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REVIEW

Recent Advances in the Study of Mechanisms of Action of Phytohormones

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Abstract—This review highlights recent advances in studies of mechanisms underlying the effects of five phytohormone groups: auxin, cytokinin, gibberellin, abscisic acid, and ethylene. The review summarizes data on receptors of all these phytohormones and the hormone signal transduction systems, which include second messengers, hormone-dependent *trans*-factors, and the genes controlled by these factors. The effects of phytohormones involve not only induction of novel protein synthesis via activation of their gene expression, but also degradation of repressor proteins through the ubiquitin system. The review contains examples of successful use of data on genes encoding enzymes of phytohormone synthesis and their receptors for development of transgenic plants with particular hormonal characteristics that provide practically valuable traits.

Key words: auxin, cytokinin, gibberellin, abscisic acid, ethylene, receptors, phytohormone signal transduction, *trans*-factors, repressors, hormone-dependent genes

The study of hormonal regulation of plant life is one of "hottest points" of world biochemistry, physiology, and plant molecular biology. All life of plants from fertilization of the egg cell up to senescence and death is controlled by phytohormones. Phytohormones also play an important role in plant responses to environmental factors and formation of plant tolerance to extreme conditions.

Here we consider recent achievements in studies of mechanisms mediating effects of five main groups of phytohormones: auxins, cytokinins, gibberellins, ethylene, and abscisic acid.

This review does not include data on the involvement of phytohormones in plant responses to abiogenic stresses and phytopathogens. These problems have recently been considered in two monographs by I. A. Tarchevsky [1, 2].

STUDY OF MECHANISMS OF AUXIN ACTION

Auxins are one of the most important plant hormone groups and indole-3-acetic acid is the main natural representative of this group. Auxin is involved in regulation of such important processes in plant life as cell division, cell growth by expansion, differentiation of root system, and gravitropic reaction of root, stem, etc. [3, 4].

Understanding of pleiotropic action of auxin and selection of definitive cell response (among diverse responses) requires elucidation of molecular mechanisms of auxin action.

Studies of mechanisms of hormonal action (for hormones in general and phytohormones in particular) generally include investigation of hormone receptor(s), signal transducing pathways, and involvement of certain genetic programs providing final cell response to this particular hormone.

Auxin binding protein 1 (ABP1) from maize coleoptile membranes is one of the best-studied putative auxin receptor proteins [5-7]. (Auxin activates maize coleoptile cell elongation.) ABP1 is a homodimer, with subunit molecular mass of 22 kD. It selectively and with high affinity binds to auxins. For physiologically active synthetic auxin analog—3-naphthyl acetic acid—the K_d value is $5 \cdot 10^{-8}$ M [5]. This glycoprotein has been sequenced, and the amino acid sequence of the auxin-binding site was established, the KDEL sequence, suggests the protein localization in endoplasmic reticulum. However, a large bulk of information exists that functionally active protein exhibiting properties of auxin receptor is located at the outer face of the plasma membrane [5-7]. ABP1 is involved in transduction of auxin signal causing membrane hyperpolarization [5-8].

Auxin effect on hyperpolarization of plant cell plasma membrane and the existence of a relationship between

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this phenomenon and activation of cell growth have been known for a long time [3, 4]. Recently it has been shown that auxin induces bioelectric potential oscillation; this tissue-specific effect depends on age and phase of cell growth [9-12]. As mentioned above, ABP1 mediates auxin signal causing membrane hyperpolarization [5, 6, 8]. In tobacco protoplasts antibodies against ABP1 inhibited the auxin-induced bioelectric reaction [6, 8].

Antibodies against ABP1 also inhibited auxin-induced activation of phospholipase A_2 (PLA_2) in isolated membranes; this suggests involvement of ABP1 in transduction of auxin signal to this membrane enzyme [13]. Increase in PLA_2 activity was detected 2 min after auxin addition to cell suspension. This means that PLA_2 activation may be considered as one of the early cell responses to auxin. Activation of PLA_2 results in formation of lysophospholipids and fatty acids [13]. Lysophospholipids activate protein kinase, which phosphorylate various proteins including H^+-ATPases. Phosphorylation of this enzyme causing increase in its catalytic activity should result in acidification of the cell wall required for its expansion during cell growth.

Thus, lysophospholipids may be considered as second messengers of auxin involved in the signal transduction downstream of ABP1. Auxin signal transduction also involves Ca^{2+} as the other messenger [14-16]. Certain evidence exists that ABP1 may also participate in this auxin effect.

An important role of ABP1 in regulation of cell elongation by auxin has recently been demonstrated in plants transformed in the *ABP1* gene; this transformation increased auxin-dependent cell growth [17]. Mutation in the *ABP1* gene of *Arabidopsis thaliana* was lethal at the globular stage of embryo development [18]. These data clearly demonstrate the pivotal role of ABP1 in plant life. Data on the crystal structure of ABP1 obtained with high resolution will help further characterization of this protein [19].

Although ABP1 binds auxin at the outer cell surface, experiments with mutants underline the importance of auxin penetration into the cell and so certain attention is paid to studies of auxin transmembrane carriers and also to auxin intra- and intercellular transport in the plant [20]. Some intracellular auxin-binding proteins are investigated as putative intracellular receptors of this hormone. For example, soluble auxin-binding protein of 57 kD was isolated from rice [21]. This protein directly interacts with plasma membrane ATPase and activates this enzyme; this results in acidification of the cell wall and cell expansion, which are typical effects of auxin [22].

We do not have any information on mechanisms responsible for transduction of auxin signal from receptor to genome. However, significant progress has been achieved in auxin-dependent regulation of gene expression. One of early cell responses to auxin consists of induction of several gene families, which are known as genes of early (or primary) response to auxin [23]. Their induction detected within 3-5 min after auxin addition was insensitive to cycloheximide (protein biosynthesis inhibitor); this suggests auxin interaction with preexisting cell proteins exhibiting properties of activators or repressors of gene expression [24]. Genes of early response to auxin include the following gene families: *Aux/IAA*, *GH3*, *SAUR* [23]. Auxin also causes induction of the gene family encoding 1-aminocyclopropane-1-carboxylic acid synthase—a key enzyme of ethylene biosynthesis, genes of *NAC-1* family, genes of glutathione-S-transferase family, etc. [24-26]. Auxin responsive element (ARE) of promoter of auxin-inducible genes contains the characteristic nucleotide sequence TGTCTC [27]. Expression of the auxin-inducible genes involves interaction of auxin responsive factor (ARF) with ARE [28]. Insertion of ARE into reporter genes is sufficient for induction of these genes by auxin [28-30]. The genome of *A. thaliana* contains at least 10 genes encoding ARF [30]. ARF proteins may undergo dimerization, and such ARF dimers interact with ARE (Fig. 1). Multiplicity of ARF suggests the existence of various ARF-dimers, which may be involved in various cell responses to auxin at the level of different auxin-inducible genes.

Theologis and colleagues discovered and studied auxin-inducible genes of the *Aux/IAA* family [23, 31-34]. Expression of genes of this family in pea and *A. thaliana* was detected within 5 min after auxin addition [23, 31, 33]. These authors also determined nucleotide sequence of promoter of auxin-inducible genes, responsible for their induction by auxin [34]. Insertion of this sequence into a genetic construction containing the *GUS* gene resulted in induction of this gene by auxin in transformed plants [35]. The highest expression of *GUS* gene was found in the root elongation zone, which corresponded to the zone of manifestation of biological activity of auxin.

Aux/IAA-proteins are related to nuclear proteins. Their genes contain a nucleotide sequence encoding a domain responsible for protein translocation into the nucleus. This sequence inserted into a chimeric genetic construct causes translocation of GUS protein into the nucleus of transformed plants [31]. Aux/IAA-proteins contain an amino acid sequence indicating their sequence-specific binding to DNA [31]; however, the latter requires direct experimental demonstration.

