (2011) pointed out that cover germination and juvenile growth were reduced when the density of vegetation increased. The steepest reduction of competition was observed at intermediate height of the snow layers. Furthermore, the interaction between the canopy density and seedling survival shifted from competition to facilitation when the height of snow coverage increased (Huelber 2011).

2.4.3 Animal–Animal

In the context of animal–animal interaction, we found a strong focus on *predator–prey* interaction within the analyzed literature. These predator–prey interactions were affected by climate change in two opposing ways: the effect of the predators was either increased or reduced.

An increased effect could be induced by an altered overlap of the seasonal peaks of predator and prey. The consequence was an intensification of the predator–prey relation that finally resulted in extinction of the prey (Costello et al. 2006). This intensification could also be triggered by the dependence of the feeding rate of the predator (Broitman et al. 2009).

A reduced effect could be induced by decreasing the maximum population density of the predator (Gilg et al. 2009) or by reducing the reproductive success of the predator (Gilg et al. 2009, Sanford 1999). Predators transferred the effects of climate change via biotic interactions. In such cases predators either buffered the effects of climate change on specific biotic interactions (Wilmers and Getz 2005) or they used climate change related advantages that led to intensified interactions (Costello et al. 2006; Owen-Smith and Mills 2006; Broitman et al. 2009).

On the base of the analyzed literature we found that it remained a challenge to disentangle top-down interactions from extrinsic influences on population dynamics, mediated through resources (Owen-Smith and Mills 2006).

Unlike in the case of predator–prey interactions, the analyzed publications provided only few examples of *competition* among animal–animal interactions in the face of climate change. Within the few publications on that specific topic we found that the studies on animal–animal competition focused on the macro-fauna. Here, the most examined interaction partners were birds (36%) and mammals (33%) (e.g., Kilpatrick et al. 2009; McEachern et al. 2009; Previtali et al. 2009).

Numerous publications applied animal–animal interaction to oceans and dealt with zooplankton, fishes, whales, and penguins.

2.4.4 Other Categories of Biotic Interactions

Within the body of analyzed literature 35 publications considered *bacteria and fungi* as interaction partners with animals. Further, only 72 articles dealt with plants as interaction partners of these organisms. Here, plant–fungi interactions had the biggest share of 84% of the previous mentioned 72 publications. Plant–fungi interactions accounted for 5% of the total number of analyzed publications.

None of the 528 reviewed papers dealt with biotic interactions under climate change between groups neither plants nor animals, like fungi–bacteria interaction. In total 28 articles reported studies on *food chains*, which were 5% of the total number of analyzed publications (not shown).

Overall it was remarkable that only eight publications dealt with *amphibians and reptiles* (2%). However, these studies commonly agreed that climate change would influence amphibians and reptiles. The first study involving reptiles was on sea turtles as an interaction partner of jellyfish (Hong et al. 2008). The second publication in this field found a negative correlation between effects of lizards on spiders and the number of days with rainfall (Spiller and Schoener 2008). The remaining six publications found that rising temperature affected amphibians in multiple ways, such as (1) decreased vulnerability to capture of tadpoles (Broomhall 2004), (2) varied survival rate correlating with tadpole density (Govindarajulu and Anholt 2006), (3) increased fecundity as maturation rate slowed, and (4) increased infectivity as growth decreases (Woodhams et al. 2008). Furthermore, it was observed that amphibians have a significantly stronger shift towards earlier breeding than all other taxonomic/functional groups, advancing more than twice as fast as trees, birds, and butterflies (Parmesan 2007).

Several *animal classes and plant orders* were not listed in Fig. 2. Only less than ten papers studied these classes and orders, which amounts to 145 in total. In detail, these classes and orders were, e.g., Anthozoa, Arachnida, Asteroidea, Cilliata, Entognatha, Gymnolaemata, Medusozoa, Reptilia, Tentaculata, Turbellaria, Alismatales, Apiales, Bryophyta, Caryophyllales, Dipsacales, Ephedrales, Genianales, Gnetales, Lichen, Myrtales, Polygonales, Polypodiales, Proteales, Ranunculales, Saxifragales, and Solanales.

Partially, publications did not offer taxonomic information but only gave categories in terms of growth form (e.g., forbs, woody plants) or in terms that did not specify a taxonomic level (e.g., herbivores). These publications could not be considered for analysis of interaction partners.

2.5 *Observation Periods, Climatic Drivers, Research Methods, and Trend Versus Event*

We also distinguish trend versus event. Here extreme weather events are proceedings restricted to several days or weeks, whereas climatic trends occur independently from those events and arise over years and decades. Hence, we distinguished studies that dealt with climatic trends such as warming and those that addressed extreme weather events such as drought, heat wave, cold spell, or heavy rain (Jentsch et al. 2009).

Articles from the body of analyzed literature that clearly reported changes in biotic interactions were distinguished into four categories: "lab," "computer modeling," "field experiment," and "observation." Further we distinguished studies according to the number of drives: "one driver," "two drivers," "three drivers," and "more than three drivers." Following our initial analysis of our data, the group "one driver" was analyzed in more detail due to a high number of publications in this category. In the group "one driver" we additionally differentiated the type of the driver: "CO_2," "precipitation," or "temperature." Additionally to prior categorization we distinguished between studies that dealt with the observation of climatic trends and those studies, which addressed extreme weather events.

From the results of our analysis we clearly saw that two types of observation periods prevailed: less than 1 year (30%) and longer than 10 years (27%). Short-term studies (duration less than 1 year) hinted that indirect effects of shifts in litter quality, which themselves were caused by shifts in community composition, had a greater impact than direct effects of increased temperature and elevated levels of CO_2 concentrations (Rouifed et al. 2010).

Long-term studies (duration longer than 10 years) were field observations in the majority. These field observations aimed to generate data on broad impacts of climate change (Brown et al. 1997; Bunce et al. 2002; Burrowes et al. 2004; Edwards and Richardson 2004; Costello et al. 2006). Manipulations that focussed on one or more climatic drivers were rare in this context. Long-term studies gave

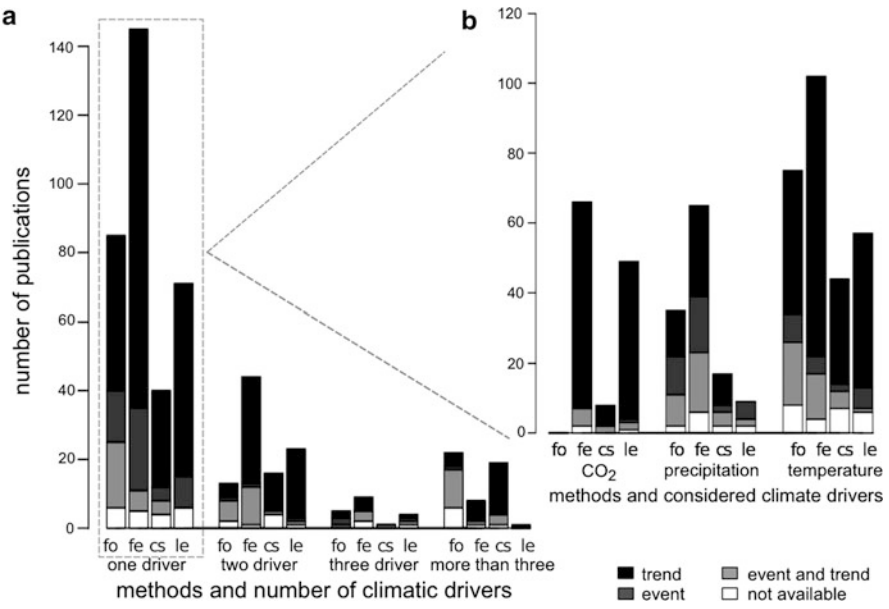

Fig. 3 Global environmental change drivers, research methods, and time periods; *fo* field observation, *fe* field experiment, *cs* computer simulation, *le* laboratory experiment; (*left*) number of studies with one, two, three, or more than three climatic drivers; (*right*) splitting of the four bars of "one driver" of the left part into the analyzed drivers CO_2, precipitation, and temperature. It is important to note that "two and more drivers" only indicates that two or more drivers were considered in the same publication. Thus, it does not necessarily mean that the interaction of these multiple drivers was investigated

evidence that changes in community composition led to a mismatch between trophic levels and functional groups (Edwards and Richardson 2004; Costello et al. 2006; Vittoz et al. 2008; Schlueter et al. 2010).

The third most abundant time period was a study duration of 1–3 years (6% of the total number of analyzed publications). Intermediate time periods were rarely examined (3–5 years, 5%). Hardly any study (2%) considered time intervals of 6–10 years.

The majority of the analyzed publications (66%) focused on one climatic driver only (Fig. 3 left). For this reason the corresponding part of the diagram (data represented in the bars for the category "one driver") was subdivided according to the before-mentioned categories CO_2, precipitation, and temperature, see in Fig. 3b (UV and N_2 not shown in Fig. 3 right). The combination of two drivers (18%) varied with the study type.

In the context of our analysis it is important to note that a combination of two or more drivers meant only that these two or more drivers are mentioned in the same publication. Therefore, "combination" does not indicate a simultaneous or correlated examination of the effects of multiple drivers on biotic interactions. In fact, this type of study is rare (e.g., Davis et al. 2010; Dukes et al. 2011; Keuper

et al. 2011, see Table 2). However, the outcome of these studies indicated that the effect of combined drivers is rarely statistically significant. Several other studies considered either an individual species with its interaction partner in relation to a single abiotic factor or an individual species in relation to several abiotic factors (Brosi et al. 2011; Henry et al. 2005; Johnson et al. 2010; Maraldo et al. 2010). The examined combinations of climatic drivers are CO_2 and temperature as well as temperature and precipitation for plant–plant interaction and temperature and precipitation for plant–fungi interaction.

Field experiments (fe) were the most frequent analyses carried out to examine biotic interactions under climate change (40%). They were followed by field observations (fo) (23%). Temperature was the most commonly observed climatic driver (54%), followed by precipitation (24%) and CO_2 (23%).

On the base of the body of analyzed literature, trends were better examined than extreme events (numbers of publications: trends = 352, events = 60). However, this ratio changed during the last years, where formerly only isolated publications about events could be found within a year; nowadays around ten publications per year can be observed.

3 Emerging Research Frontiers

On the basis of our analysis of the body of relevant publications we pointed out several gaps in research of biotic interactions under climate change. (1) Several ecozones, such as tropical forest or savannahs, have been hardly examined so far, although they cover vast regions and are crucial for global climate. (2) The frequency of analysis of different interaction partners varied a lot. Insects were the most commonly studied interaction partners. However, this did not indicate that the effects of climate change on insects were well understood since insects were the most common class. Groups that should be considered in future research are lichens, mosses, bacteria, fungi, and all other groups that were not explicitly mentioned in Fig. 2. (3) Extreme events or a combination of events and trends were subject of only few publications. The only exceptions from this rule were trends of changing precipitations and drought events. (4) The effect of more than one climatic driver on biotic interactions was another issue that is not well understood.

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From Aral Sea to Aralkum: An Ecological Disaster or Halophytes' Paradise

Siegmar-W. Breckle

Contents

Abstract The Aral Sea no longer exists. Only the Small Aral Sea has now again a constant water level. The former Aral Sea, once the fourth largest lake on the globe, is almost dry, and the desiccated seafloor is a new desert, called the Aralkum. It is

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the source of salt-enriched dust and sandstorms. These are affecting large proportions of the villages and agricultural systems in the whole region, as well as livelihood and health of the people. This new desert has developed within about 50 years. It is caused by human activities; thus, it is an artificial desert, but all the ongoing processes follow natural laws and are most interesting for science. The area can be called the largest primary succession experiment of mankind or depending on viewpoint, one of the biggest ecological catastrophes. About 70 % of the area is salt desert; thus, halophytes play a major role. One-fourth of the flora are species from Chenopodiaceae.

Within the last decades, many new developmental projects have been performed in the region. However, most of them need basic knowledge which only can be provided by scientific expertise. So it is necessary to bring together interdisciplinary and international scientists not only on ecology and geography (with remote sensing and GIS methods), but also climatologists and soil scientists, with social science and economy as well as with nature conservation organisations, developmental and health agencies, and other main stakeholders of the area. Research on the scientific aspects of the Aralkum area has been done in the last years, mainly on dust storms and climate, on soil substrate, but also on vegetation dynamics and flora, as well as on the specific site conditions in such a continental salt desert which are the basis of highly diverse and dynamic halophytic communities adjacent to the tugai woodlands, the riverine forest remnants.

1 Introduction

The Aral Sea is located in the Turan basin, a huge tectonic-based lowland in Middle Asia. It was once the fourth largest lake on the globe with a surface area of about 68,300 km. Since 1960 the water level dropped constantly from about 53 m asl to about 23 m asl in the remnant West-basin (Breckle 2011).

The Aral Sea is an endorrheic lake, meaning that it has no outlet to the ocean. Endorrheic lakes always are rather sensitive to the climatic conditions and to changes in water balance by long-term climatic effects and—more drastic—short-term human activities. This can be seen with Lake Chad, with the Dead Sea, or with the Great Salt Lake (Utah). The equilibrium between input by rivers, groundwater, and precipitation is balanced by the evaporation from the sea surface. Two main tributaries feed the Aral Sea: the Amudarya from the South and the Syrdarya from the Southwest and West (Fig. 1).

