myelination is superfluous in the mouse. Severe (but not total) CNS hypomyelination in quaking mutants allows long life (7). Rapid conduction along PNS pathways is not necessary in mice, since motor conduction velocity in *Tr/+* mice is less than 10% of normal (*17*). However, the early death of trembler-J homozygotes (*8*) and jimpy hemizygotes (7), which have extreme PNS and severe CNS hypomyelination, respectively, may be seen as evidence of myelin's utility. Either view of myelin's utility is compatible with the very different functional capacities of *Tr vt/Tr vt* and Tr^J *vt*/Tr^J *vt* mice in spite of their identical PNS appearances. Myelin could be dispensable but Tr^J *vt*/Tr^J *vt* mice might have a fatal lesion outside of the PNS; myelin could be necessary but *Tr vt/Tr vt* mice could have compensated for its lack, perhaps by redistribution of axonal membrane sodium channels (*18*). The results of failure to ever form myelin may be quite different from the effects of acute demyelination (*19*).

The few myelinated fibers in some cross sections of nerves from *Tr vt/Tr vt* mice are also of interest. It is unlikely that such myelinated fibers make any significant functional contribution, because they were less than 1% of the large-enough fibers in any nerve from any *Tr vt/Tr vt* mouse. Further, because different levels of the same sciatic nerves contained different numbers of myelinated fibers, it is unlikely that any one peripheral nerve fiber in a *Tr vt/Tr vt* mouse's nerve was myelinated completely along its length. It seems more likely that any single nerve fiber in a *Tr vt/Tr vt* mouse contained very few (if any) widely scattered myelin segments, each such segment originating from a single Schwann cell and corresponding to a single internode in a normal nerve. The small numbers of myelinated fibers in cross sections of very proximal spinal roots and cranial nerves, though extending such a short distance as to be of no functional importance, were a curiosity. That such fibers always occurred close to the spinal cord or brainstem and always occurred in clumps of contiguous or nearly contiguous fibers suggest, but do not prove, that this myelin was not of peripheral (Schwann cell) origin but was rather of central (oligodendrocyte) origin, as an irregular extension of spinal cord or brainstem myelin into the periphery.

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Naturally Occurring Auxin Transport Regulators

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The process of polar auxin transport, central to a plant's auxin relations, can be inhibited by a group of synthetic compounds that apparently act by binding to a plasma membrane protein known as the naphthylphthalamic acid (NPA) receptor. No endogenous ligand to the NPA receptor, capable of affecting polar auxin transport in plants, has yet been found. It is now shown that a group of flavonoids—including quercetin, apigenin, and kaempferol—can specifically compete with [^{3}H]NPA for binding to its receptor and can perturb auxin transport in a variety of plant tissues and transport systems in a manner closely paralleling the action of synthetic transport inhibitors. Because the active flavonoids are widely distributed in the plant kingdom and exert their effects at micromolar concentrations approximating likely endogenous levels, they may act as natural auxin transport regulators in plants.

THE PLANT GROWTH SUBSTANCE indole-3-acetic acid (IAA; "auxin") must be transported basipetally from its sites of synthesis in shoot apices and young leaves to the subapical target tissues in which it exerts its many developmental effects. The current hypothesis describing the mechanism of polar auxin transport (PAT) [the "chemiosmotic hypothesis" (*1*)] includes H^+ gradient–driven cytoplasmic auxin accumulation by diffusion of lipophilic undissociated IAA molecules ($pK = 4.7$) (*1*) and by carrier-mediated cotransport of IAA anions and H^+ ions (*2*), and a transmembrane efflux of IAA anions on a carrier preferentially localized at the basal end of cells in the transport pathway (*1, 3, 4*). A group of synthetic compounds, exemplified by naphthylphthalmic acid (NPA), can inhibit PAT apparently by blocking the polar efflux step and causing a net IAA accumulation in transporting cells (*5*). These compounds (polar auxin transport inhibitors, or PATIS) do not directly compete with IAA but act through their own receptor, the NPA receptor, in plant cell plasma membranes [dissociation constant (K_d) values for NPA reported from 2 to 500 μM (*6*)]. The NPA receptor and PATIS binding to it have been characterized (*7*), and the receptor's conformation inferred from structure-activity studies (*8*). Many PATIS compete for NPA binding with a specificity parallel to their effects on auxin transport. Yet despite the probable physiological significance of the NPA receptor, no endogenous ligands have been established. We now report that certain commonly occurring flavonoids, with specific structural requirements for activity, both inhibit auxin transport and compete for binding to the NPA receptor in the same manner as NPA in etiolated *Cucurbita pepo* L. hypocotyls and other plant shoot tissue.

