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Apparent cross-talk of two signaling pathways that regulate *Zea mays* coleoptile growth

Aparente entrecruzamiento de dos vías de señalización que regulan el crecimiento del coleoptilo en *Zea mays*

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Abstract. Auxin and insulin promote *Zea mays* embryo growth, induce S6 ribosomal protein (S6rp) phosphorylation, and promote specific protein synthesis. The objective of this research was to test a possible cross-talk between insulin and auxin transduction pathways in *Z. mays* coleoptiles, typical auxin target tissues. Auxin and insulin produced differential quantitative and qualitative stimulation of cytoplasmic and ribosomal protein phosphorylation, and specific patterns of *de novo* synthesized cytoplasmic proteins. In addition, insulin induced S6rp phosphorylation was strongly inhibited by rapamycin, indicating target of rapamycin (TOR) kinase participation; auxin-induced S6rp phosphorylation was insensitive to this inhibitor. Phosphatidic acid (PA), a second messenger of TOR in metazoan, was also tested. It produced similar results to insulin and rapamycin sensitiveness, supporting the existence of TOR pathway in plants and the participation of PA as an intermediate of insulin action. These results seem to imply that auxin and insulin induce *Z. mays* coleoptile growth through two independent signal transduction pathways.

Keywords: Auxins; Insulin; Phosphatidic acid; ZmS6K; TOR; Plant growth.

Resumen. Las auxinas y la insulina promueven el crecimiento de embriones de *Zea mays*, inducen la fosforilación de la proteína ribosomal S6 (prS6) y promueven la síntesis de proteínas específicas. El objetivo de esta investigación fue probar el posible entrecruzamiento entre las vías de transducción de insulina y auxinas en coleoptilos de *Z. mays*, tejidos blanco típicos de auxinas. Las auxinas y la insulina produjeron una estimulación diferencial cuantitativa y cualitativa de la fosforilación de proteínas citoplásmicas y ribosomales, así como patrones específicos de proteínas citoplásmicas sintetizadas *de novo*. Además, la inducción de la fosforilación de prS6 por insulina fue fuertemente inhibida por rapamicina, indicando la participación de la cinasa blanco de rapamicina (TOR), mientras que la fosforilación de prS6 inducida por auxinas fue insensible a este inhibidor. Se probó también ácido fosfatídico (PA), un segundo mensajero de TOR en metazoarios. Esto produjo resultados y sensibilidad a rapamicina similares a los de insulina, apoyando la existencia de la vía TOR en plantas y la participación de PA como un intermediario de acción de insulina. Estos datos parecen implicar que las auxinas y la insulina inducen el crecimiento de coleoptilos de *Z. mays* a través de dos vías de transducción de señales independientes.

Palabras clave: Auxinas; Insulina; Ácido fosfatídico; ZmS6K; TOR; Crecimiento vegetal.

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INTRODUCTION

Auxins regulate many processes such as growth, cell proliferation and plant development and differentiation (Dharmasiri & Estelle, 2004; Weijers & Jürgens, 2004; Overvoorde et al., 2005; Benjamins & Scheres, 2008). However, the auxin-induced signaling pathways involved in these processes are at present not fully understood. The search for auxin receptors has led to the isolation of auxin-binding proteins, the best characterized being the ABP1 (Chen, 2001; Delker et al., 2008). Overexpression of ABP1 in plant leaves has resulted in cell number reduction but increased cell size, indicating that auxins can regulate both cell elongation and cell division through different pathways (Hobbie et al., 2000). Moreover, two different ABP1 auxin receptors have been identified in *N. tabacum*, suggesting that they correspond to each of the two modes of auxin-induced cell growth, elongation or cellular division (Campanoni & Nick, 2005). Another auxin-receptor has also been reported, the transport inhibitor response 1 protein (TIR1), a member of the plant SCF complex of the proteasome. This receptor mediates auxin-induced transcriptional responses, and thereafter regulates the expression of auxin-regulated genes (Kepinski & Leyser, 2005; Badescu & Napier, 2006; Mockaitis & Estelle, 2008). All these responses seem to be tissue and developmental stage dependent events (Dharmasiri & Estelle, 2004).

