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THE ROYAL SOCIETY

Signalling of abscisic acid to regulate plant growth

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Abscisic acid (ABA) mediated growth control is a fundamental response of plants to adverse environmental cues. The linkage between ABA perception and growth control is currently being unravelled by using different experimental approaches such as mutant analysis and microinjection experiments. So far, two protein phosphatases, ABI1 and ABI2, cADPR, pH, and Ca^{2+} have been identified as main components of the ABA signalling pathway. Here, the ABA signal transduction pathway is compared to signalling cascades from yeast and mammalian cells. A model for a bifurcated ABA signal transduction pathway exerting a positive and negative control mechanism is proposed.

Keywords: abscisic acid; *Arabidopsis thaliana*; growth control; cell cycle; MAP kinase; signalling cascade

1. INTRODUCTION

ABA is involved in many physiological and developmental processes throughout the life cycle of plants. In the early phase of a plant's life, the phytohormone ABA regulates seed maturation and the maintenance of embryo dormancy. Later, at the onset of ontogenesis, it mediates several adaptational responses towards environmental cues such as desiccation, cold as well as salt stress, and acts as a negative growth regulator.

There are both fast and slow responses to ABA. The triggering of stomatal closure is the best-studied fast response to ABA and involves the orchestration of ion channel activities at the plasmalemma and tonoplast (MacRobbie 1995). The signal transduction pathway responsible for relaying the ABA signal to its targets (cation and anion channels (Blatt & Thiel 1993)) seems to be preformed and comprises an ABA-receptor(s), protein kinases and protein phosphatases (Pei *et al.* 1997).

Most ABA-mediated actions are characterized by a comparatively slow response and require differential gene regulation. A plethora of ABA-upregulated genes as well as a few ABA-downregulated genes such as *cab* and *cdc2a* (coding for the chlorophyll ab binding protein and a cell cycle-dependent kinase, respectively) have been characterized (Chandler & Robertson 1994). Several components involved in the signalling pathway responsible for modulating embryo dormancy have been described. This advance was made possible by isolating the affected genes from ABA-response mutants. Thus, the transcriptional regulators VP1 from maize (McCarty *et al.* 1991) and its homologue from *Arabidopsis* ABI3 (Giraudat *et al.* 1992), two highly homologous members of the protein phosphatases 2C family ABI1 and ABI2 (Leung *et al.* 1994, 1997; Meyer *et al.* 1994; Rodriguez *et al.* 1998; Leube *et al.* 1998), and a farnesyl transferase ERA1 (Cutler *et al.* 1996) have been identified.

Of these signalling components only the two protein phosphatases seem to act also during the vegetative growth phase of the plant. The analysis of the *abi1* and *abi2* loci supported a pleiotropic ABA-insensitive phenotype of the mutants (Koornneef *et al.* 1984; Finkelstein & Somerville 1990) and a dominant negative action of the mutant gene products associated with a strongly reduced enzymatic activity (Bertauche *et al.* 1996; Leung *et al.* 1997; Rodriguez *et al.* 1998; Leube *et al.* 1998). Both mutants are characterized by a wilty phenotype reflecting a lesion in stomatal regulation and by ABA-insensitive growth (Koornneef *et al.* 1984; Meyer *et al.* 1994).

To date, little is known about the molecular mechanism of ABA-mediated growth regulation. Here we will concentrate on this aspect of ABA responses.

2. TARGETS OF ABA ACTION MEDIATING GROWTH CONTROL

Approximately 30 years ago, it was observed that ABA inhibits DNA replication in root tips and embryos of *Fraxinus excelsior* (van Overbeek *et al.* 1967; Villiers 1968). Histochemical studies of root tissues supported the notion of an ABA-mediated inhibition of cell elongation and of an arrest in mitotic cell activity, presumably in the G1-phase (Nagl 1972; de la Torre *et al.* 1972).