We investigated phenolic compounds as PATIS because of earlier work (*9*) that recorded an increase in phenolics in tomato plants root-fed with sodium quinate, a carbohydrate precursor of phenylpropanoids and flavonoids. Such plants were dwarfed, with high levels of phenolics and reduced polar auxin movement from apical buds to roots. Their high IAA content was attributed to phenolic inhibition of both PAT and of IAA oxidase, which is antagonized in vitro by *o*-dihydric phenols, but stimulated by monohydric phenols (*10*).

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We screened phenolic compounds as potential auxin transport regulators acting by way of the NPA receptor with two tests: (i) competition with radiolabeled NPA for binding to its membrane-associated receptor, and (ii) action on IAA transport analogous to that of NPA in a well-characterized and reproducible physiological system, namely, the stimulation of net uptake of [1-^{14}C]IAA into zucchini hypocotyl segments due to inhibition of a carrier-mediated component of IAA anion efflux from segment cells (*11*). In an initial screen (Table 1) representatives of certain classes of flavonoid (structures in Fig. 1)—the flavonol quercetin and the flavone apigenin—were the only phenolics with significant NPA-like activity in either assay. Since quercetin is a widely distributed flavonoid, present in most higher plants (*10*), including *Cucurbita pepo* (*12*), it was chosen for further characterization.

Table 1. Effects of 10 μ*M* phenolic compounds on [1-^{14}C]IAA net uptake by zucchini hypocotyl segments and on [^{3}H]NPA binding to microsomal membranes from the same tissue. Net uptake of [1-^{14}C]IAA (0.3 μ*M*; 2.18 GBq mmol^{-1}; Amersham International) by 2-mm segments of dark-grown (25°C; 5 days) *Cucurbita pepo* L. (var. All Green Bush; zucchini) hypocotyls was measured at 25°C at *p*H 6.0 after 40 min as previously described (*11*). For NPA-specific binding assays, segments were ground in Grind Medium (GM) (*2*) at 0°C and filtered through a layer of fine-mesh nylon cloth. The cake was reground in GM, and the combined filtrates were centrifuged at 4500*g* for 10 min. The supernatant was spun at 90,000*g* for 45 min, and the pellet was resuspended and homogenized in Test Solution (TS; 250 m*M* sucrose, 5 m*M* $MgSO_4$, 10 m*M* citrate/NaOH, *p*H 6) to yield 1.25 g of original fresh weight per milliliter. Test compounds were added to samples of this preparation, and aliquots (1 ml) were mixed with 1 ml of TS supplemented with 1.0 n*M* [^{3}H]NPA ± 10 μ*M* unlabeled NPA. After centrifugation at 90,000*g* for 45 min at 0°C supernatants were discarded, and the pellets were transferred to 6 ml of scintillation fluid (*2*) with the aid of 2 × 200 μl ethanol before counting at 25% efficiency. Replicate samples were used for each compound. "NPA-specific binding" is defined as dpm pelleted in the presence of [^{3}H]NPA alone minus dpm pelleted in the presence of [^{3}H]NPA plus unlabeled NPA. Typically, NPA-specific binding was greater than 80% of the total binding of 1 n*M* NPA. Ethanol (reagent solvent) was kept below 0.2% (by volume) in controls and experimental samples. Phenolic compounds were obtained from Sigma or Roth, Karlsruhe. Controls in each case were with no test substance added.