On the other hand, cell growth and cell proliferation are regulated by insulin/IGFs (insulin-like growth factors) in metazoans (Meyuhas & Hornstein, 2000; Hannan et al., 2003; Oldham & Hafen, 2003). These effectors induce a signal transduction pathway known as the PI3K-TOR pathway, that targets the translational apparatus selectively inducing protein synthesis (mainly proteins of the translational apparatus) (Ruvinsky & Meyuhas, 2006; Patursky et al., 2009). A central role in this pathway is played by TOR (target of rapamycin) kinase, a highly conserved cell growth controller which regulates the downstream signals initiated by the effector (Hay & Soneneberg, 2004; Lorberg & Hall, 2004). Recently, PA has been demonstrated as a critical intermediate of mTOR pathway. Mitogenic stimulation of mammalian cells led to a phospholipase D-dependent accumulation of cellular PA (250% at 5 min of treatment), which was required for activation of mTOR downstream effectors (Fang et al., 2001). In plants, PA has been identified as an important signaling molecule, triggered in response to various biotic and abiotic stress factors; it has also been implicated in seed germination. In general, PA signal production is fast (minutes) and transient (Munnik, 2001; Testerink & Munnik, 2005; Carman & Henry, 2007).

Among the best known targets of mTOR pathway is the phosphorylation of S6rp by activation of specific S6 kinase (S6K). In mammals, S6K can be phosphorylated by at least two different protein kinases, TOR and 3-phosphoinositide dependent kinase 1 (PDK1). These kinases phosphorylate different residues of S6K; TOR commonly accepted targets are S411 and T389 (Dennis et al., 2001; Ruvinsky & Meyuhas, 2006), whereas for PDK1 are T229, T252 and T412 (Templeton, 2001; Rebholz et al., 2006). Moreover, the S6K1 phosphorylation at S411 is required for the rapamycin-sensitive phosphorylation of T389 and the subsequent activation of S6K1 (Hou et al., 2007). Recently, it has been shown that this pathway is also functional in plants. TOR and S6K plant ortholog enzymes have been reported for *Arabidopsis thaliana* (Menand et al., 2002; Turck et al., 2004) and *Z. mays* (Reyes de la Cruz et al., 2004); they demonstrated to perform similar roles and regulatory effects as in other non-photosynthetic eukaryotes (Mahfouz et al., 2006; Dinkova et al., 2007). In *Arabidopsis thaliana*, a PDK1 ortholog has been identified; this enzyme phosphorylates AtS6K2 and can be regulated by their association with different proteins (Otterhag et al., 2006).

In *Z. mays*, an insulin like growth factor (ZmIGF) has been isolated (García et al., 2001) and proved to stimulate TOR and S6K, and phosphorylate S6rp (Dinkova et al., 2007), the main S6K substrate. This pathway is also responsive to insulin in *Z. mays* (García et al., 2001; Beltrán et al., 2002).

Interestingly, recent reports have shown that auxin also induces S6rp phosphorylation in *Z. mays* (Beltrán et al., 2002), as well as in *A. thaliana* (Turck et al., 2004). This suggests that S6rp phosphorylation in *Z. mays,* and likely in other plants, might be regulated by two different effectors -auxins and insulin/ZmIGF. This opens the question of a possible "cross-talk" between these two cell growth signaling pathways. This question is very interesting since peptide-induced signal transduction pathways are at present not widely known in plants (Ryan et al., 2002). The term "cross-talk" has been proposed to refer to situations where different signaling pathways share one or more intermediates/components or have some common outputs. However, signaling pathways sharing common components may not necessarily "cross-talk" if the common components are scaffolded into distinct protein complexes (Park et al., 2003; Chinnusamy et al., 2004).