Since these investigations it has been well established that ABA generally modifies plant growth by inhibiting shoot and root growth as an antagonist of auxin (Pilet & Chanson 1981; van Volkenburgh & Davies 1983; Kutschera & Schopfer 1986; Pilet & Saugy 1987). The role of ABA in modulating the growth rate of roots has been intensely studied and was found to be concentration-dependent. Whereas a negative correlation between ABA content in the elongation zone of maize roots and growth rate was observed (Saugy *et al.* 1989), low concentrations of ABA can even stimulate root growth, thus providing a basis for the explanation of the biphasic response of roots upon application of inhibitory ABA concentrations (Pilet & Barlow 1987). Exogenous ABA application inhibited root growth after an initial phase of increased growth lasting

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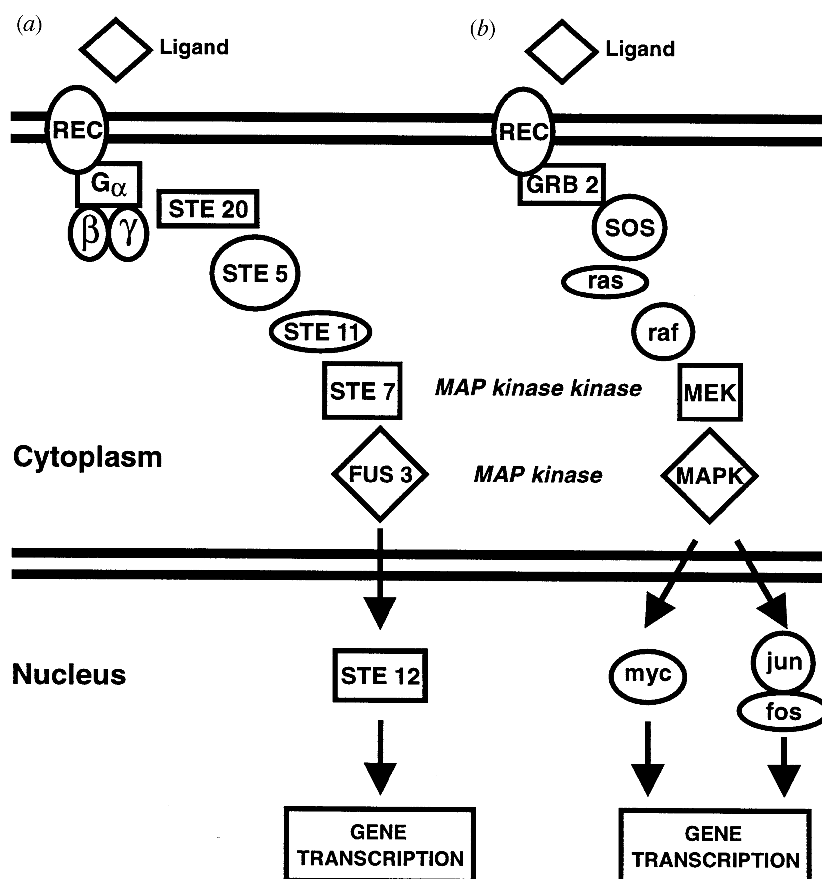

Figure 1. Schematic signalling cascade of (*a*) the pheromone response in yeast and (*b*) the mitogenic response in mammalian cells. In both cases the signal is perceived by a receptor (REC) localized at the plasma membrane and relayed via an MAPK cascade into the nucleus. See the text for details.

several hours (Mulkey *et al.* 1983; Robertson *et al.* 1990). Inhibition of ABA biosynthesis by fluoridone or ABA-deficiency due to a mutational lesion, however, did not affect root growth of maize (Moore & Smith 1984) indicating that endogenous ABA is not a central growth regulator unless ABA levels are enhanced e.g. by cold or desiccation.

The root response towards inhibitory ABA concentrations is characterized by an inhibition of cell elongation and subsequent osmotic adjustment of the root apex. The reduction in root elongation was reciprocally correlated with an increase in both osmotic potential of the medium and the endogenous ABA level (Ribaut & Pilet 1991). The same adaptational response is observed under conditions of drought stress and coincides with both increased root hair formation and decrease in the size of the meristematic zone of the root tip (Robertson *et al.* 1990). The application of ABA mimics this response of roots (Schnall & Quatrano 1992). The low water potential response of wheat roots was observed at exogenous ABA concentrations as low as 0.1 μM ABA and was sufficient to increase the turgor pressure by approximately 50% (Jones *et al.* 1987).

In summary, these data imply that ABA imposes a bimodal growth control by regulating the potential of the cell to enlarge, possibly by turgor control, and by inducing mitotic growth arrest in plants in accordance with its role as a negative growth regulator.