Geographical research in the region started only less than 200 years ago (see Uhrig 2008). Research of the last decades was mainly on the very dynamic history of the Aral Sea in historical, but also post-glacial times, on the abiotic environment, which is governed by a very continental climatic situation and geologically by a huge erosion basin which is slowly filled up by sediments from both the tributaries, and on the biotic environment of the former Aral sea (Breckle et al. 2001b; Breckle and Wucherer 2012a, d). It was a typical low saline water body with a very variable

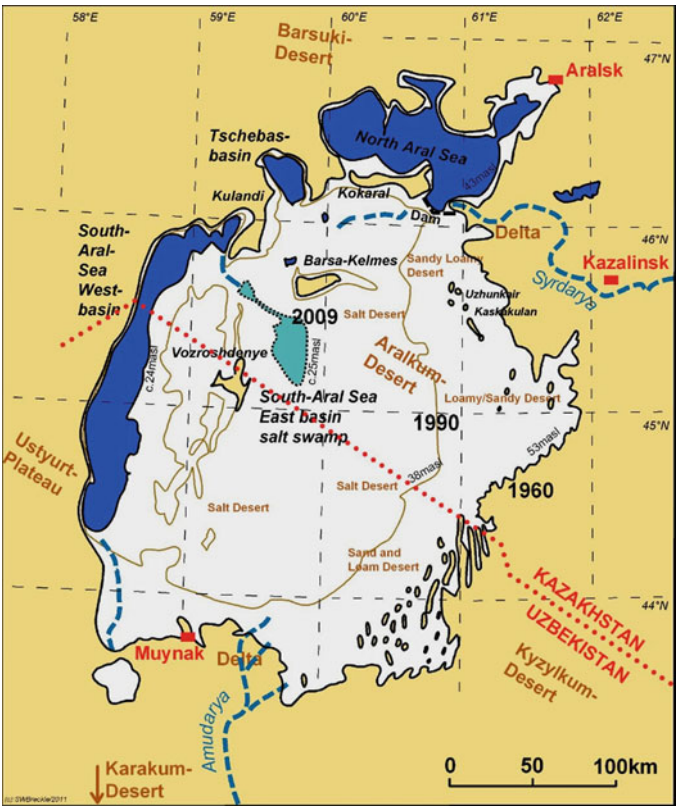

Fig. 1 Map of the Aralkum, indicating the old coastline 1960, coastline from 1990, and recent situation with the left isolated water bodies and indicating the adjacent desert regions

chemistry and influenced by the steppique and semi-desertic ecosystems in the northern half and the desertic ecosystems (Karakum and Kyzylkum) in the South-West and South-East. Both tributaries had developed rather large delta regions with riverine forests (tugai forests), also along the lower river margins.

A rapid retreat of the water level of the Aral Sea started after 1960 (Glazovskii 1990; Eliseyev 1991; Glantz et al. 1993; Zonn et al. 2009). This corresponds exactly with the largely increasing use of river water for irrigation for the enormous new cultivated areas mainly for cotton. At that time the irrigation schemes in the former Soviet-Uzbekistan, Kazakhstan, and Turkmenistan had been greatly expanded.

The new desiccated seafloor of the 1970 and the early 1980s is predominantly sandy; the later bigger area of the desiccated seafloor is governed by loamy and clayey sediments, and by the strongly increasing salinity during the desiccation process affecting the whole area (Agachanjanz and Breckle 1993; Aladin and Potts 1992; Breckle and Agachanjanz 1994; Letolle and Mainguet 1996; Breckle et al. 1998; Micklin 2007; Kostianoy and Kosarev 2010). Thus, on the new surface, mainly psammophytes and many halophytes can now be found.

Research was focussed first on the flora and the dynamics of migration and invasion of plants to the seafloor (Wucherer 1984; Wucherer and Galieva 1985), later on the successional dynamics, and the means for restoration of vegetation types that can prevent formation of sand and salt dust storms (phytomelioration) (Wucherer et al. 2012b). These applied research topics were especially promoted during the last decade (Breckle et al. 2012a).

The changes in the Aral Sea region within the last 50 years can be briefly characterised as follows (data for 2009):

- Formation of (older) sand deserts and (younger) salt deserts
- Drop of the water level by about 30 m [from 53 m above seal level (asl) to 23 m asl, at the South Aral Sea, or "Large Aral Sea"].
- Shrinking of the surface area to about one-tenth of the original area (from 67,100 to 7,000 km^2); thus, the remnant water bodies today are the North Aral Sea ("Small Aral Sea"), the West Aral Sea, the East Aral Sea, which in 2009 was almost only a huge salt swamp, but in 2010 was again a very flat sea (both forming the "Large Aral Sea", see Fig. 2) in the south, and the small Tschebas basin in the northwest.
- Retreat and change of the eastern coast by more than 100 km; the new land surface area of the dry seafloor is called the Aralkum (in 2009 about 60,000 km^2).
- Tremendous decrease of the water volume (from 1,100 km^3 to approximately 100 km^3), reaching <10 % of the 1960 value.
- Drastic increase in salinity (from 0.9 to 6–10 % for the Large Aral Sea), which means the southern remnants of the Aral Sea are hypersaline.
- Stabilisation of the water level of the North Aral Sea by a dam at 42 m asl; surface area amounts to 3,200 km^2.

2 History

2.1 Prehistoric

The Aral Sea has a complicated history. During the Mesozoicum in most parts huge sedimentation within the Tethys Ocean took place. During early Tertiary this part of the Tethys disappeared by the uplift of the old continental blocs between the Arabian and the drifting Indian plate; only some basins (the Mediterranean, the Black Sea, the Caspian, and the Aral Sea) remained as water basins. The sediments are jurassic and cretaceous and consist of limestone, clay marls, sand stones, and rather often evaporites, like salt (NaCl), gypsum, and even potassium salts, indicating various strong phases of desiccation, regressions, and transgressions (Gel'dyeva et al. 2012).

During the Tertiary some tectonic movements continued as well as uplifts and breaking of blocs. During these slight changes the various basins which exist today were formed, but slight movements changed the course of the rivers apparently

several times. The typical development of the Aral Sea started during the main melting phase of the large plateau and high mountain glaciers about 18.000–22.000 a BP. At that time the Amudarya left the lower Karakum and started to discharge to Khorezm and Uzboi and to the Caspian region. Several old riverbeds and valleys of the last few millennia are known (Mayev et al. 1983). But also an overflow of the Caspian over the Uzboi Valley to the Aral Basin took place about 5000a BP but most probably also between 8000a and 15.000a BP (Weinbergs and Stelle 1980). The very dynamic hydrology within the last few thousand years in the whole Turan area is partly explained by the changing water balance of the vast hydrological input areas but also most probably by slight tectonic movements (Svitoch 2009). Apparently also water chemistry exhibited changes (Le Callonnec et al. 2005). The Amudarya in antic times was called the Oxus river, the Syrdarya the Araxes or Jakartes, the Seravshan the Polytimetos river. Their river beds changed very often (Boomer et al. 2009; Breckle and Geldyeva 2012).

2.2 Historic

There are mainly three factors which might be responsible for fluctuations of the Aral Sea in historical times: climatic fluctuations with fluctuations of water input within the hydrotopes, development and intensity of the agricultural use of irrigation water in the oases, and variation of sediment transport and sedimentation, causing changes in river courses (Sorrel et al. 2006, 2007).

Within the last 5 decades mainly the huge irrigation projects in many parts of Middle Asia were responsible for the catastrophic desiccation of the Aral Sea. To date there are 4 separated basins left (Fig. 1), the Small Aral Sea in the north, now an own water body, since 2006 separated by an artificial dam. The western, deep basin, which will be the main remnant water body in the future, the eastern, shallow basin, which became a large salt swamp now, and the small Tschebas basin in the north-west, north of Kulandi.

During the last millennia there have been two very severe regression events at the Aral Sea, which are also important from an ecological view point (Table 1). About 400 AD the sea level sank to only 25–30 m asl, most probably by the deviation of the Amudarya to Sarykamysh and Uzboi (Nikolaev 1991; Oberhänsli et al. 2011).

During the first half of the thirteenth century AD, most of the oasis at the lower Amudarya and Syrdarya had been destroyed by the Mongolian conquerors. At that time additionally the Amudarya discharged water to the Sarykamysh again, which caused a lower water level of about 48–50 m asl. During the second half of the thirteenth century and the fourteenth century AD, the Amudarya discharged totally to the Aral See, and the level rose to 52–53 m asl. This time is also characterised by another prosperous oasis culture. At the end of the fourteenth century the Timur wars brought devastation. During the fifteenth and sixteenth century AD, the water discharge of the Amudarya was very variable, as well as the sea level of the Aral Sea. Most time the level was about 43–44 m asl, but it is under dispute, how long this medieval regression took place.

Table 1 The main regressive phases of the Aral Sea during the late Pleistocene and the Holocene (Breckle and Geldyeva 2012)

Nomenclature	Period (years BP)	Dating	Altitude (m asl)
Timuridica	400–600	Historical documents	43–44 m
Late Antica	1,600	1,590 ± 140 (14 C)	30–35 m
Neolithica	3,500	3610 ± 140 (14 C)	?
Paskevitshic	10,000–22,000		<25 m

After Weinbergs and Stelle 1980; Mayev et al. 1983; Nikolaev 1991

Since the seventeenth century AD the Amudarya completely discharged to the Aral Sea, but only since the eighteenth century the sea level became stabilised at about 53 m asl., which was defined as the official mean water gauge. Since then the sea level fluctuated less than 3 m, caused by climatic fluctuations. L'vov (1959) and Berg (1908) have reconstructed the water gauge fluctuations.

In general, we see that the Aral Sea is a very sensitive and dynamic lake. It is an endorrheic water body in the centre of the extensive hydrotope. The recent development of the Aral Sea is perfectly documented by satellite images between 2000 and 2011 (http://earthobservatory.nasa.gov/Features/WorldOfChange/aral_sea.php; see Fig. 2a–c). Between 2000 and 2009, the Aral Sea steadily shrank. In 2006, following a severe drought, very little water reached the Aral Sea in 2007, and nothing flowed from the Amudarya to the Aral Sea in 2008 and 2009 (Micklin 2010). Without water from the Amudarya, the southern Aral Sea rapidly dwindled; the eastern part disappeared in 2009 (Fig. 2a).

In 2010, however, the drought ceased. Snow in the Pamir Mountains was above normal, and enough water flowed into the Amudarya, so that the river reached the Aral Sea. The muddy pulse of water settled in a shallow layer over the bed of the eastern flat basin of the South Aral Sea, making it looking much larger than it appeared in 2009 (Fig. 2b). In 2011, the eastern basin was less filled than in 2010 (Fig. 2c), indicating a new very low hydrological equilibrium.

3 Research on the Abiotic Environment

3.1 Hydrology

The Aral Sea until 1960 had an area of about 68,300 km^2 together with the islands (water level of 53.0 m asl). The area of the sea water surface was 66,100 km. The water volume accounted to about 1.100 km (Bortnik et al. 1991; Bortnik 1996). The then established huge canals and irrigation systems diverted most of the water of the two tributaries Syrdarya and Amudarya. Thus, the catastrophic rapid desiccation of the Aral Sea started. Since 1960 about 90–92 % of its water volume and about 85–88 % of the sea surface area has been lost.

The first half of the twentieth century has been a stable phase for the Aral Sea, with a rather constant water level of 53 m asl (Oberhänsli and Zavialov 2009).

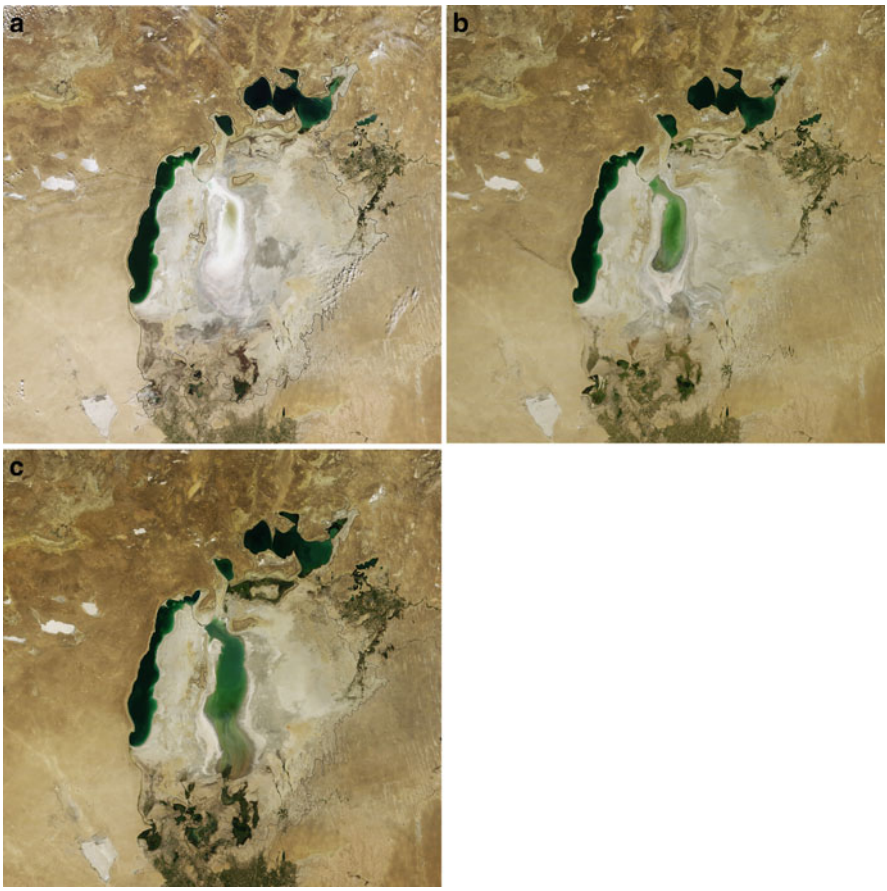

Fig. 2 Recent satellite images of the ever increasing Aralkum (NASA). (**a**) From 16 August 2009—with very low water level in shallow East basin forming a salt swamp (http://earthobservatory.nasa.gov/images/imagerecords/39000/39944/aral_sea_20090816_lrg.jpg). (**b**) From 26 August 2010—a very wet winter season has brought some inflow from Amudarya from the south to the shallow East basin (http://earthobservatory.nasa.gov/IOTD/view.php?id = 46685); (**c**) From 11 September 2011—east basin again almost dry (http://eoimages.gsfc.nasa.gov/images/imagerecords/52000/52002/aralsea_tmo_2011227_lrg.jpg)

The two tributaries, Syrdarya and Amudarya, are coming from the mountains of the Pamiro-Alai, the Hindu Kush, and the Inner Tian Shan in Tajikistan, Afghanistan, and Kyrgyzstan. The total discharge per year is estimated to ca.120–130 km^3 (Kirsta 1989). The discharge to the Aral Sea before the desiccation started has been ca. 60–70 km^3. In the last decades, however, it dropped to about 3–20 km^3. This discharge of water is still present, but the water is diverted to fields, fish ponds and new water bodies, new reservoirs and ditches, and thus evaporates before reaching the former lowest point in the hydrotope, the Aral Sea. A model of the whole water and salt balance has been presented by Benduhn and Renard (2004).