Dimerization is a characteristic feature of Aux/IAA proteins. They can form not only homodimers, but also heterodimers with various members of this protein family (Fig. 1). *A. thaliana* contains at least 24 members of Aux/IAA-proteins; this allows the formation of diverse heterodimers in the cell. Homo- and heterodimerization involve III and IV domains at the C-end of the protein [36]. Most ARF *trans*-factors also contain similar III and IV domains at the C-end. This allows Aux/IAA-proteins to form heterodimers with ARF-proteins. We have already mentioned that induction of auxin-inducible

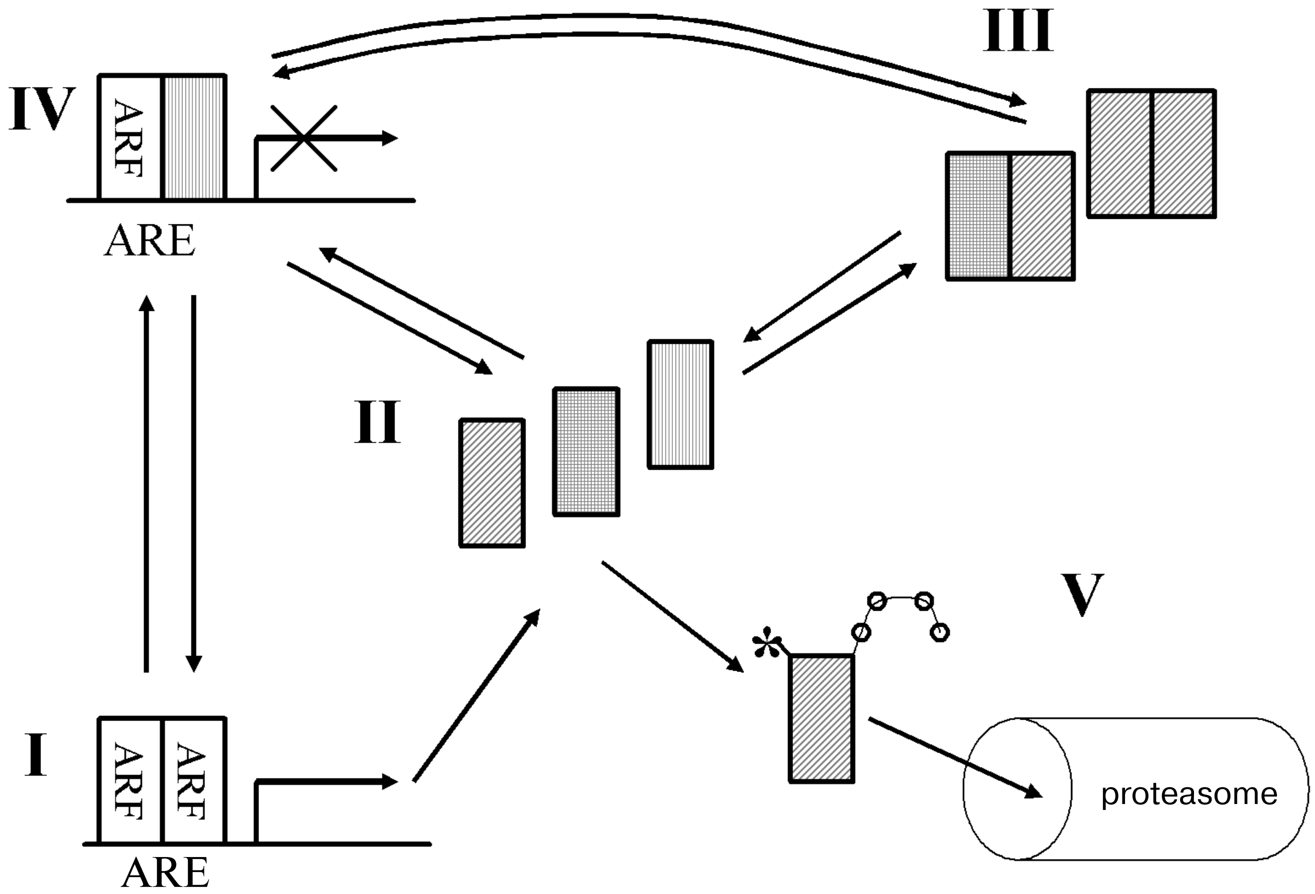

Fig. 1. Scheme illustrating regulation of gene expression by auxin involving ARF and Aux/IAA-proteins. I) Auxin induction of gene expression involving dimer of *trans*-factor ARF; II) cellular pool of various Aux/IAA-proteins; III) homo- and heterodimers of Aux/IAA-proteins; IV) repression of auxin-inducible genes by a heterodimer of Aux/IAA-proteins and ARF; V) degradation of Aux/IAA-proteins involving ubiquitin and proteasomes. Rectangles with various hatches show various types of Aux/IAA-proteins. Circles show ubiquitin molecules. The asterisk marks modification of Aux/IAA-protein targeted for degradation. ARE and ARF are auxin responsive element of promoter and auxin responsive *trans*-factor, respectively.

gene involves interaction of ARF dimer with ARE promoter of these genes. Dimerization of Aux/IAA-proteins with ARF should prevent formation of ARF dimer suppressing expression of auxin-inducible genes (Fig. 1). Thus, Aux/IAA-proteins may act as repressor of auxin-dependent transcription.

Aux/IAA-proteins are short-living proteins [32]. Their instability is determined by a so-called "degron", a 13-member amino acid sequence located in domain II [20, 37-39]. Mutations in this domain cause increase in lifetime of such proteins and impaired plant response to auxin [37]. Fusion of the luciferase gene with nucleotide sequence of *Aux/IAA* encoding domain II results in luciferase destabilization in the transgenic plant. This convincingly demonstrates that instability of Aux/IAA-proteins is determined by domain II [37]. A large bulk of experimental data demonstrates that instability of Aux/IAA-proteins is important for auxin effect on plants [20, 32], and auxin itself accelerates degradation of Aux/IAA-proteins [20].

Using mutations in *Arabidopsis* it was demonstrated that degradation of Aux/IAA-proteins involves their ubiquitinylation followed by subsequent degradation in proteasomes. Good evidence exists that Aux/IAA-protein interacts with ubiquitin protein ligase E3 (SCF^{TIR1}), which selects Aux/IAA-protein for subsequent degradation in proteasomes [20, 37]. Thus, accelerating degradation of Aux/IAA-proteins auxin abolishes their repressor effect (Fig. 1).

Auxin-induced protein degradation involving the ubiquitin/proteasome system implies protein modification, which targets a protein for this system. Protein phosphorylation is the most common modification. There are indications in the literature that a cascade of mitogen-activated protein (MAP) kinases participates in cell response to auxin [40]; however, the Aux/IAA degron lacks conservative phosphorylation sites. Leiser et al. [20] suggest that auxin signal causes protein modification by hydroxylation of proline residues in the degron sequence.

It should be noted that *Aux/IAA* genes differ in kinetics of auxin-induced expression [41] and various proteins are characterized by different affinity to their partners during formation of homo- and heterodimers between members of Aux/IAA family and ARF. This may also cause variations of cell response to auxin.

The large number of various Aux/IAA-proteins and ARF factors and their corresponding combinations may also contribute to distinct expression of different auxin-inducible genes and, consequently, to realization of different physiological programs of cell response to auxin [36].

Subsequent studies in this field will open the possibility for selective regulation by auxin of various programs (activation of some programs and suppression of others).

RECENT ADVANCES IN CYTOKININ RESEARCH

Cytokinins play an important role in the whole life of plants. They are involved in regulation of cell division and differentiation of chloroplasts; they induce stem morphogenesis and attenuate leaf senescence. Cytokinins participate in regulation of transport of various metabolites into plants; the root system regulates functional activity of above ground organs (e.g., leaves) in a cytokinin-dependent manner [42]. It should be noted that until recently genes encoding cytokinins had not been found in plants.