3. PARADIGMS OF CELL CYCLE CONTROL

Mitotic growth arrest is well-known to be induced under adverse conditions in animal cells by preventing the transition of G1 into the S-phase of the cell cycle (Ron 1994). The tumour suppressor p53 (Baker *et al.* 1990) and the retinoblastoma protein pRb (Weinberg 1995) were identified as central links in the pathway. Extensive phosphorylation of pRb at a checkpoint of the cell cycle (the restriction point) within the mid-G1 phase inactivates its growth-inhibitory action (Bartek *et al.* 1996). The multistep phosphorylation is initiated and controlled by the cell cycle machinery and provides a means to integrate internal and external signals. Central components of the mammalian cell cycle are the cyclins and their specific kinases (cdks), the proliferating cell nuclear antigen, and the cdk inhibitors such as p16, p21 and p27 (Sherr & Roberts 1995; Coats *et al.* 1996). Among the key regulators of S-phase entry are the cyclinD-cdk4/6 as well as cyclinE-cdk2 complexes that promote cell cycle progression by specific protein phosphorylation (Morgan 1995).

The pathway leading to mitotic growth arrest in the mating response of *Saccharomyces cerevisiae* has been unravelled through extensive mutant analyses (Levin & Errede 1995). A schematic presentation of the pathway is given in figure 1*a*. During mating of yeast cells, the release of a pheromone from a mating partner triggers a signalling cascade from the plasma membrane to the nucleus that finally activates a transcription factor and FAR1. The signalling pathway is activated by the pheromone acting on a membrane receptor that in turn activates a trimeric G-protein. The G-protein transmits the signal by initiating a phosphorylation cascade leading from STE20 and STE5 to the serine–threonine protein kinases STE11 and STE7, and subsequently to the kinase FUS3 that finally phosphorylates the transcription factor

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STE12 and FAR1. FAR1 blocks DNA replication by inhibiting the cyclin–cdc2 kinase complex while STE12 regulates response-specific transcription.

The last steps of the signalling cascade are similar to the mitogenic response of mammalian cells that is induced by epidermal growth factor (figure 1*b*) and results in the opposite response, i.e. mitotic cell division (Robinson & Cobb 1997). Here, activation of a membrane-bound tyrosine receptor kinase activates, via the involvement of two receptor-associated proteins GRB2 and SOS, the small G-protein ras that activates a serine–threonine phosphorylation cascade by recruiting raf to the plasma membrane. The raf protein phosphorylates the mitogen activated protein kinase kinase (MEK) that regulates the activity of the MAP kinase (MAPK). The MAPK controls the heterodimeric transcription factor consisting of jun and fos but also regulates protein biosynthesis via the cell cycle-controlled S6 protein kinase (Blenis 1993). MEK and MAPK are structural homologues of STE7 and FUS3; hence both responses, cell cycle inhibition in yeast and cell cycle activation in mammals, rely on a conserved cascade of protein kinases.

In addition to the mitogenic response, mammalian MAPK pathways also regulate stress responses resulting in growth inhibition e.g. by the transforming growth factor TGF-β (Waskiewicz & Cooper 1995; Yamaguchi *et al*. 1995). Thus, distinct MAPK cascades emerge as general signalling pathways that regulate cell cycle progression.

4. SIGNALLING OF GROWTH INHIBITION

The question of how ABA could exert control of the cell cycle in plants is currently unresolved. One likely possibility is through a mechanism analogous to the mammalian system involving the regulation of the phosphorylation state of pRb and an MAPK pathway. In plants, indeed, the presence of the components of the cell cycle machinery (Doonan & Fobert 1997) as well as of MAPK cascades (Mizoguchi *et al*. 1996, 1997; Ligterink *et al*. 1997; Machida *et al*. 1997) have been unequivocally demonstrated. The observed structural conservation of the proteins is reflected by either functional complementation or protein–protein interactions among animal or yeast forms and plant components (Hirt *et al*. 1991; Dahl *et al*. 1995; Ball & Lane 1996; Ach *et al*. 1997*a*). This supports the view that cell cycle control mechanism are conserved throughout the eukaryotes.

Protein kinases homologous to Cdc2 were initially cloned from alfalfa (Hirt *et al*. 1991) and *Arabidopsis* (Ferreira *et al*. 1991; Hirayama *et al*. 1991) and identified as key components of the plant cell cycle machinery required for G1 to S-phase transition and for entry into mitosis (Martinez *et al*. 1992). By using a reporter gene under control of a cdc2 promoter, it was shown that Cdc2 expression is not always linked to cell proliferation but it always precedes cell division (Hemerly *et al*. 1993). Treatment of the transgenic plants with ABA resulted in the strong transcriptional down-regulation of the reporter gene. In contrast, auxin increased cdc2 gene expression within minutes after hormone application (John *et al*. 1993).

