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KINETIN EFFECTS ON THE FORMATION OF FLAVONOIDS IN BUCKWHEAT COTYLEDONS DEPENDING ON THE TIMING AND ILLUMINATION CONDITIONS OF THE TREATMENT

In the last two decades kinetin, a N-furfurylderivative of adenine, has been frequently used as a highly active regulatory agent which is able to stimulate a wide range of physiological and biochemical processes of plants and cultured plant cells. In many cases, among other effects, an increase in the formation of anthocyanins was observed (Mishra, 1966; Ibrahim et al., 1971; Straub, Lichtenthaler, 1973; Servettaz et al., 1975; Nakamura et al., 1980). Evidence is accumulating that, in fact, also formation of other flavonoids can be stimulated by kinetin (Miller, 1972; Samejima et al., 1982; Загоскина, Запрометов, 1983).

In the majority of experiments carried out so far in this field, kinetin served as a permanent component of growth or incubation media. The observed effects thus actually arose in response to a rather long-term action of kinetin making it difficult to understand what the intimate metabolic mechanisms responsible for the stimulation might be. However, in 1974 R. C. Pecket and T. A. H. Bassim demonstrated that in dark-grown red cabbage seedlings a marked increase in the accumulation of anthocyanins can be achieved in result of only a single short (15 min) treatment with kinetin. In a study of this laboratory carried out with buckwheat cotyledons a similar short treatment induced even an up to 9-fold (!) stimulation of anthocyanin synthesis and enhanced formation of rutin and C-glycosylflavones as well (Margna, Vainjärv, 1983; Маргна et al., 1985). A 20-minute treatment with kinetin proved to be highly effective in augmenting flavonoid accumulation also in primary leaves of barley (Laanest, Margna, 1985; Маргна et al., 1985). In both buckwheat and barley an increase in the incorporation of exogenous L-phenylalanine into flavonoids was observed after kinetin treatment (Margna, Vainjärv, 1983; Laanest, Margna, 1985).

The aim of this contribution was to study the action of kinetin on the incorporation of that precursor in some more detail and to clarify general characteristics of kinetin effects on the formation of flavonoids depending on the timing and illumination conditions of the treatment.

Material and methods

The experiments were carried out with isolated buckwheat (*Fagopyrum esculentum* Moench) cotyledons excised from 80 h old etiolated seedlings grown in water. The excised material was either i) immediately subjected to a single 15 min treatment with a saturated solution of kinetin followed by a 16 h incubation in distilled water in the dark or in the light, or ii) was at first incubated in water (for 16 h in the dark or in the light) and only thereafter exposed to a similar treatment with kinetin. In both cases the treated material was then transferred to a 10 mM solution of labelled L-phenylalanine (spec. act. 9.96 GBq/mol) and incubated there for 24 h either in the dark or in the light. For the labelling a radioactive preparation of [1-^{14}C]-D,L-phenylalanine was used.

For illumination white fluorescent tubes were employed, light intensity 28 $W \cdot m^{-2}$, temperature 25 °C. In the experiments involving incubations in the dark the excision of cotyledons and their preincubation treatments with kinetin were performed under dim green safelight.

Depending on the type of the experiments the content of flavonoids in the cotyledons was determined either at the end of the first 16 h or at the end of total 40 h period of incubation. Parallel to that the initial content of flavonoids in the excised material was determined. The measurements were carried out spectrophotometrically after paper chromatographic separation of individual flavonoids (rutin and four C-glycosylflavones orientin, iso-orientin, vitexin, and isovitexin) as described elsewhere (Margna, Margna, 1969; Margna, Vainjärv, 1983). Radioactivity of flavonoids was assayed in the ethanolic eluates using a Beckmann LS-100C liquid-scintillation counter.

Treatment effects were compared on the basis of net synthesis of flavonoids during incubation.

