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Impairment of Polar Auxin Transport by Protein Kinase Inhibitors in Etiolated Pea Seedlings

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Treatment of *Pisum sativum* tissue with the protein kinase inhibitor staurosphorine resulted in impairment of ^{3}H-indoleacetic acid transport in etiolated stem segments. The transport inhibition was accompanied by an increase in net uptake of labeled auxin in the tissue. The magnitude of auxin accumulation in tissue treated with the phytotropin N-1-naphthylphthalamic acid (NPA) which specifically blocks the efflux of auxin in the plasma membrane was reduced by the protein kinase inhibitor, suggesting that inhibition of protein phosphorylation could lead to hindrance of the auxin-exporting function of NPA receptors. The flavonoid genistein which is also known to inibit protein kinase likewise reduced NPA-induced auxin accumulation. However, the flavonoid did not bring about auxin accumulation by itself, nor did it inhibit auxin transport. In view of the finding that the flavonoid also competes with NPA for a common binding site, a mechanism for the flavonoid effect on the NPA action will be proposed.

Keywords : staurosporine, genistein, NPA, auxin transport, *Pisum sativum*

Auxin transport can be altered by a variety of endogenous and environmental factors giving rise to physiological manifestations such as tropism, epinasty and breaking of apical dominance (Burg and Kang, 1993). Two distinct carrier-mediated transport of auxin molecules across the plasma membrane, namely uptake of auxin into and its efflux out of the cell, accounts for the cellular basis of the continuity of transport stream (Sussman and Goldsmith, 1981; Hertel, 1983; Hertel *et al.*, 1983;). The latter represents the rate-limiting element of the auxin transport system.

N-1-naphthylphthalamic acid (NPA) which is a potent and specific inhibitor of auxin transport binds with a high affinity to the putative auxin efflux carrier (Katekar, 1985; Katekar *et al.*, 1987). NPA and related compounds were originally coined phytotropins by Katekar and Geissler (1980). Kinetics of phytotropin binding to membrane vesicles in vitro is thoroughly characterized and its kinetic parameters well established (Katekar and Geissler, 1977; Jacobs and Hertel, 1978; Trillmich and Michalke, 1979; Michalke *et al.*, 1992). Decreases in the affinity of the NPA receptor to its ligand by means of treating membrane vesicles with the lipophilic agent diethyl ether were suggested to be causally related to the ether effect to enhance polar auxin transport in tissue segments *in vivo* (Kang, 1987). Decay in the auxin transport capacity in tissue segments following excision (Yoon and Kang, 1992) and by ethylene pretreatment (Kang, 1987; Suttle,1988) was attributable to a gradual decrease in the number of NPA binding sites.

In a previous report (Nam and Kang, 1995), we established in pea seedlings that modulation of the NPA receptor as disclosed by increased affinity of the receptor to the phytotropins NPA and 2-(1-pyrenoyl) benzoic acid (PBA), respectively, was brought about by inhibition of protein phosphatase activity with NaF and/or ATP. The receptor modulation was thought to lead to an enhancement of polar auxin

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transport in the stem segments. We tested in the present work is inhibition of protein kinase activity would give rise to reversed effects, and the results obtained from this work are presented.

MATERIALS AND METHODS

Plant material

Subapical third internodal tissue of etiolated seedlings of *Pisum sativum* L. grown in complete dark for 5 days were used for both *in vivo* auxin transport and *in vitro* NPA binding.

Chemicals

NPA was generously provided by Drs. Wolfgang Michalke and Rainer Hertel, both of University of Freiburg, Germany. Radio-labelled (2,3,5-^{3}H)-NPA (55 Ci/mmol) was purchased from American Radiolabeled Chemicals. Staurosporine and genistein were Sigma products, and all other chemicals were reagent grade coming from various commercial sources.