The area of the dry sea floor of the Aral Sea, called the Aralkum desert (Breckle and Agachanjanz 1994; Breckle et al. 2001a; Dech and Ressl 1993), is still increasing. About 60,000 km^2 of the former sea floor have been fallen dry. This is inclusive the islands area (Peneva et al. 2004). In 2009 the maximum of desiccation could be observed (Fig. 2a). In this remotely taken view even the faintest glimmers of blue-green had disappeared in the eastern basin, and earth tones predominate, surrounded by a ghostly film of pale beige and white. Lake sediments from this depleted water body have provided ample material for frequent salt dust storms. In 2010, the huge, but very flat, Eastern basin was filled up somehow by strong winter and spring rains in the whole area, becoming a very unstable huge salt swamp, indicating a very strongly fluctuating hydrological dynamic equilibrium (Fig. 2b).

The dry seafloor of the Aral Sea has developed to a new geographical object, a new desert, that has a strong environmental impact on the surroundings of the Aral Sea, which quite often is called catastrophic (Breckle et al. 1998, 2001a, b; Micklin 2007; Erdinger et al. 2011).

3.2 Soils

The dry seafloor is normally a loamy flat plain, surrounded by sandy beach lines and including vast saline (solonchak) plains. Whether and how the remnants of water plants (*Zostera*) or the mats of algae and diatoms from the hypersaline water (Sapozhnikov et al. 2009) are influencing the further development of biotic crusts or soils is an open question. Most decisive is the colonisation by plants. This is again depending on the salinity and particle size distribution of the new soils as the main factors for flora and vegetation development. From the former coastline (1960 line) to the interior, to the retreating recent coastline, we find a zonation of ecotopes. Rather regularly the substrate material becomes finer and salinity is increasing along this directional gradient. The main soil types are marshy solonchaks, coastal solonchaks, degraded and sand covered coastal solonchak, and takyr soils. Distribution of soil material and vegetation changes often discontinuously and stripes of plants can be seen corresponding with seasonal desiccation fluctuations. But the temporal sequence of vegetation and soils is not corresponding with the spatial sequence of plant associations. This is most conspicuous in regard to the contrasting desiccated seafloor areas from the 1960–1970 to the 1980–1990 years.

On the desiccated seafloor with sandy substrates from the 1960 and 1970 rather soon by aerodynamic processes, barkhanes and other sand dune types develop. Mainly two types can now be distinguished: loose open sand dunes with a barkhan cover of 10–50 %, and those with a dense barkhan cover of normally over 60 %. The most intensive development of dune fields could be recognised along the vast stretches of the former E coast, where near Kaskakulan it was obvious that they spread to S and SE.

In accordance with the present erosion catena, the lowest parts presently are characterised by crystalline sediments of the desiccated salt lakes and by solonchak soils. Loam, sandy loam, and silt are mixed with salts, and pure salt layers are

included. Sometimes salt is covering the surface. These mixed layers may have a thickness of up to 8 m. If the groundwater level is rather high, less than 1.5 m from surface, the formation of solonchak soils is prominent. If it is deeper than 1.5 m, crusty-puffy solonchaks are formed.

The desiccated seafloor is a huge open, often bare surface rich in salt. The salt crusts are rather alkaline (with sulphates and carbonates) and may also partly be contaminated by various pesticides and their decay derivatives as well as with radioactive material (Friedrich 2009). It is now one of the main sources of salt dust storms and salt blowouts, and, as a consequence, salt contamination of the adjacent cultivated lands in Kazakhstan and Uzbekistan, being a health hazard for the people.

3.3 Climate

The Aral Sea and the Aralkum are located within the Asiatic desert belt (Walter 1974; Walter and Breckle 1994). The climate of the surroundings of the Aralkum can be characterised being very continental with very cold winters and very hot summers. The consequences of the desiccation of the Aral Sea on climatic conditions have still to be estimated. Current climatic data do only reveal a slight shift to a more accentuated continentality (Breckle and Wucherer 2012b). However, it is fact that with respect to the whole region the loss of water surface area is totally compensated or even overcompensated for by the development of other widespread evaporation surface areas—fish ponds, reservoirs, new canals, irrigation for paddy fields and many other crops, etc.—at other places. Thus, it cannot be expected that there is a general change of influence on the water balance by the regional atmospheric processes (Breckle and Wucherer 2012b). The overall water dynamic of the atmosphere seems not to be altered in general, but an intensification of the water cycling by global change overlaying the local and regional desiccation processes may be observed (Giese and Moßig 2004).

The temperature amplitude during the year, expressed by the relevant monthly means, can reach almost 40 K, the amplitude of the absolute temperature extremes may reach more than 85 K (Fig. 3) (Ginzburg et al. 2003). The Aral Sea region is intermediate between the N steppe region with summer rains and the S desert region with Mediterranean influenced winter rains. This intermediate position leads to rather strong changes, if the atmospheric circulation changes somewhat (Lioubimtseva and Henebry 2009). However, the variability from year to year allows only rough conclusions, since the available observation periods are often too short.

The meteorological data (Muminova and Inogamova 1995) indicate that in the whole Middle Asia region the annual precipitation has increased within the last 50 years. This is due to all parts of the turanian plain, as well as to the high mountains at the margins (Khan and Holko 2009).

Directly at the remnants of the Aral Sea, and at the Aralkum, at the stations of the former islands (Barsa-Kelmes, Lazarev, Vozrozhdenie, Tigrovni) this trend cannot be significantly evidenced. However, those stations mostly have been closed since

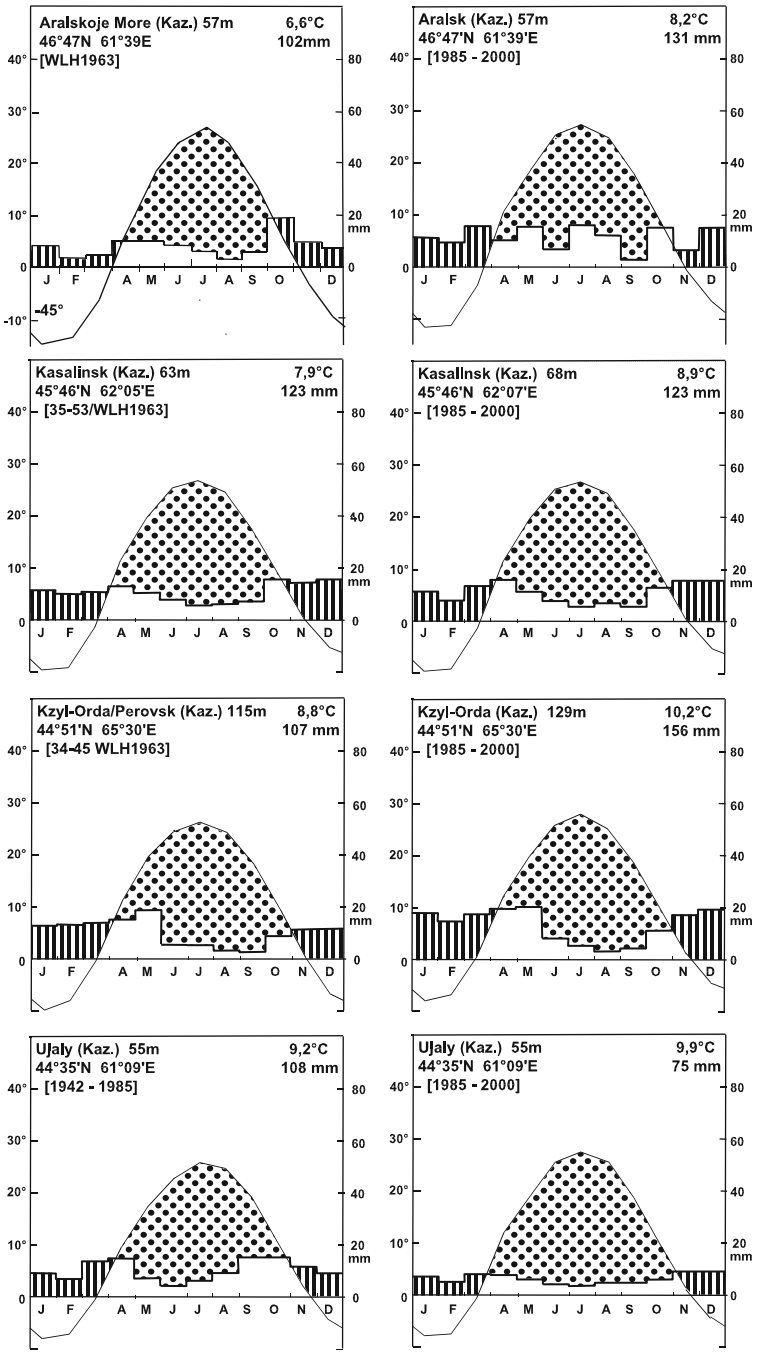

Fig. 3 Ecological climatic diagrams from 4 stations of the Aralkum area from two different time periods, indicating slight changes within the last decades (Breckle and Wucherer 2012b). *Left side*: data from mid-1990s (WLH, data from Walter and Lieth 1967); *right side*: data from late 1990s (various sources, Breckle and Wucherer 2012b)

the mid or end of the 80s. Thus, just the last 15 or 20 years of observations would have been especially worthwhile. Only the station Aralsk clearly indicates an increase in precipitation within the 50 years, but within the last 40 years the mean of precipitation seems stable (Fig. 3). The data from Aralsk from 1937 until 2005, which were checked by Kuz'mina (2007), also indicate a rather distinct rise in annual precipitation (with a correlation line of: $y = 1.0003\ x = 1{,}858$; $r = 0.40$).

3.4 Dust Storms

The general atmospheric circulation and the macrorelief are responsible for the length and intensity of dust storms. The average number of days with strong winds (>15 m s^{-1}) in most of the Aral Sea region is about 15 days, in some parts up to 25–29 days, and the meteorological station from Barsa Kelmes even reported up to 44 days (Galayeva et al. 1996).

The arid climatic conditions and the open surface with fine grain sizes (Fig. 4) are favourable for the development of regular dust storms in the Aralkum area. However, there are still uncertainties on the meteorological conditions causing dust emissions, as Darmenova and Sokolik (2007) had shown.

According to particle size we have to distinguish dust storms and sand storm (Yang et al. 2002). Sand particles are between 0.1 and 1 mm in diameter and are transported close to the surface, and their transport per day may rarely reach 1 km, normally forming sand dunes 10 or 50 m by creeping saltation. Dust particles are much smaller and are transported by wind often to rather high parts of the atmosphere, thus reaching transport distances of hundreds of km (Razakov and Kosnazarov 1987; Orlovsky and Orlovsky 2002; Semenov et al. 2006; Spivak et al. 2012). Many satellite images had been made public by NASA showing dust storms in the Aral Sea region. One of the biggest storms on April 29, 2008, across the whole area to the SW is to be seen at the following Web link: http://earthobservatory.nasa.gov/NaturalHazards/natural_hazards_v2.php3?img_id=14808

From the data and model calculations (Semenov 2012) the whole amount of transported material from the desiccated seafloor can be extrapolated. For the period 1966–1979 an average transportation of dust and sand from the area between the former coastline and the 10-m-isobathe-line was calculated, amounting to 7.3 Mio t of sand and 70,000 t of salt per year (but without significant probability). From satellite images 15–75 Mio t have been estimated (Grigorjev and Lipatov 1982). This is a rather strong difference in numbers which seem to be rather arbitrary. The upper number would almost correspond with figures of dust output from the Sahara (Semenov 1995). The aerosol output from the former coastline is also very variable, but considerably high. The main dust storm sources have been shown by Spivak et al. (2012) to be the eastern dry seafloor (Fig. 5).

Since the environmental effects of the salt dust storms are one of the many health impacts in the region, it would be strongly necessary to indicate the medicinal factors of the dust particles which affect health of the people (Winckler et al 2012),

Fig. 4 Typical deflation pattern by wind from puffy solonchak surface with powdery salt structure. Desiccated seafloor of the Aral Sea between Barsa Kelmes and Kaskakulan (Photo: Breckle, June 2004)

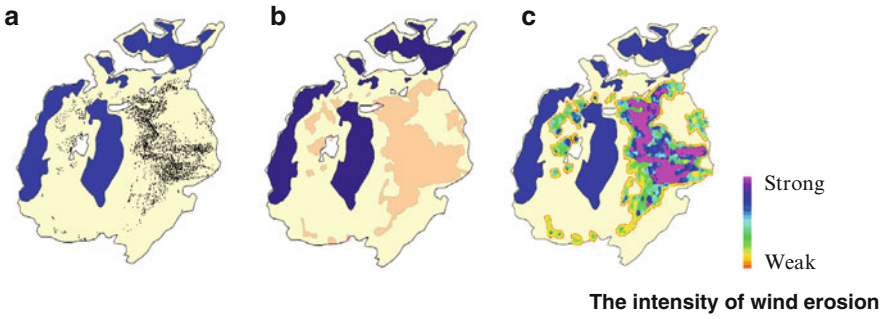

Fig. 5 The results of space monitoring of dust storm events from the dried bottom of Aral Sea in 2008. (**a**) Results of expert estimation of single dust storm events on satellite scenes for 2008; (**b**) Outlines of zones of intensive dust removal (the state of the water mirror is corresponding to the satellite data, May 2008); (**c**) The intensity of the process of dust removal (intensity of wind erosion averaged over the year data) (Spivak et al 2012)

even more since the area of salt dust as a source for dust storms has tremendously increased in the Eastern basin. It is clear that the arid climate and the structure of the desiccated seafloor with the puffy salt crusts are very favourable for the development of far-reaching salt dust storms. A detailed study on the effectiveness of dry weather, low humidity, soil dynamics, hygroscopicity of dust, and chemical compounds of crust and dust particles, on the particle size distribution and physical factors enhancing dust storms, as well as on the kinematics of mass transport of the lower atmosphere is strongly needed.