Results

Material initially incubated in the dark

As can be seen from Table 1, a 15 min treatment with kinetin carried out immediately after excision lead to an about 2.5-fold increase in the accumulation of rutin but had no influence on the formation of C-glycosylflavones during the following 16 h incubation of cotyledons in water in the dark. When the material was thereafter transferred to a 10 mM solution of labelled L-phenylalanine for further 24 h incubation in the dark even a 4-fold increase in the content of rutin was observed in the kinetin-treated cotyledons by the end of incubation and a 75% rise was observed in the content of C-glycosylflavones. However, kinetin was also able to markedly enhance flavonoid production when the cotyledons, during their final 24 h incubation in the presence of L-phenylalanine, were exposed

Table 1

Kinetin effects * on the total production of flavonoids in excised buckwheat cotyledons kept during the first 16 h incubation in water in the dark

Regime, flavonoid	Control (not treated with kinetin)	Treated with kinetin	
		before the first 16 h incubation in water	before the final 24 h incubation in the presence of Phe
Only 16 h incubation in water **in the dark**			
rutin	8.4	21.4	—
C-glycosylflavones	102	101	—
+24 h incubation in the presence of Phe: either **in the dark**			
rutin	15.1	59.9	46.0
C-glycosylflavones	126	220	206
or **in the light**			
rutin	76.4	143	159
C-glycosylflavones	233	320	296

* In all Tables the numbers express production of flavonoids in nmols per pair of cotyledons. Phe — [1-^{14}C]-L-phenylalanine; C-glycosylflavones — sum of vitexin, isovitexin, orientin, and iso-orientin.

to the light. Though the relative effects of kinetin, due to high normal level of flavonoids in the illuminated material, remained under these treatment conditions comparatively moderate, the absolute increase in the content of both rutin and C-glycosylflavones was here, in fact, of the same magnitude as (or even greater than) was the increase induced by kinetin in the dark series.

Table 2

Kinetin effects * on the production of flavonoids from exogenous [1-^{14}C]-L-phenylalanine (Phe) in excised buckwheat cotyledons kept during the first 16 h incubation in water in the dark

Regime, flavonoid	Control (not treated with kinetin)	Treated with kinetin	
		before the first 16 h incubation in water	before the final 24 h incubation in the presence of Phe
16 h incubation in water **in the dark** followed by a 24 h incubation in the presence of Phe:			
either **in the dark**			
rutin	9.5	25.3	25.6
C-glycosylflavones	21.6	36.6	48.1
sum	31.1	61.9	73.7
or **in the light**			
rutin	38.2	60.5	73.5
C-glycosylflavones	51.0	51.9	64.1
sum	89.2	112	138

* See footnote in Table 1.

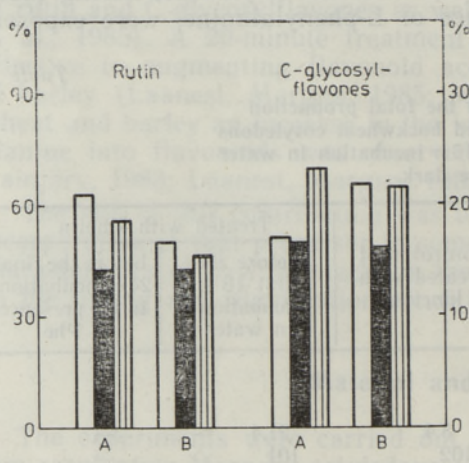

Fig. 1. Relative amount (% of the total) of flavonoids synthesized from exogenous [1-^{14}C]-L-phenylalanine (Phe) in the kinetin-treated buckwheat cotyledons incubated for 16 h in water **in the dark** and thereafter for 24 h in the presence of Phe either in the dark (A) or in the light (B). White bars — controls, black-bars — treated with kinetin before the first 16 h incubation in water, shaded bars — treated with kinetin before the final incubation in the persence of Phe.

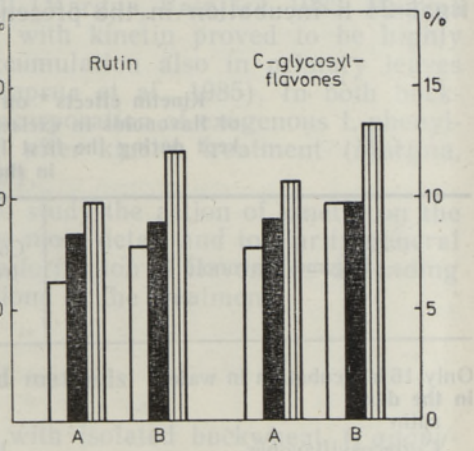

Fig. 2. Relative amount (% of the total) of flavonoids synthesized from exogenous [1-^{14}C]-L-phenylalanine (Phe) in the kinetin-treated buckwheat cotyledons incubated for 16 h in water **in the light** and thereafter for 24 h in the presence of Phe either in the dark (A) or in the light (B). Bar marking as in Fig. 1.

