Mechanism of kinetin-induced death of *Vicia faba* ssp. minor cortex cells

Andrzej Kaźmierczak (✉ andrzej.kazmierczak@biol.uni.lodz.pl)

Univeristy of Łódź

Anita Kunikowska

Univeristy of Łódź

Magdalena Doniak

Univeristy of Łódź

Andrzej Kornaś

The Pedagogical University of Cracow

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Abstract

Cell death (CD) takes part in the control of all the steps of plant development. It may be induced by endogenous or exogenous factors. This paper presents the results related to mechanism of CD regulation induced by kinetin (Kin) in root cortex of *Vicia faba* ssp. *minor*.

To explain the process 6-(2-hydroxy-3-methylbenzylamino)purine (PI-55), adenine (Ad), 5'-amine-5'-deoxyadenosine (Ado) and N-(2-chloro-4-piridylo)-N'-phenylurea (CPPU) were applied to (i) block cytokinins (CKs) receptors and to inhibit the activity of (ii) kinases, (iii) oxidases and (iv) phosphoribosyltransferase, respectively.

Moreover, ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), lanthanum chloride (LaCl_3) and cyclosporine (CsA) were applied to inactivate calcium ion (Ca^{2+}) channels (i) across all membranes (ALM- Ca^{2+}), including (ii) endoplasmic reticulum (ER- Ca^{2+}) and (iii) mitochondria (MIT- Ca^{2+}), respectively.

The effects of these factors were measured by estimation of vitality of cortex cells and amounts of cytosolic Ca^{2+} .

Additionally, the role of Sorafenib, inhibitor of RAF kinase, on vitality of cortex cells and ethylene amount as well as the activities of Raf-like kinase and MEK2 with Syntide and Mek2 as substrates, were studied.

The results of the paper clarified the suggestion that Kin was converted directly or indirectly to appropriate ribotides (5'-monophosphate ribonucleotides) which cooperated with ethylene and calcium ions signaling pathway activating CD by Kin (Kin-ECD) exogenously (E).

Based on the present and previously published results, the scheme of the crosstalk between ethylene signaling MAP kinases, CKs receptor and enzymes of their metabolism in induction of Kin-ECD is proposed.

Introduction

Cell death (CD) is a developmentally and environmentally (biotically or abiotically) induced process in all organisms¹. During plant tissue and organs differentiation, i.e., developmental CD (dPCD), according to Galluzzi *et al.* (2018)² such type of CD is mentioned as programmed CD (PCD). It occurs *via* modification of cell functions or cell elimination, control of formation of vegetative (e.g., xylem and phloem tissues) and generative (e.g., embryos)¹ organs. One of the factors, which applied exogenously induced cell death, is Kin^{3,4,5}. Remaining consistent with suggestion of Galluzzi *et al.* (2018)² that CD induced by exogenous factors is a Regulated CD (RCD), authors of the paper will use the term "kinetin-exogenously induced-CD" (Kin-ECD).

Kin-ECD is of special interest because Kin is a differentiation regulator, naturally occurring in plant and animal organisms⁶. The process of CD induction by Kin was firstly discovered in cortex of 2-cm apical parts of *V. faba* ssp. *minor* (faba bean) seedling roots⁷.

Hallmarks of Kin-ECD were extensively^{3,4,5,7,8,9} and thoroughly¹⁰ scrutinised. One of the effects of Kin-ECD is degradation of about 50% of the cortex cells in faba bean seedling roots making cell-free spaces known as aerenchyma^{3,7,15/8}. Aerenchyma is an important plant tissue allowing plant to exchange respiratory gases (among them oxygen and carbon dioxide) between shoots and roots in aquatic and wetland environments¹¹. The most common morphologic features of Kin-ECD during aerenchyma formation consist in degradation of some or all cellular compartments^{3,4,5}, where nuclei^{4,5} and/or cell walls⁹ are degraded as the last ones. Its progress is directly induced by ethylene (ETH)¹¹ or by exogenously applied 1-aminocyclopropane-1-carboxylic acid (ACC)¹², which is a direct precursor of ETH¹¹.

Kin induced elimination of cortex cells⁷, but not whole roots and/or seedlings^{3,4}. It indicates that this developmental regulator plays a dual role, i.e., it triggers disruption of some of the destined-to-die cells and simultaneously stimulates mechanisms preventing death in the other ones. The latter was evidenced by greater activity of catalases and superoxide dismutases, the enzymes of ROS metabolism scavenging⁹ and by elevated amount of cellulose, callose and other cell wall bounded sugars, in the living, not destined for death, cortex cells, leading to thickening of their walls³. Moreover, transmission electron microscopy showed absence of plasmodesmata connection in the walls of living cortex cells which is the probable result of greater amount of callose clogging them. This may lead to isolation of dying cells from non-dying ones⁹, likely by blocking transport of Kin, its metabolites and other signal molecules. It is in accordance with the data indicating that cytokinins (CKs) are transported by symplasmic connections within the plants¹⁴.

Kin-ECD was also hallmarked by lower number of mitochondria and by their malformed morphology as the effect of ROS overproduction^{9/12}, by nuclear chromatin condensation and chromatin fragmentation with exo-/endonucleolytic enzymes^{4,5}. Finally, enormous acidic lytic vacuoles appeared⁷. During Kin-ECD, the amount of proteins was unchanged, while activities of serine- and cysteine-dependent proteases fluctuated. Moreover, treatment with N-ethylmaleimide (NEM), phenylmethylsulfonyl fluoride (PMSF) and Z-Leu-Leu-Nva-H (MG115) decreased the number of dying cortex cells. These facts and the relationships between NEM concentration and protein amounts as well as changes in β 1 proteasome subunit activity and inhibiting influence of MG115 on its activity indicated that proteolytic activities played important role, mimicking caspase-like signaling elements, in the signal transduction pathway during of Kin-ECD⁸ like in other instances of processes¹³.

Similarly, to the results of studies of apoptosis in *Caenorhabditis elegans*¹⁴ specification, executive and degradation phases of the Kin-ECD^{5,6} were distinguished. The phases of the process may last from the 0

to 3rd h, from 6th to 18th and from 24th to 96th h, respectively^{5,6}. In the specification (signaling) phase of Kin-ECD, the total and cytosolic levels of calcium ions (Ca^{2+}) in the cortex of apical parts of faba bean seedling roots and in cortex cells respectively, as well as the amount of ACC in these fragments, were the greatest³. Whereas in the executive phase, the reactive oxygen species (ROS) amounts were the greatest and in the degradation phase of the process the activity of the ROS metabolism enzymes and the amount of free sugars were the greatest^{3,9}.