The main objective of this research was to test whether insulin and auxin induce signaling pathways cross-talk at common steps, to understand the meaning that this phenomenon might have in regulating plant cell growth. The participation of PA as an intermediate of TOR pathway in plants was also analyzed. Since *Z.mays* coleoptiles are typical targets of auxin responsive tissues (Woodward & Bartel, 2005), they were chosen as the model system to pursue this investigation. The results from the present study demonstrated that PA is triggered by both effectors (auxin and insulin) and that auxin, insulin or PA confluence at the phosphorylation of S6rp. However, insulin induced S6rp phosphorylation was strongly inhibited by rapamycin, supporting the involvement of TOR kinase. At the same time, S6rp phosphorylation induced by auxin was insensitive to this inhibitor, suggesting a TOR kinase independent pathway. The rapamycin inhibition of PA

induced S6rp phosphorylation supports the participation of PA as an intermediary of insulin pathway. Furthermore, the output events induced after S6rp phosphorylation by either effector were not biochemically equivalent.

MATERIALS AND METHODS

Biological material and treatments. Seeds from *Z. mays* L. var. Chalqueño were disinfected with 10% (v/v) sodium hypochlorite solution and imbibed in sterile water for 22 h in darkness at 25 ˚C. Embryonic axes were manually dissected and incubated for 2 h at 25 ˚C in Murashige Skoog medium containing any of the following effectors: 200 µmol/L auxin (indole acetic acid, IAA), 200 µU insulin or 100 µmol/L PA. Rapamycin (100 nmol/L) was also added for some experiments. At the end of the treatment, coleoptiles were excised, frozen in liquid nitrogen and stored at -70 ˚C until used. Auxin and insulin concentrations and time of treatments have been previously demonstrated to be effective in inducing coleoptile growth (Beltrán et al., 1995; Reyes de la Cruz et al., 2004). Due to its availability, insulin was used to stimulate *Z. mays* coleoptiles. The concentration of PA has been used in mammalian cells to stimulate TOR pathway (Fang et al., 2001).

Quantification of PA concentration. Phospholipids were isolated and separated according to Munnik et al. (1995). Embryonic axes from 22 h germinated seeds were excised and then labeled with 3.7 x 10^6 Bq [^{32}P]-orthophosphate. They were then incubated with either of the above mentioned effectors and 1% (v/v) butanol for 5, 10, 15 and 30 minutes. After this incubation period, coleoptiles (200 mg) were dissected, pulverized with liquid nitrogen, and frozen powder was homogenized with 6 mL of extraction buffer (250 mmol/L sucrose; 3 mmol/L EDTA; 2 mmol/L EGTA; 14 mmol/L 2-mercaptoethanol; 2 mmol/L DTT; 30 mmol/L Tris-HCl, pH 7.4). The homogenate was centrifuged at 100000 g at 4 ˚C for 1 h. The pellet was resuspended in Hepes 50 mmol/L, and the lipids were extracted with 1.5 mL of $CHCl_3$ / methanol (1:2 v/v), 0.5 mL of HCl 2.4 mol/L and 0.5 mL of $CHCl_3$, vortexing for 30 s. A two-phase system was observed by adding 2 mL of HCl 1 mol/L / methanol (1:1 v/v). Organic phase was recovered and dried under nitrogen atmosphere. [^{32}P]-Phospholipids were separated on heat-activated silica 60 thin layer chromatography (TLC) plates (20 cm x 20 cm; Merck) with a mixture of ethyl acetate / iso-octane / formic acid / water (13:2:3:10 v/v). Radiolabeled phospholipids were visualized by autoradiography and bands corresponding to PA or PA-BuOH were recovered and quantified by scintillation counting. PA can be produced by two ways: (1) phospholipase C (PLC) in combination with diacylglycerol kinase (DAGK) and (2) phospholipase D (PLD).