Substance* added (10 μ*M*)	[1-^{14}C]IAA net uptake (% of control)†	Residual specific NPA binding (% of control)‡
NPA	191.0 ± 11.3	0
Salicylic acid	113.8 ± 6.0	87.8 ± 9.5
Protocatechuic acid	99.6 ± 3.3	105.4 ± 20.3
Phenylacetic acid	94.0 ± 3.7§	102.6 ± 17.9
Caffeic acid	97.8 ± 8.7	91.2 ± 15.6
Esculetin	104.9 ± 1.9	81.9 ± 7.2
Naringenin	108.8 ± 6.8	79.1 ± 13.7
Apigenin	155.1 ± 11.5	35.8 ± 6.0
Quercetin	141.8 ± 4.3	21.5 ± 1.4
Rutin	104.3 ± 3.3	79.5 ± 13.4

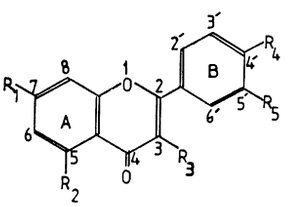
*Salicylic acid, 2-hydroxybenzoic acid; protocatechuic acid, 3,4-dihydroxybenzoic acid; caffeic acid, 3,4-dihydroxycinnamic acid; esculetin, 6,7-dihydroxycoumarin; rutin, quercetin-3-rutinoside. Flavonoid structures in Fig. 1. †Means ± SEM, *n* = 3 (experimental and controls); typical control, 23.2 ± 0.8 dpm mg^{-1} (fresh weight) per 40 min. ‡Means ± SEM, *n* = 2; control value, 17.2 ± 3.4 dpm mg^{-1} (fresh weight). §Ineffective up to at least 1 m*M* at *p*H 6.0.

In a dose-response study of quercetin's ability to increase the net uptake of [1-^{14}C]IAA by hypocotyl segments (Fig. 2), quercetin's stimulation reached a plateau at 10 μ*M*, giving about half the maximum NPA effect. In separate tests (*13*), 10 μ*M* quercetin increased IAA uptake throughout a time course (0 to 60 min, *p*H 6.0) and at *p*H 4, 5, and 6 with a fixed uptake period of 40 min. IAA uptake was less sensitive to the flavanone naringenin (Fig. 2), but we found that the corresponding flavone, apigenin (see Fig. 1), gave the same stimulation as quercetin. The apigenin glycoside rhoifolin had no effect. Quercetin and NPA effects were additive at suboptimal levels (Fig. 2), but not at 10 μ*M* NPA when quercetin no longer stimulated. This pattern suggests a common target.

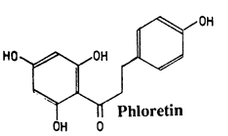
Quercetin stimulation is apparently not due to increased tissue sink capacity or to nonspecific plasma membrane damage. Uptakes of the ^{14}C-labeled weak acids DMO (5,5-dimethyloxazolidine-2,4-dione; Fig. 2), a Δ*p*H probe, and the plant hormone abscisic acid were not increased by up to 30 μ*M* quercetin. This indicates that quercetin does not stimulate net IAA uptake by raising intracellular *p*H and that general permeabilization had not occurred. Quercetin appears not to create a metabolic sink: (i) it is ineffective when 10 μ*M* NPA is present (Fig. 2); (ii) it elicited no detectable metabolites of labeled IAA after a 60-min incubation of hypocotyl segments with or without the flavonol [methods of (*11*)]; (iii) it can stimulate net uptake of [^{3}H]NAA (1-naphthaleneacetic acid), a synthetic auxin that is not a substrate for IAA oxidase (*14*); and (iv) the specificity pattern for phenolic modulation of IAA oxidase (*10*) is quite distinct from that found for phenolic effects on IAA net uptake (Table 1 and Fig. 3). Finally, quercetin did not alter the nonspecific retention of [1-^{14}C]IAA by tissues killed by freezing and thawing.