Genes encoding isopentenyl transferase (IPT), key enzyme of cytokinin biosynthesis, were discovered in the genome of *A. thaliana* only in 2001 [43, 44].

Isolation of these genes opens the possibility for investigation of their tissue-specific expression in ontogenesis and the search for factors regulating their expression.

Discovery of membrane cytokinin receptors. In 2001 studies by two independent Japanese laboratories found membrane receptors for cytokinins in *Arabidopsis* [45, 46]. These membrane receptors representing a bicomponent regulatory system are widespread in bacteria [47]. This bicomponent system consists of two proteins: 1) transmembrane sensor protein containing histidine protein kinase at the cytoplasmic C-end (this protein kinase autophosphorylates a conservative histidine of this protein in response to external signal); 2) cytoplasmic protein, known as response regulator; in a signal receiver domain this protein contains conservative aspartate residue, which accepts the phosphate group from the histidine residue of the sensor protein. This activates the response regulator, which "passes" the accepted signal further (and causes regulation of gene expression, in particular).

A bicomponent regulatory system also exists in yeast, where it is involved in osmoregulation [48]. However, in the yeast system sensor protein and the response regulator are fused into one protein molecule. It is known as hybrid or fused histidine protein kinase. In this fused protein, there is an intramolecular phosphate group transfer from histidine to aspartate. Genes encoding cytokinin membrane receptors have been isolated and sequenced. These include *CRE1*, *AHK2*, *AHK3*, and *AHK4*. Genes *CRE1* and *AHK4* are analogous. The earlier isolated *WOL* gene regulating formation of vascular system in *Arabidopsis* root [49] is also analogous to *CRE1* and *AHK4*, and so the cytokinin receptor-encoding gene is often called *CRE1/AHK4/WOL* [50, 51]. These genes encode the transmembrane protein that is the fused sensor histidine kinase. At the N-end of this molecule there are transmembrane domains flanking at both sides extracellular CHASE domain, which is probably responsible for cytokinin binding [52]. Substitution of one amino acid residue in the CHASE domain results in a loss of cytokinin binding by CRE1 *in vitro*; *in vivo* it is accompanied by loss of biological function [53]. This suggests that the CHASE domain actually binds cytokinin and this is important for signal transduction.

Cytokinin interaction with its binding site activates histidine kinase, located in cytoplasmic part of the receptor. This results in autophosphorylation of this protein at conservative histidine (His459) of the transmitter domain of the receptor kinase followed by subsequent transfer of the phosphate group to conservative aspartate (Asp973) of the receiver domain of the regulatory response part of this receptor (Fig. 2). The latter transduces signal to other intracellular structures responsible for triggering of cytokinin programs. Involvement of CRE1 in phosphate cascade triggering was demonstrated using mutant yeast with damaged osmoregulator histidine kinase. Transformation of such yeast with the *A. thaliana CRE1* gene resulted in CRE1 functioning in mutant yeast cells as cytokinin-dependent histidine kinase [43].

Arabidopsis cells with damaged *CRE1* gene did not respond to cytokinin by cell divisions, callus greening, and shoot differentiation; this underlines the role of CRE1 in cytokinin signaling [43].

Transformation of mutant yeast cells by *AHK3* gene also provided convincing evidence that this gene encodes functionally active histidine kinase responsible for phosphate group transfer from histidine to aspartate residue in mutant yeast cells lacking their own osmosensor histidine kinase [44]. Sensor AHK4 histidine kinase from *A. thaliana* was also able to carry out cytokinin-dependent phosphate group transfer in *Escherichia coli* cells transformed with the *AHK4* gene [54].

Thus, cytokinin membrane receptor CRE1/AHK4 isolated in 2001 was able to bind cytokinin; being inserted into regulatory cascades of yeast and bacterial cells, this receptor functioned in transfer of the signal phosphate group [50, 51, 55-57].

Genes of primary response to cytokinin and signal transduction to them from membrane receptor. Recently a whole group of genes involved in primary response to cytokinin has been discovered. Activation of these genes did not require preceding activation of other genes. These include maize *ZmCip1* gene [58] and the family of *ARR* (Arabidopsis Response Regulator) genes [50, 55, 59, 60]. These genes encode proteins corresponding to response regulators of the bicomponent regulatory system. These proteins contain a characteristic receiver domain with highly conservative aspartate residue, which might be involved in phosphate group transfer from phosphorylated histidine. The *Arabidopsis* genome contains 22 *ARR* genes, which are subdivided into two groups, denominated as groups A and B. The N-terminus of ARR molecules of A and B groups contains the conservative receiver domain, whereas the C-terminus significantly differs in proteins from A and B groups. In the ARR proteins of group A, this domain is shorter than in group B proteins, and it contains less than 100 amino acid residues; in group B proteins this domain contains up to 500 amino acid residues [60]. The amino acid sequence of group B ARR proteins suggests their nuclear localization and functioning as transcription factors. Nuclear localization of ARR proteins of both groups was demonstrated in experiments when genes encoding these proteins were fused with the gene encoding jellyfish green fluorescent protein (GFP). During introduction of such construction into plant cells fluorescence was detected in the nuclei; this means that the fused GFP protein was translocated into the nucleus by group A and B ARR proteins [61]. Cytokinins induce genes encoding only the A group ARR proteins [55, 59, 60].

Treatment of *A. thaliana* leaves with cytokinin followed by subsequent isolation of nuclei from these leaves and analysis of group A *ARR* gene expression in "run-on" experiments revealed that cytokinin induction of expression of these genes was noted after 5 min and maximal expression was detected after 10 min, and this was accompanied by rapid termination of this effect [60].

ARR proteins of type B are regulators of transcription of cytokinin-inducible type A *ARR* genes. This was demonstrated in direct experiments [62]. ARR1 and ARR2 proteins (type B) contain a "GARP" domain, which binds to regulatory element of promoter of type A *ARR* genes. The N-terminal receiver domain of type B ARR1 protein containing conservative aspartate is involved into cytokinin signaling. In the absence of cytokinin, this domain represses ARR1 activity in regulation of transcription, and cytokinin abolishes this negative effect [62].

Promoter of the *ARR* gene type A contains several copies of AGATT sequence, which interacts with "GARP" domain of ARR1 *trans*-factor during cytokinin-dependent expression of type A *ARR6* gene [62]. ARR1 and ARR2 are *trans*-factors for a wide spectrum of cytokinin-dependent type A *ARR* genes: *ARR4-ARR9* [62].

Insertion of genes encoding cytokinin membrane receptor (*CRE1/AHK4/WOL*, *AHK2*, *AHK3*) into *Arabidopsis* protoplasts increased expression of marker luciferase gene under promoter of cytokinin-dependent *ARR6* gene [61]. Mutations in genes encoding cytokinin receptor, which influenced conservative histidine or aspartate involved in phosphate group transfer, inhibited induction of marker genes under cytokinin-dependent promoter [61]. This demonstrates that cytokinin signal to expression of type A *ARR* genes involves sensor histidine kinase of the receptor and phosphate group transfer from histidine residue of transmitter to aspartate residue of response regulator [61].