Isolation of microsomal membranes

All operations were carried out 0 to 4C throughout. Three to four grams of internodal tissue were chopped with a razor blade in a plastic container on ice and ground gently with a motar and pestle with four weight equivalents of extraction medium, which consisted of 25 mM N-2'-hydroxyethyl-piperazine-2-ethanolsulfonic acid (HEPES), 3 mM ethylenediaminetetraacetic acid (EDTA), 250 mM sorbitol, 25 mM 1,3-bis-tris(hydroxymethyl)-methylaminopropane (BisTrisPropane, or BTP), and 1 mM KCl at pH 7.8. Immediately before use, 0.1% bovine serum albumin (BSA), 1 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonylfluoride (PMSF) were added to the extraction medium. The crude homogenates were squeezed through a nylon cloth to remove cell wall and unbroken cells. The filtrate was centrifuged at a low speed (6000 rpm) for 20 min to remove nuclei and most of the mitochondria. The supernatant was centrifuged at 38,000 rpm for 20 min with an ultracentrifuge. The resulting microsomal pellets were washed twice with the extraction medium before resuspending with test medium (10 mM Na citrate-citric acid, 5 mM $MgCl_2$, 250 mM sorbitol, at pH 5.5) for binding assay.

NPA binding

Microsomal membrane vesicles resuspended in test medium to which 0.98 nM ^{3}H-NPA and varying concentrations of cold ligands (either NPA or genistein) added were equilibrated with or without test substances at 0℃ for 30 min. The binding mixture had a volume of 500 μL. The tubes were centrifuged at 15,000 rpm for 15 min at 4℃. The supernatants were decanted and pooled together, and radioactivity of both the supernatant (free ^{3}H-NPA) and the pellets (bound ^{3}H-NPA) was determined after extraction of radioactivity from the pellets with MeOH for 30 min.

^{3}H-IAA transport

Auxin transport through internodal segments was tested using agar blocks, 3 mm×3 mm×1 mm (1.5% buffered with 50 mM Na phosphate at pH 5.0), containing 18.2 nM ^{3}H-IAA as a donor and plain buffered agar blocks as a receiver. Ten individual segments, 6 mm in length, were vertically placed, the basal end down, between donor and receiver blocks. Test substances were applied in the receiver block attached to the basal end of the segment. At the end of a transport period of 4 h, radioactivity in the receiver blocks as well as that remaining in the tissue were counted. Each experiment was repeated at least three times with consistent results. Data from a representative experiment are presented as cpm values for 5 segments in duplicate.

RESULTS AND DISCUSSION

Effect of staurosporine

In view of our previous findings that auxin transport is promoted by agents inhibiting protein dephosphorylation (Nam and Kang, 1995), it might be predicted to produce a reversed effect, namely inhibition of auxin transport, with protein kinase inhibitors. Staurosporine is a microbial alkaloid which is known to inhibit Ca^{2+}/phospholipid dependent protein kinase (protein kinase C) which catalyzes phos-

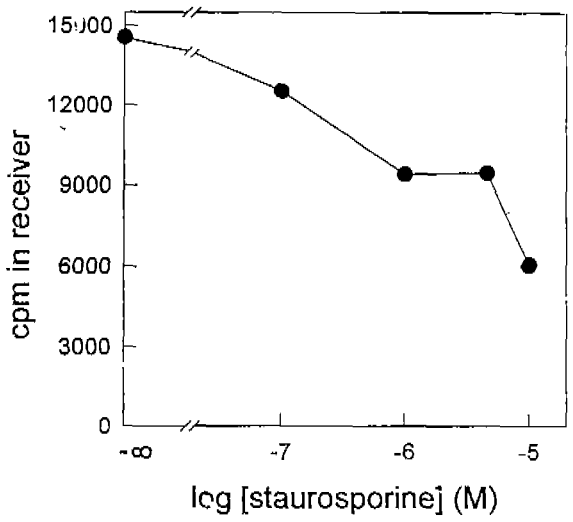

Fig. 1. Concentration dependency of staurosporine on ^{3}H-IAA transport in third internode segments. Radioactivity transported through 6 mm segments for 4 h is expressed as cpm values.

Table 1. Effect of staurosporine (STA) on net uptake of ^{3}H-IAA in epicotyl slices (1.5 mm) in the absence or presence of NPA (1 μM). Eight slices were incubated in 300 μL medium containing labelled auxin with or without test substences for 30 min

STA (μM)	cpm in tissue		NPA-induced uptake (cpm)	Percent of NPA effect
	−NPA	+NPA		
0	4561	7452	2891	63.4
5	5712	7641	1929	33.8
10	5342	7061	1719	32.2

phorylation of proteins on serine/threonine residues (Tamaoki *et al.*, 1986; Tamaoki, 1991). Data illustrated in Fig. 1 indicate that staurosporine applied in receiver agar blocks attached to the basal cut end of stem segments inhibited polar transport of labelled auxin. The inhibitory effect clearly appears dose-dependent.