Parallel to stimulation of the total synthesis of flavonoids kinetin also induced a rise in the incorporation of exogenous L-phenylalanine into these compounds (Table 2). However, that increase was always relatively smaller than the increase in the total production of flavonoids. As a result, decreased relative levels of labelled flavonoids (i. e. of the fraction of flavonoids synthesized from exogenous [1-^{14}C]-L-phenylalanine) were characteristic of the treated cotyledons (Fig. 1). It indicated that under these particular conditions kinetin preferentially favoured biosynthesis of flavonoids from precursor molecules of endogenous origin.

Kinetin also remained highly effective in stimulating flavonoid accumulation when the cotyledons, after a preceding 16 h incubation in water in the dark, were subjected to the treatment before their final 24 h incubation in the presence of L-phenylalanine. Although the cotyledons now experienced favouring action of kinetin at a later stage of incubation and the favourable conditions created could have an influence on the formation of flavonoids during a much shorter time period, the final content of both rutin and C-glycosylflavones was raised here almost to the same high level as it occurred exposing cotyledons to kinetin by 16 h earlier (Table 1). However, under these conditions of treatment exogenous L-phenylalanine clearly became more accessible for flavonoid biosynthesis and incorporated into these compounds in greater amounts than it occurred in the former case (Table 2, Fig. 1).

Material initially incubated in the light

When the excised cotyledons, instead of being firstly incubated in the dark, underwent an initial 16 h period of incubation in water under continuous illumination, a 15-min preincubation treatment with kinetin again led to a marked increase in the accumulation of only rutin but not C-glycosylflavones by the end of that first incubation period while being clearly stimulatory for the formation of both of them by the end of the following 24 h period of incubation of cotyledons in the presence of L-phenylalanine (Table 3). However, in contrast to the former case, illumination conditions during the final 24 h incubation made now practically no difference for the final levels of flavonoids (cf. Tables 1 and 3).

Interestingly enough, a preincubation treatment of cotyledons with kinetin now preferentially favoured synthesis of flavonoids from the exogenous L-phenylalanine: at the background of a general stimulation caused by kinetin (Table 4) the levels of the labelled fraction of flavonoids showed somewhat higher increases than was the rise observed in the total production of flavonoids (Fig. 2).

The most surprising finding, however, was that kinetin, when used for a treatment after a preceding 16 h exposure of cotyledons to the light (i. e. before the final 24 h period of incubation in the presence of L-phenylalanine) remained practically ineffective in promoting flavonoid production (Table 3). It clearly indicated that the conditions which were necessary for flavonoid biosynthesis and were favoured by the light during a preceding incubation of cotyledons under continuous illumination could not be further positively modified by a later kinetin treatment. Kinetin, nevertheless, retained its ability (although somewhat reduced in the case of rutin) to stimulate incorporation of exogenous L-phenylalanine into flavonoids (Table 4). So, the relative amount of labelled flavonoids was even further increased under these treatment conditions (Fig. 2).

It must be emphasized that although the effects of kinetin were essentially similar for both rutin and C-glycosylflavones, in all series of experiments typical quantitative differences still occurred permanently.

Table 3

Kinetin effects * on the total production of flavonoids in excised buckwheat cotyledons kept during the first 16 h incubation in water in the light

Regime, flavonoid	Control (not treated with kinetin)	Treated with kinetin	
		before the first 16 h incubation in water	before the final 24 h incubation in the presence of Phe
Only 16 h incubation in water **in the light**			
rutin	89.7	144	—
C-glycosylflavones	265	260	—
+ 24 h incubation in the presence of Phe:			
either **in the dark**			
rutin	118	207	119
C-glycosylflavones	350	429	394
or **in the light**			
rutin	117	238	148
C-glycosylflavones	362	448	376

* See footnote in Table 1.