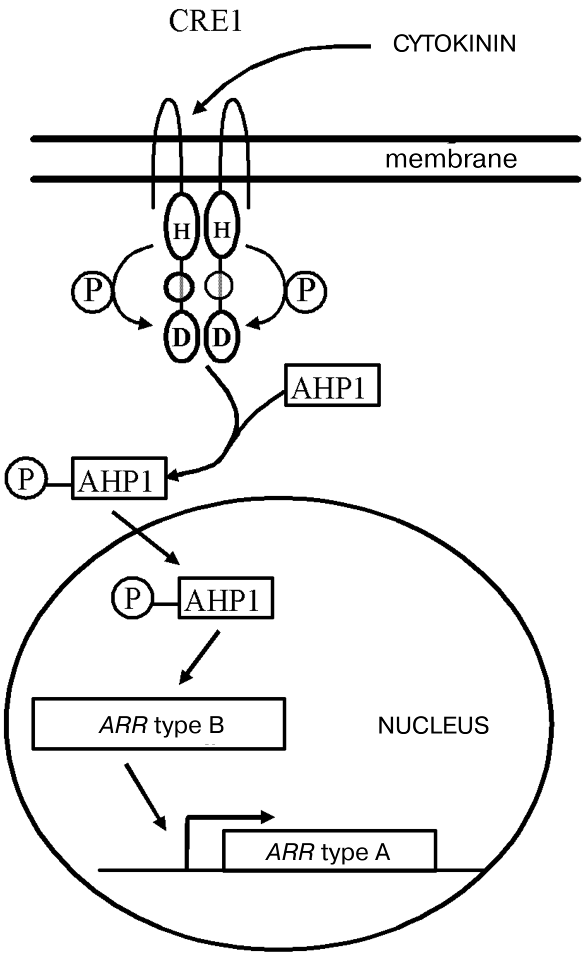

Fig. 2. Involvement of membrane cytokinin receptor in regulation of type A *ARR*-gene expression. Abbreviations: CRE1, membrane receptor; H, conservative histidine residue in domain of histidine protein kinase; D, conservative aspartate residue in response regulator domain; AHP1, histidine phosphotransmitter; *ARR* type B, cytokinin-dependent *trans*-factor; *ARR* type A, genes of primary response to cytokinin; P, phosphate group.

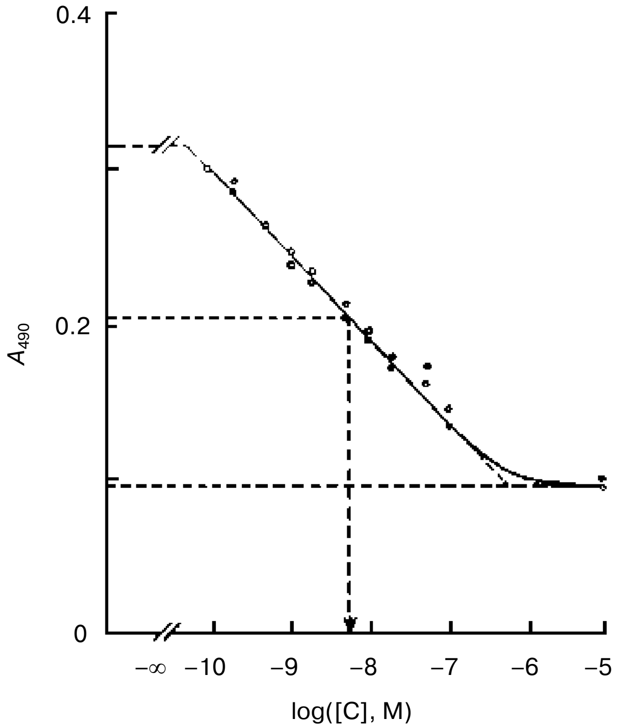

Fig. 3. Competitive inhibition of antiidiotype antibody ($AB_{a\text{-}i}$) binding to CBP-67 by *trans*-zeatin. $AB_{a\text{-}i}$) antiidiotype antibodies against antibodies (AB) to *trans*-zeatin. Data were obtained using competitive ELISA. The arrow indicates the concentration of *trans*-zeatin required for 50% inhibition of $AB_{a\text{-}i}$ interaction with CBP-67 [72].

It should be noted that besides cytokinin, *ARR* genes type A are sensitive to other stimulations. For example, cytokinin-dependent *ARR4* and *ARR5* genes also respond to low temperature, high salinity, and dehydration [63].

Signal transduction from sensor histidine kinase to nuclear ARR *trans*-factors type B, which trigger expression of type A *ARR* genes, may involve special proteins. In yeasts, the phosphate group is transferred from sensor histidine kinase to histidine phosphate transmitter (HPt) proteins, which transfer phosphate group to the receiver domain of the response regulator protein. In the *Arabidopsis* genome there are five genes encoding histidine phosphate transmitter proteins, AHPs [64]. These proteins contain a phosphate transmitter domain. The latter contains a conservative histidine residue, which represents a potential site for phosphorylation. Exogenous cytokinin stimulates translocation of fused AHP1–GFP and AHP2–GFP proteins (which were expressed in *Arabidopsis* protoplasts) from cytoplasm into nucleus [64]. This suggests involvement of these proteins in cytokinin-dependent phosphate group transfer from sensor histidine kinase to nuclear ARR *trans*-factors type B.

Figure 2 shows a putative phosphate cascade triggered by cytokinin interaction with its receptors, which results in induction of type A *ARR* genes.

Components of this signaling chain were discovered during the last two-to-three years. However, important features of this process still require detailed investigation. 1) It is known that mutation in genes encoding phosphotransmitters (AHP1 and AHP2) by conservative histidine (involved in phosphate group transfer) do not prevent cytokinin-induced AHP translocation into the nucleus. This means the existence of other mechanisms of cytokine recognition by AHP. 2) Mutation of the aspartate residue in the receiver domain of cytokinin-dependent ARR2 *trans*-factors does not influence the *trans*-factor mediated activation of luciferase expression under cytokinin-induced promoter of *ARR6* gene type A [61]. This means that *trans*-factor ARR2 may accept cytokinin signal bypassing the phosphate cascade. 3) Insertion of type A genes into *A. thaliana* mesophile protoplasts inhibited cytokinin-induced transient expression of luciferase gene under promoter of *ARR6* gene, and this inhibition was not abolished by mutation of the conservative aspartate in the receiver group of type A ARR protein [61]. This suggests that type A ARR proteins block expression of their genes, and the repressing effect may occur without involvement of the phosphate cascade. In accordance with these data, transformation of *A. thaliana* by *ARR6* gene attenuated cytokinin effects [61]. Consequently, type A genes of primary response to cytokinin (or at least some of them) act as repressors of cytokinin effect, and genes responsible for the positive effect of cytokinin remain to be determined.

Thus, consideration of recent data suggests the existence of alternative mechanisms of cytokinin signaling in the cell. For example, expression of the *GUS* gene under control of promoter of cytokinin-dependent *ARR6* gene in transformed *Arabidopsis* plants depends on activity of membrane-bound phospholipase D [65]. However, the position of this enzyme in cytokinin signaling mechanism remains to be determined. It was recently recognized that 3 min after cytokinin addition to suspension culture, plant cells release NO molecules; this also indicates involvement of NO-dependent signaling system in propagation of cytokinin signal [66]. Besides membrane cytokinin receptor, nuclear cytokinin receptor has also been recognized [57, 60, 67]. This requires investigation of integration of cell signals involving membrane and nuclear receptors.

The possibility of the existence of nuclear and membrane receptors and also complex systems integrating receptors at various levels has already been demonstrated for animal glucocorticoid hormones [68].

Nuclear cytokinin receptor. The membrane receptors of cytokinin considered in the previous section accept signals from exogenous cytokinins. However, there are intracellular cytokinins. They have been found in cytoplasm, nucleus [69], and chloroplasts [70]. This suggests the existence of intracellular molecular targets for cytokinins. In fact cytokinin binding proteins (CBP) with properties of cytokinin receptors involved in cytokinin-dependent regulation of transcription have been isolated from barley leaves, where cytokinin delayed senescence [71, 72]. Subsequently, it was found that CBP of 67 kD (CBP-67) with properties of cytokinin receptor was localized in nuclei of leaf cells [67]. This protein exhibited high specificity with respect to cytokinin and it did not bind its inactive analogs. CBP-67 exhibits high affinity to natural cytokinin, *trans*-zeatin. The concentration of *trans*-zeatin required for 50% displacement from the complex with CBP-67 of antiidiotype antibodies $AB_{a\text{-}i}$ (obtained against AB to zeatin) was $5\cdot10^{-9}$ M (Fig. 3) [72].