Slices (1.5 mm thick) of the epicotyl tissue comprise only several cell layers and provide an excellent system for studies on cellular net uptake and efflux of labelled auxin *in vivo*. Treatment of these thin slices with NPA results in an increase in net uptake of labelled auxin from the bathing medium by specifically blocking auxin efflux with auxin entry unaffected (Yoon and Kang, 1992). Results from experiments employing thin slices presented in Table 1 indicate that staurosporine also increased net uptake

Table 2. Effect of STA on ^{3}H-IAA transport in internode segments in the absence and presence of NPA. Data are presented in radioactivity (cpm) transported through 6 mm segments for 4 h and collected in receiver blocks, and that remaining in the tissue

Treatment		cpm	
STA (μM)	NPA (μM)	Receiver	Tissue
0	0	3256	12734
0	10	203	15386
5	0	1785	15311
5	10	367	18776

of labelled auxin suggesting that the auxin efflux is specifically blocked by the protein kinase inhibitor. The data also indicate that in the presence of NPA where auxin efflux is fully inhibited, staurosporine exerted no further effect on the net uptake of auxin. Consequently the magnitude of the NPA effect was significantly reduced by staurosporine treatment.

Polar auxin transport through 6 mm epicotyl segments was likewise strongly inhibited by NPA and partially inhibited by staurosporine (Table 2). Also the presence of NPA nullified the influence of staurosporine on auxin transport. However, radioactivity remaining in the tissue whose cells total 4 times more than those in the 1.5 mm slice was further increased by staurosporine in the presence of NPA as well. The discrepancy cannot be accounted for at present, but it appears apparent from the results of these experiments that inhibition of protein phosphrylation could lead to impairment of auxin transport possibly through modulation of auxin efflux carriers.

Agents known to inhibit protein phosphatases, namely NaF (Pesi and Villa-Moruzzi, 1990) and/or ATP (MacKintosh *et al.*, 1991), promote polar auxin transport in the pea epicotyl, and this has been suggested to be attributable to covalent modification of the auxin efflux carrier through inhibition of protein dephosphorylation (Nam and Kang, 1995). Incubation of membranes with NaF and/or ATP in the presence of Triton X-100 results in increased affinity to both NPA and 2-(1-pyrenpyl)benzoic acid (PBA). However, treatment of membrane vesicles with staurosporine under the same conditions as described by Nam and Kang (1995) produced no appreciable effect on NPA binding in the present work (data

Table 3. Effect of genistein on ^{3}H-IAA transport in internode segments. Data are presented in radioactivity (cpm) transported through 6 mm segments for 4 h and collected in receiver blocks, and that remaining in the tissue.

Genistein (μM)	Receiver	Tissue
0	4359	11712
1	4605	12307
5	3989	11249
10	4048	14141

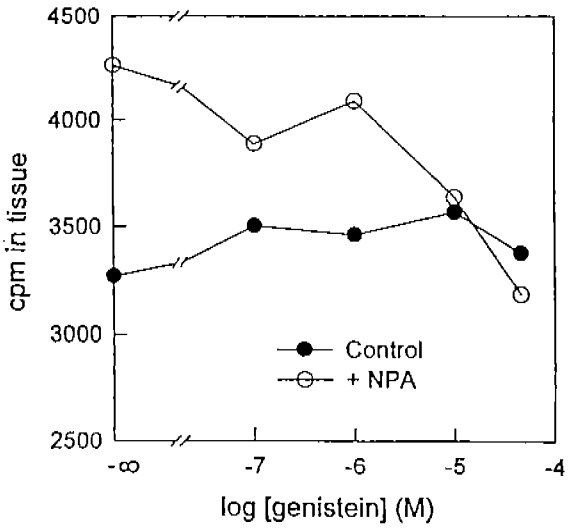

Fig. 2. Effect of genistein on net uptake of ^{3}H-IAA in the absence (closed circles) or presence (open circles) of 5 μM NPA. Epicotyl slices (1.5 mm) were incubated in a medium containing ^{3}H-IAA with various concentrations of genestein in the absence or presence of 5 μM NPA.

not shown) for reasons unknown at present.