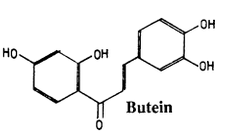
We confirmed directly that efflux of [1-^{14}C]IAA from preloaded hypocotyl segments was inhibited by 10 μ*M* quercetin. Compartmental analysis (*15*) revealed that quercetin reduced efflux differentially from a putative "cytoplasmic" compartment, lowering the rate constant (0.113 min^{-1}) by about 60%. Quercetin does not stimulate the IAA uptake carrier (*1, 2*): when NPA blocks the efflux carrier, quercetin does not increase [1-^{14}C]IAA uptake (Fig. 2) or alter the extent of competition for the uptake carrier by nonradioactive IAA (*13*). Quercetin and NPA stimulation of net IAA uptake into segments was completely reversible with a thorough washing to facilitate efflux (three rinses, and a 30-min incubation, in 1.5% sucrose at 25°C); after being washed, the tissue retained its sensitivity to both additives.

A dose-response curve (Fig. 4) shows that 50 μ*M* quercetin completely inhibits specific NPA binding in zucchini hypocotyls. In contrast, galangin (a flavonol with no B-ring hydroxylation) and naringenin are about one-tenth as effective, and caffeic acid has essentially no effect (Fig. 4). Lineweaver-Burk analysis of NPA binding (K_s = 5.25 ± 0.85 n*M*) suggests that inhibition by quercetin is competitive (Fig. 5), with an inhibition constant (K_i) of 1.88 ± 0.52 μ*M*. The inhibition by 10 μ*M*



	C_2-C_3	R_1	R_2	R_3	R_4	R_5
Naringenin	><	OH	OH	H	OH	H
Apigenin	>=<	OH	OH	H	OH	H
Galangin	>=<	OH	OH	OH	H	H
Kaempferol	>=<	OH	OH	OH	OH	H
Kaempferide	>=<	OH	OH	OH	OCH_3	H
Quercetin	>=<	OH	OH	OH	OH	OH
Taxifolin	><	OH	OH	OH	OH	OH
Isorhamnetin	>=<	OH	OH	OH	OH	OCH_3
Tamarixetin	>=<	OH	OH	OH	OCH_3	OH
Fisetin	>=<	OH	H	OH	OH	OH





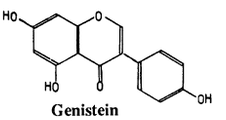


Fig. 1. Structures of the flavonoids used in the experiments.

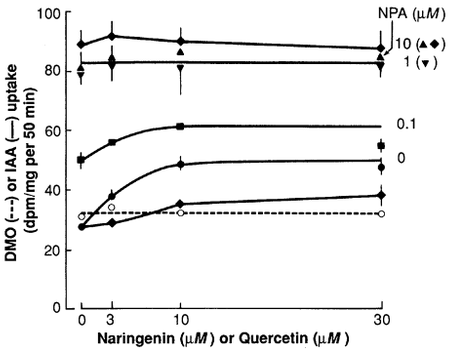


Fig. 2. Characterization of flavonoid effects on net [1-^{14}C]IAA uptake by zucchini hypocotyl segments. All measurements of uptake (protocol in Table 1) are means of three replicates ± SEM, shown where it exceeds the size of the indicating symbol. Effect of varying quercetin concentration on [2-^{14}C]DMO (0.3 μ*M*, 2.04 GBq mmol^{-1}, Amersham International) uptake (○) or on [1-^{14}C]IAA uptake (0.3 μ*M*) in the absence (●) and presence of NPA (0.1 μ*M*, ■; 1 μ*M*, ▼; 10 μ*M*, ▲). Also, effect of 0 to 30 μ*M* naringenin (◆) plus and minus 10 μ*M* NPA.

