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Regulation of the activity of N-1-naphthylphthalamic acid binding protein by ATP and phosphatase

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Abstract

N-1-naphthylphthalamic acid (NPA), an auxin transport inhibitor, was found to bind specifically to a crude membrane preparation from sugar beet seedling leaf cell suspension cultures. The dissociation constant (Kd) and binding protein concentration were found to be 1.71 μmol dm^{-3} and 220 pmol g^{-1}(membrane protein), respectively. The amount of specific ^{3}H-NPA binding was significantly increased by adding Mg^{2+}ATP to the binding assay solution. Treatment of membrane preparations with acid phosphatase, prior to the NPA binding assay, resulted in lower specific binding. ATP activation and phosphatase inactivation were culture stage dependent. Although a considerable effect could be detected when using cells from day 8 (representing the linear phase), the same treatment did not alter the binding if cells from day 1 (representing lag phase) or day 14 (representing the stationary phase) were used. These observations have strongly highlighted the possible involvement of a phosphorylation and dephosphorylation mechanism *in vivo* in the regulation of the activity of the NPA binding protein. High phosphatase activity was found in the supernatant, but not in the membrane pellet, after 50 000 *g* centrifugation. Our present study has indicated that receptor activity could be regulated by a phosphorylation and dephosphorylation mechanism in plants.

Additional key words: auxin transport, *Beta vulgaris*, receptor phosphorylation.

Introduction

According to the chemiosmotic model of auxin transport, polarity of transport is achieved by efflux of auxin anions from the cell via specific, saturable auxin efflux carriers that are located in the plasma membrane preferentially (in basipetal transport) at the basal ends of auxin transporting cells (Rubery and Sheldrake 1974). Synthetic auxin transport inhibitor N-1-naphthylphthalamic acid (NPA) is thought to act by blocking the auxin efflux carriers (Palme and Gälweiler 1999). Genetic studies have revealed critical roles of NPA binding proteins in auxin transport. The *Arabidopsis pin1* mutant showed dramatic morphological aberrations and these were easily phenocopied when wild-type *Arabidopsis* was grown on media containing NPA (Gälweiler *et al.* 1998). The inflorescences of the *pin1* mutant showed a significant reduction in basipetal auxin transport. Cloned *AtPIN1* gene encodes a 622 amino acid protein with 12 putative transmembrane segments that shared similarities with prokaryotic and eukaryotic transporters (Gälweiler *et al.* 1998). Immunolocalization study indicated that *AtPIN1* was associated with the basal end of elongated parenchymatous xylem and cambial cells of *Arabidopsis* inflorescence axes (Gälweiler *et al.* 1998). Also, several members of the *PIN* gene family in *Arabidopsis* have been identified, indicating that there are multiple auxin efflux carriers with distinct expression patterns (Palme and Gälweiler 1999). Another study has shown that the *tir3* (*t*ransport *i*nhibitor *r*esponse 3), an *Arabidopsis* mutant, is much less sensitive to NPA than is the wild type, and it exhibits a reduction in NPA binding and polar auxin transport (Ruegger *et al.* 1997). The *tir3*

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Abbreviations: ABA - abscisic acid; 2,4-D - 2,4-dichlorophenoxyacetic acid; IAA - indole-3-acetic acid; 1-NAA - 1-naphthylacetic acid; 2-NAA - 2-naphthylacetic acid; NPA - N-1-naphthylphthalamic acid; TIBA - 2,3,5-triiodobenzoic acid.

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gene may encode either the NPA binding protein itself, or a gene required for the functioning of the NPA binding protein. A recent analysis of numerous lines of evidence points towards the involvement of three different families of membrane proteins in catalyzing auxin transport in *Arabidopsis* (Luschnig 2002).