Isolation and analysis of phosphorylated ribosomal and cytoplasmic proteins. Embryonic axes from 22 h germinated seeds were excised and then labeled *in vivo* with 7.4 x 10^6 Bq [^{32}P]-orthophosphate and incubated with either of the above mentioned effectors for 2 h. For some experiments, 100 nmol/L rapamycin was also included in the incubation medium. After this incubation period, the embryonic axes were dissected and their coleoptiles separated. The coleoptiles (200 mg) were homogenized in liquid nitrogen, and the frozen powder was mixed with three volumes of extraction buffer (50 mmol/L Tris-acetic acid (pH 8.2); 50 mmol/L KCl; 5 mmol/L magnesium acetate; 250 mmol/L sucrose; 5 mmol/L 2-mercaptoethanol; 5 mmol/L NaF, and 1 mmol/L PMSF). The ribosomal and cytoplasmic proteins were obtained as reported by Beltrán et al. (1995). The homogenate was centrifuged at 27000 g for 30 min. The supernatant was centrifuged during 4 h at 280000 g in 1.6 mol/L sucrose cushion to isolate the ribosomal fraction. The ribosomal pellet was resuspended in 1 mL buffer (20 mmol/L HEPES; 20 mmol/L KOH; 5 mmol/L magnesium acetate; 125 mmol/L potassium acetate, and 6 mmol/L 2-mercaptoethanol). Ribosomal proteins were extracted with acetic acid-magnesium acetate precipitated with acetone and centrifuged at 3000 g. Pellets were dried at room temperature and resuspended in water. The supernatant of the 240000 g centrifugation was treated with cold TCA [(10% (w/v) final concentration]. Precipitated cytoplasmic proteins were centrifuged at 3000 g; washed again with cold TCA [10% (w/v)] recentrifuged at 3000 g; the pellet was resuspended in water, and neutralized with 1mol/L KOH for further analysis. Protein concentration was determined according to Bradford (1976), using bovine serum albumin as standard. Scintillation counting was used to determine [^{32}P] incorporation. Labeled ribosomal proteins were resolved on one dimensional 12% acrylamide gels; cytoplasmic proteins were separated by 2-D gel electrophoresis using IPG ReadyStrip with 5 to 8 pH gradient (BIO-RAD) and 12% SDS polyacrylamide gels. Labeled proteins were analyzed by Phosphorimager (BIO-RAD GS-525).

2-D gel electrophoresis of newly synthesized proteins. Embryonic axes from 22 h germinated seeds were excised and incubated with 1.48 x 10^7 Bq [^{35}S] methionine for 2 h, and exposed to the effectors as mentioned above. After incubation at 25 ˚C, the coleoptiles were separated. Thereafter, cytoplasmic proteins were isolated as described above, and analyzed by 2-D gel electrophoresis as indicated. Labeled proteins were analyzed by Phosphorimager (BIO-RAD GS-525).

Statistical analysis. All experiments were repeated at least three times using extracts from different biological samples. [^{32}P]-Orthophosphate and [^{35}S]-methionine incorporation values were analyzed by ANOVA and Tukey's mean comparisons ($p<0.05$ or $p<0.01$), using SPSS (Statistical Package for the Social Sciences) version 16.0.

RESULTS

PA levels. Recently, it has been shown that PA is an important component for the mTOR signaling pathway and for protein synthesis in mammalians cells (Fang et al., 2001;

Foster, 2007; Toschi et al., 2009). Moreover, PA has been implicated in the regulation of seed germination and as an intermediary in the response to several stresses (Munnik, 2001; Testerink & Munnik, 2005). Therefore, the levels of this phospholipid were quantified in *Z. mays* coleoptiles exposed to IAA or insulin for different time periods. Incubation with butanol allowed the identification of the PA originated from PLD activity, since this phospholipid binds to the alcohol, while when it is originated from the combined actions of PLC and DAGK this binding does not occur. Results of the present research indicate a higher contribution (almost 80%) of PLD in PA biosynthesis. A significant transient increase in total PA levels was observed at 5 and 10 min with IAA or insulin treatments ($p<0.01$); however, this stimulation was higher with insulin (175%), than with auxin (120%) in comparison to the control (Fig. 1). This stimulation is in the same order of that reported in mitogen activated mammalian cells (Fang et al., 2001). The induction effect diminishes after 15 minutes of treatment. These results support the participation of PA as an intermediary on the insulin and auxin transduction pathways in *Z. mays*.