The studies which results are presented in the paper were undertaken to prove the hypothesis suggested in Kunikowska et al. (2013)⁷. Authors of that paper suggested that Kin induced CD after its conversion with phosphoribosyl transferase to corresponding monophosphates which are mentioned as the purine specific ligands^{15,16} for one or two histidine kinases (HKs) CK receptors. This hypothesis is based on the data described in several papers showing that exogenously applied free CK bases into organisms are rapidly converted into their nucleosides and nucleotides^{16,17,18}. Then they triggered apoptosis in human leukemia cell lines (HL-60 cell line)^{16,19}. Moreover, treatment of the cell cultures of *Arabidopsis thaliana* with benzyl adenine (BA) induced CD only in the presence of HK4 receptors²⁰ although BA had low affinity to them²¹. In *A. thaliana* HK3 had about 10-fold lower affinity to isopentenyladenine and its riboside, but higher affinity than HK4 to dihydrozeatin and zeatin and isopentenyl adenine/cytokinin ribosides and *cis*-zeatin^{26,27}.

This fact confirmed that CK ribosides and their monophosphates are purine ligands allegedly inducing CD *via* HK4²⁰. This receptor can cooperate with HK3 one^{14,20,22,23,24}, which together with HK2 are plasma- and endoplasmic reticulum (ER)-localized transmembrane proteins with extracellular and intracellular domains. These facts were evidenced in *A. thaliana*²³, *Zea mays*¹⁴ and in other plants²⁴.

The main steps of cytokinin transport and signaling cascade involve purine permeases (PUP; a transporter of free cytokinins) equilibrating nucleoside transporters (ENT), subfamilies of HK-cytokinin receptors^{14,23-25}, His phosphotransfer proteins (HPT1-5) and two types (A and B) of cytokinin gene response regulators (RR).

HPTs, small (app. 16 kDa) monomeric proteins, together with RR, initiate cytokinin signaling. The A-type RRs are stabilized by their phosphorylation and they are negative regulators of cytokinin response, but B-type of RRs, which can bind to DNA and start expression of cytokinin-sensitive genes after their phosphorylation^{14,22-29}.

We proposed that monophosphates interacting with HK4 or, eventually with HK3, whose presence in faba bean we suggested, evoking efflux of Ca^{2+} , activated ETH-dependent cell death process regulators. This hypothesis was based on the data showing that (i) ETH and CKs interacted at the level of signal transduction^{29,30} as well as Kin (ii) elevated ACC amount³, and (iii) evoked efflux of Ca^{2+} to cytosol³, finally forming aerenchyma^{3,6}.

To prove the mentioned hypothesis the effects of factors controlling perception and metabolism of CKs in Kin-ECD and amount of cytosolic Ca^{2+} the following were analysed: (i) 6-(2-hydroxy-3-methylbenzylamino purine (PI-55)^{22,22,30} the specific cytokinin histidine kinase receptor inhibitor and (ii) adenine (Ad), 5'-amino-5'-deoxyadenosine (Ado) and N-(2-chloro-4-piridyl)-N'-phenylurea (CPPU) the inhibitors of adenine phosphoribosyl transferase (APRT)^{31,32-34}, adenosine kinases (ADK)^{16,35,36} and CK oxidases (CKO)³⁶, respectively.

Authors of the paper would like to find the answer to question related to assumption whether MAP kinases being the crucial elements of ethylene-dependent signaling pathways are engaged in Kin-ECD. Thus, the effect of plant Raf-like kinase (CTR1) inhibitor (Sorafenib)^{37,38} on vitality of cortex cells as well as MEK2 and Raf-like kinase activities were measured using its substrates, i.e., Syntide³⁸ and Mek2³⁹.

To find the source of cytosolic Ca^{2+} taking part in Kin-ECD, the stream of its destinations were studied by the effects of the of all (ALM- Ca^{2+})⁴⁰, ER-⁴¹ and mitochondria-⁴² membrane channel inhibitors i.e., ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetra-acetic acid (EGTA), lanthanum chloride (LaCl_3) and cyclosporine (CsA) respectively on vitality of cortex cells and cytosolic amount Ca^{2+} .

Results

Analysis of the double stained nuclei showed that after 72 h in Ctrl about 4% of cells were dying or dead, while in the same time, after Kin treatment number of these cells significantly increased to about 48% (Fig. 1A,C – 5A,C). The cytophotometric analysis showed that the amounts of cytosolic Ca^{2+} differed between series, and were about 10 000 a.u. and 14 000 a.u. in Ctrl and in the Kin-treated cells, respectively (Fig. 1B,D – Fig. 5B,D).

Effect of PI-55, inhibitor of cytokinin perception, and of Ad, Ado and CPPU, cytokinin metabolism inhibitors, on Kin-ECD and the amount of cytosolic Ca^{2+} . It was showed that PI-55-5 did not induce cell death compared to Ctrl, while PI-55-10 did. The number of dying cells in the latter case was about 20%. In Kin/PI-55-5 cell death was almost completely inhibited in comparison with Kin (Fig. 1A). At the same time, the amounts of Ca^{2+} in PI-55-5 and PI-55-10 were significantly greater, about 2.5- and 2.0-fold, than in Ctrl and about 2.0- and 1.5-fold than in Kin, respectively. In Kin/PI-55-5, the amounts of Ca^{2+} were significantly greater, about 3.0- and 2.5-fold, than in Ctrl and Kin, respectively (Fig. 1B).

Both Ad-50 and Ad-100 did not increase the number of dying cells compared to Ctrl. However, to study its effect on Kin-ECD, the Ad-100 was used. In Kin/Ad-100 the number of Kin-ECD-dying cells was like in Ctrl (Fig. 2A). While the amounts of Ca^{2+} in Ad-50 and Ad-100 were significantly greater, about 2.0-fold, in comparison with Ctrl and about 1.3-fold in comparison with Kin. In Kin/Ad-100 variant the amount of Ca^{2+} was like in Kin (Fig. 2B).

Ado-50 did not increase the number of dying cells compared to Ctrl, but Ado-10 increased their number to 12%, thus Ado-50 was used to study its impact on Kin-ECD. It was showed that in Kin/Ado-50 variant the

number of dying cells was like in Ctrl (Fig. 2C). While in Ado-10 and Ado-50 variants the Ca^{2+} amounts were significantly greater, by about 2.0- and 3.0-fold, in comparison with Ctrl, respectively, and by about 1.5- and 2.5-fold, in comparison with Kin variant respectively, while in Kin/Ado-50 series, the amount of Ca^{2+} was like in Kin (Fig. 2D).