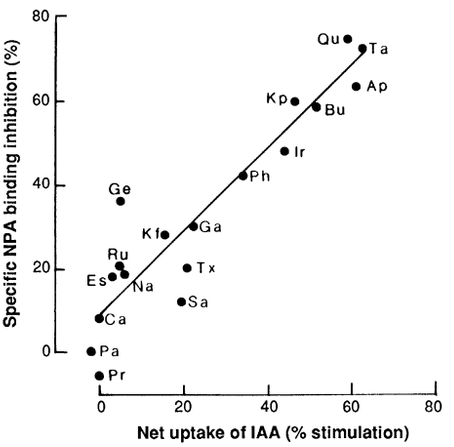


Fig. 3. Correlation of effects of phenolic compounds on [1-^{14}C]IAA net uptake and on specific [^{3}H]NPA binding, assayed as described in Table 1. Percent stimulation or inhibition is relative to controls with no test phenolic added. Straight line drawn after least-squares linear regression. Abbreviations: Ap, apigenin; Bu, butein; Ca, caffeic acid; Es, esculetin; Ga, galangin; Ge, genistein; Ir, isohamnetin; Kf, kaempferide; Kp, kaempferol; Na, naringenin; Pa, phenylacetic acid; Ph, phloretin; Pr, protocatechuic acid; Qu, quercetin; Ru, rutin; Sa, salicylic acid; Ta, tamarixetin; and Tx, taxifolin. Flavonoid structures in Fig. 1.

quercetin remains at about 70% at *p*H 5, 6, and 7, although the amount of specific NPA binding declines with increasing *p*H as found previously (*6*). Thus, quercetin has the potential to interact with domains of the NPA receptor exposed to either the cell wall (*p*H 5) or the cytoplasm (*p*H 7). The inhibitions of [^{3}H]NPA binding by quercetin and nonradioactive NPA are at least partially reversible (*16*).

We found that flavonoids also have NPA-like effects in [^{3}H]NPA binding and segment uptake tests using light-grown zucchini hypocotyls, light-grown pea internodes, and etiolated maize coleoptiles. There were some species differences in the order of effectiveness of specific active flavonoids but the active compounds (quercetin, apigenin, fisetin) again included widely distributed flavone and flavonol aglycones.

Having investigated the effects specifically of quercetin, we examined the flavonoids more systematically for NPA-like activity, comparing 10 μ*M* test compound and NPA at *p*H 6.0. There was a high positive correlation between effects on net IAA uptake and on NPA binding (Fig. 3). There appear to be particular structural (Fig. 1) requirements for maximum NPA-like activity. (i) Hydroxyl groups are required on both A and B rings of the flavonoids, but not on the central pyran ring. Thus, galangin (unsubstituted B ring) and kaempferide (4′-OCH_3) are both less active than kaempferol (4′-OH); tamarixetin (3′-OH, 4′-OCH_3) and isorhamnetin (3′-OCH_3, 4′-OH) are as effective as quercetin (3′-OH, 4′-OH); apigenin (a flavone) and the corresponding flavonol (kaempferol) are equally effective. (ii) The C2–C3 bond in the pyran ring should be unsaturated. Reduction of apigenin to the flavanone naringenin and of quercetin to the flavanonol taxifolin causes a substantial loss of activity. C2 is now tetrahedral rather than planar, and conjugation is reduced. (iii) The quercetin glycoside rutin is inactive in both tests, which suggests that the large polar substituent interferes with binding rather than simply preventing tissue penetration. However, the isoflavone genistein inhibits NPA binding without stimulating IAA uptake, the only such mismatch that we found. Poor uptake or rapid metabolism could be responsible. (iv) The high activity of the chalcone butein suggests that the pyran ring oxygen is unnecessary. Also the dihydrochalcone phloretin is more active than the corresponding flavanone, naringenin, perhaps because of its greater conformational flexibility.