4 Research on the Developing Flora and Vegetation

4.1 Biotic Crusts

The desiccated seafloor from the 70s and 80s has predominantly a sandy surface. Aeolian processes have formed various types of sand dunes. From an ecological point of view, sand deserts offer more favourable conditions for plant cover and species diversity than do other desert types. This is due to the specific characteristics of sand, namely low water absorption, rapid infiltration rate, and low evaporation losses (because capillary threads are only 10–20 cm long). Thus, in arid regions sandy areas represent water-storing bodies. This is rather old ecological knowledge (e.g. Walter 1960). The only limitation for plant establishment and plant cover is a high frequency of extreme wind speeds that limit surface stability. In areas where extreme wind speeds are less frequent, the sand surface is relatively stable. Under such conditions, the establishment of biological topsoil crusts (microbiotic crusts by cyanobacteria) and a plant cover takes place, increasing surface stability by reducing wind speed and sand mobility (Belnap and Lange 2001; Breckle et al. 2008; Yair 2008; Veste et al. 2008; Kidron et al. 2009). In the Aralkum this is only found on few places and has not yet been studied intensively.

4.2 Succession

The dry seafloor of the Aral Sea is a new surface, where terrestrial plants (including seed banks) and animals have not existed before. It is now actively populated by organisms. The formation of plant communities, soils, a new groundwater level, aquifers, and all components and processes of ecosystems is occurring more or less simultaneously. It is a typical primary succession (Wucherer 1979; Breckle 2002a, 2008a; Dimeyeva 2007a; Wucherer et al. 2012a). The succession on the dry seafloor has continued for the last 50 years. The distribution and dynamics of the vegetation and ecosystems were surveyed along transects. On average, the succession on loamy stands can be described by two to four stages and on sandy soils by three to five stages. The existence of a distinct stage is a consequence of the ecological conditions and stability, and thus might range between 2 and 30 years.

The succession on the *sands* (Table 2) in the starting phase is caused by exogenous factors. The replacement of the *Salicornia* and *Suaeda* phases by annual psammophytes is caused by exogenous factors, like water availability. The further process of the succession after the settlement of *Stipagrostis pennata* is more endogenous and is caused by biological mechanisms (speed of the dispersal of the annual and perennial species, their ability to establish themselves on the open sand surface).

The succession on the *loamy* soils (Table 3) is mainly caused also by exogenous factors. The Aralkum vegetation is very dynamic in composition. This leads to unexpected combinations of species and interrelationships. There are many examples

Table 2 Succession on sandy substrates in the Aralkum

Phase	Time (years)	Depth of water table (m)	Dominant species	Main ecosystems	Substrate type
0	0	0	*Zostera* Cyanobacteria	Wet mud	Wet marshy solonchak
1	1–3	0.3–0.7	*Salicornia europaea* *Suaeda acuminata* *Atriplex pratovii*	Dense cover of annuals, very variable from year to year	Marshy solonchak
2	3–7	0.7–1.2	*Atriplex pratovii* *Suaeda acuminata* *Climacoptera aralensis*	Open cover of annuals, mixed with bare open sand desert and small mobile dunes	Coastal solonchak, degraded solonchak
3	7–20	1.2–1.7	*Horaninovia ulicina* *Salsola paulsenii* *Agriophyllum spp.* *Stipagrostis pennata* *Calligonum spp.* *Haloxylon aphyllum*	Patches with open mobile sand dunes, mixed with vegetation islands of perennials in dune valleys	Coastal solonchak, degraded solonchak
4	20–50	1.7–3	*Calligonum spp.* *Astragalus spp.* *Eremosparton aphyllum* *Haloxylon aphyllum* *Corispermum spp.* *Heliotropium spp.*	Open shrubby perennial vegetation between bare open sand dune desert	Sandy soils and salt hummocks
5	>40	>3	*Haloxylon aphyllum* *H.persicum* *Calligonum spp.* *Carex physodes*	Open, sandy desalinized semi-desert with scattered, sometimes dense occurrence of perennials (only fragments and local)	Arenosol

of rapid changes of plant communities and ecosystems with unique composition by various species from the psammophytic, halophytic, and hygrophytic units. Species which under natural conditions cannot be found together with other species despite an apparently similar ecological behaviour form new vegetation units here. As an example, *Climacoptera aralensis* occurs on crusty and puffy solonchaks as well as on secondary solonchaks in fields in the Kyzyl–Orda district. It forms isolated, monotonous units, sometimes together with a few salt meadow halophytes. *Petrosimonia triandra* is distributed very locally only and prefers moderate marsh conditions or slightly saline meadows. Outside the Aralkum both species do not occur together at the same localities. However, on the dry seafloor they meet and form extensive stands. *Petrosimonia triandra* is dispersed faster and colonises the coastal

Table 3 Succession on loamy and clayey substrates in the Aralkum

Phase	Time (years)	Dominant species	Main ecosystems	Substrate type
0	0	*Zostera* Cyanobacteria	Wet mud	Wet marshy solonchak
1	1–3	*Salicornia europaea* *Suaeda crassifolia* *Tripolium vulgare*	Often dense cover of annuals, very variable from year to year	Marshy solonchak
2	3–10	*Climacoptera aralensis* *Petrosimonia triandra*	Open cover of annuals, mixed with bare open salt desert	Coastal solonchak
3	10–20	*Climacoptera aralensis* *Climacoptera ferganica* *Halocnemum strobilaceum* *Halostachys caspica*	Large patches with open salt desert, mixed with annual vegetation and islands of perennials	Coastal solonchak, degraded solonchak
4	20–50	*Halocnemum strobilaceum* *Halostachys caspica* *Haloxylon aphyllum* *Climacoptera ferganica* *Climacoptera brachiata* *Petrosimonia brachiata*	Shrubby perennial vegetation with therophytes mixed with bare open salt desert	Degraded solonchak, takyr soil
5	>40	*Artemisia terrae-albae* *Anabasis salsa* *(Haloxylon aphyllum)*	Open, partly desalinized semi-desert with scattered occurrence of perennials (only fragments and local)	Xerosol (Burozem)

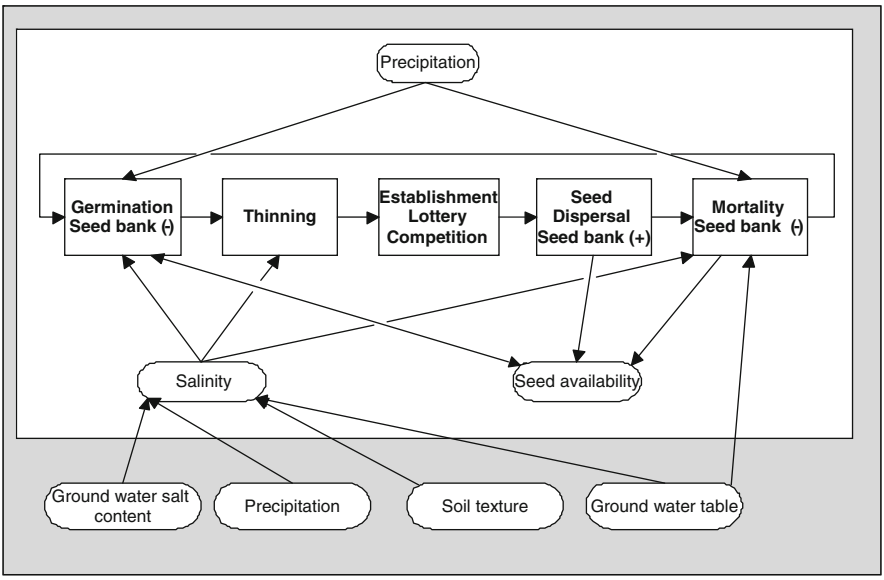

Fig. 6 Overview of the processes considered (*bright* background). Germination, thinning, establishment, seed dispersal, and mortality are simulated each year depending on precipitation, salinity, and seed availability. Salinity depends on additional abiotic factors (e.g. groundwater salt content and grain size distribution in the soil—*dark* background) which are not considered explicitly in the model (modified after Groeneveld et al. 2005)

solonchaks first. One to 2 years later, *Climacoptera aralensis* follows. Because of winged fruits, this latter species has a high potential for wide range dispersal, but in comparison with *Petrosimonia* it always remains subdominant. Both species form mixed stands with a high coverage of about 60–80 %. If the salinity of the topsoil increases, then the frequency of *Petrosimonia* decreases, but *Climacoptera* remains. The ecological range of both species apparently overlaps broadly, but some of their life strategies are different. *Petrosimonia triandra* has a more rapid dispersal ability; *Climacoptera aralensis* is more salt resistant. Grid modelling by Groeneveld et al. (2005, 2012) revealed this dynamic pattern along the salt gradient during desiccation and ongoing succession focussing on the main additional plant functional types and using their ontogeny (Fig. 6).

On the sandy substrate around the island of Barsa-Kelmes and at the southeastern coast a plant unit often can be found that is composed either by *Stipagrostis pennata* and *Halocnemum strobilaceum* or by *Stipagrostis pennata* and *Phragmites australis*. This latter combination is very peculiar. The sandy coastal solonchaks are first colonised by *Halocnemum strobilaceum*. With further decrease of the groundwater table, the upper sandy layer becomes slowly desalinised within 8–10 years down to about 1 m depth. The main active roots of *Halocnemum strobilaceum* are at a depth of 1–2 m. *Stipagrostis pennata*, however, has its main roots in the upper sandy soil. This vegetation unit is actually an intermediate succession type.

Table 4 The dominant plant families of the flora of the Aralkum [the desiccated seafloor of the Aral Sea; after Dimeyeva et al. (2012a)]

Plant families	Genera	Species	Species (%)
Chenopodiaceae	31	90	24.3
Asteraceae	25	48	13.0
Polygonaceae	5	39	10.5
Brassicaceae	24	34	9.2
Fabaceae	9	23	6.2
Poaceae	14	22	6.0
Boraginaceae	9	13	3.5
Tamaricaceae	1	10	2.7
Ranunculaceae	7	8	2.2
Zygophyllaceae s.l. (3 families s. str.)	3	7	1.9
Caryophyllaceae	3	6	1.6
Cyperaceae	4	6	1.6
Apiaceae	2	5	1.4
Additional families (29)	42	58	15.7
Total: 44	174	370	100

But with stabilisation of the seawater level, it might be possible for such a vegetation unit to become rather permanent. In any case, the separation of the root systems preferring different soil depths is a precondition for the co-existence of the two species.

The rather rapid changes of vegetation cover and of seasonal vegetation depending on varying precipitation had been checked also by remote sensing techniques (Nezlin et al. 2004, 2005; Micklin 2008; Löw et al. 2012).

4.3 Flora of Vascular Plants

The vascular plants of the Aralkum consist of about 370 species belonging to 43 families and 178 genera. The leading 13 vascular plant families (Table 4) comprise 310 species (84.2 % of the flora) belonging to 136 genera (76.4 %). Species from Chenopodiaceae, Asteraceae, Polygonaceae, Brassicaceae, and Poaceae families prevail. The most important genera are *Calligonum* (35 species), *Artemisia* (14 species), *Salsola* (13 species), *Atriplex* (12 species), *Astragalus* (11 species), *Tamarix* (10 species), *Suaeda* (9 species), *Climacoptera* (5 species), and *Corispermum* (5 species) (Dimeyeva et al. 2008, 2012a). The biodiversity of vascular plant species related to the whole area is relatively low, as generally in deserts. However, the diversity of halophytes is rather high. Related to the available water input, the resource-related diversity is very high (Breckle 2006).

4.4 Higher Vegetation: Dominant Elements and Physiognomy

4.4.1 Psammophytic Vegetation

The area of sandy and sandy-loamy sediments is about 12,000 km^2 of the whole Aralkum, mainly in the oldest parts of the desiccated seafloor. This means that sandy deserts with psammophytes are the most important vegetation around the old coastline, to a great extent at the SE and NE coasts and around the former islands Barsa-Kelmes and Vozhrozhdenie. For the whole old coastline sands are characteristic with a particle size of about 0.1–0.5 mm. Coarser sands are found adjacent to the Syrdarya delta. This sand is predominantly made up of quartz. However, in some parts, limestone particles from sea shells can contribute to 30–70 % of total mass. The sandy soils exhibit no or only a very slight formation of horizons, yet. Wind erosion causes the development of a complicated relief with all kind of sand dunes. At the E coast the upper limit of sand deposits is at about 43–46 m asl, at the N coast at 48–50 m asl, and at the S coast it goes down to about 33–36 m asl.

The physiognomy of this sand desert in the Aralkum is dominated by the rather high growing *Haloxylon aphyllum* and *H. persicum, Salsola arbuscula, S. richteri,* and *S. paletzkiana* (Chenopodiaceae), by *Calligonum* species (Polygonaceae), *Astragalus brachypus* and *A. ammodendron, Ammodendron bifolium,* and *A. conollyi, Eremosparton aphyllum* (Fabaceae), *Stipagrostis pennata* (Poaceae), *Artemisia arenaria* (Asteraceae), and some species from other families (Kurochkina 1978, 1979; Wucherer and Breckle 2001; Breckle et al. 2012b). Average canopy height is about 1.0–2.0 m, but in extremes it can reach 5 m (by *Haloxylon* species, Fig. 7a). Characteristic therophyte species are *Horaninovia ulicina*, *Agriophyllum squarrosum*, *Salsola paulsenii,* and *Corispermum* species, all Chenopodiaceae, about 20–40 cm high. Typical perennial species are *Allium sabulosum, Artemisia santolina, Chondrilla brevirostris*, *Heliotropium arguzioides, Astragalus lehmannianus*, and some other species. The cover of the psammophytic plant communities is about 10–40 %, rarely up to 80 %. The psammophytic vegetation is dominant on the dry surface areas from the 1960s, partly from the 1970s and rare from the 1980s (Dimeyeva 2004).