Effect of genistein

Genistein, a flavonoid naturally occuring in plants, is known to inhibit tyrosine-specific protein kinase (Akiyama and Ogawara, 1991). We tested the inhibitor for possible action on auxin transport. The data presented in Table 3 indicate that genistein had no appreciable effect on polar auxin transport in the pea system. Experimental results for net cellular uptake of auxin using the 1.5 mm slices, however, revealed an interesting point to be noticed as shown in Fig. 2. The flavonoid by itself had no significant effect on the net uptake of auxin, but it clearly suppressed NPA-induced accumulation of labelled auxin in the tissue. The finding suggests that genistein might somehow interact with NPA for binding to the receptor.

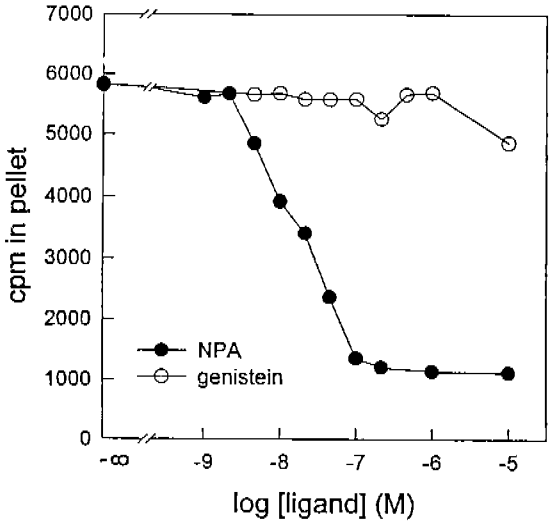

Fig. 3. Displacement of bound NPA by cold ligands (NPA and genistein). To the binding mixture containing 0.98 nM ^{3}H-NPA, various concentrations of cold ligands were added, and after a 30 min equilibrium period, the tubes were centrifuged at 15,000 rpm for 15 min and data were presented as radioactivity bound in the pellets.

A group of naturally occuring flavonoids including genestein can compete with NPA for binding to its receptor, and these compounds were suggested to act as endogenous ligands for the NPA receptor and to play a role as natural auxin transport regulators (Jacobs and Rubery, 1988; Rubery and Jacobs, 1990). Fig. 3 illustrates saturation kinetics of NPA binding with a K_D of 2×10^{-8} M for NPA. Genistein at a high concentration (10 μM) slightly but consistently displaced labelled NPA bound in the pellet. Genistein at this concentration increased the K_D value for NPA (i.e. decreased the affinity) as shown on a Scatchard plot in Fig. 4. There is a close correlation between biological activity disclosed as percent stimulation of net uptake of auxin and inhibition of NPA binding among the phenolic compounds tested, but genistein is the only compound straying considerably from the line of correlation (see Fig. 3 in Jacobs and Rubery, 1988). The data indicate that for its inhibitory activity on NPA binding, genistein has a negligible activity to stimulate net uptake of auxin. This could be interpreted as binding of genistein to the NPA receptor with a low affinity without having biological consequences on auxin transport. Our results are in excellent agreement with