CPPU-5 did not increase the number of dying cells compared to Ctrl but in CPPU-10 the number of dying cells was like in Kin. Thus, to study its effect on Kin-ECD, CPPU-5 was used. In Kin/CPPU-5 the number of Kin-ECD-dying cells was significantly lower, about 30%, in comparison with Kin (Fig. 3A). Analyses of the levels of Ca^{2+} in CPPU-5 and CPPU-10 showed that its amount were significantly greater, about 2.0- and 1.5-fold, in comparison to Ctrl, and about 1.5- and 1.3-fold greater, in comparison to Kin, respectively, while in Kin/CPPU-5 the amount of Ca^{2+} was significantly greater, about 1.6-fold, in comparison with Kin and like in CPPU-5 (Fig. 3B).

Effect of the EGTA, La^{2+} and CsA inhibitors of ALM- Ca^{2+} , ER- Ca^{2+} , MIT- Ca^{2+} -dependent channels, respectively, on Kin-ECD and the amount of cytosolic Ca^{2+} . EGTA-10 and EGTA-50 did not increase the number of dying cells compared to Ctrl. To study its effect on Kin-ECD, EGTA-10 was used. It was showed that in comparison with Kin in Kin/EGTA-10 the number of dying cells was significantly lower, about 38% (Fig. 4A). The amounts of Ca^{2+} in EGTA-10 and EGTA-50 were significantly greater, about 1.5- and 2-fold, in comparison with Ctrl, respectively, and about 1.3- and 1.5-fold greater, in comparison with Kin, respectively, while in Kin/EGTA-50 the amount of Ca^{2+} was like Ctrl (Fig. 4B).

La-5 and La-25 did not increase CD index compared to Ctrl. Therefore, La-5 was used to study its effect on Kin-ECD. It was observed that in comparison with Kin in Kin/La-5 the number of Kin-ECD-dying cells was significantly lower; there were only 10% of them (Fig. 4C). The amounts of Ca^{2+} in La-5 and La-25 were significantly greater, about 2- and 1.5-fold, in comparison with Ctrl and Kin, respectively. In Kin/La-5 the amount of Ca^{2+} was significantly greater, about 1.5-fold, in comparison with Kin (Fig. 4D).

CsA-5 significantly increased the number of Kin-ECD-dying cells in comparison with Ctrl while CsA-25 did not. Therefore, CsA-25 was used to study its effect on Kin-ECD. It was observed that in comparison with Kin, in Kin/CsA-25 the number of dying cells was significantly lower, about 30% (Fig. 5A). The amounts of Ca^{2+} in CsA-5 and CsA-25 were significantly greater, by about 3.0- and 2.0-fold, in comparison with Ctrl, respectively, and by about 2.0- and 1.5-fold greater in comparison with Kin, respectively. In Kin/CsA-25 the amount of Ca^{2+} was significantly greater, by about 2.0-fold, in comparison with Kin (Fig. 5B).

Effect of Sorafenib on Kin-ECD, ethylene amount as well as Raf-like and MEK2 activity. To study the effect of Sorafenib on cell death, the 1 mM concentration of this compound was used. The number of living cells in Sorafenib and Sorafenib-Kin series remained at the same level like in Ctrl series. In Kin series the number of living and dying cells was about 45 % and 55 % respectively (Fig. 6A).

In Ctrl series amount of ETH was about 5 ppm while after treatment with Kin for 72 h its amount was statistically significantly greater by about 30 %. Treatment with Sorafenib of Ctrl seedlings (Sorafenib

series) showed that ETH amount per six seedlings was about 20 % lower compared to Ctrl. In combination of 1 mM of Sorafenib with Kin (Sorafenib-Kin series), the amount of ETH was about 30 % and 40 % greater compared to Ctrl and Sorafenib series respectively and was like in Kin (Fig. 6B).

Analyses of kinase activities showed that activities of Raf-like kinase, with Syntide substrate, and MEK2, with Mek2 substrate, in Ctrl series was about 2.5 mg and 1 mg of ATP per mg of protein, respectively. After treatment with Kin for 72 h their activities were about 20 % greater and 20 % lower respectively compared to Ctrl series (Fig. 6C).

Discussion

Scientific reports indicate that 10–30 μM concentrations of cytokinins (CKs), among them Kin, induced CD neither in animals^{6,16,43,44} nor in plants^{20,45,46}. In HL-60 cells, 50 μM Kin inhibited growth⁴³, but even 100 μM Kin or BA did not induce their death¹⁶. However, death caused by 13 μM , 27 μM and 44 μM BA as well as by 46 μM Kin of cells was induced in *A. thaliana* plants²⁰ and in carrot (*Daucus carota*) and *A. thaliana* suspension cultures⁴⁷ as well as in cortex cells of apical parts of faba bean seedling roots^{3,4,5,8,9}, respectively.

Results of scientific studies indicated that cytokinin ribosides, at 10–30 μM concentrations are the active cell death inducers in plants^{45,48,49} and in animals^{16,19,20,44}.

These facts allowed us to suggest that CD depends on an organism and the concentration of CKs which was decided on sufficient amount of free bases of CKs important for synthesis of their ribosides and their phosphorylated derivatives both in animal (HL-60)^{16,19} as well as plant cells, e.g., in *Arabidopsis* sp. suspension cell culture²⁰ and in *Gerbera* sp. callus where the free zeatin became conjugated with ribose or ribose phosphate to produce the physiologically-active compounds, i.e., zeatin-9-riboside and zeatin-9-ribotide⁵², respectively.

The studies of Kin-ECD showed that Kin in faba bean roots induced two responses³. The first one is related to development of the protective mechanisms, acting against death. It is known that Kin is an antioxidative factor which strongly inhibits oxidative and glycoxidative protein-damage¹¹. Such antioxidative effect is observed in apical parts of faba bean seedlings during Kin-ECD as the increase and then decrease in ROS amount as the results of increase in activities of the enzymes of their metabolism⁹. CK in Kin form is the factor which can delay the senescence of detached *Raphanus sativus* L leaf discs⁵². It inhibits the leaf senescence *via* activation of cytokinin receptor (AHK3), the type-B response regulator (ARR2) and the cytokinin response factor (CRF6)⁵³.