At the SE coast perennial psammophyte communities are found, mainly with grasses like *Stipagrostis pennata* (Fig. 7a), and also shrubs as, e.g. *Eremosparton aphyllum, Haloxylon aphyllum, Astragalus brachypus*, and *Calligonum* species, etc. (Wucherer and Breckle 2003).

4.4.2 Halophytic Vegetation

The halophytic vegetation is present on most of the dry seafloor of the 1970s, 1980s, and 1990s and especially the more recent desiccated areas. This vegetation is the most prominent now all over the Aralkum. The halobiomes (salt deserts) exhibit a typical eu- or hemihalophytic vegetation on more or less saline substrates. A rather considerable portion of the flora is halophytic, mainly from Chenopodiaceae, which

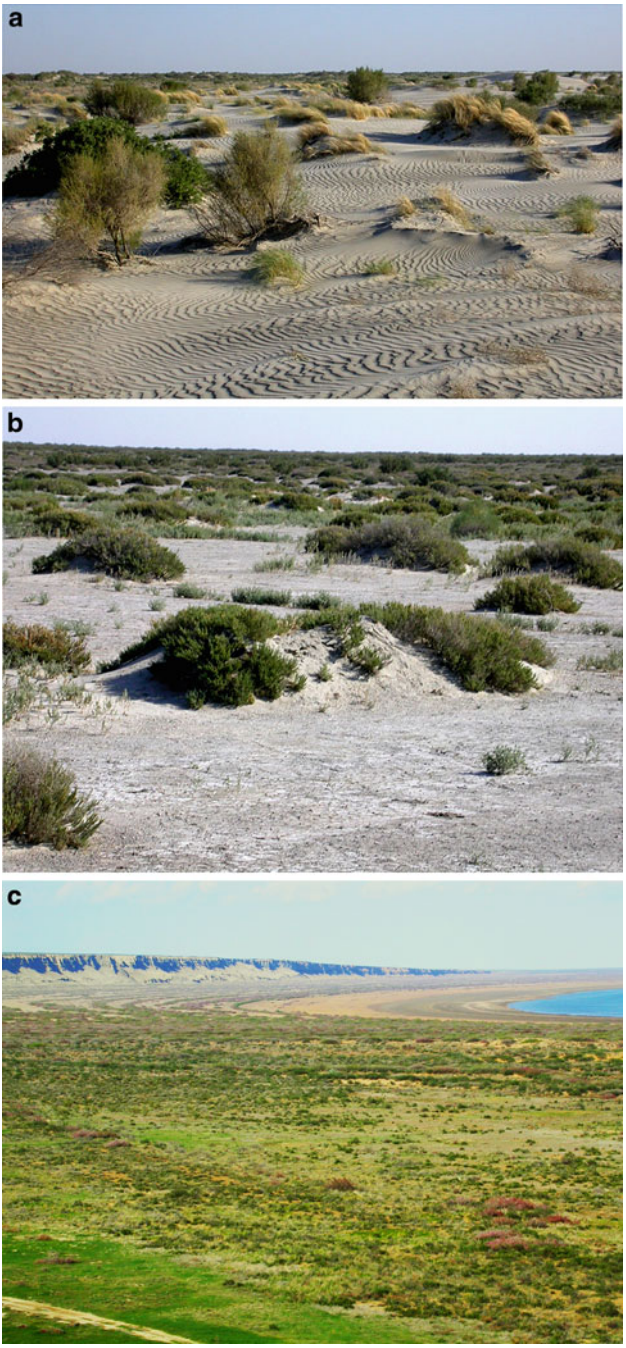

Fig. 7 (continued)

Fig. 7 (**a**) Psammophytic vegetation on the dry seafloor of the 1960s at the SE coast: *Stipagrostis pennata* plant community with *Haloxylon aphyllum* (Photo: Wucherer). (**b**) Perennial halophytic vegetation on the dry seafloor of the 1970s at the E coast (Transect Kaskakulan): hummock-forming *Halocnemum strobilaceum* plant community (Photo: Wucherer). (**c**) Steep North coast (chinks) of Aralkum at Shevshenko Bay with retreating Aral Sea and distinct *Tamarix* stripes (Photo: Breckle 2003). (**d**) *Haloxylon aphyllum* plantings (12 years old) on the Kaskakulan transect (Photo: Wucherer, Sept 2003)

are the most dominant plant family with about 25 % of the whole Aralkum flora. Their ecology and ecophysiology are characterised by the fact that they can fulfil their whole life cycle under saline conditions (Breckle 2002b, 2008b; Breckle and Wucherer 2012c). Life forms are shrubs, semi-shrubs, dwarf shrubs, perennial and annual herbs, mainly from the Chenopodiaceae (*Halostachys, Halocnemum, Salicornia, Suaeda*), but also from Tamaricaceae, Limoniaceae, and some other plant families. Canopy height is normally below 1 m, but can exceed with adult saxaul (*Haloxylon*) shrubs also 3 m. Cover percentage also varies considerably between 10 and 100 %. A rather high variability in shape of this halophytic vegetation is correlated with the very variable salinity in soil and the various soil horizons. Typical soils for halophytic vegetation in the Aralkum are various types of solonchaks.

Annual Vegetation

Close to the retreating water level of the Aral Sea very often a rather dense belt of therophytic carpets with *Salicornia europaea*, *Suaeda* species, and *Tripolium vulgare* is developed. The canopy is about 20–60 cm high, with *Tripolium* growing also up to 120 cm. Cover percentage of therophytic plant communities varies between 20 and 100 %. *Salicornia* is the main component of the annuals close to the water. But also some *Suaeda* species (*S. acuminata* and *S. crassifolia*) are mixed. *Tripolium vulgare* is only present with isolated stands. At the E coast near

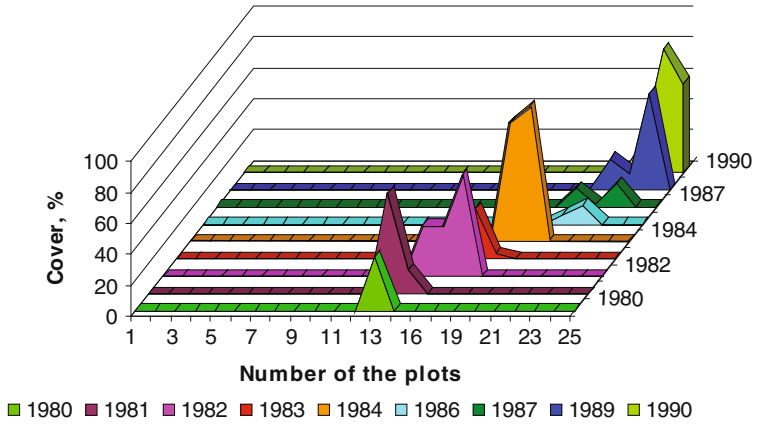

Fig. 8 Replacement succession of *Salicornia* belts on the marshy solonchaks from year to year on the Bajan transect, indicating the migrating *Salicornia* belt along the retreating water level

Barsa Kelmes aggregations of *Bassia hyssopifolia* can be found, and adjacent to the delta regions of rivers mixed stands of *Salicornia europaea* and *Phragmites australis* or *Bassia hyssopifolia* and *Phragmites australis* can be observed. Seeds of *Salicornia* can withstand high salinities, and germination can take place under euhaline conditions. Close to the water level the irregular inundations of the marshy solonchaks are favourable for those annuals. Dead remnants of plants from preceding years can be found everywhere. The development of the therophytic vegetation belt moves according to the retreat of the water level, indicating a typical replacement succession (see Fig. 8).

On the desiccated seafloor about 3–10 years after it was fallen dry, we can find quite often a second wave of annuals developing, mainly with *Climacoptera aralensis, Petrosimonia triandra, Bassia hyssopifolia*, and *Atriplex pratovii* on the coastal solonchaks. The canopy is about 20–60 cm high, with *Atriplex* reaching up to 100 cm. Those sites are already under the predominant influence of the zonal climatic conditions. This causes rather irregular establishment and varying growth rates from year to year. Cover percentage of plant communities varies between 5 and 100 %.

Perennial Vegetation

At the E coast of the former Aral Sea on the older seafloor from the 1960s and 1970s salinity is moderate or low. Here plant communities with *Haloxylon aphyllum, Halocnemum strobilaceum*, *Kalidium caspicum*, *Limonium suffruticosum,* etc. are found (Fig. 7b). They form stands with a cover percentage of 20–40 %. On the sand covered coastal solonchaks rather dense stands with a cover percentage up to 80 % can be observed. However, these are small portions in the whole mosaic and not

Table 5 Percentage of dominant plant communities and landscapes

Plant communities/landscapes	Transect Kabanbai (SW coast of the Aral Sea), (1994)	Transect Kaskakulan between old coastline and the former island Kaskakulan (E coast of the Aral Sea), (1994)
Salt desert without vegetation (bare desert)	43.1	–
Salicornia plant communities	14.8	–
Barkhanes (open sand dunes)	29.8	–
Astragalus-Haloxylon plant communities	6.9	–
Stipagrostis pennata plant communities	5.4	–
Haloxylon plant communities	–	48.3
Halocnemum plant communities	–	34.9
Kalidium plant communities	–	10.7
Halostachys plant communities		6.1

representative for the whole coast. *Haloxylon* (Saxaul) is used by the people for fuel. In 1998 there were massive "deforestations" of saxaul.

Almost no perennials except for some *Tamarix* or *Halostachys belangeriana* shrubs grew on the desiccated seafloor from the 1980s and 1990s. Invasion of the former sea areas by the perennial vegetation is definitely slower than the retreat of the sea level. The conditions for germination and establishment of the perennials are quite favourable on the marshy solonchaks a few years after desiccation, but in later phases they become much slower and often seem to be hindered.

In 1998, the perennial vegetation was investigated on the transects of Karabulak, Bayan, and Kaskakulan. The share of plant communities varies widely. In the southern transects (Kabanbai, Table 5) bare open areas predominate; in northern transects vegetation often has higher cover values (Table 5, Kaskakulan transect). Especially the communities with *Tamarix* which are common along the narrow desiccated coasts of the Small (Northern) Aral Sea are conspicuous. They indicate the retreat of the coastline by almost annual germination events, later forming distinct stripes of bushes (Fig. 7c).

Salt Deserts Without Vegetation

The whole seafloor which desiccated since 1980 can be regarded as a huge salt desert flat. It is about 70–80 % of the present Aralkum. The therophytic vegetation is spatially and temporarily very instable; they do affect the soil surface only superficially. Thus, denudation of the desiccated soil surface amounts to about 2 mm per annum; in other words, within 30 years about 6 cm of loose substrate may have been blown away. This was very obvious in the 1980s, when formation of solonchaks just had started. The process seems to be even intensified, because the

Fig. 9 Salt desert on the dry seafloor of the 1990s at the E coast (Transect Bajan) with crusty solonchak without any vegetation (Photo: Wucherer)

present open and often puffy salt crust surface can exhibit windblown losses of 3–7 cm per year (Semenov 2012).

This makes it important to know which annuals and which perennials are present on those rather open sites of the 1970s and 1980s. However, those areas are not easy accessible. Within the transect Bayan between the former islands Barsa-Kelmes and Kokaral, it was possible to reach the actual coastline in the 1990s. Here, a mosaic of pure open salt desert (Fig. 9) and of patchy therophytic stands was present. More important therophytes that were found are *Suaeda acuminata, Petrosimonia triandra*, and *Climacoptera aralensis*.

4.4.3 Tugai Vegetation

The woody vegetation on alluvial soils adjacent to river valleys or delta areas is called tugai forests. The tugai biome along the lower parts of the rivers is characterised mainly by *Salix songarica, Salix wilhelmsiana, Elaeagnus oxycarpa*, and shrubs from the Tamaricaceae. The shallow groundwater level and rather often periodic inundations enable good growing conditions for woody plants. Floristically those stands are rather poor. Soils can be somehow saline and then halophytes are mixed. Canopy height may reach 8 m and cover percentage 50–100 %. Most stands are heavily endangered and need special care with additional measures for water supply by groundwater (Novikova 2001; Ogar 2001).

In former centuries these tugai forests had been the centre of rich wildlife, including tigers, leopards, Bukhara-deer (*Cervus elaphus bactrianus*), etc. In 2007, a group of deer was released in Uzbekistan's Zarafshan Nature Reserve.

In the lower Amudarya River, tigers sometimes preyed on jackals, jungle cats, and locusts, around the Aral Sea in Kazakhstan the tiger fed on boar, saiga, goitered gazelle, wild horses, Mongolian Wild Ass, and mountain sheep (Heptner et al. 1992). But this is history of last century, since most tugai forests got lost. Thus, dense Tugai forests with adult trees of *Populus* species (called Turanga forests) are rather rare nowadays. Absence of spring-summer flooding and river channel straightening are limiting factors for establishment of a floodplain forest on the dry seafloor. Often the original tugai forest along rivers around the former Aral sea is replaced by a dense scrub of *Elaeagnus* and *Tamarix* (*T. laxa, T. elongata, T. hispida, T. ramosissima*). *Elaeagnus oxycarpa* forms small vegetation fragments and is very local. *Tamarix laxa* and *T. elongata* established locally and are characteristic of the dry seafloor of the 1960s and 1970s, *T. hispida* at seafloor sites desiccated in the 1980s and 1990s, and *T. ramosissima* occurs at the dry seafloor of the delta areas. There, a *Tamarix* community is present which spreads also to most of the dunes along the E- and S-coast. Canopy height may reach 3 m and cover percentage 30–80 %.