Table 4

Kinetin effects * on the production of flavonoids from exogenous [1-^{14}C]-L-phenylalanine (Phe) in excised buckwheat cotyledons kept during the first 16 h incubation in water in the light

Regime, flavonoid	Control (not treated with kinetin)	Treated with kinetin	
		before the first 16 h incubation in water	before the final 24 h incubation in the presence of Phe
16 h incubation in water **in the light** followed by a 24 h incubation in the presence of Phe:			
either **in the dark**			
rutin	14.8	34.9	23.4
C-glycosylflavones	27.1	38.8	42.2
sum	41.9	73.7	65.6
or **in the light**			
rutin	18.3	42.2	35.9
C-glycosylflavones	35.2	43.6	49.9
sum	53.5	85.8	85.8

* See footnote in Table 1.

Responses at the level of rutin were always relatively much more pronounced and often greater even in absolute terms than were the comparable responses at the level of C-glycosylflavones. The relative size of changes induced by kinetin in the accumulation of individual C-glycosylflavones (orientin, iso-orientin, vitexin, and isovitexin) remained, however, quite alike and no firm tendencies allowing to differentiate between them by their responsiveness could be observed. It justified the presentation of data over these flavonoids in a summarized form.

Discussion

The results obtained provide further convincing evidence that kinetin is an extremely effective promoter of flavonoid biosynthesis in buckwheat cotyledons. A single short (15 min) treatment with kinetin was powerful enough to induce a drastic (up to 4-fold) increase in the accumulation of flavonoids in the dark and a considerable, though somewhat less relatively, increase in the production of these compounds in the light. In both cases kinetin also markedly increased accessibility of exogenous L-phenylalanine for flavonoid biosynthesis. High stimulatory potential of kinetin was best illustrated by the fact that in the dark increased levels of flavonoids remained only slightly lower than were their normal levels in the untreated cotyledons continuously exposed to the light. Moreover, depending on the conditions of the posttreatment incubation, kinetin could even induce, in certain particular cases, higher final levels of flavonoids in the treated material kept in the dark than were the levels of flavonoids in the untreated cotyledons exposed to continuous illumination. Thus, in promoting flavonoid biosynthesis, kinetin as a stimulatory agent (i) almost equals the light with respect to its general efficiency as well as to the magnitude of its final effects, and (ii) may, at least partly, substitute for the light. When applied together, kinetin and light, in general, act synergistically.

Close similarity of kinetin and light effects indicates that both factors, independent of what might be the intimate mechanisms of the initial stages on their action, ultimately lead to a similar critical change in the intracellular conditions favourable for flavonoid biosynthesis. This point was clearly supported by an almost complete loss of the ability of kinetin to stimulate flavonoid accumulation when the treatment was carried out after a preceding 16 h exposure of cotyledons to the light (Table 3). Thus, the metabolic or structural rearrangements in the cells responsible for a favoured biosynthesis of flavonoids were obviously developed to the full extent in the light and could not be further modified by an additional treatment with kinetin at a later stage of incubation.

As can be deduced from a much increased incorporation of exogenous L-phenylalanine into flavonoids in the treated cotyledons, increased levels of flavonoids caused by kinetin resulted, in fact, from an improved access of precursor (L-phenylalanine) molecules to the sites of their use for building flavonoids. Accessibility of exogenous as well as of endogenous L-phenylalanine was generally improved under all treatment conditions and here again kinetin action resembled the action of the light. However, as the relative amount of flavonoids synthesized in the treated cotyledons from exogenous [1-^{14}C]-L-phenylalanine markedly varied depending on the conditions of illumination, kinetin somewhat differed from the light by its ability to control distribution and channelling of endogenous L-phenylalanine. This difference was clearly evidenced by the following facts: (i) at the background of a general increase in the availability of both endogenous and exogenous precursors for building flavonoids, a treatment with kinetin followed by an immediate exposure of cotyledons to the light preferentially stimulated synthesis of flavonoids from L-phenylalanine supplied exogenously while in cotyledons kept after the treatment in the dark, kinetin preferentially favoured production of these compounds from the endogenous pools of that precursor; (ii) kinetin, when applied after a preceding 16 h incubation of cotyledons in the light, could not further increase the production of flavonoids from endogenous precursors although it fully retained its ability to increase their synthesis from L-phenylalanine of exogenous origin. These differences unequivocally indicate that L-phenylalanine supplied exogenously does not equilibrate with the endogenous

pool(s) of that amino acid. Both pools obviously remain more or less strictly compartmentalized within the cells and are apt, for that reason, to a different control by metabolic and environmental factors.