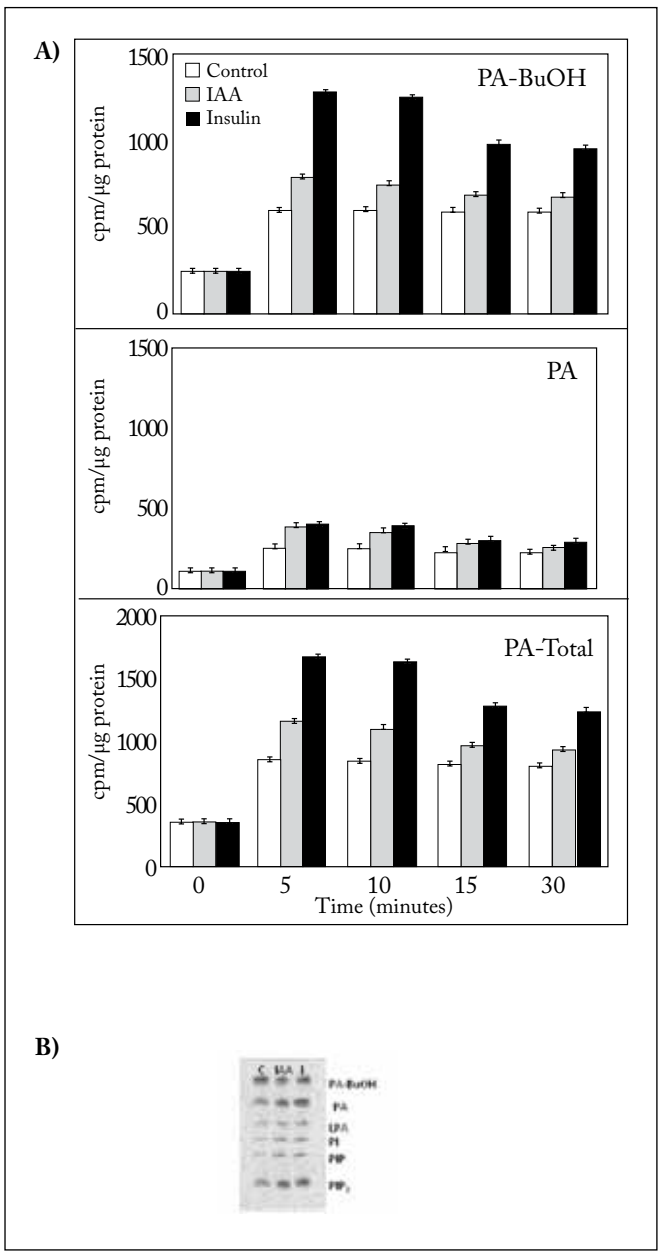
Auxin, insulin or PA phosphorylate cytoplasmic proteins. The effect of auxins, insulin or PA on cytoplasmic protein phosphorylation was determined in *Z. mays* coleoptiles to track the phosphoproteins involved in the signaling pathways activated by these effectors. [^{32}P]-Orthophosphate was applied to the 24 h germinated maize axes, previously stimulated with either of the effectors. Coleoptiles were excised and the [^{32}P]-orthophosphate incorporation into the cytoplasmic proteins was measured. Results indicated a general increase of [^{32}P] incorporation into these proteins, which was significantly more pronounced for insulin- or PA- (approximately 80%) than for the auxin-stimulated coleoptiles (approximately 35%) in relation to the control treatment ($p<0.05$) (Table 1).

Table 1. Auxin, insulin and phosphatidic acid effect on cytoplasmic and ribosomal protein phosphorylation from maize coleoptiles. Results are shown as mean values ± 1 SD of n=3.
Tabla 1. Efecto de auxina, insulina y ácido fosfatídico en la fosforilación de proteínas citoplásmicas y ribosomales de coleoptilos de *Zea mays*. Los resultados se muestran como los valores de las medias ± 1 DE de n=3.