Several different cyclins including type A, B and D have been isolated (Hemerly *et al*. 1992; Day & Reddy 1994; Renaudin *et al*. 1996). Interestingly, several cyclins of the D-type revealed a pRb interaction motif (Fürst *et al*. 1996; Dahl *et al*. 1995; Soni *et al*. 1995). In fact, pRb proteins of maize have been identified and were capable of interacting with this cyclin type (Xie *et al*. 1996; Ach *et al*. 1997*b*) pointing to a similar role for these cyclins in cell cycle control as in the animal system. Recently, cdk inhibitors of plants were identified by their specific interaction with Cdc2 (Wang *et al*. 1997; de Veyler *et al*. 1997).

To understand the molecular mechanisms for the ABA-mediated control of plant growth and mitotic growth arrest, our group took a genetic approach to identify loci providing insensitivity to the growth inhibitory action of ABA in *Arabidopsis*. Mutants of *Arabidopsis* insensitive to the germination-inhibiting action of ABA have already been characterized as representatives of five independent loci, *abi1* to *abi5* (Koornneef *et al*. 1984; Finkelstein 1994). In addition, response mutants hypersensitive towards ABA have been characterized (Cutler *et al*. 1996). However, of these mutants only *abi1* and *abi2* seem to reveal an altered ABA sensitivity during vegetative growth. Additional ABA-insensitive *Arabidopsis* mutants were isolated by screening more than 1×10^5 seedlings of the chemically mutagenized M2 generation in the presence of growth-inhibiting concentrations of ABA. The intention was to identify all the signalling loci involved in the pathway leading to the growth inhibition by ABA (T. Ehrler, M. Iten and E. Grill, unpublished results). Genetic complementation analyses of the 22 identified mutant plants revealed lesions in eight new loci, *GCA1* to *GCA8* (growth control exerted by ABA). Physiological analyses, including stomatal regulation as well as dormancy of the mutant seeds, enabled the successful isolation of two additional pleiotropic mutants *gca1* and *gca2* that phenotypically resemble *abi1* and *abi2*. All other loci of the mutants, *gca3* to *gca8*, affect, monotropically, the growth response.

Identification of the corresponding genes certainly will aid our understanding of how the hormonal signal controls cell growth and division. So far there is no evidence to support the involvement of an MAPK cascade in this response. However, the cascade has been implicated in the ABA-mediated antagonism of gibberellin action during barley seed germination (Knetsch *et al*. 1996)—a process in which a phospholipase D has recently been postulated to be involved (Ritchie & Gilroy 1998). In yeast and *Arabidopsis*, PP2Cs have been identified to counteract the activation of an MAPK cascade involved in osmoregulation and stress signalling, respectively (Maeda *et al*. 1994; Meskiene *et al*. 1998). Thus, the characterization of ABI1 and ABI2 as two PP2Cs (Leung *et al*. 1994, 1997; Meyer *et al*. 1994; Rodriguez *et al*. 1998; Leube *et al*. 1998) could suggest a similar role of these phosphatases as negative regulators of a MAPK cascade.

The unresolved question of ABA signal transduction has recently been tackled by the identification of cyclic ADP ribose as a second messenger of ABA action capable of inducing specifically ABA-dependent gene transcription (Wu *et al*. 1997). In detailed experiments Wu and coworkers demonstrated that NAD might serve as a precursor of cADP ribose and that an inhibitor of cADP ribose, 8-amino-cADP ribose, blocked both ABA signalling as well as cADP ribose action. cADP ribose

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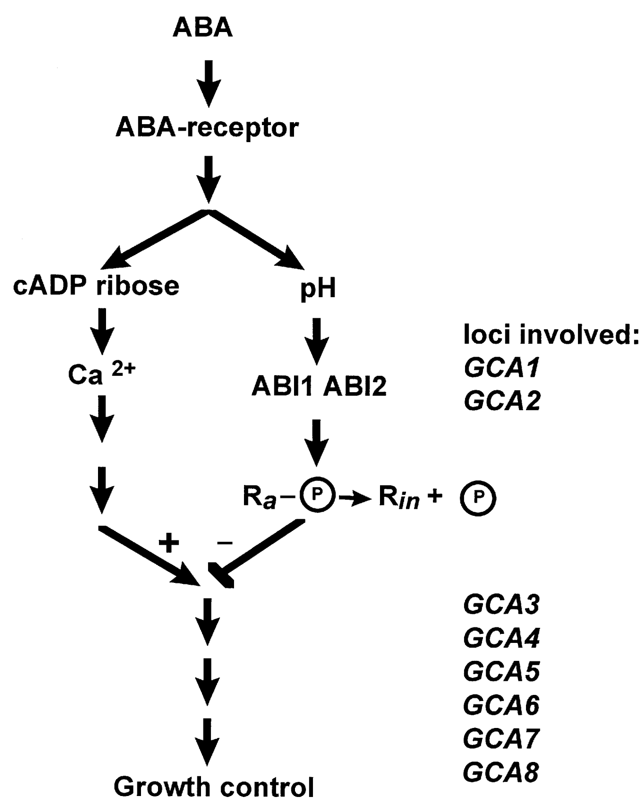