4.4.4 Salt Meadows

Some intermediate salt meadows with reed vegetation and perennial hemicryptophytes (*Puccinellia* and *Limonium* species, *Phragmites australis*, *Aeluropus littoralis*, *Karelinia caspica,* etc.) can withstand rather high salinities. *Phragmites australis*, together with *Puccinellia dolicholepis*, forms plant communities at the chink coasts (steep coasts at the former coastline) on the dry seafloor of the 60s with cover percentage of 60–80 %. On the meadow solonchaks at the delta areas a very characteristic *Limonium otolepis–Aeluropus littoralis* plant community with cover percentage of 40–80 % can be found.

4.5 Fauna

The actual fauna of the Aralkum has been studied only partly. Lists of mammals of the Kazakhstan part of the Aral Sea region, the migratory breeding bird species and rare winter visitors, the resident breeding bird species, passage visitors (birds), vagrant birds, reptiles collected around Aral Sea in 2002–2004, and taxonomical diversity of insect orders and other groups are documented by Joger et al. (2012). The ecological disaster of the Aral Sea reduced the faunistic diversity of the area in a selective manner. Aquatic and semiaquatic animal species such as fish-eating birds, waterfowl, amphibia, water snakes, and aquatic insects suffered dramatic reductions in numbers. Some freshwater species and species of riverine forest died out (see above) or left the area completely. On the other hand, desert species and certain eurybionts were able to extend their ranges into the Aralkum (Joger et al. 2012).

In the hypersaline remnant water bodies, only few animals survive, mainly *Artemia*, the salt brine shrimp (Arashkevich et al. 2009), which in the future may even have some economic role as a protein source.

The succession needed about 3 decades after drying to establish stable communities. The situation is less simple with typical steppe animals, such as certain mammal and reptile species. Some of them (rodents, small carnivores, lizards, snakes) were able to colonise new habitats on the former seafloor, others remained in preserved steppe habitats, and a third group suffered from population reduction. The latter fact must be stated for mammals of the former island of Barsa-Kelmes, a nature reserve which used to be protected from outside influences when it was an island, but which lost its unique characteristics when it became connected to the mainland.

Further monitoring of the fauna of the Aralkum is strongly recommended to document the very active migrations to and invasions of the dynamic new ecosystems.

5 Ecology of Halophytes and Psammophytes

In arid sites with a continental climate, various types of salinity are known (chloride, sulphate, carbonate, magnesium, and boron), more variable than along ocean coasts, depending on geology, soil properties, climatic conditions, and ecosystem processes. The presence of excessive amounts of ions in such ecosystems dominates over many other environmental factors. Only the supply of water is the other decisive factor in the development of such ecosystems.

The colonisation of the desiccated seafloor of the former Aral Sea by halophytic species occurs under climatic conditions which are rather variable from year to year (Breckle et al. 2012b). The halophytic species, nevertheless, are on the other hand indicators of the degree of salinity at their respective growing sites, and thus can be used to monitor salinity. A novel list of indicator values for salinity is presented by Breckle and Wucherer (2012c). Relevant knowledge is also a precondition for the necessary means of phytomelioration.

It is obvious that leaf succulents and stem succulents, such as species from the genera *Suaeda, Salicornia*, and *Halocnemum*, accumulate considerably more Na^+ and Cl^- (3,000–5,000 mmol/kg) in comparison with other species. The ionic contents (Na^+ and Cl^-) of *Climacoptera* species and of *Ofaiston monandrum* are lower (2,000–3,500 mmol/kg) in comparison with those of species from *Salicornia* and *Suaeda*. Even lower are the values from *Petrosimonia triandra*. On the other hand, the Na^+ and Cl^- accumulation of pseudohalophytes such as *Euclidium syriacum* and *Stipagrostis pennata* is very low.

Not only salt accumulation is important but also discrimination between ions. Most halophytes discriminate between Na^+ and K^+ and only few species are really sodiophilic (Moore et al. 1972). To demonstrate the characteristics in K^+/Na^+ discrimination, it is necessary to have the relevant soil samples from the

Table 6 Ion pattern of some common halophytic species of the Aralkum analysed from hot water extracts

Species		n	Locality	Cl^-	Na^+	K^+	Ca^{2+}	Mg^{2+}	Na^+/K^+
Climacoptera aralensis	LSu	8	3×Ba, 5×Ka	2,780 ± 531	3,360 ± 1,100	573 ± 199	3.55 ± 1.29	142 ± 46	5.9
Petrosimonia triandra	LSu	4	3×Ba, 1×Ka	600 ± 109	1,020 ± 289	570 ± 54	13.0 ± 8.3	306 ± 77	1.8
Suaeda acuminata	LSu	10	1×Ba, 9×Ka	4,410 ± 810	4,200 ± 597	697 ± 138	5.66 ± 2.9	236 ± 70	6.0
Suaeda crassifolia	LSu	2	2×Ka	4,480 ± 579	3,460 ± 298	427 ± 0.7	20.3 ± 6.3	545 ± 105	8.1
Ofaiston monandrum	SSu	2	2×Ka	2,200 ± 837	2,180 ± 1,290	423 ± 142	120 ± 149	532 ± 59	5.2
Salicornia europaea	Ssu	2	2×Ba	4,290 ± 157	3,860 ± 335	428 ± 132	5.4 ± 1.8	168 ± 23	9.0
Halocnemum strobilaceum	Ssu	1	1×Ba	3,040	3,750	506	2.02	133	7.4
Halostachys caspica	Ssu	1	1×Ba	1,090	2,090	509	2.61	60.0	4.1
Euclidium syriacum	Ps	1	1×Ka	314	127	497	142	95	0.26
Malcolmia africana	Ps	1	1×Ka	451	648	998	328	148	0.65
Eremosparton aphyllum	NoH	1	1×Ba	155	32.7	324	52.9	78.5	0.10
Stipagrostis pennata	NoH	1	1×Ba	78.0	44.7	327	141	43.4	0.32

From Bajan—Ba and from Karabulak—Ka; *n* number of samples, ion content as mMol kg^{-1} and standard deviation. For each species the halophyte type is indicated (second column: *Lsu* leaf-succulent Eu-halophytes; *SSu* stem-succulent Eu-halophytes; *Ps* pseudohalophytes; *NoH* non-halophytes

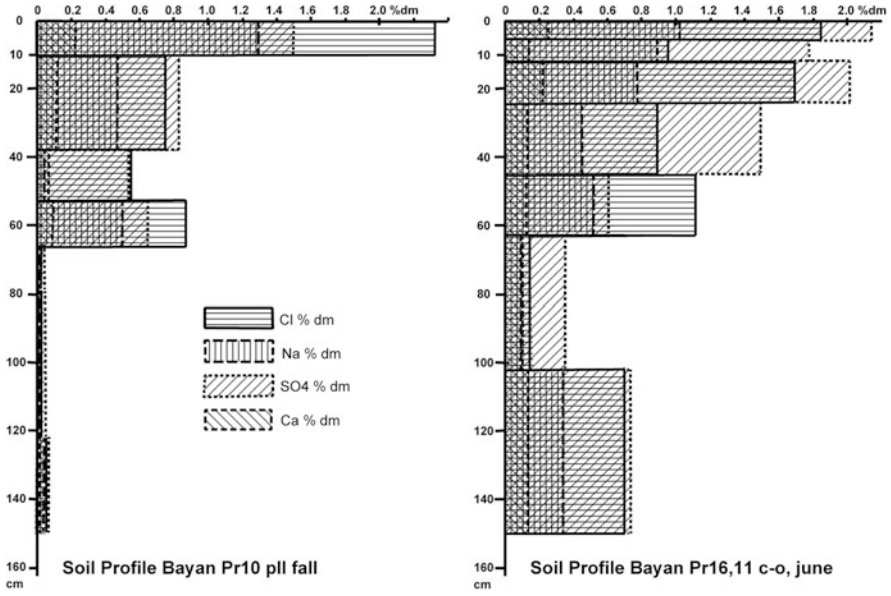

Fig. 10 Ion content in soil horizons of Aralkum. *Left*: Soil Profile Bayan Pr10 with main salt accumulation, mainly chloride in top soil. *Right*: Soil Profile Bayan Pr16 with salt, mainly sulphate accumulation in top soil and in lower horizons

rhizosphere of the respective plants. Then the accumulation factor for sodium in comparison with potassium can be calculated. It is easily seen that most species under a wide range of given cation ratios in the soil favour potassium uptake. The widespread Chenopodiaceae *Salicornia europaea* and *Suaeda maritima* can be termed sodiophilic, and so can *Climacoptera aralensis* and *Suaeda acuminata* (Table 6), whereas *Petrosimonia triandra* exhibits a rather balanced Na^+/K^+ ratio. In contrast, *Puccinellia distans, Stipagrostis pennata*, and *Eremosparton aphyllum* very selectively accumulate potassium by a factor of 10–100 according to the soil Na^+/K^+ ratio; even in saline soils their Na^+/K^+ ratio is between 0.10 and 0.40. Slightly more sodium is accumulated in some Brassicaceae, e.g. in *Malcolmia africana*. All other Chenopodiaceae are more or less halophytic and exhibit rather high Na^+/K^+ ratios, which is not really very different when results are compared from hot water extracts and from acidic extracts, respectively.

The data on soil indicate that along the Karabulak transect all sites are rather alkaline. Salinity is very variable between sites and with concern to its distribution between different soil horizons. The amount of salt depends on season, as salinity increases with high evaporative demands in summer along the very long capillaries in loam and clay to the upper horizons. This may lead to formation of a salt crust. However, lower horizons also often have a rather high salinity level, whereas middle horizons may store less saline water from winter snow or rains. This is shown by two examples of soil profiles (Fig. 10). In both soil profiles it is obvious

Table 7 Control mechanisms enabling growth of halophytes on saline sites (Breckle 1995, 2002b; Breckle and Wucherer 2012c) with main morphological strategy type indicated

	Halophyte type
A. Avoidance	
a Growth only during favourable seasons (time niche)	NoH, Ps,Su
b Growth only on favourable sites (site niche)	Ps, NoH
c Limitation of root growth and absorption activity to distinct soil horizons (site niche)	Ps, NoH
B. Evasion and adaptation processes	
a Selectivity against Na and Cl	NoH, Ps
b Leaching of salt from shoots	NoH, Ps
c Removal resp. Exclusion of salt from assimilating tissues	Ps
d Compartmentation of salt within plant, within tissues, within cells	All plants
e Accumulation of salt in xylem parenchyma in roots and shoots	All halophytes
f Synthesis of organic solutes	All plants, Su
g Retranslocation of salt to roots and recretion by roots	Halophytes
h Disposal of older plant parts ("salt-filled organs")	Ps, all halophytes
i Recretion by gland-like structures on shoots	
1. By salt glands	EX
2. By salt bladders	NX
C. Tolerance	
a Increase of salt tolerance of tissues, cells, organelles (e.g. by compatible solutes, uptake of salt)	LSu,SSu,NX,EX,Ps
b increase in halo-succulence	LSu, (Ps)
1. Increase in leaf succulence	SSu
2. Increase in stem succulence, reduction of leaves	

Abbreviations of last column, see also text: *EX* exocrinohalophytes; *LSu* leaf-succulent Eu-halophytes; *NoH* non-halophytes; *NX* endocrinohalophytes; *Ps* pseudohalophytes; *SSu* stem-succulent Eu-halophytes; *Su* xero-succulents

that the sulphate salinity is as high as or even higher than the chloride salinity, but differing in the various horizons.

There are many indications that also in the stem- and leaf-succulent halophytes, in the exocrino-, and in the pseudohalophytes from the dry Aral Sea floor, different mechanisms and strategies for the adjustment and regulation of the salt concentration in the plant tissues are operating (Table 7) and thus a differing salt tolerance in the various species leads to a specific pattern of species and halophyte types along salt gradients.

The sequence of species along the salt gradient in a rich halophytic area, as it is in the Central Asian deserts, reveals a typical sequence of the dominating halophyte types. Along the salt gradient (Breckle 1986), which can be derived from salinity measurements in a mosaic vegetation, it is obvious that the stem succulents (see Fig. 14a) and then the leaf succulents (Fig. 14b) play the major role close to the saline lakes or basins, where salinity is high. The recreting halophytes (exocrinohalophytes, Fig. 14c, endocrinohalophytes, Fig. 14d, e) dominate in the middle part of the transect, where salinity is more variable as is water supply (Breckle 1995,

Table 8 Definitions of the S-value, the ecological salinity-indicator value (see Breckle 1985; Breckle and Wucherer 2012c; Ellenberg et al. 1991)

S-value	Definition
S0	Not salt tolerant, species never in brackish soils [NaCl content in soil <0.01 %], very sensitive to salt, strong non-halophytes
S1	Almost not salt tolerant, very rare in brackish soils [NaCl content in soil <0.05 %]
S2	Similar as S1, but more often in slightly brackish soils [oligohaline, c. 0.05–0.3 % Cl^-] slightly salt-tolerant species, which can withstand some salinity, but most frequently occur in non-saline soils ("pseudohalophytes", exhibiting no special morphological or anatomical features, also possible for higher S-values)
S3	Species indicating salinity, may also grow in soils with low salinity ("facultative halophytes", "accidental halophytes" in ecological sense) [ß-mesohaline, c. 0.3-0.5 % CI^-]
S4	Similar as S3 [α/ß-mesohaline, c. 0.5–0.7 % CI^-], exhibiting some salt tolerance and longer survival under salinity
S5	Species normally only on saline soils [α-mesohaline, c. 0.7–0.9 %CI^-], can withstand moderate salinities
S6	Typical halophytes, indicating salinity, rare in non-saline soils [α -meso-/ polyhaline, c. 0.9–1.2 %CI^-]
S7	Similar as S6, but very salt tolerant, never in non-saline soils (often "obligatory halophytes" in ecological terms) [polyhaline, c. 1.2–1.6 %CI^-], species indicating moderate to rather high salinities in soil
S8	Typical halophytes, indicating high salinity, very salt tolerant [euhaline, c. 1.6–2.30 % CI^-], typical salt plants, indicating high salinities, only growing on severely saline sites (obligatory halophytes, Eu-Halophytes)
S9	Extreme halophytes, in soils with very high—during dry periods extremely high—salinity ("obligatory halophytes" in ecophysiological terms) [euhaline/hypersaline, >2.30 %CI^-], found only on and always indicating very strongly saline and salt-crust soils. Species, which can fulfil their whole life cycle on highly saline sites
X	S-value very variable, broad, indistinct, species found from non-saline to very saline sites
–	S-value not yet known, most probably S0 or S1

2002b). This part of the transect is characterised by less water availability and often here a much higher proportion of C4 plants occurs. This is also the case on the less saline side, where the pseudohalophytes and finally on almost salt-free substrates the non-halophytes predominate and other ecological factors, such as water availability, water supply, and nitrogen source, govern the vegetation mosaic. However, on the desiccated seafloor of the Aral Sea an equilibrium of halophyte types has not yet been reached; the dynamics of changing ecological conditions from year to year are so drastic that only by chance a mixture of more or less adapted species is found, which in part resemble the sequence of the halophyte types discussed.