The complex of CBP-67 with cytokinin can activate transcription *in vitro* in experiments with isolated nuclei or chromatin preparation from barley leaves [67, 71-73]. It was demonstrated that cytokinin–protein complex activated elongation of transcription by RNA polymerases I and II (Table 1) [71]. In this connection, it should be mentioned that in animal systems elongation of transcription plays an important role in regulation of gene expression, and many *trans*-factors involved in this regulation have been discovered [74, 75].

CBP-67 was isolated from barley leaves exhibiting high sensitivity to cytokinin (cytokinin delayed their senescence). Subsequently, a similar protein was isolated from aging leaves of the dicotyledonous plant *A. thaliana*. The protein from senescent *Arabidopsis* leaves in the complex with cytokinin activated transcription in model systems *in vitro* containing chromatin and RNA polymerase I from *Arabidopsis* or barley leaves [73].

Thus, nuclear cytokinin receptor demonstrates high selectivity in binding of natural physiologically active cytokinins. In the complex with cytokinin, it can regulate transcription in model systems containing nuclei or chromatin from leaves of both mono- and dicotyledons. This indicates a universal mode of nuclear cytokinin receptor action in leaves.

A related protein of 70 kD CBP with properties of cytokinin receptor was isolated from etiolated maize seedlings by Brovko et al. [76]. This protein belongs to the same family of cytokinin receptors as the 67-kD CBP from barley leaves. Both proteins are localized in the nucleus, and they share common immune determinants and identical functional activity—regulation of transcription in the complex with cytokinin, as demonstrated in model transcription systems containing chromatin [72]. This suggests the existence of a whole family of nuclear cytokinin receptors involved in regulation of transcription in complex with hormone.

Monoclonal antibodies were obtained against maize seedling CBP-70 and these antibodies were used for sandwich-variant ELISA analysis in combination with the biotin–streptavidin system. Its use revealed preferential localization of CBP-70 in etiolated maize seedlings in the zone of root cell division [77]. This suggests involvement of CBP-70 in regulation of cell divisions by cytokinin.

Table 1. Effect of cytokinin-binding protein (CBP) and *trans*-zeatin (10^{-7} M) on RNA synthesis in isolated nuclei from barley leaves in reaction medium optimized for RNA polymerases I and II

CBP	*Trans*-zeatin	α-Amanitin, 4 μg/ml	Incorporation of [α-^{33}P]AMP into RNA, cpm per 20 μg DNA	
			RNA polymerase I	RNA polymerase II
–	–	–	5236 ± 121	6203 ± 423
+	–	–	4974 ± 211	5940 ± 501
–	+	–	4597 ± 270	6761 ± 328
+	+	–	17590 ± 525	21710 ± 1008
+	+	+	14929 ± 994	2171 ± 116
–	–	+	4922 ± 164	3169 ± 289

Chloroplast cytokinin binding protein with properties of receptor or cytokinin-dependent *trans*-factor. Chloroplasts are organelles responsible for photosynthetic activity of green plants. They are characteristic features of green plant cells. Chloroplasts have their own genetic system and protein synthesizing machinery. Biogenesis of chloroplasts is under double genetic control of the nucleus and chloroplast.

Regulation of chloroplast biogenesis is one of the most pronounced manifestations of biological activity of cytokinins. Each complex of the chloroplast electron-transport chain contains protein whose expression is controlled by cytokinin [78]. Cytokinin also induces synthesis of enzymes involved in formation of chloroplast pigments [79]. Recently, a wide spectrum of cytokinins typical for plants has been found in chloroplasts [70]. This raises a reasonable problem on the existence of the own chloroplast system responsible for cytokinin signal reception. In fact, cytokinin-binding protein, which specifically regulates (in complex with cytokinin) RNA synthesis in chloroplast transcription system, has been identified; this protein did not influence the nuclear transcription system (Table 2) [67, 80, 81]. This implies that the chloroplast receptor or *trans*-factor in complex with cytokinin is involved in regulation of the chloroplast genome.

It is shown that functional activity of chloroplast CBP (chlCBP) is not species-specific and manifested in chloroplasts of both monocotyledons and dicotyledons whereas it sharply changes with age [81]. This is promising for the elucidation of the role of chlCBP in regulation of chloroplast biogenesis by cytokinin.

Table 2. Effect of chloroplast (chlCBP) and cytoplasmic (cytCBP) cytokinin binding protein on *in vitro* RNA synthesis in systems containing chromatin bound RNA polymerase I or chloroplast lysate

Source of CBP	*Trans*-zeatin, 10^{-7} M	Incorporation of [α-^{33}P]AMP into RNA, cpm per 50 μg DNA	
		chromatin RNA polymerase	chloroplast RNA polymerase
Control	—	2446 ± 231 (100)	2846 ± 114 (100)
chlCBP	+	3046 ± 142 (125)	8622 ± 628 (303)
cytCBP	+	9902 ± 320 (405)	3469 ± 169 (120)

Thus, new important data on membrane, nuclear, and chloroplast cytokinin receptors have been accumulated during recent years (Fig. 4). Subsequent studies will clarify the integration of these receptors into pathways of the concerted cell response to cytokinin.

RECENT ADVANCES IN STUDIES OF GIBBERELLIN SIGNALING

Gibberellins are diterpenoid plant hormones. They are involved in stimulation of stem growth, transition to flowering of so-called "long day plants", and seed and tuber dormancy release [82]. Recently, very important results have been obtained in elucidation of the mechanism of action of these hormones.

Mutations in genes determining regulation of stem growth by gibberellin have been obtained. In wheat, these genes are known as *RHt* (Reduced Height) genes, whereas in *Arabidopsis* they are known as *GAI* (Gibberellic Acid Insensitive) and *RGA* (Repressor of GA) genes. Recent studies revealed that proteins (RHt, GAI, RGA) encoded by these genes share homology, nuclear localization, and "membership" in the groups of transcription factors [83]. They encode repressor, blocking activation of stem growth. Gibberellin can abolish this repression [83]. Gibberellin signal is transduced to repressor via an N-terminal amino acid sequence that is known as DELLA and comprises 17 amino acid residues. Mutations in this region make this protein insensitive to gibberellin, which