The second responses³ of Kin in faba bean roots is related to induction of ECD^{5,8,9} in which Kin may be degraded to adenine and ribosylated to its ribosides by adenosine phosphorylase. This enzyme was identified in wheat (*Triticum aestivum*) germs⁴⁹. To obtain phosphorylated forms of CKs, the adenosine kinase catalysed formation of the adenosine monophosphate from adenosine. Adenosine kinase was

found both in human¹⁶ and in tobacco BY-2 cells¹⁷ as well as in Arabidopsis³⁶. The next step of adenine metabolism is related to conversion of 5'-monophosphate of zeatin to its i.e., *trans* or *cis* forms. The fact that Ad and Ado, adenosine phosphorylase^{31,32-34} and adenosine kinase^{16,35,36} inhibitors, respectively, completely suppressed Kin-ECD in root cortex confirmed occurrence of such enzyme in faba bean roots and worked according to their functions.

Ad inhibited formation of AMP in human²⁷ although in *A. thaliana* not³⁵. Its application to faba bean seedlings inhibited the CD induced by Kin. This fact suggested that the Ad inhibiting activity of APRTs broke the CKs synthesis pathway therefore lower amount of CK monophosphates, which are suggested as ligand for CKs receptors, were released in the CK-dependent pathway.

Inhibition of adenosine phosphorylase and cytokinin kinases activities might enhance the inhibitory effect on Kin-ECD because the results showed that an inhibitor of cytokinin oxidase, i.e., CPPU, suppressed Kin-ECD. Since adenine, which is a direct product of CKs degradation by cytokinin oxidase and inhibitor of adenine phosphoribosyl transferase activities^{27,31} acted against Kin-ECD⁵. It indicated that the level of Kin or other CK riboside monophosphates, as the results of the activity of ATPR, may decreased. Thus, its CD-inducing effects can be decreased.

It is suggested that riboside monophosphates of CKs could activate faba bean HK4 receptors in ER-membrane^{20,45,46}. The monophosphorylated forms of cytokinin ribosides have greater affinity for HK4^{16,20,21} than HK3 and free cytokinin bases however both receptors can interact with each other²¹. This might result from the fact that PI-55 completely inhibited the death of cortex cells. After dimerisation and autophosphorylation of HKs, the phosphate group is translocated from histidine to aspartic acid of a regulatory domain of the receptor. Then histidine of faba bean homologues of *A. thaliana* HPT carriers and type B of RR^{15,20} can activate the cytokinin-dependent response (Fig. 7). The earlier results showed that during Kin-ECD the level of ATP decreased⁴. It suggests that decreased level of ATP amount after Kin treatment was related to the fact that ATP was used as the phosphate group for synthesis CKs riboside monophosphates and/or with the mitochondria destruction, observed in apical parts of faba bean roots as the reduction of their number and structure malformation caused by ROS⁹, but also on lower activities of cellular dehydrogenases¹⁵ and histone kinase activities⁴. It may be assumed that, during Kin-ECD the cytotoxic N⁶-furfuryladenine (Kin-riboside) may be synthesized and depleted ATP amounts, like in human cancer cell lines⁴⁸.

The extensive studies delivered important results suggesting that ETH may be the effector of Kin-induced death signal transduction pathway³. The loss of HK4 function in *ahk4* mutant decreased ETH response to CKs, indicating that the *A. thaliana* HK4 receptor is probably a primary contributor to ETH biosynthesis responding to CKs^{29,30}. Therefore, *A. thaliana* HPT can transduce signals from CKs (Fig. 7)^{15,23} to ETH receptors (ethylene triple response, ETR1 and 2; 1; ethylene insensitive 4, EIN4; ethylene response sensor 1 and 2 ERS1 and 2) in ER membranes^{28,29,38,40,56}. Moreover, CKs can post-transcriptionally increase the activity of ACS4,5 (ACC synthases 4,5) gene products^{29,56}, leading to increase in activities of these

enzymes in ethylene synthesis, thus it can synergistically increase ETH amount (Fig. 6,7). This suggestion was confirmed by the fact that in the Kin-treated seedlings in apical parts of faba bean roots, the ACC¹² and ETH amounts increased. It can activate EIN2, ER- and nuclear- ETH-dependent membrane receptors and induce ETH response elements (ERE)^{28,29}. The fact that after Kin-^{3,7} and ACC-induced¹² death of cortex cells of faba bean roots aerenchyma was formed^{3,7,12} also confirmed the hypothesis, because ETH seemed to be direct hormonal factor involved in the process³.

It cannot be excluded that another ETH-CKs pathway in Kin-ECD exists. The MAP kinases are considered regulators of ETH signal transduction pathway. One of them in plants is CTR1, a structural equivalent of RAF kinase. It is assumed that CTR1 acts in the ETH signaling that is MAP-kinase-dependent cascade. Results showed that Sorafenib suppressed the Kin-ECD because the number of living cells in Kin series were at the same level as in the Ctrl. However, the Raf-like kinase activity in root of Kin series was greater than in Ctrl series while the MEK2 activity was lower compared to Ctrl series. Sorafenib, an inhibitor of ATP-competitive kinase can affect activity of other kinases, e.g., ERK (extracellular signal-regulated kinase)⁵⁰ one. These facts clearly indicated that in faba bean roots both kinases exist.

The results showed that decrease in the number of dying cells is dependent on the effect of Raf-like kinase activity increase and of MEK2 activity inhibition. Moreover, the results confirmed that Kin-ECD is the process controlled by cooperation of ETH-dependent MAP kinases signaling pathway. The crosstalk between Kin and ETH might take place at the level of ETH and CK receptors because ETH receptors are members of HK family proteins⁵⁷. It was proved by the appearance of the triple ethylene response (cell length and width changes as well as root apical hook formation)^{3,7,59} in the faba bean seedlings after Kin treatment which features were inhibited by 2,5-norbornadiene (NBD), the ETR3 and ETR4 receptors inhibitor³.

The results presented by Scharein and Groth (2011)⁵⁹ confirmed the existence of ETH-CKs crosstalk. The phosphorylation between ETR1 and AHP1 complexes play a crucial role in this process. When both are either in phosphorylated or non-phosphorylated states, the affinity between them decreased. On the other hand, the affinity between the two partners is greater when one of them is phosphorylated and the other one is not. The additional reason that this process might exist in faba bean is the fact that both ETH and CK signaling pathways work using the transduction factors being the two-component systems^{15,57}.

The present results showed that the amounts of cytosolic Ca²⁺ in the root cortex cells of faba bean seedlings in PI-55-5 and CPPU-5 were similar to Kin/PI-55-5 and Kin/CPPU-5 but in Ad-100 and Ado-50 they were greater, in comparison with Kin/Ad-100 and Kin/Ado-50; the levels of cytosolic Ca²⁺ in Kin/PI-55-5 and Kin/CPPU-5 were greater than in Kin while in Kin/Ad-100 and Kin/Ado-50 were similar to Kin series.