The biological activity of membrane receptors is highly regulated, and this regulation plays a major role in modulating the sensitivity of cells to extracellular signals. Protein phosphorylation of receptors is one of the primary mechanism of regulation of receptor function (Cadena *et al.* 1994, Krupnick and Benovic 1998). Recent studies suggested that auxin transport is modulated by phosphorylation, and protein phosphatase activity is involved in regulation of auxin transport streams in roots (Muday and DeLong 2001, DeLong *et al.* 2002). *Arabidopsis* mutant *rcn1* (*r*oots *c*url in *N*PA) exhibits an enhanced sensitivity to NPA. The cloned *RCN1* gene showed close relation to the regulatory subunit of protein phosphatase 2A (Garbers *et al.* 1996). The phenotypic alterations in this mutant are due to reductions in protein phosphatase activity, because treatment with the phosphatase inhibitor cantharidin produces a phenocopy of *rcn1* (Deruère *et al.* 1999). Studies using *rcn1* mutant and wild-type *Arabidopsis* has indicated that genetic and chemical reductions in protein phosphatase 2A activity produced auxin transport abnormalities that impeded gravity response and altered lateral root growth (Rashotte *et al.* 2001).

Polar auxin transport mechanisms are often studied at the level of organs or tissues and auxin efflux machinery is generally depicted within a cell in the root or stem *in situ*. Plant cell suspension cultures provide a uniform system in which the correlative influences present in the intact plant are removed, and in which differential patterns of distribution of nutrients and hormones between cells are essentially eliminated (Dixon 1985, Zhang *et al.* 1993). Cell suspension cultures are widely used as model systems for studying many aspects of biochemistry (Dixon 1985). Our previous work in suspension cultured *Acer pseudoplatanus* L. cells suggested that the NPA binding protein functions as a regulatory element in the control of auxin transport and that NPA binding could be involved in the regulation of intracellular IAA levels and cell division (Elliott *et al.* 1988, Fowler *et al.* 1998, Xing and Zhang 1997). Further studies indicated that NPA binding protein activity is regulated by chemical modification (Xing *et al.* 1996). Here we reported our study of ATP and phosphatase effect on NPA binding proteins from suspension cultured sugar beet cells with emphasis on *in vitro* biochemical analysis.

Materials and methods

Plants: Sugar beet (*Beta vulgaris* L.) cell suspension cultures from leaves of cv. Regina seedlings were produced from the callus which developed on MS medium without added plant growth regulators. The stock cultures were grown in 250 cm^3 narrow necked Erlenmeyer flasks (20 cm^3 inoculum and 80 cm^3 medium) and subcultured every 16 d. All the culture flasks were incubated in the continuous light at temperature of 25 °C on flat bed reciprocating shakers at 120 cycles per minute. Batch culture propagation was used in this study.

Membrane isolation: Cells (day-5 to day-10 unless indicated) from suspension cultures were harvested by filtering under reduced pressure and rinsed twice with ice cold distilled water. The tissue was homogenized with pestle and mortar in an equal volume of isolation buffer (50 mM Tris-base, 1 mM Na_2EDTA, 0.1 mM $MgCl_2$ and 0.25 M sucrose, adjusted to pH 8.0 with KOH) for 5 - 6 min. The homogenate was centrifuged at 4 000 *g* for 10 min. The pellets were discarded and the supernatant was centrifuged at 50 000 *g* for 45 min. The pellet was then resuspended in assay buffer (50 mM Na_3 citrate/citric acid, 5 mM $MgCl_2$, 0.25 M sucrose, pH 4.5) at a concentration of 2 g initial fresh mass per cm^3. The whole isolation procedure took place at 4 °C.

Binding assays: For binding affinity analysis, the membrane suspension (0.5 cm^3) was incubated with a fixed amount (0.1 mM final concentration) of ^{3}H-NPA (specific activity 1.81 TBq $mmol^{-1}$, *Amersham,* Buckinghamshire, UK) and different concentrations of unlabelled NPA, from 0 to 0.1 mM in polycarbonate tubes giving a final assay volume of 2.5 cm^3. The samples were incubated with shaking for 60 min at 4 °C and then centrifuged at 75 000 *g* for 30 min. The supernatants were decanted and 1 cm^3 ethanol was placed in each tube. After 16 h at room temperature or at least 30 min at 60 °C, the ethanol together with the pellets were transferred to scintillation vials for counting. The calculation for Scatchard analysis (Scatchard 1949) was made using the revised *SCAPRE* program (distributed by Institute of Nuclear Medicine, The Middlesex Hospital Medical School, London, UK).

For competition experiments, the membrane suspension and 0.4 mM ^{3}H-NPA were incubated with different concentrations of unlabelled competitors. Bound and free NPA were separated by the centrifugation method described above. The Kd was determined as the concentration of competitor which displaced 50 % of the labelled NPA from the binding sites.