The results of the present study do not allow any direct conclusion about the immediate mechanisms of kinetin action underlying the improvement of conditions for intracellular transport. However, all observed effects fully agree with the idea that the mechanisms involve an increase in the permeability of cell membranes as a decisive factor facilitating movement of precursor molecules to the site of flavonoid biosynthesis (Pecket, Bassim, 1974; Bassim, Pecket, 1975; Nakamura et al., 1980; Laanest, Margna, 1985; Margna et al., 1985). In several recent works direct evidence for a high capacity of kinetin in increasing membrane permeability has been obtained (Stillwell, Hester, 1983; Szweykowska, Schneider, 1986).

REFERENCES

Bassim, T. A. H., Pecket, R. C. The effect of membrane stabilizers on phytochrome-controlled anthocyanin biosynthesis in *Brassica oleracea* // Phytochemistry, 1975, **14**, 731—733.

Ibrahim, R. K., Thakur, M. L., Permanand, B. Formation of anthocyanins in callus tissue cultures // Lloydia, 1971, **34**, 175—182.

Laanest, L., Margna, U. Kinetin as an efficient stimulator of the formation of C-glycosylflavones in barley (*Hordeum vulgare* L.) // Plant Science, 1985, **41**, 19—22.

Margna, U., Margna, E. A suitable chromatographic method for quantitative assay of rutin and flavone C-glycosides in buckwheat seedlings // ENSV TA Toim. Biol., 1969, **18**, 40—50.

Margna, U., Vainjärv, T. Kinetin-mediated stimulation of accumulation of buckwheat flavonoids in the dark // Z. Naturforsch., 1983, **38c**, 711—718.

Miller, C. O. Modification of the cytokinin promotion of deoxyisoflavone synthesis in soybean tissue // Plant Physiol., 1972, **49**, 310—313.

Mishra, D. Formation of anthocyanin in detached wheat leaves by kinetin treatment // Naturwissenschaften, 1966, **53**, 307—308.

Nakamura, N., Nakamae, H., Maekawa, S. Effects of light and kinetin on anthocyanin accumulation in the petals of *Rosa hybrida* Hort. cv. Ehigasa // Z. Pflanzenphysiol., 1980, **98**, 263—270.

Pecket, R. C., Bassim, T. A. H. The effect of kinetin in relation to photocontrol of anthocyanin biosynthesis in *Brassica oleracea* // Phytochemistry, 1974, **13**, 1395—1399.

Samejima, M., Yamaguchi, T., Fukuzumi, T., Yoshimoto, T. Effects of phytohormones on accumulation of flavanols in callus cells of woody plants // Plant Tissue Culture 1982. (Proc. 5th Int. Congr. Plant Tissue a. Cell Culture, Tokyo a. Lake Yamakava, July 11—16). Tokyo, 1982, 353—354.

Servettaz, O., Castelli, D., Longo, C. P. The effect of benzyladenine on anthocyanin accumulation in excised sunflower cotyledons // Plant Sci. Letters, 1975, **4**, 361—368.

Stillwell, W., Hester, P. Kinetin increases water permeability of phosphatidylcholine lipid bilayers // Plant Physiol., 1983, **71**, 524—530.

Straub, V., Lichtenthaler, H. K. Die Wirkung von Gibberellinsäure A_3 und Kinetin auf die Bildung der Photosynthesepigmente, Lipochinone und Anthocyane in *Raphanus*-Keimlingen // Z. Pflanzenphysiol., 1973, **70**, 308—321.

Szweykowska, A., Schneider, J. Effect of kinetin on the electrolyte efflux from cucumber cotyledons // Folia Physiologica, Cytologica et Genetica, 1986, **1**, 89—95.

Загоскина Н. В., Запрометов М. Н. Влияние кинетина на образование фенольных соединений в длительно пассируемой культуре ткани чайного растения // Физиол. биохим. культ. растений, 1983, **15**, 250—253.

Маргна У. В., Вайнярв Т. Р., Лаанест Л. Э. Кинетин — эффективный стимулятор образования флавоноидных соединений в проростках гречихи и ячменя // Физиол. растений, 1985, **32**, 1127—1136.