The fact that Ca²⁺ migrated from ER, which is important for Kin-ECD induction, was suggested previously³. The present research showed that application of La²⁺, inhibitors of ER-membrane Ca²⁺

channels^{40,60}, in a nitrate³ or chloride form, inhibited the process. The results of the present paper showed that migration of Ca^{2+} *via* plasma membrane- (from outside the cell) or mitochondria-channels was also important for Kin-ECD because application of EGTA or CsA suppressed Kin-ECD. Cytosolic levels of Ca^{2+} in the cortex cells of seedling roots in La-5 and La-25 μM , EGTA-10 and EGTA-50, CsA-5 and CsA-25 and Kin/La-5 were greater in comparison with Ctrl and in Kin series. Thus, Ca^{2+} may not be the direct transducer of Kin-dependent signals, as it was suggested in Doniak *et al.* (2017)³ and Kunikowska *et al.* (2013)⁷, but it seems that their amounts are important for activation Kin-ECD enhancing ACC and ETH synthesis *via* calcium-dependent ACC synthase (ACS) enzymes.

The fact that in Kin/EGTA-5 the amount of Ca^{2+} was like in Ctrl confirmed that induction of Kin-ECD depended on direct migration and on the type of Ca^{2+} channels. Thus, when in mitochondria⁶¹ and in ER membranes CNGCs (cyclic nucleotide gated channels)^{40,62} were blocked by CsA or La^{2+} , Ca^{2+} were transported to cytoplasm *via* plasma membrane channels leading to elevation of cytosolic Ca^{2+} (Fig. 7). It is confirmed by the results showing that EGTA (an inhibitor of all Ca^{2+} channels)³⁹ applied to the culture solution blocking plasma membrane channels lowered the influx of Ca^{2+} into cytosol.

Thus, it is possible that ETH initiated expression of protein kinases and/or their activities, especially of H1 and core histones⁴ and induced ETH-positive feedback *via* Ca^{2+} -dependent ACC synthase (Fig. 7) important for the regulation of ETH synthesis. Moreover, phosphatidyl inositol 4,5 bisphosphate and inositol 1,4,5 trisphosphate may increase ETH amount^{11,58}.

The results indicating that ethylene is involved in Kin-ECD control were also confirmed by the studies revealing that inhibitors of ACC synthesis and its conversion to ETH suppressed Kin-ECD³. Moreover, Kin-ECD triggered aerenchyma formation accompanied ETH-dependent triple-response^{3,7,58}.

The results of studies related to male sex determination of *Anemia phyllitidis* gametophytes, where ETH is the gibberellin secondary signal, additionally confirm that reorganization of metabolism and of cell wall structure in plant depends on ETH⁵⁹.

Conclusions

The fact that some of the results of the paper are partially based on usage of factors such as Sorafenib, Syntide and Mek2, the inhibitor of RAF kinase^{37,50} as well as Raf-like and MEK2 substrate related to the animals³⁷, plants³⁵ and bacteria³¹ it was the crucial for explanation Kin-ECD progress.

First and foremost is the fact that Kin is the factor playing important role in the controlling of differentiation both in plants and animals⁶. Secondly, CTR1 kinase (in plants called Raf-like³⁸) is a homologue of RAF kinase, existing in animals³⁷. Additionally, in plants, animals and bacteria the similar histidine kinases forming the transmembrane receptors for ETH and CKs were found^{28,56}.

Therefore, taking all results into account, we can conclude that the Kin-dependent signal by the phosphorylated forms of Kin or other CKs ribosides may activate CD-dependent HK4 (strongly suggested as the cell death receptor activated by CKs)^{20,21} receptors and generate pathways *via* HPT^{20,46} enhancing expression of *ACS4,5* genes^{15,55,56} and activities of Ca²⁺-dependent ACSs⁵⁸, increasing ACC and then ETH amounts (Fig. 7).

Then the signal is transferred onto specific ETH-dependent genes and it induces: (i) the expression of elements initiating the executive phase of Kin-ECD among them serine and caspase-like proteolytic⁸ and nucleolytic^{4,5} machinery as well as (ii) cell wall compound metabolism³ which finally lead to complete degradation of some cells in the degradation phase of Kin-ECD^{5,8} resulting in aerenchyma formation^{3,7}. Kin generates two signals, the death one results in cells selection, *via* ETH, and the pro-life one against death in other cells, *via* activation of ROS enzyme metabolisms and *via* maintaining the appropriate concentration of cytosolic Ca²⁺ and/or its destination^{40,60}.

During the morphologic and metabolic studies, we demonstrated that perception and metabolism of CK inhibitors completely suppressed Kin-ECD, but they did not reduce the Kin-elevated amount of cytosolic Ca²⁺ in the cortex of apical roots of faba bean seedlings. Moreover, ER- and mitochondria-membrane Ca²⁺ channel inhibitors reduced Kin-elevated amount of cytosolic Ca²⁺ in the cortex cells of seedling roots while the total-membrane ones did not.

The fact that CKs and ETH can crosstalk is crucial for the initiation of transducing CD execution signals. ACC-synthases and -oxidases elevate ETH which causes cortex cell degradation and aerenchyma formation in Ca²⁺-dependent pathway.

Material And Methods

Plant material, chemicals, and experiments. *V. faba* ssp. *minor* cv. Bobas (Danko, Sobiejuchy 2 88-400, Żnin Sobiejuchy 2, Poland; www.danko.pl).

88-400 Żnin seeds (20) were germinated for 3 days in Petri dishes (15 cm in diameter and 3 cm high) on two blotting papers with distilled water (the seeds were half submerged) in a dark breeding room. For analyses 6 of the 3-d-old seedlings with nearly equal root length (2.0 ± 0.3 cm) were transferred into a glass container (8 cm in diameter and 4 cm high) with two blotting papers moistened with 10 cm³ of water (Ctrl) or adequate solutions of chemicals and cultivated at 23 ± 1 °C and at $92\% \pm 2\%$ of relative humidity exactly for 72 h and then used for analyses. The types and sources of factors originally used in the studies are presented in Table 1.

First, the impact of CK perception and metabolism regulators as well as Ca²⁺channel inactivators at two selected concentrations (Table 1) without Kin on CD and cytosolic Ca²⁺ amounts in faba bean seedling root cortex was tested. Then the concentration of the factor which did not induce, or slightly induced cell

death compared to the other one was used to analyse its respective influence on vitality and amount of cytosolic Ca^{2+} in cortex cells during Kin-induced CD.