Binding assays in the presence of Mg^{2+} ATP or acid phosphatase: The membrane suspension was divided into

two equal portions. In one portion, 0.5 cm^3 of 25 mM Mg^{2+} ATP (*SigmaUltra grade, Sigma*, Dorset, UK) or 0.5 cm^3 buffer containing 1 unit acid phosphatase (10 units mg^{-1} solid from potato, *Sigma*) was added to the binding assay mixture, giving a final assay volume of 2.5 cm^3, unless otherwise indicated, while the other was run as a control. Two fixed concentrations of unlabelled NPA, 0.01 μM (for total binding) and 100 μM (for non-specific binding) were used (together with 0.04 pM ^{3}H-NPA) as a relative measure for specific NPA binding.

Determination of endogenous phosphatase activity: An aliquot of 0.1 cm^3 supernatant was added to 0.9 cm^3 *p*-nitrophenyl phosphate (pNPP) solution dissolved in isolation buffer (pH 8.0). Hydrolysis of pNPP was measured over a minute period. The endogenous phosphatase activity in the sample was determined from the absorbance change at 410 nm.

Protein quantification: Protein concentration was determined according to Bradford (1976).

Results

Determination of binding kinetics: The affinity constant for NPA binding was determined by Scatchard (1949) analysis (Fig. 1), which indicates a Kd of approximately 1.71 μM. The steep part of the plot (left hand side) represents high affinity with low capacity binding, whereas the horizontal part indicates nonspecific binding with low affinity. The concentration of the binding sites was 220 ± 7 pmol g^{-1}(membrane protein).

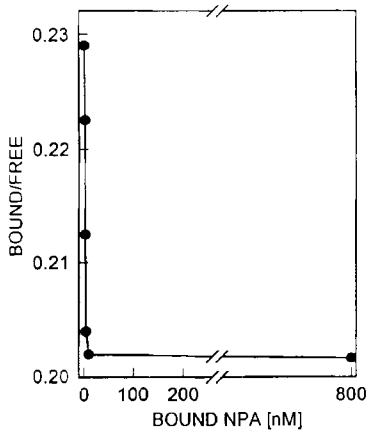

Fig. 1. Scatchard analysis of NPA binding protein. Isolated membrane preparations were incubated with a fixed concentration of ^{3}H-NPA and an increasing concentration of unlabelled NPA. After incubation at 4 °C for 60 min. Bound, free, and bound/free were calculated using *SCAPRE* program.

Examples of displacement curves are given in Fig. 2. Auxins (2,4-D, 1-NAA, 2-NAA and IAA) were all able to compete with ^{3}H-NPA for the NPA binding site. While the affinity constant Kd for others were 1 - 10 μM, IAA was shown to have only weak interaction with the NPA binding site, with a Kd over 500 μM. Tryptophan, kinetin, zeatin and ABA had no significant competition, even at 1 mM. TIBA was found to be effective in displacing ^{3}H-NPA with a Kd of 1 - 10 μM. Since the concentration of competitor which gives 50 % displacement concentration is a measure of the Kd value, these data also indicated the affinity of the NPA binding site for different compounds.

Activation of NPA binding with Mg^{2+} ATP: Cells were harvested 5 to 10 d after subculture. The membrane pellets

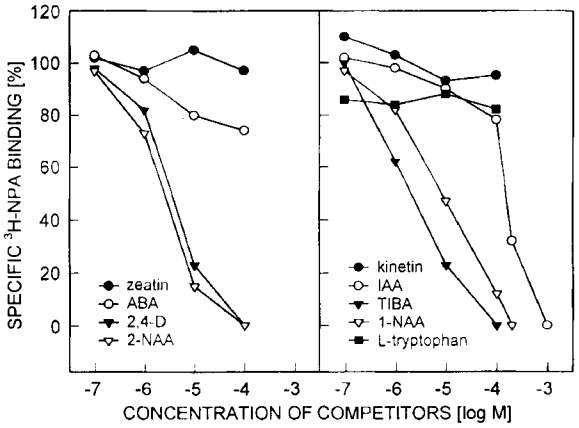

Fig. 2. Competition experiments. The isolated membrane preparations were incubated with a fixed concentration of ^{3}H-NPA and different concentrations of unlabelled competitors. Bound and free NPA were separated and Kd was determined: *A* - zeatin, ABA, 2,4-D and 2-NAA; *B* - kinetin, IAA, TIBA, 1-NAA and L-tryptophan. Values were means taken from four different experiments. SE values did not exceed 8.7 % for each of the means and were omitted from the figure.