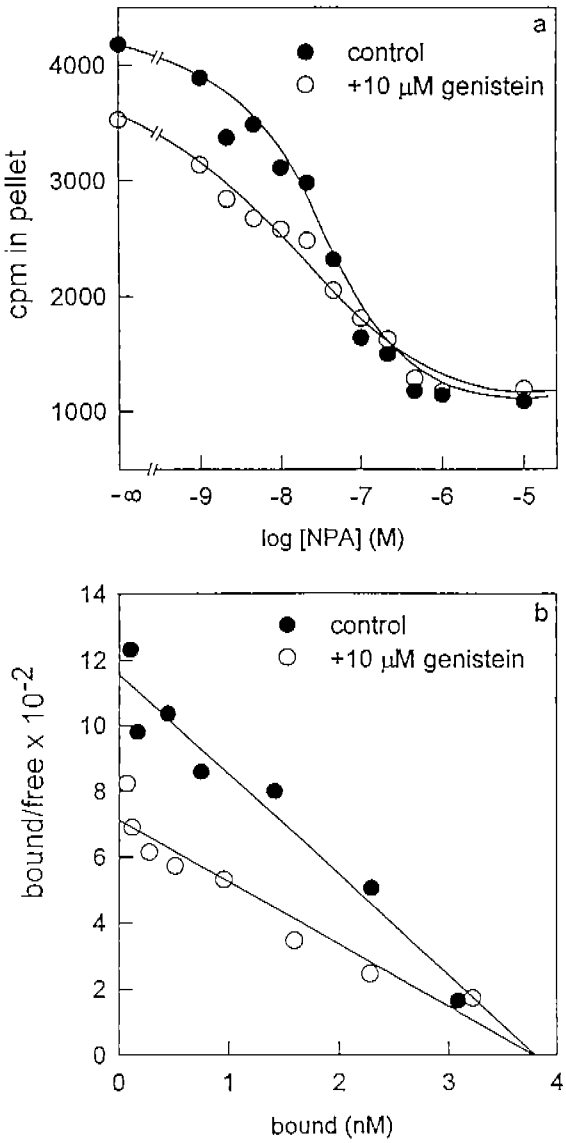

Fig. 4. (a) Displacement of bound NPA by cold NPA at various contrations in the absence or presence of 10 μM genistein. (b) Scatchard plots of data presented in (a).

this notion.

The NPA receptor has been implicated as being linked to auxin action on cell elongation (Hertel, 1983), and this was supported by experimental data (Kang *et al.*, 1992). The receptor was recently identified as a 23-kDa protein from maize coleoptile plasma membrane (Zettle *et al.*, 1992).

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LITERATURE CITED

Akiyama, T. and H. Ogawara. 1991. Use and specificity of genistein as inhibitor of protein-tyrosinekinase. *Methods in Enzymol.* **201**: 362-370.

Burg, S.P. and B.G. Kang. 1993. Gravity dependent ethylene action. *In* Cellular and Molecular Aspects of the Plant Hormone Ethylene. J.C. Peck, A. Latche, and C. Balague (eds.). Kluwer Academic Pub., Dordrecht, pp. 335-340.

Hertel, R. 1983. The mechanism of auxin transport as a model for auxin action. *Z. Pflanzenphysiol.* **112**: 53-67.

Hertel, R., T.L. Lomax and W.R. Briggs. 1983. Auxin transport in membrane vesicles from *Cucurbita pepo* L. *Planta* **157**: 193-201.

Jacobs, M. and R. Hertel. 1978. Auxin binding to subcellular fractions from *Cucurbita* hypocotyls: *In vitro* evidence for an auxin transport carrier. *Planta* **142**: 1-10.

Jacobs, M. and P.H. Rubery. 1988. Naturally occuring auxin transport regulators. *Science* **241**: 346-349.

Kang, B.G. 1987. Modification of auxin efflux carrier in the auxin transport system by diethyl ether and ethylene. *In* Plant Hormone Receptors. Vol. 10. D. Klambt (ed.). Springer Verlag, Berlin Heidelberg, pp. 113-123.

Kang, B.G., W.J. Park, M.H. Nam and R. Hertel. 1992. Ethylene-induced increase of sensitivity to auxin in *Ranunculus* petioles and its implications regarding ethylene action on adaptation. *In* Progress in Plant Growth Regulation. C.M. Karssen, L.C. Van Loon and D. Vreugdenhil (eds.). Kluwer Academic Pub., Dordrecht, pp. 248-253.

Katekar, G.F. 1985. Interaction of phytotropins with the NPA receptor. *Biol. Plant.* **27**: 92-99.

Katekar, G.F. and A.E. Geissler. 1977. Auxin transport inhibitors III: Chemical requirements of a class of auxin transport inhibitors. *Plant Physiol.* **60**: 826-829.

Katekar, G.F. and A.E. Geissler. 1980. Auxin transport inhibitors IV: Evidence of a common mode of action for a proposed class of auxin transport inihibitors. *Plant Physiol.* **66**: 1190-1195.

Katekar, G.F., A.E. Geissler, C.H.L. Kennard and G. Smith. 1987. Recognition of phytotropins by the receptor for 1-N-naphthylphthalamic acid. *Phytochem.* **26**: 1257-1267.

MacKintosh, C., J. Coggins and P. Cohen. 1991. Plant protein phosphatases: Subcellular distribution, detection of protein phosphatase 2C and identification of protein phosphatase 2A as the major quinate dehydrogenase phosphatase. *J. Biochem.* **273**: 733-738.