Quantification of cell viability. Analyses of viability of cells were made in cortex of untreated plants (Ctrl) and those treated with PI-55 (5 μM and 10 μM , series PI-55-5 and PI-55-10), Ad (50 μM and 100 μM , series Ad-50 and Ad-100), Ado (10 μM and 50 μM , series Ado-10 and Ado-50), CPPU (5 μM and 10 μM , series CPPU-5 and CPPU-10), EGTA (10 μM and 50 μM , series EG-10 and EG-50), CsA and La^{2+} (5 μM and 25 μM , series CsA-5 and CsA-25 and 25 La -5 and La -25), Kin (46 μM 0.1 M HCl solution) with 5 μM PI-55 (series Kin/Pi-55-5), Kin with 100 μM Ad (series Kin/Ad-100), Kin with 50 μM Ado (series Kin/Ado-50) as well as Kin with 5 μM CPPU (series Kin/CPPU-5), Kin with 10 μM EGTA (series Kin/EG-10), Kin with 25 μM CsA (series Kin/CsA-25) and Kin with 5 μM La^{2+} (series Kin/La-5).

Viability (Figure 1S). of cells was also analysed after Sorafenib treatment, a Raf-like kinase inhibitor, at 1.0 μM concentration with treated and untreated by Kin *V. faba* ssp. *minor* seedlings (Figure 2S).

Additionally, the effects of solvents, used for PI-55 and CsA, Kin as well as for CPPU preparation, i.e., the 0.05% DMSO (Avantor), 0.01 N HCl (Avantor) and 0.1% methanol and mixture of HCl and DMSO as well as HCl with methanol (Avantor) in distilled water, respectively, on viability of cortex cells were tested and the average values were used to complete the figures. Moreover, effects of the mentioned solvents on fluorescence intensity of CTC/cytosolic Ca^{2+} complexes used to determine the relative amounts of Ca^{2+} were assessed and the results of studies were referred to the respective Ctrl values.

To detect and measure the intensity of CD (percentage number of dying cells) in root cortex, apical fragments of roots were cut off from the seedlings, washed twice with 0.1 M PHB (Na phosphate buffer pH 7.4; Avantor), stained with the mixture of 100 $\mu\text{g cm}^{-3}$ acridine orange (AO; Sigma-Aldrich) and 100 $\mu\text{g cm}^{-3}$ ethidium bromide (EB; Sigma-Aldrich) in PHB, washed in PHB two times and fixed with 2% solution of glutaraldehyde in PHB. Then, the handmade thin sections of long axes of 2-cm apical part of roots were analysed and photographed under the blue light of fluorescence filter (B2A) of Optiphot-2 (Nikon, Japan), epi-fluorescence microscope equipped with DXM1200 digital camera and Act-1 (Precoptic, Poland) software.

Estimation of the numbers of living, dying and dead cortex cells was carried out according to the method described in Doniak et al. (2016)³, Byczkowska et al. (2013)⁶ and Kunikowska et al. (2013)⁸ using the specially prepared calibration curve which presents relations between fluorescence intensity (FI) of dyes and amount of nuclear chromatin. Measurements of FI were carried out using the Scion Image (Scion Corporation) software. During measurements, each stained nucleus was separately outlined using the threshold function, then the values of fluorescence intensity in arbitrary units were read and compared to the calibration curve Byczkowska et al. (2013)⁶.

This method uses the properties of EB-migration through damaged plasma and nuclear membrane, which amount in nuclei increases proportionally with the CD-induced permeabilisation. OA-migration

through all types of membrane do not depend on their conditions. Thus, the changing colour of nuclear chromatin ranging from green to orange-red is related to increasing fluorescence intensity (FI; Figure 3S; A)⁵.

Bright-orange (Figure 3S; A,A1) and orange-red (Figure 2S; A,A2) colours indicate dead cells (FI values > 46 a.u.), yellow (Figure 3S; A,A3) and yellow-orange (Figure 3S; A,A4) indicate dying ones (FI values 34–55 a.u.), while green-yellow (Figure 2S; A,A2) green and indicates living cells (RFI values 10–35 a.u.) whereas (Figure 3S; A,A1),

FI values of viability were reported as indices. Data represent the mean $\pm$ SE of two replicates of three independent experiments (n = 3) from each of about 450–550 cells.

Estimation of cytosolic calcium ions content. Analyses of cytosolic Ca^{2+} amounts were made in cortex of untreated plants (Ctrl) and those treated with PI-55 (5 μM and 10 μM , series PI-55-5 and PI-55-10), Ad (50 μM and 100 μM , series Ad-50 and Ad-100), Ado (10 μM and 50 μM , series Ado-10 and Ado-50), CPPU (5 μM and 10 μM , series CPPU-5 and CPPU-10), EGTA (10 μM and 50 μM , series EG-10 and EG-50), CsA and La^{2+} (5 μM and 25 μM , series CsA-5 and CsA-25 and 25 La -5 and La -25), Kin (46 μM 0.1 M HCl solution) with 5 μM PI-55 (series Kin/Pi-55-5), Kin with 100 μM Ad (series Kin/Ad-100), Kin with 50 μM Ado (series Kin/Ado-50) as well as Kin with 5 μM CPPU (series Kin/CPPU-5), Kin with 10 μM EGTA (series Kin/EG-10), Kin with 25 μM CsA (series Kin/CsA-25) and Kin with 5 μM La^{2+} (series Kin/La-5).

To measure cytosolic Ca^{2+} amount in the cortex cells, 2-cm long apical parts of faba bean roots (between the 4th and the 20th mm from apex) were fixed with 2 % solution of glutaraldehyde (POCH) in PHB for 1 h and stained with 100 μM chlortetracycline (CTC; Merck-Sigma) and longitudinal handmade (about 300–400 μm thick) sections were prepared. Then analyses were carried out under B2A filter of an epi-fluorescence microscope (Figure 3S; B,B1–B6), photos were taken and total green fluorescence intensity (TFI) of Ca^{2+} -CTC complexes was cytophotometrically measured using the Scn Image software³.

During measurements, each stained cell was separately outlined using the threshold option, then the values of fluorescence intensity in a.u. were read and used for calculation of the Ca^{2+} amounts. The decreasing amount of Ca^{2+} was related to the values of green TFI of Ca^{2+} -CTC complexes (Figure 3S; B,B1–B6). Data represent the mean $\pm$ SE of two replicates of three independent experiments (n = 3) from about 500–600 cells.