By comparing many sites with different salinities, one can evaluate the distinct ecological optimum of the co-occurring taxa (not ecophysiological optimum without competition, which can be rather different: many plants grow better under low salinities but are pressed to higher salinity sites by competition, where they can grow, but not optimally). This ecological optimum can be used to grade the

Table 9 Number of species of halophytic strategy types and related salinity indicator values for the halophytic flora of the Aralkum

Halophytic strategy-type	S1	S2	S3	S4	S5	S6	S7	S8	S9	X	Σ
Non-halophytes (NoH)	**42**	18	0	0	0	0	0	0	0	0	60
Pseudohalophytes (Ps)	4	**30**	**42**	**15**	8	4	1	0	0	4	108
Xero-Succulents (Su)	0	0	0	0	0	0	0	0	0	1	1
Leaf-succulent Eu-halophytes (LSu)	0	2	2	5	**14**	**7**	**22**	**9**	1	0	62
Stem-succulent Eu-halophytes (SSu)	0	0	0	1	1	1	1	0	**2**	0	6
Endocrinohalophytes (NX)	0	0	2	**5**	1	2	0	0	0	0	10
Exocrinohalophytes (EX)	0	0	0	0	0	9	**7**	**4**	0	0	20
Hydro-halophytes (HH)	0	0	0	1	0	0	0	0	1	0	2
Σ	46	50	46	27	24	23	31	13	4	5	269
Not determined strategy type	104										
Σ Σ											373

ecological salinity tolerance by an indicator value (*S* value, see Table 8). Such indicator values are used rather widely in various regions for various ecological parameters, e.g., pH, nitrogen supply, drought tolerance, and heat tolerance (Ellenberg et al. 1991).

For salt tolerance a scale from 0 to 9 can be used (Breckle 1985), where $S = 9$ means the highest salt tolerance. In contrast to many other indicator values, the distribution of the *S* values over the whole scale is oblique since most species belong to the non-halophyte group, which has an indicator value of $S = 0$ or $S = 1$. By long-term observations and comparing many sites, one can define *S* values for many species. A few species are very variable in their adaptation to saline site conditions, and those species have no definite *S* value ($S = X$). For others, their typical site conditions are not known exactly ($S = –$) and will be revealed only in the future. It should also be kept in mind that the *S*-value list is only valid for a distinct region; it depends on the whole given flora and the respective competitive plant communities.

The Aralkum flora is very rich in halophytes; thus, the percentage of species with high *S* values (above 4) is rather high in several plant families. Other plant families are represented in the Aralkum by a quite high number of species, too, but still prefer mainly sites with low salinity (Polygonaceae, Brassiceae, Fabaceae). It is easily recognisable that the halophytic type and the S-value are rather strongly correlated (see Table 9).

On the sand dune areas a characteristic set of psammophytes is found. This is part of the adjacent flora of the Kyzylkum and the Karakum. The occurrence of a high number of *Calligonum* species is very remarkable (Dimeyeva 2004; Dimeyeva et al. 2008, 2012a).

Fig. 11 Plantings of *Haloxylon aphyllum* on poorly to moderately salinated sand soils. Plantings in March 2004, Record in June 2004 (Photo: Wucherer)

6 Conclusions

6.1 Phytomelioration

Regarding the present ecological and environmental situation in the Aralkum basin, it is obvious that phytomelioration is one of the main and urgent tasks to minimise the formation of salt dust storms from the desiccated seafloor. Only few shrubs are suitable for this purpose, the best results were achieved with black saxaul (*Haloxylon aphyllum*, Fig. 7d) plantings by saplings.

Establishment and the growth of *Haloxylon* is dependant on the weather conditions, the hydrological conditions and the season of the plantings. Lake deposits or soils of which the topsoil is lightly sandy are favourable for the plantings. Establishment of saxaul saplings was successful on the poorly to moderately saline sandy soils and the loamy coastal solonchaks with sand cover, with low salinity of the topsoil. The establishment rate from the plantings is higher in spring compared to autumn plantings (Fig. 11).

On the crusty-puffy coastal solonchaks, however, the rate of establishment of *Haloxylon* is only up to 10 % and the vitality of the young plants is very bad (Fig. 12). On the crusty-puffy coastal solonchaks *Haloxylon* plantings can only be successfully performed with an improvement of the soil conditions (e.g. planting in furrows or pits filled with sand). Some seedlings can reach the generative phase, however, rather fast, but a second plant cohort does not establish, since the seeds of *Haloxylon* do not germinate on coastal solonchaks. Therefore, plantings on crusty-puffy coastal solonchaks are only worth to be carried out on locations where a

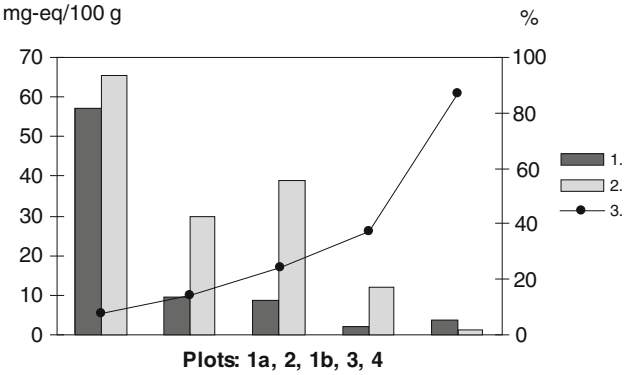

Fig. 12 Survival rates of saxaul saplings (*Haloxylon aphyllum*) on saline soils (in root-inhabited horizon) with differing physical–chemical site characteristics 1—total concentration of toxic ions [mg equiv./100 g of soil]; 2—content of silt and clay [%]; 3—survival rate [%] in first vegetation season

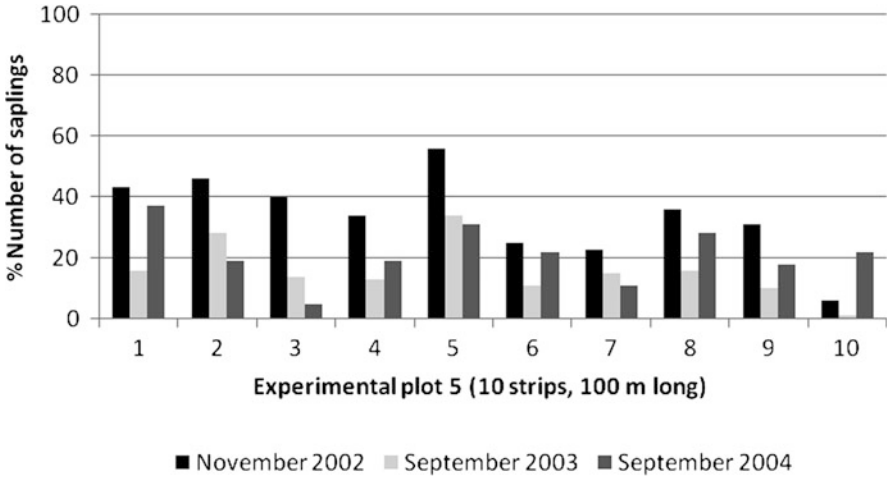

Fig. 13 Percentage number of established saplings and seedlings of *Haloxylon aphyllum* on loamy coastal solonchaks with a sand layer, ten strips, 100 m long (Planting in November 2002, record in September 2003 and 2004; Wucherer et al. 2012b)

natural desalinisation of the topsoil can be expected, and where sand islands are present (Fig. 13).

The species list to select for the plantings must hence be extended to more eu-halophytic species, which can withstand more salinity in the soil, such as *Halocnemum strobilaceum* (Fig. 14a). There are many other perennial eu-halophytes from the genera *Halostachys, Kalidium, Salsola,* and *Suaeda* in the region. However, planting technology for those species is practically unknown and must still be developed. The already tested technologies (construction of furrows,

Fig. 14 (**a**) *Halocnemum strobilaceum*, young shoots (Photo: Breckle, May 2004). (**b**) *Suaeda acuminata*. Remnants from last year, new seedlings and saplings. North Aral Sea (Photo: Breckle, May 2003). (**c**) *Frankenia hirsuta*, in flower with many dry recreted salt crystals (Photo: Breckle, May 2004). (**d**) *Atriplex pratovii*. Intact bladders from *lower* side of leaves (Photo: Breckle, May 2003. (**e**) Crushed bladders from *upper* side of leaves of *Atriplex pratovii* after wilting, forming a layer of salt crystals. North Aral Sea (Photo: Breckle, May 2004)

mechanical enforcement of the topsoil, removing the salt crust, minicatchment construction, etc.) should also be used for planting of eu-halophytes in other parts of the Aralkum, considering the specific habitats and the biological properties of the

Table 10 Sand accumulation by various plant bushes at Southern Aralkum (Novitskiy 2012)

Plant	Age of plant (years)	Height of plant (cm)	Circumference of plant (cm)	Amount of accumulated sand (m^3)	Cover of stems (%)
Haloxylon aphyllum	7	265	320	10.6	28
Salsola richteri	Not known	220	260	5.6	23
Calligonum caput-medusae	Not known	110	210	6.9	55

reforestation species and the climatic conditions (Kuz'mina and Treshkin 2012). It has been shown on several sites that phytomelioration by permanent plantings is an effective procedure to decrease the spreading of salt and dust of the desiccated seafloor and open salinised fields (Breckle 2003; Breckle and Wucherer 2006; Hüfler and Nowitzkiy 2001; Kaverin and Salimov 2000; Kaverin et al. 2005; Novitskiy 1997; Singer et al. 2003; Wucherer and Breckle 2005; Wucherer et al. 2005a, b). The goal of phytomelioration must be the preferential support of the natural development of a vegetation cover and the initiation of a natural reproduction with seeds.

In the course of re-vegetation on the desiccated Aral seafloor in the South (Novitskiy 2012) the following woody plants are used: *Haloxylon aphyllum*, *Salsola richteri*, and *Calligonum caput-medusae*. For pastures and fodder crops the following plants are used: *Ceratoides papposa*, *Salsola orientalis*, *Aellenia subaphylla*, *Kochia prostrata*, *Salsola arbuscula*, and *Aristida karelinii*. It also was shown (Novitskiy 2012) that plantings have a good potential for reducing sand movements (Table 10).

The salt-rich soils of the younger seafloor are a mosaic of shallow deflation basins within an absolutely vegetation free salt desert. Here, plantings are urgently needed (Wucherer et al. 2012b). The area is, however, by far too large, to be planted in a reasonable time. Thus, many smaller areas should be chosen to initiate core areas of vegetation from which it may spread and which, from the beginning, may start to act as wind shelters, thus preventing formation of salt dust clouds.

Plantations are thus needed in several parts of the desiccated seafloor. Then, the ecological situation may improve, additional pastures will be formed, and new opportunities for cattle breeding and bee keeping may arise. Thus, plantations also contribute to poverty reduction. On the long run, it may be appropriate even to use saline water for irrigation in specific agricultural purposes on soils which are anyhow saline (Breckle 2009) for specific grazing needs and for stabilisation of surface by halophytic meadows.

6.2 Nature Conservation

The island of Barsa-Kelmes was the fourth protected area in Kazakhstan in 1939. Since that time, typical ecosystems of northern Turanian deserts have been kept in natural conditions and as a base of life for various protected mammals (Demchenko

1950). Starting from the 1960s, the area of the island has been involved in the Aral ecological crisis. Barsa-Kelmes Nature Reserve is the only nature reserve in the world located in a zone of an ecological catastrophe on a global scale. Monitoring studies are a basis for long-term observation of natural processes (Afanasiev and Smirnov 1985; Kuznetsov 1995, 2007). It is important for Barsa-Kelmes Nature Reserve to monitor the dynamics of territory changes of the Aral Sea dry seabed. It represents great scientific importance as a unique worldwide nature laboratory to study global desertification processes.

The problems of nature conservation in the Aralkum region as a whole are described by Dimeyeva et al. (2012b), taking the former island Barsa-Kelmes as an example. The territory of the nature reserve of Barsa-Kelmes was expanded in 2006 by almost 10 times (160,826 ha), including territories of the dry seafloor. The area now consists of two cluster areas (1) the former island of Barsa-Kelmes with the surrounding dry seafloor; (2) the former islands of Kaskakulan and Uzun-Kair with the surrounding dry seafloor. These are presently the main habitats of onager (kulan; *Equus hemionus onager*) and Persian gazelle (jairan; *Gazella subgutturosa*), to where they migrated after the area became dry. The flora and vegetation represent typical combinations of plants and vegetation types for the region as well as unique trends of the successional development on the desiccated seafloor of the Aral Sea (Dimeyeva 2007b).