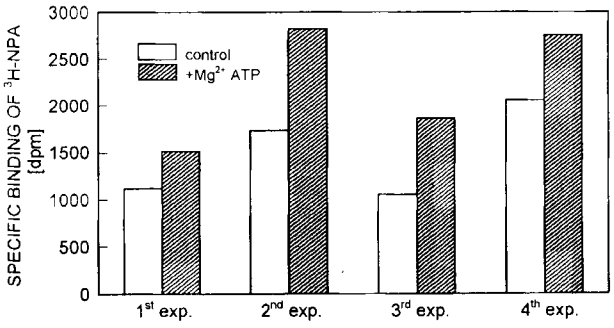

Fig. 3. Effect of Mg^{2+}ATP on specific NPA binding. The isolated membrane preparations were incubated with a fixed concentration of ^{3}H-NPA and a fixed concentration of unlabelled NPA in the presence of Mg^{2+}ATP. Relative levels of specific bindings were determined.

were divided into two equal portions. Mg^{2+} ATP was added in one portion, giving a final concentration of 2 mM while the other was run as a control. In all four different experiments, higher specific NPA binding was found in the assays with Mg^{2+} ATP (Fig. 3). When expressed as a percentage of the specific binding, the extent of the increase in specific binding resulting from addition of Mg^{2+} ATP to the binding assays was always found to be greater than 30 %. The difference among experiments could be the result of the ages of the cells used.

Inactivation of NPA binding with acid phosphatase: Both NPA binding and acid phosphatase activity are likely to be influenced by temperature. Binding assays with the addition of acid phosphatase were performed at three temperatures, *i.e.* 4 °C, which was optimal for NPA binding (data not shown), 37 °C, at which acid phosphatase has maximal activity, and 20 °C as a compromise condition. Acid phosphatase did not affect NPA binding at 4 °C, whilst at 37 °C binding in neither the control nor enzyme-treated samples could be detected. However, acid phosphatase had a clear effect at 20 °C, even though the specific binding of the control was shown to be less than that at 4 °C (Fig. 4).

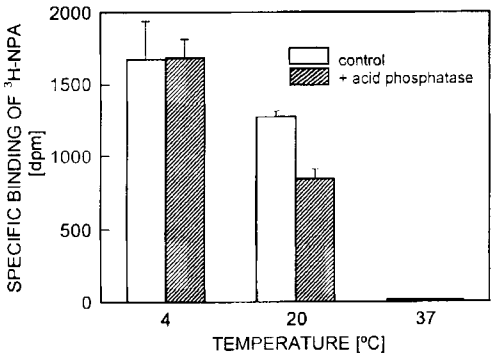

Fig. 4. Effect of acid phosphatase on NPA binding at different temperatures. After treatment with acid phosphatase at different temperatures, the isolated membrane preparations were incubated with a fixed concentration of ^{3}H-NPA and a fixed concentration of unlabelled NPA. Relative levels of specific bindings were determined. Values are the mean ± SE (n = 4).

At this temperature, specific binding, under the effect of acid phosphatase treatment, decreased to 67 % of that of the control. Further experiments on the effect of acid phosphatase were performed at 20 °C. The effect of this enzyme on NPA binding was also shown to be concentration-dependent, with increase in enzyme concentration resulting in decreased specific NPA binding. Extended treatment also led to greater reduction of the specific binding (Fig. 5). Boiled acid phosphatase (20 min) had no effect on specific NPA binding (data not shown).

Endogenous phosphatase may represent an internal mechanism which could dephosphorylate NPA binding proteins. After isolation of the membrane preparation, both the membrane pellet and the supernatant were collected. Phosphatase activity could not be detected in the membrane fractions, but high levels of the activity were reproducibly detected in the supernatant, using pNPP as its substrate at pH 8.0. The activity was shown to be 1.63 units per cm^3 of the supernatant (data not shown). Considering the volume ratio of plant material and isolation buffer (1:1), the activity in the cytosol of intact cells can be expected to be even higher and exceed the concentrations of acid phosphatase that were used in the *in vitro* NPA binding assay. These observations highlight the possibility that the activity of the NPA binding protein could be regulated *in vivo* by phosphatases.