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KINETIINI TOIME FLAVONOIDIDE MOODUSTUMISELE TATRA IDULEHTEDES OLENEVALT TÖÖTLEMISE AJAST JA VALGUSTINGIMUSTEST

On näidatud, et kinetiini stimuleeriv toime rutiini ja C-glükosüülflavoonide moodustumisele tatra idulehtedes on võrreldav valguse toimega samale protsessile. Seejuures on mõlemal juhul stimulatsiooni tõenäoliseks põhjuseks rakusisesed muutused, mille tulemusel paraneb flavonoidide lähtesubstraadi — L-fenüülalaniini — kättesaadavus biosünteesikeskmetele. Kinetiin suudab toimefaktorina suurel määral asendada valgust, nende koosmõju on üldjuhul sünergeetiline. Mõjutustingimuste varieerimisel tuli aga ilmsiks, et valgus modifitseerib mõnevõrra kinetiini toimet substraadi kasutamisele flavonoidide biosünteesiks. Kui materjali pärast lühiajalist (15 min.) töötlemist kinetiiniga inkubeerida pimedas, siis substraadi kättesaadavuse üldise suurenemise taustal kinetiin eelistatult soodustab endogeense päritoluga substraadi kasutamist flavonoidide biosünteesiks. Tingimustes, kus töötlemisele järgneb materjali valgustamine, avaldub kinetiini toime endogeensele substraadile nõrgemini ning suhteliselt reljeefsemaks kujuneb flavonoidide biosünteesi stimulatsioon eksogeenselt manustatud L-fenüülalaniini arvel. Kui aga materjali töödelda kinetiiniga pärast eelvalgustamist (16 t.), siis kinetiin endogeense substraadi kasutamisele enam üldse mõju ei avalda, kuigi ta endiselt stimuleerib eksogeense L-fenüülalaniini lülitumist flavonoididesse. Need erinevused näitavad, et indutseeritud muutused, mis rakus tingivad substraadi parema kättesaadavuse flavonoidide biosünteesiks, kujunevad idulehtedes täiel määral välja juba ainuüksi valguse toimel ega ole hilisemal kinetiiniga töötlemisel enam oluliselt suurendatavad. Võib oletada, et substraadi rakusisese transpordi paranemine kinetiini (ja ka valguse) toimel tuleneb rakumembraanide läbilaskvuse suurenemisest.

Удо МАРГНА, Тийу ВАЙНЯРВ

ВЛИЯНИЕ КИНЕТИНА НА ОБРАЗОВАНИЕ ФЛАВОНОИДОВ В СЕМЯДОЛЬНЫХ ЛИСТЬЯХ ГРЕЧИХИ В ЗАВИСИМОСТИ ОТ ПРИУРОЧЕННОСТИ ОБРАБОТКИ И УСЛОВИЙ ОСВЕЩЕНИЯ

Стимулирующее действие кинетина на образование рутина и С-гликозилфлавонов в семядольных листьях гречихи сравнимо с действием света, причем в обоих случаях причиной стимуляции, по всей вероятности, являются внутриклеточные изменения, в результате которых улучшается доступ исходного субстрата — фенилаланина — к центрам биосинтеза этих соединений. Кинетин в качестве стимулирующего агента способен заменять свет, взаимодействие кинетина и света имеет, как правило, синергетический характер. При варьировании условий обработки, однако, выяснилось, что свет модифицирует действие кинетина на использование субстрата для образования флавоноидов. На общем фоне повышенного использования как экзогенного, так и эндогенного фенилаланина обработка кинетином при последующей инкубации материала в темноте преимущественно стимулировала образование флавоноидов из эндогенного субстрата, в то время как в семядолях, экспонированных после обработки на свету, кинетин больше стимулировал продукцию за счет экзогенного фенилаланина. Обработка же материала кинетином после предварительного освещения (16 ч) не вызывала повышенного использования эндогенного фенилаланина, хотя она по-прежнему стимулировала биосинтез флавоноидов из экзогенного фенилаланина. Эти различия показывают, что метаболические или структурные изменения в клетке, обусловливающие повышенную доступность субстратов для указанного биосинтеза, в полной мере формируются в семядолях уже под влиянием света и не могут быть существенно углублены более поздней обработкой освещенного материала кинетином. Предполагается, что в основе улучшения кинетином (а также светом) условий внутриклеточного транспорта субстратов лежит увеличение проницаемости клеточных мембран.