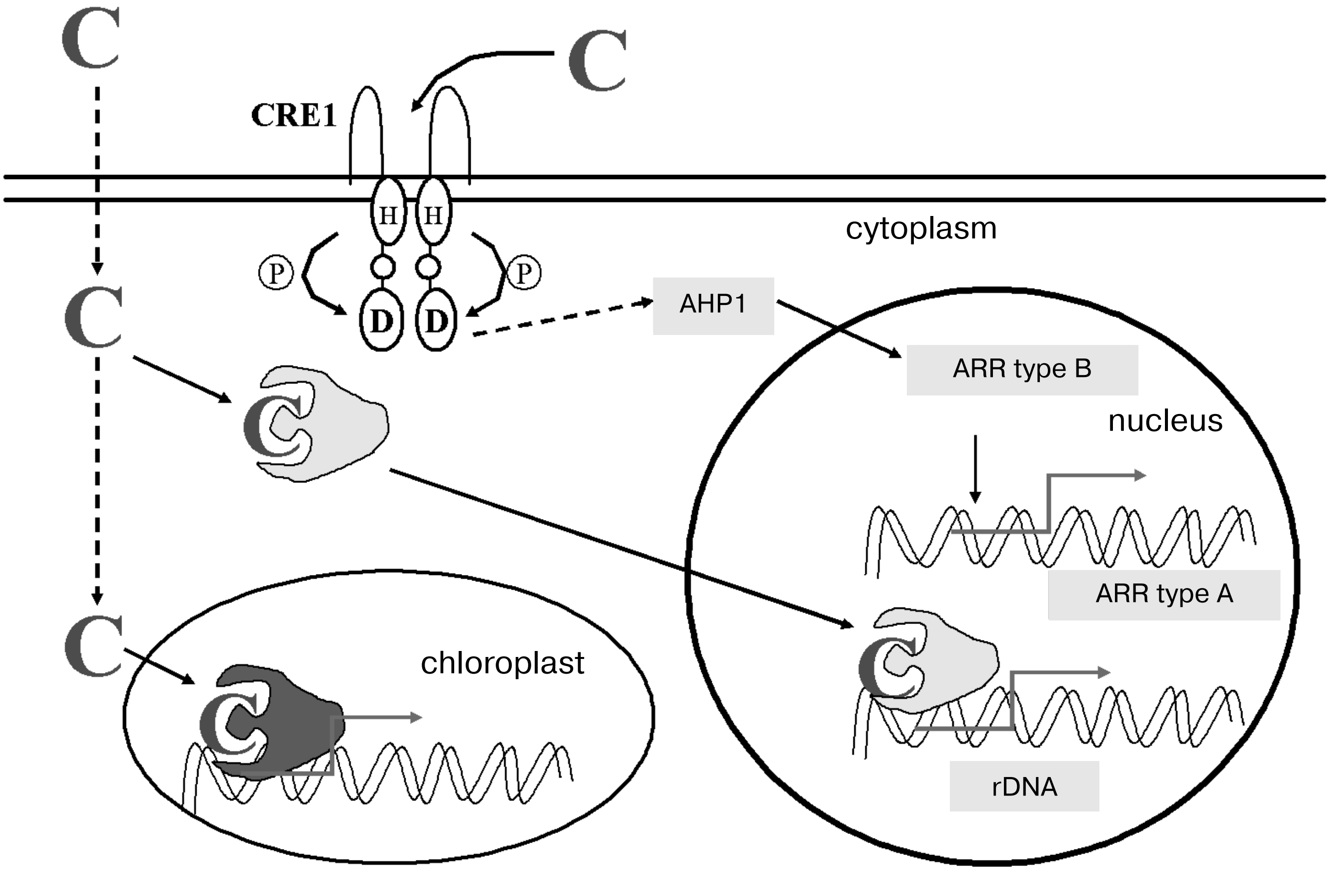

Fig. 4. Hypothetic scheme of cytokinin signal cell perception by membrane, nuclear, and chloroplast receptors (C is cytokinin; all other designations are the same as in Fig. 2).

does not abolish repression of genes responsible for stem growth in these mutants. This leads to formation of plants with shortened and thickened stem; these plants are resistant to rain- or wind-caused lodging and, consequently, they may provide for significant improvement in cereal crops defined as the "green revolution" [83]. Mutated genes are known as dwarfism genes. Isolation and investigation of dwarfism genes opened the possibility of development of dwarf, lowly sensitive to gibberellins and lodging-resistant high-yielding plants by gene-engineering methods without a long-term breeding process. Transformation of a rice plant by the *gai* dwarfism gene from the *Arabidopsis* genome represents a successful example of this approach [83].

Gibberellin causes degradation of protein repressors of its signal, and N-terminal DELLA sequence and the C-end of the repressor molecule are important for manifestation of this effect [84, 85]. This was demonstrated for *Arabidopsis* RGA-repressor [84] and was studied in detail using barley aleurone layer cells, where gibberellin induced α-amylase synthesis [85]. Induction of *GAMYB* gene expression resulting in accumulation of protein product activating the α-amylase gene is one of the early responses of aleurone layer cells to gibberellin. However, these cells contain SLN1, a nuclear protein blocking gibberellin-dependent induction of GAMYB *trans*-factor. Gibberellin causes SLN1 degradation, and this overcomes the repression [85].

As in the case of auxin, gibberellin signal transduction involves modulation of the functioning of the ubiquitin system responsible for selective degradation of protein repressors. This new (and rather unexpected) aspect of hormone signaling has been intensively studied during the last two-to-three years.

The system of reception and transduction of gibberellin signal involved in degradation of some proteins and induction of other proteins has not been characterized yet. Some evidence exists indicating that perception of gibberellin signal occurs at the outer surface of the plasma membrane [86]. Gibberellic acid (GA) causes increase in Ca^{2+} in aleurone cells, decrease in pH, and increase in calmodulin and cGMP concentrations [87]. It was also reported that GA influenced protein phosphorylation in aleurone layer cells, and this was an important event for induction of α-amylase. There was a prolonged lag period between GA-induced disappearance of SLN1 and expression of GAMYB; this suggests involvement of a chain of processes between these events. cGMP might be one of the components involved in these processes because there is a correlation between transient increase in cGMP concentration and onset of increase in GAMYB content [88]. Recently, it was demonstrated that in potato plant cells GA induced translocation of regulatory protein PHOR1 into the nucleus. This protein shares homology with *Drosophila* "armadillo" protein and its homolog in man, β-catein. In the nucleus, this protein forms a complex with *trans*-factors, and this results in induction of gene expression [89].

Thus, in spite of significant progress in gibberellin signaling research many aspects of the mechanisms underlying the hormone action require further investigation. Discovery of dwarfism genes encoding gibberellin repressors clearly demonstrates the importance of studies of mechanisms of gibberellin action not only for solution of basic problems, but also for practical goals.

STUDY OF MECHANISMS OF ABSCISIC ACID ACTION

Abscisic acid (ABA) plays an important role in the life of plants [82]. It is involved in regulation of formation and accumulation of storage compounds in plant seeds, dehydration at later stages of embryogenesis, seed maturation, and their transition to dormancy. ABA is also involved in dormancy induction in buds, tubers, bulbs, and roots. ABA plays an important role in plant response to external factors such as their survival under drought, salinity, and cold [90]. ABA causes stomatal closing, and this prevents transpirational water loss by the plant [91]. As in the case of other hormones, the most impressive results were obtained during investigation of ABA signal transduction mechanisms [92] using *Arabidopsis* mutants with reduced, absent, or increased sensitivity to ABA or mutants with knockout genes involved in signaling. One of the most popular experimental models for studies of ABA action is stomatal guard cells; in these cells ABA stimulates processes resulting in stomatal closing and decrease in transpiration.

A protein with properties of ABA receptor was isolated from the lower epidermis of broad bean leaves in 2002 [93]. This protein of 42 kD specifically recognized (+ABA) natural form but not (–ABA) form. This suggests that the hormone binding is stereospecific. The ABA-binding protein is membrane protein. The ABA-binding site is exposed on the outer surface of the plasma membrane. In the presence of ABA, the protein activated phospholipase D (PLD) in protoplasts of stomatal guard cells, i.e., it transduced ABA signal to PLD. The latter hydrolyzes membrane phospholipids with formation of phosphatidic acid, which acts as a second messenger and provides downstream signal transduction. These data on involvement of putative ABA receptor in ABA activation of PLD are consistent with previous results on ABA-induced activation of PLD in aleurone layer cells [94]. *In vitro* ABA activated PLD in plasmalemma-enriched fraction. Heterotrimeric G-protein and GTP are involved in signal transduction to PLD. Thus, these results suggest the following chain for ABA downstream signal transduction: membrane receptor → heterotrimeric G-protein → PLD → phosphatidic acid (as second messenger) and its interaction with possible target systems.

Analysis of the contemporary literature suggests the existence of many targets that might represent a whole network for ABA effect rather than a chain (as was thought earlier) [92]. Since ABA receptor was discovered only in 2002, it is too early to draw final schemes for its participation in a complex signaling network of ABA action. So, we will briefly consider only several main directions in modern studies of ABA action on cells.