Estimation of ETH amount, Raf-like kinase and MEK2 activities and protein amount. ETH measurements were carried in Erlenmeyer flasks before sample preparations. Erlenmeyer flasks were sealed with aluminum foil (to keep seedlings in the dark) and tightly closed with cap with the clogged pipette tips. After 30 min of incubation, handheld ETH analyzer (SCS56, Storage Control System, United Kingdom), equipped with a pump. was connected to pipette tips *via* flexible tube directly before measurement (Figure 2S). Then the pump was turn on and measurements were conducted for 30 sec. Results between

20th to 30th sec, when the values reached the plateau, the five readings from monitor were written in a spreadsheet of MS.Excel and taken to calculate the ETH amount in ppm per six seedlings.

To estimate Raf-like and MEK2 kinase activities, the one third of the length of apical parts of roots were homogenised and reextracted in 0.04 M Tris-HCl pH 7.5 buffer (Sigma-Aldrich) containing 20 mM MgCl₂, 10 µg ml⁻¹ BSA (bovine serum albumin; Sigma-Aldrich) and 1 mM PMSF (phenylmethylsulphonyl fluoride; Sigma-Aldrich) in 1.5 ml Eppendorf-like tubes with the plastic mortar and centrifuged at 5 000 g for 10 min⁸.

The reaction mixture for kinase activity analyses was prepared by sequentially adding to 2-ml tubes: the extract (20 µl), extraction buffer (1035 µl), of ATP (5 µl), of Syntide or Mek2 (25 µl; 1 µg protein per 1 ml of buffer) as substrates and of Kinase-Glo Reagent (50 µl; Promega), containing Ultra-Glo™ Luciferase and luciferin. After mixing, the luminescent signals were measured in semi-micro fluorometer cell with Teflon Stopper by Fluorescence/Luminescence Spectrophotometer F – 2500 (Hitachi) at 458 nm, every for 2.5 min with 30 s intervals. The kinase activities were calculated as the difference between the luminescence of the samples without substrates and the luminescence of the amount of ATP not utilized for kinase substrates phosphorylation. The kinase activity was expressed in relative light units (RLU), indicating RLU amount of ATP utilized by kinases in 1 mg of protein,

To estimate the protein amounts, the apical parts of roots were homogenised with 100 mM Tris-HCl (pH 7.4) buffer in Eppendorf-like tubes using a plastic pestle (4–8 °C), then centrifuged at 5000 × g for 10 min (4 °C) and residues were re-extracted. Combined supernatants were used to measure protein amounts in the reaction mixture containing, in 2-ml Eppendorf-like tubes, extract (10–100 µl), extraction buffer (90–0 µl) and Coomassie Brilliant Blue G-250 reagent⁵ (1.4 ml). Absorbance was measured at 595 nm after 10 min of incubation (Amersham Biosciences Ultrospec 1100 Pro UV-VIS spectrophotometer with semi-micro cell).

To calculate protein amount, standard, i.e., BSA dissolved in PHB, was prepared in a range of 5 to 100 micrograms of protein in 100 µl volume and measurements were carried out according to above description. The prepared calibration curve was used to read the amount of protein in the sample and final calculations.

Reagent to protein determination was prepared by oneself with Coomassie Brilliant Blue G-250 (100 mg) diluted in 95 % ethanol (50 ml) and 85% H₃PO₄ (100 ml) and 950 ml of distilled water and saved in dark bottle.

Statistics and software. Three biological replicates, at least in tri-, duplicate and more random samples were analysed. The samples were prepared at least from six plants. The results of measurements were statistically verified by Mann–Whitney U test and/or Student's t-test using the MS.Excel of the Microsoft 365® Software (licensed) by independent step by step analyses of each column of results. It allowed to

show the significant differences between results at $P \leq 0.05$. Calculations, all charts and table have been prepared using MS.Excel of the Microsoft 365® Software.

To estimate the vitality of cells, by counting the number of alive, dying, and dead cells, the Optiphot-2 epi-fluorescence microscope (Nikon) with a blue filter (B2A) equipped with a digital camera (DXM1200) and objectives (10x, 20x, 40x) Act-1 software (Precoptic, Poland; <http://www.precoptic.pl>) and Scion Image (Scion Corporation) software (open source; <http://www.scioncorp.com>) were used.

The CorelDraw Graphics Site X7 EduLic (<https://www.coreldraw.com/pl/product/coreldraw>) or Inksape (open source; <https://inkscape.org/release/inkscape-1.0.1>) were used to prepare figures and images planes in tiff extensions.

The BioRender (<https://biorender.com>) software was used to prepare the Figure 7.

Declarations

Authors confirm that all experiments were performed in accordance with relevant guidelines and regulations in accordance with operation instruction as well as protection against hazard and all methods were performed in accordance with the relevant guidelines and regulations.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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Authors contributions

A.Ka. conceived the idea of the paper, participated in its coordination, took part in performance of experiments, compiled the data, wrote the manuscript, prepared almost all figures and statistical analysis. **A.Ku.** and **M.D.** performed experiments. **A.Ko.** improved deeply final version of the manuscript. **A.Ka., M.D.** and **A.Ko.** approved final version of the manuscript.

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Competing interests

No competing interests have been declared.

Additional information

Supplementary Information The online version contains supplementary material available at:

Abbreviations

ACC – 1-aminocyclopropane-1-carboxylic acid; ACS – Ca^{2+} -dependent ACC synthase; Ad – adenine; Ado – 5'-amine-5'-deoxyadenosine; AO – acridine orange; BA – benzyl adenine; CDKs – cyclin dependent kinases; CHK – cytokinin receptor; CK/CKs – cytokinin/cytokinins; CNGC – cyclic nucleotide gated channels; CPPU – N-(2-chloro-4-piridylo)-N'-phenylurea; CsA – cyclosporine; CTC – chlortetracycline; CTR1 – serine/threonine-protein kinase, Raf-like kinase; DMSO – dimethylsulfoxide; dPCD – developmental PCD; EB – ethidium bromide; EGTA – ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid; EIN – ethylene receptor (ethylene insensitive); ENT – nucleoside transporters; ER – endoplasmic reticulum; ERE – ethylene response element; ERK – extracellular signal-regulated kinase; ERS – ethylene receptor, i.e., ethylene response sensor; ETH – ethylene; ETR – ethylene receptor, i.e., ethylene triple response; HK – histidine kinase; HPT – His-containing phosphotransmitter; Kin – kinetin; KR – kinetin riboside; KRMP – kinetin riboside monophosphate; LaCl_3 – lanthanum chloride; MEK2 – mitogen-activated protein kinase 2; MG115 – Z-Leu-Leu-Nva-H; NBD – 2,5-norbornadiene; NEM – N-ethylmaleimide; PCD – programmed cell death; PHB – Na phosphate buffer; PI-55 – 6-(2-hydroxy-3-methylbenzylamino)purine; PMSF – phenylmethylsulfonyl fluoride; PUP – purine permease; RCD – Regulated Cell Death; RFI – resultant fluorescence intensity; ROS – reactive oxygen species; RAF – mitogen-activated protein factor; RR – cytokinin gene response regulator; SOD – superoxide dismutase; TFI – total (integrated) fluorescence intensity