Michalke, W., G.F. Katekar and A.E. Geissler. 1992. Phytotropin-binding sites and auxin transport in *Cucurbita pepo*: Evidence for two recognition sites. *Planta* **187**: 254-260.

Nam, M.H. and B.G. Kang. 1995. Modulation of phytotro-

pin receptors by fluoride and ATP. *J. Biochem. Mol. Biol.* **28**: 552-555.

Pesi, R. and E. Vilia-Moruzzi. 1990. Efect of NaF on type-1 phosphatase aggregation. *Biochem. Biophys. Res. Comm.* **171**: 362-368

Rubery, P.H. and M. Jacobs. 1990. Auxin transport and its regulation by flavonoids. *In* Plant Growth Substances 1988. R.P. Pharis and S.B. Rood (eds.). Springer-Verlag. Berlin. pp. 428-440.

Sussman, M.R. and M.H.M. Goldsmith. 1981. The action of specific inhibitors of auxin transport on uptake of auxin and binding of N-1-naphthylphthalamic acid to a membrane site in maize coleoptiles. *Planta* **152**: 13-18.

Suttle, J.C. 1988. Effect of ethylene treatment on polar IAA transport, net IAA uptake and specific binding of N-1-naphthylphthalamic acid in tissues and microsomes isolated from etiolated pea epicotyls. *Plant Physiol.* **88**: 795-799.

Tamaoki, T. 1991. Use and specificity of staurosporine, UCN-01, and calphostin C as protein kinase inhibitors. *Methods in Enzymol.* **201**: 340-347.

Tamaoki, T., H. Nomoto, I. Takahashi, Y. Kato, M. Morimoto and F. Tomita. 1986. Staurosporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. *Biochem. Biophys. Res. Comm.* **135**: 397-402.

Trillmich, K. and W. Michalke. 1979. Kinetic characterization of N-1-naphthylphthalamic acid binding sites from maize coleoptile homogenates. *Planta* **145**: 119-127.

Yoon, I.S. and B.G. Kang. 1992. Autoregulation of auxin transport in corn coleoptile segments. *J. Plant Physiol.* **140**: 441-446.

Zettle, R., J. Feldwisch, W. Boland, J. Schell and K. Palme. 1992. 5'-azido-(3,6-$^3H_2$)-1-naphthylphthalamic acid, a photoactivatable probe for naphthylphthalamic acid receptor proteins from higher plants: Identification of a 23-kDa protein from maize coleoptile plasma membranes. *Proc. Natl. Acad. Sci. USA* **89**: 480-484.

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황백화된 완두 유식물에서 Protein Kinase 억제물질에 의한 옥신의 극성 이동의 저해

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적 요

황백화된 완두의 제3절간 조직에 protein kinase의 활성을 억제하는 것으로 알려진 staurosporine을 처리하였더니 ^{3}H-IAA의 이동이 억제되었다. 이와 동시에 조직에 축적된 ^{3}H-IAA의 양은 증가하였다. Staurosporine은 조직박편으로의 ^{3}H-IAA의 축적을 증가시켰으며, 원형질막상에 위치한 옥신 유출 운반 복합체의 기능을 억제하는 것으로 알려진 phytotropin의 일종인 1-N-Naphthylphthalamic acid(NPA)에 의하여 유발되는 ^{3}H-IAA의 순유입량의 증가를 감소시켰다. 이러한 결과는 단백질의 인산화/탈인산화가 NPA 수용체와 연관을 지니고 있을 것으로 알려져 있는 옥신 유출 운반 복합체의 기능에 관여하고 있음을 시사한다. 식물체에 널리 존재하는 flavonoid의 일종으로서 동물에서 protein kinase의 기능을 억제하는 것으로 알려진 genistein은 NPA에 의한 ^{3}H-IAA의 순 유입량의 증가를 감소시켰다. 그러나 그 자체만으로는 ^{3}H-IAA의 유입이나 이동에 아무런 영향을 주지 않았다. Genistein이 NPA 수용체와 결합할 수 있다는 사실과 연관하여 NPA 수용체에 미치는 genistein의 작용기작을 분석하였다.

주요어 : staurosporine, genistein, 옥신이동, 완두

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