Inositol triphosphate (IP_3) has also been recognized as a messenger of ABA signal; this suggests involvement of PLC in ABA signaling [92]. IP_3 activates Ca^{2+} channels of endoplasmic reticulum (ER) and tonoplast. This results in Ca^{2+} release from ER and vacuoles and increase in cytosolic Ca^{2+}, which then activates ion channels and efflux of K^+, Cl^-, and organic osmolytes from the guard cells. All these processes cause a decrease in turgor pressure in stomatal guard cells, followed by narrowing stomatal aperture, i.e., stomatal closing and transpiration decrease.

ABA-induced response of guard cells also involves reversible phosphorylation/dephosphorylation of proteins. There are ABA-dependent (serine-threonine type) protein phosphatases (protein phosphatase type 2C) [95, 96] and ABA-dependent protein kinases [92, 96]. Mutations of genes encoding ABA-sensitive protein phosphatases (abi1-1 and abi2-1) are accompanied by insensitivity of *Arabidopsis* to this phytohormone [95]. It is suggested that protein phosphorylation is involved in ABA signal transduction related to activation of ion channels and efflux of K^+ and Cl^- from cells, which is required for stomatal closing [96]. ABA-dependent protein kinases act as negative regulators of ABA signals. However, in another model system, barley aleurone layer cells, ABA blocks gibberellin-induced α-amylase by inducing specific protein kinase PKABA1; the latter is involved in manifestation of ABA inhibitory effect on α-amylase synthesis [88].

The stomatal model of ABA effects also involves reactive oxygen species (ROS) for transduction of ABA signal [92]. ABA increases ROS content in stomatal guard cells; this activates Ca^{2+} channels and results in the increase in cytosolic Ca^{2+} and causes all subsequent events (activation of ion channels, stomatal closing) [97]. Originally, it was suggested that H_2O_2 is the ABA messenger activating Ca^{2+} channels. However, in 2003 it was published that ABA and H_2O_2 effects on stomatal closing occur in different ways [98]. Both factors activate Ca^{2+}-channels and cause Ca^{2+} oscillation, which differs during the action of these factors. Parameters of ABA-induced oscillation of Ca^{2+} concentrations are important for manifestation of its effect on stomas, whereas changes of these parameters in *Arabidopsis* mutants result in their insensitivity to ABA [99].

It has just recently been discovered that ABA signaling in stomatal guard cells involves NO [100]. The latter activates guanylate cyclase and causes cGMP accumulation, which in its turn activates synthesis of cyclic ADP-ribose inducing Ca^{2+} channel opening. This increases concentration of cytosolic Ca^{2+}, central messenger of ABA effects on stomatal guard cells, and leads to a chain of abovementioned events leading to stomatal closing.

There is increasing evidence for the existence of alternative transduction of ABA signals in stomatal guard cells. There are Ca^{2+}-dependent and Ca^{2+}-independent pathways [101, 102]. Multiple signal transducing pathways involve phosphatidic acid, IP_3, Ca^{2+}, NO, cGMP, cADPR, and such phospholipid as sphingosine-1-phosphate [103].

Analysis of ABA-induced changes in stomatal guard cells suggests [92] that ABA signal transducing pathways represent a network rather than a chain. The ABA signaling network allows alternative downstream signals and cross talks between ABA and other hormonal signals. This might result in well known physiological phenomena: 1) antagonism in effects of various hormones; 2) mutual augmentation of hormonal signals; 3) partial "substitution" of the effect of one hormone by another; 4) necessity of concerted action of several hormones for certain physiological effects.

Besides stomatal closing, ABA regulates many other physiological programs in plants. Using microchip technique it was demonstrated that ABA causes induction of 382 genes in *Arabidopsis*. Several types of *cis*-elements of promoters of ABA-dependent genes and several types of *trans*-factors (ABI3, ABI4, ABI5) involved in ABA-dependent gene expression have been discovered [104, 105].

The expression of various ABA-dependent genes requires the involvement of different combinations of these *trans*-factors; this provides multiplicity of responses to ABA [105]. ABA regulates gene expression not only at transcriptional level. Recently a modulator of ABA signaling, ABH1 protein, was discovered. This protein is a homolog of subunits of nuclear heterodimer complex representing a component of cap-structure of mRNA [106]. A mutant deficient in this protein exhibits hypersensitivity to ABA. This suggests a negative role of this protein in ABA downstream signaling and a relationship between mRNA processing and ABA signaling.

Thus, results of recent studies open new aspects in the mechanisms of ABA signaling. The huge amount of experimental material accumulated during recent years demonstrates that in stomatal guard cells ABA induces a complex cascade of processes involving many signal molecules and alterations of such large blocks as state of membranes and ion channels, cytoskeleton, and reprogramming of genome activity [92].

ABA plays an important role in plant responses to various unfavorable external factors; however, we do not consider this aspect in the present review.

ADVANCES IN RECEPTION AND TRANSDUCTION OF ETHYLENE SIGNAL

Although the structure of the gaseous compound ethylene is very simple, it is an important phytohormone. It activates processes related to fruit maturation and accelerates senescence; ethylene induces defoliation, abscission of flowers and fruits, formation of adventitious roots. This phytohormone also regulates growth processes in plants (see for review [82]). As signal molecule ethylene is involved in plant response to pathogen: ethylene may increase or decrease plant resistance to various pathogens [1, 2]. Ethylene is also involved in plant response programs to abiotic stresses [107, 108]. The hormonal action of ethylene was discovered by D. N. Nelyubov (St. Petersburg University) in 1901.

During recent years, significant progress has been achieved in studies of ethylene signal reception and transduction [109].

The era of ethylene receptor study began with development of ethylene insensitive mutants [110]. Cloning and sequencing of genes responsible for loss of sensitivity to ethylene culminated in discovery of a whole family of ethylene receptors. The first member (ETR1) was discovered by the Bleecker's group in 1993 [111]. This receptor belongs to a family of bicomponent regulatory systems that also includes cytokinin membrane receptors.

The hydrophobic N-terminus of the ETR1 molecule contains transmembrane domains responsible for binding to membrane. The N-terminal sequence lacks analogs in protein databases. The N-terminus is responsible for ethylene binding. The first 128 amino acid residues of this receptor protein are important and sufficient for ethylene binding [112]. Copper localized at the N-terminus of ETR1 also participates in ethylene binding [109, 113]. ETR exhibits histidine kinase activity *in vitro* [114], but its role in ethylene signaling remains unclear. Although it also remains unclear whether ethylene binding to ETR results in activation or inhibition of histidine kinase activity, there is increasing evidence that ethylene binding is accompanied by inhibition of receptor histidine kinase [109].

ETR1 forms a homodimer stabilized by a disulfide bridge. Ethylene receptors represent a family of five proteins: ETR1, ERS1, ETR2, ERS2, and EIN4 [109, 113]. Genes encoding these proteins have been cloned and sequenced. Some members of this family can physically interact with each other. However, the functional specificity of these proteins remain unclear.

Figure 5 shows that the next component of downstream signal transduction is CTR1 [109, 115]. Its mutation caused constitutive response to ethylene. Consequently, CTR1 acts as a negative regulator of the ethylene signal.

It has been suggested that ETR1 operates in complex with CTR1 and blocks ethylene signal transduction [116]. Physical interactions between these proteins have been demonstrated in direct experiments. This suggests that ethylene inhibits rather than induces receptor histidine kinase activity; this abolishes the blocking effect of ETR1–CTR1 complex on downstream components involved in ethylene signal transduction (i.e., ethylene binding to its receptor derepresses a regulatory chain of response to this phytohormone) (Fig. 5).

CTR1 is related to Raf-kinase of mammals, which activates MAP-kinase cascade and is a kinase kinase MAP-kinase (MAPKKK). This suggests the participation of MAP-kinase cascade in ethylene signal transduction [109].