We examined the effects of the active flavonoids on PAT in whole stem segments. The standard method for demonstrating PAT by excised stem tissue is to follow the basipetal transport of labeled IAA from an apical agar donor block to a basal receiver block (*3*). Incorporation of NPA and other transport inhibitors in the receiver block slows IAA arrival. However, quercetin and other flavonoids bind strongly to cellulose and, unlike NPA, do not diffuse away from a local application site (*17*). Thus, quercetin inhibits IAA efflux only from cells close to the receiver, while NPA is more mobile and intercepts the polar transport stream away from its site of application (*17*). We were able to circumvent the apparent immobility of quercetin by applying it or NPA as small, local doses halfway up 20-mm-long subapical zucchini hypocotyl segments. The segments were almost bisected with a transverse cut, leaving a hinge of tissue. A 1-μl droplet of water, 10 μ*M* NPA, or 10 μ*M* quercetin was applied to the cut surfaces, which were then reappressed. The treated segments were mounted vertically and a ^{14}C-labeled IAA solution was applied to the apical end. Basal halves of the segments were assayed for ^{14}C after a 6-hour transport period. Both substances inhibited basipetal auxin transport significantly compared to control treatments, NPA by 30% and quercetin by 28% ($P < 5\%$ by Dunnett's multiple comparison test).

The close correspondence between the effects of NPA and the active flavonoids suggests that the latter may act as natural regulators of PAT and auxin efflux from cells in plants. As such, the flavonoids would influence auxin distribution and local concentrations and hence could modulate auxin-related phenomena from gene expression and ion transport to cell and organ differentiation (*18*). Some characteristics of the flavonoids are attractive for such a role.

1) They are ubiquitous in higher plants. Several hundred flavonoid aglycones are known (*10*). Any higher plant tissue is likely to contain one or more of the most common flavonol (kaempferol, quercetin, myricetin) or flavone (apigenin, luteolin) nuclei (*10*). It is specifically these widely distributed flavonoids that are the ones we have found to be particularly effective (*19*).

2) Flavonoid skeletons are often present at concentrations within or above the range we find here to be effective. However, most estimates of flavonoid levels concern glycosides, which are inactive in our assays and usually confined to the vacuole. Little quantitative information is available for free aglycones, which are cytoplasmic precursors of glycosides, but have been most studied as secretory products (*20*). Tissues exposed to pathogens can accumulate both free flavones and antimicrobial isoflavonoid phytoalexins (*21*). The "trace amounts" of free aglycones often reported could easily encompass the micromolar flavonoid concentrations needed to affect auxin transport, particularly

since flavonoids are often concentrated in only a few cell layers of the plant organs from which total aglycones are extracted.

3) Phenolic biosynthesis is intensely responsive to environmental stimuli (*22*). It seems unlikely that compounds once stigmatized as secondary would be so elaborately controlled unless they have some important functions. Recently discovered roles in the control of *Rhizobium* nodulation for free flavones and flavonols released by pea and clover roots (*23*) illustrate specific flavonoid functions at the molecular level.

4) Factors such as light and wounding that can inhibit PAT (*24, 25*) often also increase flavonoid synthesis (*26*). Auxin can promote its own polar transport (*3, 5*) and inhibit flavonoid synthesis (*26*). After wounding, a rapid rise in free flavonoids due to glycosidase action could be followed by increased biosynthesis in cells adjacent to those directly damaged. Then, flavonoid immobility would be highly adaptive, serving to "hold" auxin within cells near to the wound by inhibiting the efflux carrier while not affecting auxin transport in unwounded portions of the plant.

In testing this hypothesis, it will be important to closely link changes in PAT or in more local auxin distribution to changes in levels of specific active flavonoids. Although measurement of free flavonoid levels in small, circumscribed amounts of plant tissue is difficult, the potential elaboration of an endogenous system regulating auxin transport in plants is a strong incentive.