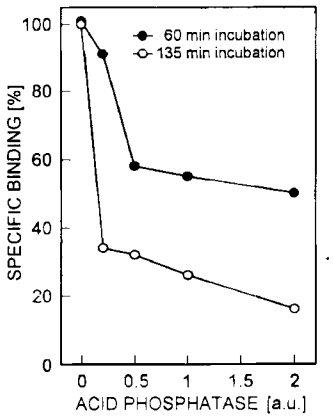

Fig. 5. Effect of acid phosphatase concentration (unit) and time on specific binding of NPA. After treatment with acid phosphatase for different times, the isolated membrane preparations were incubated with a fixed concentration of ^{3}H-NPA and a fixed concentration of unlabelled NPA. Relative levels of specific bindings were determined.

Culture stage dependence of Mg^{2+} ATP and acid phosphatase effects on NPA binding: The effects of Mg^{2+} ATP and acid phosphatase on NPA binding strongly imply that a phosphorylation/dephosphorylation mechanism operates in the regulation of the activity of NPA binding proteins. The level of NPA binding proteins was not always constant, as indicated in our previous studies in *Acer pseudoplatanus* L. cells (Elliott *et al.* 1988). One of the regulatory mechanisms could well be the extent of phosphorylation of this protein. Hence, the response of NPA binding to Mg^{2+} ATP and acid phosphatase treatment was investigated in the growth cycle of the sugar beet cell suspension culture. Three representative stages were studied. These include day 1 (within the lag phase), day 8 (within the linear phase) and day 14 (within the stationary phase). While the effect could be reproducibly observed when using cells from day 8, there seemed to be no response to the same treatment when using cells from day 1 or day 14 (Fig. 6).

Discussion

Scatchard analysis of membrane-bound NPA binding data indicated a high affinity binding protein with a dissociation constant (Kd) of 1.71 μM. This value was similar to those reported in maize coleoptiles (Sussman and Gardner 1980), but was higher (lower affinity) than those found in *Acer pseudoplatanus* L. cells (Elliott *et al.* 1990). In competition experiments designed to determine the specificity of the NPA binding protein, 1-NAA, 2-NAA and 2,4-D were found to be able to compete with NPA (Kd from 1 to 10 μM), whilst IAA was shown to have only weak interaction with the NPA binding protein (Kd > 100 μM). Tryptophan, kinetin, zeatin and ABA were virtually not competitive. Similar results demonstrating weak competition by IAA for NPA binding to membranes have been reported in maize coleoptile (Sussman and Gardner 1980) and *A. pseudoplatanus* cells (Elliott *et al.* 1990).

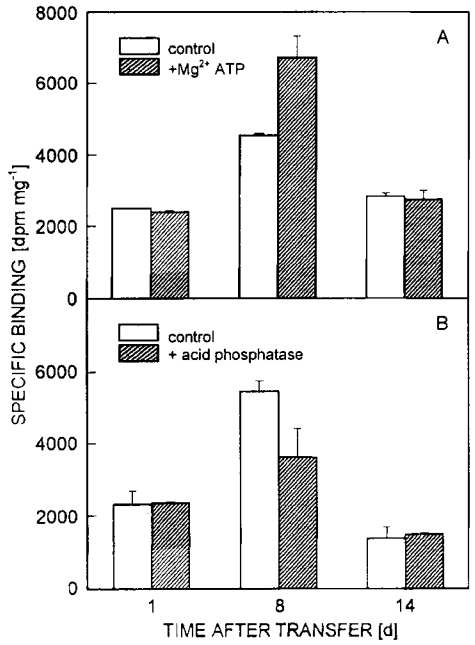

Fig. 6. Influence of time following cell transfer on effect of Mg^{2+}ATP and acid phosphatase on specific NPA binding. Membranes were prepared from cells harvested 1, 8 or 14 days after batch culture transfer. They were incubated with a fixed concentration of ^{3}H-NPA and a fixed concentration of unlabelled NPA in the presence of Mg^{2+}ATP (A) or after treatment with acid phosphatase (B). Relative levels of specific bindings were determined. Values are the mean ± SE (n = 4).