Thus, ethylene signal transduction in plants involves initially the prokaryotic bicomponent regulatory system and the purely eukaryotic system of MAP-kinase cascade. Details of this cascade functioning in transduction of ethylene signal remain unknown. According to a notion of a negative role of CTR1 in ethylene signal transduction, ethylene might block MAP-kinase activity. However, some evidence exists that ethylene activates MAP-kinase; this effect was noted in pea epicotyls and *Arabidopsis* leaves within several minutes after beginning of the phytohormone action [117-119]. The kinetics of MAP-kinase response to ethylene coincided with kinetics of response of monomeric G-proteins to this hormone. Activation of GTP binding to these proteins also represents early cell response to ethylene [119-122]. This suggests the existence of an alternative pathway of ethylene signal transduction relative to that presented in Fig. 5.

Ethylene signal transduction may also involve EIN2, a homolog of a metal transporter [123]. Being involved in cellular Cu^{2+} influx, EIN2 can regulate biogenesis of copper-containing receptor proteins [109]. The C-terminus of EIN2 contains a large cytoplasmic domain lacking homology with metal transporters. Some evidence exists that EIN2 may participate in ethylene signaling [123], but the mechanism of downstream signal transduction from ETR–CTR complex to EIN2 remains unknown. It has been suggested that EIN2 signal is "perceived" by nuclear EIN3 protein, which is related to *trans*-factors. EIN3 activates expression of genes encoding *trans*-factors of the ERF1-family [124]. ERF1 interacts with ethylene-dependent regulatory promoter element of genes included into the program of ethylene response.

In vitro homodimer of EIN3 interacts with a regulatory element of the *ERF1* gene. Consequently, regulation of gene expression by ethylene involves a transcriptional cascade providing induction of ethylene-dependent genetic programs (Fig. 5).

Thus, recent years are characterized by discovery of new components involved in perception and transduction of ethylene signal [109]. These include a family of transmembrane receptor proteins exhibiting histidine kinase activity (ETR1, etc.), CTR1 protein related to MAP-kinase cascade (which is negative regulator of ethylene response), EIN2, and, finally, sequentially acting *trans*-

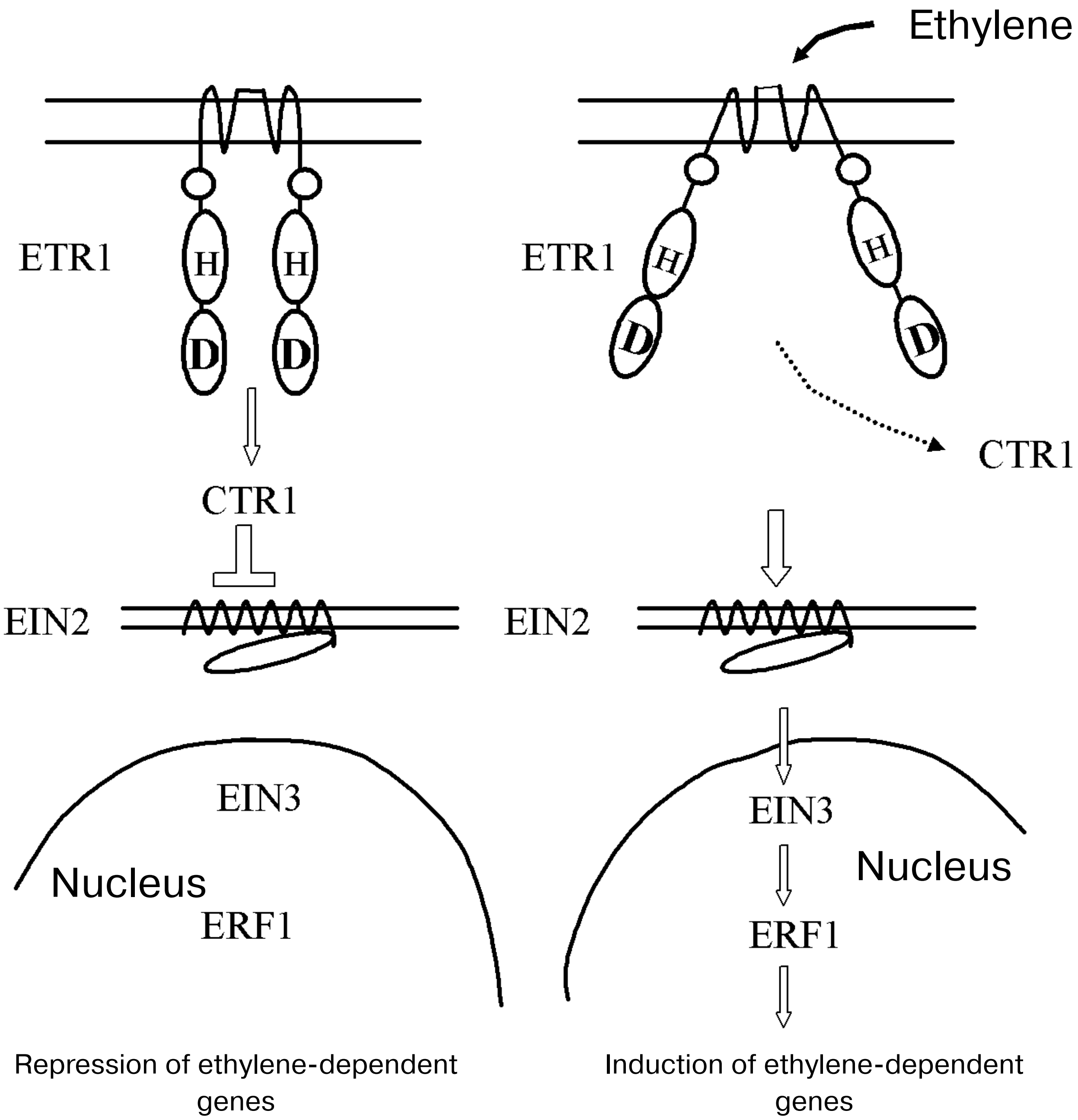

Fig. 5. Putative downstream transduction of ethylene signal in the cell. ETR1) ethylene membrane receptor; CTR1) RAF-like protein kinase; EIN2) protein of metal transporter family; EIN3 and ERF1) transcription factors.

factors EIN3 and ERF1. Discovery of these components involved in perception and transduction of ethylene signal represent a great achievement of plant biochemistry and molecular biology.

However, many aspects of these regulatory chains require further investigation. It is possible that alternative pathways of ethylene signal transduction exist [119].

Study of the biochemistry and molecular genetics of ethylene biosynthesis, reception, and transduction of its signal represents a good example of the importance of theoretic studies for solution of practical problems. Since ethylene regulates fruit maturation, characterization of stages of its biosynthesis has resulted in development of transgenic plants with prolonged period of maturation and storage of tomatoes. This was achieved by inserting a genetic construct containing antisense sequence of cDNA of gene encoding oxidase of ethylene precursor, 1-amino-cyclopropane-1-carboxylic acid (ACC). Catalyzing ACC oxidation, this enzyme is responsible for ethylene formation in cells. Antisense sequence of ACC oxidase cDNA inhibits biosynthesis of this enzyme, and this results in inhibition of ethylene formation. This approach extends the period of tomato storage by one month [125]. Tomato plant transformation by antisense ACC-synthase cDNA completely blocked ethylene biosynthesis in fruits and their maturation; however, fruit maturation could be induced by treatment of the fruit with an ethylene producer [126]. Transformation of petunia plant with mutated *etr1* gene of ethylene receptor significantly delayed wilting and abscission of flowers [109].

All these considerations clearly demonstrate that studies of biochemical pathways of biosynthesis and of mechanisms of phytohormone action open important perspectives in development of agriculturally valuable and ecologically safe plant genotypes on the basis of altered intensity of expression of their own genes.

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