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Table

Factor/concentration	Destination	Origin
6-(2-hydroxy-3-methylbenzylamino purine (PI-55) – 5 and 10 μ M	Inhibitor of cytokinin receptors	Laboratory of Growth Regulators, Palacký University
Kinetin (Kin) – 46 μ M	inducer of cell death in root cortex cells	Sigma-Aldrich Company
Adenine (Ad) – 50 and 100 μ M	inhibitor of adenine phosphoribosyl transferase (APRT)	Sigma-Aldrich Company
5'-amino-5'-deoxyadenosine (Ado) – 10 and 50 μ M	inhibitor adenosine kinases (ADK)	Sigma-Aldrich Company
N-(2-chloro-4-piridylo)-N'-phenylurea (CPPU) – 5 and 10 μ M	inhibitors CK oxidases	Sigma-Aldrich Company
Cyclosporine (CsA) – 5 and 25 μ M	blocker of mitochondria membrane calcium channels	Sigma-Aldrich Company
Ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetra-acetic acid (EGTA) – 10 and 50 μ M	blocker of all membrane calcium channels	Sigma-Aldrich Company
Lanthanum chloride (LaCl_3) – 5 and 25 μ M	blocker of endoplasmic reticulum membrane calcium channels	Sigma-Aldrich Company
Kinetin (Kin) – 46 μ M	inducer of cell death in root cortex cells	Sigma-Aldrich Company
Sorafenib 1 μ M	RAF kinase inhibitor	Selleckchem
Mek2 1 μ g per 1 ml	MEK2 kinase substrate	Selleckchem
Syntide 1 μ g per 1 ml	RAF kinase substrate	Selleckchem

Table 1. The types and concentrations as well as experimental destination and origin of the modulator of cytokinin reception and metabolism as well as blocker of calcium ions channels and inhibitor Raf-like kinase activates and RAF and MEK2 substrate applied in the studies.

Figures

Figure 1

Effect PI-55 on death (A) and on cytosolic calcium ion (Ca^{2+}) amounts (B) of cortex these of *Vicia faba* ssp. minor seedling roots. Bars indicate $\pm$ SE of the results from three biological replicates. Means of the results with the same letter under the column are not significantly different at $P \leq 0.05$.

Figure 2

Effect of Ad (A,B) and Ado (C,D) on death (A,C) and on cytosolic calcium ion (Ca^{2+}) amounts (B,D) of cortex cells of *Vicia faba* ssp. minor seedling roots. Bars indicate $\pm$ SE of the results from three biological replicates. Means of the results with the same letter under the column are not significantly different at $P \leq 0.05$.

Figure 3

Effect of CPPU on death (A) and on cytosolic calcium ion (Ca^{2+}) amounts (B) of cortex cells of *Vicia faba* ssp. minor seedling roots. Bars indicate $\pm$ SE of the results from three biological replicates. Means of the results with the same letter under the column are not significantly different at $P \leq 0.05$.

Figure 4

Effect of EGTA (EG; A,B) and LA (C,D) on death (A,C) and on cytosolic calcium ions (Ca^{2+}) amounts (B,D) of cortex cells of *Vicia faba* ssp. minor seedling roots. Bars indicate $\pm$ SE of the results from three biological replicates. Means of the results with the same letter under the column are not significantly different at $P \leq 0.05$.

Figure 5

Effect of CsA on death (A) and on cytosolic calcium ion (Ca^{2+}) amounts (B) of cortex cells of *Vicia faba* ssp. minor seedling roots. Bars indicate $\pm$ SE of the results from three biological replicates. Means of the results with the same letter under the column are not significantly different at $P \leq 0.05$.

Figure 6

The number (%) of living and dead cells (A), ethylene amount after treatment with sorafenib (B), whole root length (C) as well as CTR1/Raf-like and MEK2 activity with Syntide and Mek2 substrate, respectively (D) in apical parts of roots of 72-h Kin-treated of *Vicia faba* ssp. minor seedlings. Bars indicate $\pm$ SE of the results from three biological replicates. Means of the results with the same letter under the column are not significantly different at $P \leq 0.05$.

Figure 7

Scheme of mechanism of Kin-ECD signaling pathway based on the suggestion presented by Doniak et al. (2016)³ and Kunikowska et al. (2013)¹⁵ and the results described in the present paper in root cortex of *Vicia faba* ssp. minor seedlings. Kin transported to the cell through purine transporter (PUP) is converted to riboside and then to ribonucleotide (rybotide). The rybotide activates histidine kinase (HK) receptors which activates the ethylene (ETH)-dependent signaling pathway using histidine-containing

phosphotransmitter (HPT) and Ca^{2+} -dependent channels. These channels are needed to release Ca^{2+} (also from mitochondria), which is important for activation of Ca^{2+} -dependent ACC (1-aminocyclopropane-1-carboxylic acid) synthase (Ca^{2+} -ACS). Then the action of serine and next cysteine proteases and exo-/endonucleases of execution/dying phase led to protoplast and cell wall degradation. It appears as aerenchyma formation. Simultaneously HPT may stimulate a cytokinin response regulator (RR-B) and synthesis of Ca^{2+} -dependent ACS, which may enhance ECD-effect by activation of ETH-dependent genes through the ethylene response elements (ERE). Moreover, by stimulation of enzymes of ROS metabolism with HPT (CKs-dependent genes), the protective role of Kin against Kin-ECD might be activated. 1, cytokinin histidine kinase 4 (HK4) receptor; 2, ETH histidine kinases receptors (ETR/ERS/EIN4); 3, receptor activating constitutive triple response 1 (CTR1), a Raf-like serine/threonine (Ser/Thr) kinase; 4, ethylene insensitive (EIN2) transmembrane protein; 5, plasma membrane CNGCs (cyclic nucleotide gated channels) of calcium ions; 6, mitochondrial membrane CNGCs; 7, ER membrane CNGCs. Ado, 5'-amine-5'-deoxyadenosine; CPPU, N-(2-chloro-4-pyridyl)-N'-phenylurea; CsA, cyclosporine; EG, EGTA – ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid; La^{2+} , LaCl_3 – lanthanum chloride

Supplementary Files

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- [Table1.docx](#)
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