Our previous work with *A. pseudoplatanus* L. cell suspension cultures suggested that NPA binding could be involved in the regulation of intracellular IAA levels and cell division (Elliott *et al.* 1988). These findings may indicate that the NPA binding protein functions as a regulatory element in the control of auxin transport. As the initial point of interaction of growth regulators, regulation of the receptors themselves are of particular importance. Genetic studies in *Arabidopsis* mutants suggested that the function of IAA efflux carriers could be regulated by phosphorylation (Garbers *et al.* 1996, Deruère *et al.* 1999, Rashotte *et al.* 2001). Here, we examined the possible role of phosphorylation/dephosphorylation in the regulation of the activity of NPA binding proteins with *in vitro* biochemical analysis.

This regulatory mechanism was first investigated by additions of Mg^{2+} ATP to the binding assay. The treatment was found to considerably affect NPA binding. Addition of Mg^{2+} ATP to *in vitro* NPA binding assay increased specific NPA binding by over 30 % (Fig. 3). As protein kinases have substrate specificity (Hardie 1999), it is hard to determine what kinases should be examined by adding them into NPA binding assay. However, it has been found that some protein kinases are located on cellular membranes (Hardie 1999) and some protein kinases, such as calmodulin domain-like protein kinase (CDPK), probably could translocate between cytosol and plasma membrane (Xing *et al.* 1997, 2001). It is thus possible that the kinase(s) that regulate NPA binding site activity may well associate with plasma membrane or arise from trace amount of cytosol remained in microsomal preparation. This could be true in our case that ATP added to the binding assay was utilized by the kinases and promoted phosphorylation.

Pretreatment of membrane preparations with acid phosphatase led to significant decrease of specific NPA binding (Fig. 4 and 5). High phosphatase activity was also detected in cell cytosols. It is likely that the activity of NPA binding protein could be regulated by the endogenous phosphatase in the intact cells. The effect of Mg^{2+} ATP on the reactivation or recovery of this binding strongly implies that phosphorylation (presumably through protein kinase action) as well as dephosphorylation may cooperate in regulating NPA binding activity.

Effects of Mg^{2+} ATP and acid phosphatase on NPA binding were examined at three representative periods of the batch culture cycle. It is notable that neither Mg^{2+} ATP nor acid phosphatase caused any change of specific NPA binding when using cells from day 1 or day 14 of the batch culture, whilst they substantially altered the activity when using cells from day 8 (Fig. 6). In our previous study in suspension-cultured *A. pseudoplatanus* cells, we observed that the number of active NPA binding proteins varied during the 14-d culture passage (Elliott *et al.* 1988). One peak arose from day 0 to day 4 and the other occurred at day 14, leaving a minimum around day 9. Taking both the content of NPA binding proteins and the content of intracellular IAA into account, it was apparent that the IAA level tended to increase while the amount of specific binding of NPA decreased. It seems possible that the

intracellular IAA contents are intrinsically linked to the status of NPA binding proteins. The intracellular IAA peak appeared to be just within the exponential phase of the batch culture cycle. These studies not only have indicated the importance of NPA binding proteins in the regulation of IAA transport and hence cell division but may also suggest that the properties and the function of NPA binding proteins were controlled by some yet unidentified mechanisms. Although the profile of NPA binding capacity in batch culture of sugar beet cells is different, it is certainly possible that some unidentified mechanisms exist and regulate the response of NPA binding proteins to phosphorylative regulation, especially as indicated in our study in *A. pseudoplatanus* cells, where the status of NPA binding proteins is involved in regulation of intracellular IAA levels and cell division.

In animal systems, at least two major effects of receptor phosphorylation have been identified, *i.e.* regulation of receptor function and regulation of receptor distribution. The latter was found with the β-adrenergic receptor, and receptors for insulin, epidermal growth factor, insulin-like growth factor II and transferrin (Sibley *et al.* 1987). Although the role of phosphatases in altering auxin transport, gravity response and lateral root growth has been demonstrated (Muday and DeLong 2001, Rashotte *et al.* 2001, DeLong *et al.* 2002) and in NPA binding capacity in our present study, there is no evidence yet to indicate the role of phosphorylation in the distribution of NPA binding proteins in the cells. It would be interesting to find whether phosphorylation can regulate the distribution of NPA binding proteins or auxin efflux carriers and hence determine the polarity of auxin transport.

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