The ecosystem approach is a modern method for research, monitoring, and preservation of the environment and as a tool for nature conservation. Ecosystems of the new land should be distinguished as an area of extraordinary scientific interest. The rate of desiccation, the change of the hydrological regime, the salinity of sediments, the presence of a seed bank and a gene fund of biota are the limiting conditions for the formation of new land ecosystems within the enlarged nature reserve. The stability of ecosystems (degree of equilibrium) is a task for discussion in a framework of biological self-regulation. It is dependent on the stage of formation of biological and soil components of ecosystems during the change of the hydromorphic regime. The time of formation of stable ecosystems and their dynamics are associated with increasing environmental problems in climatic regimes of deserts (Glantz 1995).

Future studies should continue on the basis of maps of different scale. Mapping on a large scale will give the overall spatial pattern of distribution of the ecosystems. The most important aspect of nature chronicling should be selection of key species and communities. Each protected area existing in isolation from human impact can be automatically compared with similar contiguous ecosystems, first of all according to vegetation. Key objects should be chosen according to their significance, value of resources, their vulnerability, and their zonal and representative species according to global, national, and local importance. Advanced ecological monitoring is related to reorganisation of the nature reserve into a biosphere reserve as the most efficient form of regulation of nature conservation and socio-economic activities in the territory. The direction towards assessment and realisation of sustainable functioning of wetlands will best meet the requirements

of the Ramsar Convention, after the area in the mouth of the Syrdarya has joined the nature reserve as a cluster core area.

It seems reasonable to reconstruct the tugai ecosystems (Kuz'mina and Treshkin 2012), which perished because of man-made regulation of the river flows of the Amudarya and the Syrdarya, into halophytic productive pastures on desiccated delta sites. Shrubs such as saxaul (*Haloxylon aphyllum*), cherkez (*Salsola richteri*), and teresken (*Krascheninnikovia ceratoides*) are recommended for planting to improve the plant communities that are presently under degradation. This may also be reasonable if the forecasts of a decreased river flow of the Amudarya and the Syrdarya in view of changing climatic conditions and mainly of the growing deficit of water resources in Central Asia come to reality (Kuz'mina and Treshkin 2009). The mixed halophytic–tugai ecosystems formed in such a way (turanga–saxaul, tamarisk–black saxaul and others) will be conducive in maintaining the productivity of ecosystems and the main elements of tugai flora to be human-modified analogues of relict tugai halophytic ecotones. In the case of an increase of the natural moisture conditions or improvement of water management situation, they can be restored or rehabilitated into typical tugai ecosystems in the nearest future.

6.3 Developmental Projects

Winckler et al. (2012) present a synthesis on the complexity of the social and economic situation in the post-Soviet states as well as an approach to the applications of scientific results to combat desertification also regarding the issue of the sensitivity of the Aralkum area to climate change. An important issue will certainly be the rebuilding of efficient agriculture with water-saving techniques and plant crops for advanced irrigation on sites with low salinity (Müller et al. 2006; Breckle and Küppers 2011; Breckle et al. 2011). Tree establishment under water stress conditions was checked by Khamzina et al. (2008).

Breckle et al. (2012a, d) have brought together some important data and basic scientific knowledge in an interdisciplinary and international approach not only by scientists from the fields of ecology and geography, but also by social scientists and economists. To improve the regional situation, cooperation of nature conservation organisations, developmental and health agencies (Erdinger et al. 2011), and other main stakeholders in the area with administrations, decision makers, and regional and national politicians is an urgent need and precondition for sustainable international water management between the relevant states (Cai et al. 2003; Fang et al. 2005; UNESCO 2000, see also Giese et al. 1998). It is quite obvious that the present situation cannot be turned back to a new Aral Sea despite many hypothetical attempts and proposals (see below).

The northern Small Aral Sea has been allowed to refill from the inflow of the Syrdarya, and though it is never expected to regain its former extent, the refill was faster than planners had expected and enough to support a robust fishing again. It should also help to stabilise the continental climate—smoothing out winter–-summer temperature extremes, and suppressing dust storms.

6.4 Restoration of the Old Aral Sea

Expectation is unrealistic that the entire Aral Sea will return to its 1960s state, and it is an open question, if it is desirable. The annual inflow from the Syrdarya and Amudarya rivers would have to be quadrupled from the recent average of 13 km^3. The only means would be to curtail irrigation, which accounts for 92 % of water withdrawals (Micklin and Aladin 2008). Yet four of the five former Soviet republics in the Aral Sea basin (Kazakhstan is the exception) and Afghanistan intend to expand irrigation, mainly to feed growing populations. Switching to less water-intensive crops, such as replacing cotton and rice with winter wheat, could help, but the two primary irrigating nations, Uzbekistan and Turkmenistan, intend to keep cotton to earn foreign currency. The extensive irrigation canals could be greatly improved; many are simply cuts through sand, and they allow enormous quantities of water to seep away. Modernising the entire system could improve the water budget, but would be very costly. The Aral basin states do neither have the money nor the political will. The importance of the Aralkum as a huge new desert with the remnant water bodies for the Central Asian states may be seen as an economic and ecological challenge, as an area for gas and oil mining, and also as an area where nature may have a chance to develop rich semi-desert and desert ecosystems as basic parts of a protected nature reserve or a national park.

Revival of parts of the Aral Sea has been successfully demonstrated with the damming of the Northern part. Kazakhstan has thus partially restored the northern Aral Sea by a 13 km long earthen dike with a gated concrete dam for water discharge, completed in November 2005. Heavy runoff from the Syrdarya in the ensuing winter jump started the North Aral Sea's recovery. The North Aral Sea, which, in the extreme, had receded almost 100 km south of the port-city of Aralsk, is now merely 25 km away from this city. The water rose to 42 m asl—the intended design height—in only 8 months. The area increased by 18 percent, and salinity has dropped steadily, from roughly 20 to less than 10 g NaCl/l today (Micklin and Aladin 2008).

A second dam is to be built based on a World Bank loan to Kazakhstan, with the start of construction planned for 2009, to further expand the shrunken Northern Aral, eventually reducing the distance to Aralsk to only 6 km. Then, it is planned to build a canal spanning the last 6 km, to reconnect the withered former port of Aralsk with the sea (Fletcher 2007). This second phase has started in 2009, but the first phase alone is revitalising Aralsk. Fishermen have returned (Middleton 2005). A large new fish processing plant is working at full capacity. A new fish hatchery will release 15 million fingerlings into the northern Aral every year, and reintroducing sturgeon in future is planned. Another new factory is building fibreglass fishing boats. Aralsk processed 2,000 tonnes of fish in 2009—enough to export some to Georgia, Russia, and Ukraine. So fishers are once again catching several fish species in substantial numbers (Micklin and Aladin 2008).

Irrigation in Central Asia has been sustained by large diversions of water from the Amudarya and Syrdarya Rivers, reducing the volume of freshwater reaching the Aral Sea and causing extensive secondary salinisation of agricultural lands (Kharin

1997; Spoor 1998; Wichelns 1999). Already Levintanus (1992) proposed to reduce the irrigation demands for cotton by 50 %, and thus to save 18–24 km^3 water. So certainly water-saving techniques should be introduced as well as modernisation of the whole irrigation systems (Kirsta 1989). This would probably be possible with less money than all the big macroprojects would need, proposed during the last decades. There were plans to use water from Ob and Irtysch ("Sibaral project"), water from Issik-Kul, or even from the Indus.

There are again proposals of macroprojects published with operational scenarios which include macroengineering solutions (Badescu and Cathcart 2011a, b, c; Cathcart and Badescu 2011). These are interesting scenarios, but are they realistic? They bear big uncertainties and deny the present situation, which has not only disadvantages but also offers opportunities for the future. Those macroprojects include huge canals for the deviation of Caspian water or the transport of Siberian water from Irtysch (Schuiling and Badescu 2011), which is just an interesting modification of the former Sibaral project, with the intention to use the Balkhash Lake as an additional reservoir. The danger of damage to the west-siberian bog-region, the largest bog-region of the world, with the eventual consequence of setting free huge amounts of greenhouse gases (methane) is ignored. According to Letolle and Mainguet (1996) long-distance diversion of water from Siberian rivers or from the Caspian is outdated. There might be also the risk of unforeseeable aquatic invasions (Dumont et al. 2004). It is, however, a great interest to control the sea level fluctuations of the Caspian which in the last years was rising alarmingly. In this respect, it could checked, if by renewable energy sources the water pumping to the Aral Sea via the Uzboi might be feasible (Cathcart and Badescu 2011) and if a connection with the Black Sea is necessary.

In this respect it is interesting that on 10 June 2007, in St. Petersburg it was proposed by Kazakhstan to construct a 650 km-long "Eurasia Canal" between the Black Sea and the Caspian Sea. The Eurasia Canal could require 3–5 years to build and cost >6 billion US$. As proposed in 2007, the Eurasia Canal would be 80 m-wide and have a standard vessel navigational depth of 16.5 m; theoretically it should be capable of carrying ships of up to 10,000 metric tons, allowing cargo delivery schedules of 9–12 days with a cargo traffic capability more than twice the Volga-Don Canal. This new proposed Eurasia Canal, however, would utilise the navigable freshwater reservoirs in the Kuma-Manych Depression of southern Russia which would shorten the shipping route by ~1,000 km, transforming landlocked Kazakhstan and all Central Asian ecosystem states into maritime nations (http://www.daviddarling.info/encyclopedia/A/Aral_Sea_refill.html). This macroengineering proposal apparently opens again thoughts for a macroproject speculation related to the "Aral Sea Refill" seawater pipeline! From an environmental viewpoint the dilution of salty water by freshwater in any cases should be avoided. Freshwater is too worthy and the best precondition for a sustainable agriculture. If those canal constructions once will come to reality, then not only the economic aspect should be taken into account to avoid another ecological disaster.

It is obvious that the ongoing desiccation has recently led to the decoupling of the Western and the Eastern basin of the former South Aral Sea. The Western basin

will have a much longer lifetime, because it is much deeper and its slopes are much steeper than in the Eastern basin. It is expected that it will not disappear but that it will transform into a basin of the type of the Dead Sea (Stanev et al. 2004; Benduhn and Renard 2004). However, it is expected by Shermatov et al. (2004) that through natural fluctuations within the watersheds and improved irrigation techniques the run-off of the Amudarya may slightly increase again. Calculations by Salokhiddinnov and Khakimov (2004) show that at least 7.6 km^3 additional discharge per year would be necessary to preserve the present water levels of the two basins of the South Aral Sea. Consequently, the preservation of the Eastern basin is not possible, as was observed in 2009, but the current water flux is sufficient to preserve the West Sea, including the preservation of its circulation. The present situation could be used by Uzbekistan to use the Eastern basin as a salt sink, surrounded by some halophytic vegetation and this is perhaps the best role it can play in the future. This option also would need in Karakalpakstan a newly constructed system of waterways to bring all drainage water of the area to the Western deep basin and to envisage a rise to about 30 m asl. Such inflow would allow achieving a stable trend towards salinity decrease by an overflow at the northern end to the Eastern basin.

6.5 Final Remarks

The gigantomanic projects of the former Sovet Union have caused the catastrophic situation of the Aral Sea and its disappearance. The development from Aralsea to Aralkum took just 50 years, losing a reservoir of more than 1,000 km^3 of water. This is an ecological disaster. The complexity of the problem cannot be solved by easy means. However, it seems appropriate to adapt to the present situation and to see it as a challenge. The present status can be improved with many small steps by involving all stakeholders instead of new gigantic canals with uncertain results.

Some examples of necessary tasks (Breckle et al. 2001a) had been and will be:

1. Phytomelioration with salinity tolerant shrubs of the area (e.g. *Haloxylon, Halocnemum*, *Halostachys, Tamarix*, etc.): Wucherer et al. (2005a, b, 2012b). Truly salinity tolerant woody species are sparse; only *Halocnemum strobilaceum* is rather salinity tolerant, but slowly growing and reaches only 60–80 cm in height.
2. Wind shelter belts around villages
3. More efficient water use and energy supply
4. Sustainable agriculture by modern irrigation techniques and drainage systems
5. Management systems for controlled grazing
6. Participation of village people and capacity building to adopt new planting projects and other environmental initiatives

7. Normative nature conservation through:

- Preserving and enhancing biodiversity
- Developing a plausible strategy for nature conservation
- Securing and enlarging the conservation area around the nature reserve Barsa Kelmes and other parts of the North Aral Sea (Kaskakulan, Syrdarya delta, fossil-rich north coast chinks (steep coasts)
- Establishing a national park or a biosphere reserve.

The economies of the five independent Central Asian countries and Afghanistan are highly interconnected by water discharge of the Syrdarya and Amudarya and other transboundary rivers. The inhabitants along the lower reaches of the rivers in Central Asia need substantial quantities of water for their irrigated agriculture (Winckler et al. 2012). The inhabitants of the upper reaches, however, are aiming to use hydropower more intensively as a source of energy. One of the crucial challenges therefore is sustainable and equitable water management in the region, in order to avoid conflicts over water issues, and integration of water issues with energy and climate issues.

The Central Asian states involved are strongly advised to fulfil their mutual treaties (Winckler et al. 2012) as well as to use international help and funds for joint development of adapted water and energy supplies for the people living adjacent to the Aralkum. The Aralkum is a focal point for combating desertification on many levels. Joint programmes for social and economic development of the area with promotion of education and capacity building of the people provide a great chance for a better future by minimising the negative effects of the irreversible desiccation of the Aral Sea and by accepting the new desert Aralkum as an opportunity for the next generations. This includes to use the ”paradise of halophytes” also for agriculture, livestock, economy, and tourism.

The complex problems of the Central Asian region will continue to have a decisive influence on the ecological and socioeconomic situation in the Aralkum and determine its economic future. Sustainable solutions require not only money and innovation but must be politically, socially, and economically practical (Micklin and Aladin 2008). In other words, the situation in the Aralkum will only have a chance of a positive evolution if solutions for the structural problems on all levels which threaten the future of the Central Asian region as a whole can be found.

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