Figure 2. Model of a bifurcated ABA signal pathway exerting plant growth control. The binding of ABA to a specific receptor results in the generation of cADP ribose and a concomitant rise of the pH in the cytosol. cADP release triggers an increase in the concentration of cytosolic calcium. The pH signal activates the protein phosphatases ABI1 and ABI2 which dephosphorylate a postulated active repressor (R_a) of the signalling cascade thereby generating the inactive form R_{in}. The other branch of the pathway exerts a positive regulation and is activated by the released calcium ions. This leads to the presence of inactivated repressor and by several, so far unknown, steps to the control of cell enlargement and cell division. The gene products of the identified loci *GCA1* to *GCA8* represent candidates involved in this signalling pathway. *GCA1* and *GCA2* loci pleiotropically affect the ABA response while *GCA3* to *GCA8* seem to modulate specifically the ABA-mediated growth control.

seems to act by triggering the release of calcium ions into the cytosol (Allen *et al.* 1995; Wu *et al.* 1997) and by a second hitherto unknown mechanism, thereby providing specific regulation of ABA-modulated genes.

The specific signature of ABA and cADP ribose action observed via calcium release could be provided by the regulation of the proton concentration in the cytosol. ABA application to plant cells results within minutes in the alkalization of the cytosol by approximately 0.2–0.4 pH units, whereas an acidification concomitant with calcium release has been demonstrated after auxin administration (Gehring *et al.* 1990; Irving *et al.* 1992). The alkalization triggered by ABA would be sufficient to increase the PP2C activity of ABI1 and ABI2 by a factor of more than two (Leube *et al.* 1998, A. Himmelbach, unpublished results) and thereby could change the phosphorylation state of substrates critical for ABA signal relay. Guard cells of plants expressing the mutant abi1 phosphatase, although ABA-insensitive, do still respond to ABA by a pH shift in the cytosol (Armstrong *et al.* 1995). Provided that these guard cells also show ABA-dependent calcium mobilization, then cADP ribose appears to act upstream of the protein phosphatases. Furthermore, because both ABI1 and ABI2 are involved in mediating ABA growth responses, cADP ribose must also be capable of regulating this response. Thus, CADP ribose emerges as a central player of ABA action.

In a speculative scenario, the signalling events leading to ABA-mediated growth control could be envisaged in the following way (see figure 2): ABA interacts with (a) receptor(s) generating by unknown steps a shift of the cytosolic pH into the alkaline and the formation of cADP ribose which results in an increase in the concentration of cytosolic calcium. The pH-shift activates ABI1 and ABI2 which in turn dephosphorylate a postulated negative regulator, a repressor, of ABA-signalling. This step inactivates the repressor and allows ABA-signal progression.

The behaviour of mutant forms of abi1 and abi2 that act dominantly and provide ABA-insensitivity while revealing a strongly reduced enzymatic activity could be explained by sequestering the active repressor and not being able to inactivate it (Bertauche *et al.* 1996; Leung *et al.* 1997; Rodriguez *et al.* 1998; Leube *et al.* 1998; Sheen 1998). In accordance with the model, the rescue of the ABA-response by the application of serine–threonine kinase inhibitors to abi1-expressing guard cells (Armstrong *et al.* 1995; Pei *et al.* 1997) could be explained by the generation of the dephosphorylated (inactivated) repressor. Interestingly, ABA signalling still required the presence of ABA in these experiments. Thus, ABA and possibly cADP ribose seem to act on a bifurcated signalling pathway by deactivating a negative control mechanism via ABI1 and ABI2, as well as by activating a positive control pathway in which calcium ions are involved as secondary messengers.

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