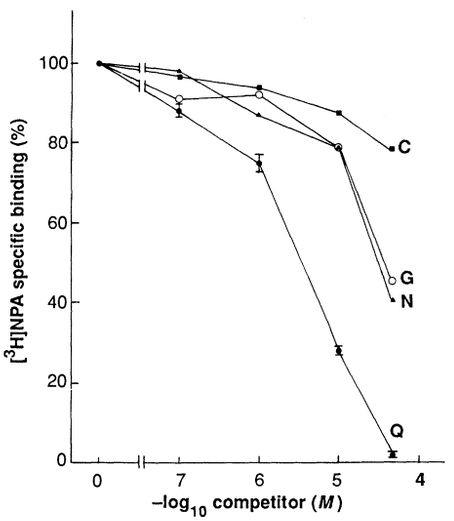


Fig. 4. Dose-response curves for the effects on NPA specific binding of caffeic acid (C), galangin (G), naringenin (N), and quercetin (Q). Assay was performed as described in Table 1. The ranges of duplicate values, shown for quercetin, varied from 0.5 to 2% of the mean for all points.

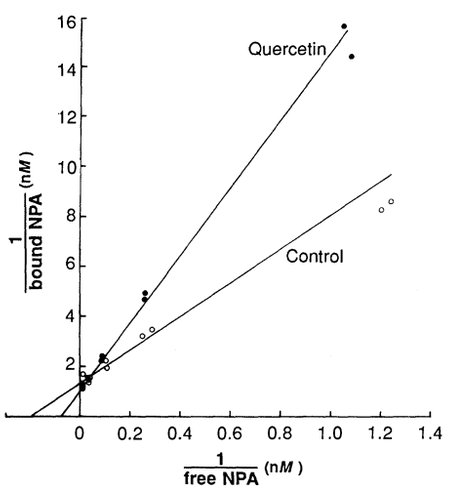


Fig. 5. Effect of quercetin (3 μ*M*) on the concentration-dependence of NPA-specific binding. The assay procedure was as in Table 1. Amounts of [^{3}H]NPA in both supernatants and pellets were measured in two series of tubes (±3 μ*M* quercetin); each tube contained membranes prepared from 1.12 g of fresh tissue and 1 n*M* [^{3}H]NPA together with increasing concentrations of unlabeled NPA. Nonspecific binding was subtracted and the K_s (±SD) was estimated by weighted linear regression (weighting factor was [specifically bound NPA]^3) of a plot relating reciprocal bound and free NPA concentrations.

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14. We used the uptake protocol of Table 1 but [^{3}H]NAA instead of [1-^{14}C]IAA; quercetin increased net uptake of NAA from a control value of 102.0 ± 1.6 dpm/mg (fresh weight) per 40 min to 116.9 ± 4.8 (means ± SEM, n = 3).
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16. Earlier workers [K.-S. Thomson *et al.*, *Planta* **109**, 337 (1973)] concluded that NPA bound its receptor reversibly and noncovalently. Our attempts to show reversibility were complicated by time-dependent loss of binding during the washing procedure. Nevertheless, a minimum of about 50% reversal of quercetin and nonradioactive NPA inhibition could be shown. This finding and results that suggest competitive inhibition (Fig. 5) indicate that the interaction of both substances with the receptor is not irreversibly covalent.
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18. Up-to-date accounts of plant hormones in P. J. Davies, Ed., *Plant Hormones and Their Role in Plant Growth and Development* (Nijhoff, Dordrecht, 1987).
19. We confirmed [compare (*12*)] by paper chromatography of acid hydrolysates that light-grown zucchini seedlings do contain quercetin and kaempferol. We have also demonstrated that dark-grown zucchini hypocotyl segments can convert [3-^{14}C]cinnamic acid to quercetin and kaempferol as well as to hydroxycinnamic acids.
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