[^{32}P] Incorporation into cytoplasmic and ribosomal proteins (cpm x 10^3 /mg protein)				
	CONTROL	**IAA**	**INSULIN**	**PA**
Cytoplasmic	500.8 ± 55	687.3 ± 51	881.1 ± 54	955.7 ± 57
Ribosomal	375.3 ± 50	845.3 ± 53	1539.4 ± 59	1612.4 ± 52

Furthermore, autoradiographies of 2D gel electrophoresis of the phosphorylated cytoplasmic proteins were performed. They showed that application of IAA also induced changes in the phosphorylated protein patterns with respect to the control (arrow heads), although different from those promoted by insulin (arrows with close head) or PA (arrows) (Fig. 2). Even though these effectors stimulated the phosphorylation of some common proteins, PA and insulin were more similar than that produced by auxin. These data suggest that insulin- and auxin-induced coleoptiles do not internalize the signal through the same pathway. On the other hand, these results further suggest the role of PA as an intermediary on the TOR pathway in *Z. mays* tissues.

Fig. 1. Auxin and insulin effects on PA levels from *Zea mays* coleoptiles. A) PA-BuOH, PA and PA-total levels are shown as mean values ± 1 SD (n=3 measurements of the incorporated [^{32}P]-orthophosphate into phospholipids). B) Autoradiography of a TLC plate of [^{32}P]-phospholipids obtained at 10 minutes after treatment.
Fig. 1. Efecto de auxinas e insulina en los niveles de PA en coleoptilos de *Zea mays*. A) Los niveles de PA-BuOH, PA y PA total se presentan como los valores de las medias ± 1 DE (n=3 mediciones de [^{32}P]-ortofosfato incorporado en fosfolípidos). B) Autoradiografía de una placa de TLC de los [^{32}P]-fosfolípidos obtenidos a los 10 minutos de tratamiento.



Ribosomal protein phosphorylation after auxin, insulin or PA induction. Auxin and insulin are known to induce phosphorylation of the S6rp in *Z. mays* and *A. thaliana* (Beltrán et al., 2002; Turck et al., 2004), a protein known to be targeted by activation of the PI3K-TOR pathway in mammals. Thus, the possibility of cross-talk at this step by the auxin- or insulin-induced signaling pathways was analyzed. With this purpose, the ribosomal fraction from *Z. mays* axes [^{32}P] labeled and stimulated by either auxin, insulin or PA was isolated, and the ribosomal proteins were obtained. For some samples rapamycin, an inhibitor of TOR kinase, was applied to the tissues in combination with the effector. [^{32}P] incorporation was determined as mentioned above. Significantly higher [^{32}P] incorporation (approximately 400% with respect to the control) was found in ribosomal proteins from insulin or PA-stimulated coleoptiles than for the auxin-treated coleoptiles (approximately 225% with respect to the control) (Table 1).

The phosphorylated ribosomal proteins were further resolved by SDS-PAGE (Fig. 3A). The correspondent autoradiographs showed again three different patterns, one for each effector (auxin, insulin or PA). A phosphorylated 32 kDa band was observed in all samples, which corresponded to S6rp identified by Western blot (data not shown). The densitometric analysis of this protein indicated a significantly higher level of [^{32}P]-incorporation (approximately 250%) in the insulin or PA-stimulated samples, whereas the stimulation was significantly lower (150%) in the auxin-induced ones, relative to the control (Fig. 3B). Regarding the rest of the phosphorylated ribosomal proteins, important qualitative differences were also observed. Insulin or PA induced phosphorylation of 42 kDa and 51 kDa ribosomal proteins. Auxin induced phosphorylation of a ribosomal protein of approximately 24 kDa, and of a smaller one (14 kDa), which possibly corresponded to the so called acidic ribosomal phosphoproteins (Montoya et al., 2002; Santos et al., 2004) (Fig. 3A). Addition of rapamycin to the axes resulted in a dramatic decrease of the insulin or PA-induced S6rp phosphorylation, but almost no effect was observed in the S6rp phosphorylated protein induced by auxin (Fig. 3A and 3B). This suggests that TOR pathway is involved on insulin and PA action, and that PA is an intermediary of the insulin effect.

Auxin, insulin and PA effect on protein synthesis of *Z. mays* coleoptiles. TOR activation of S6K by insulin is known to target the translational apparatus regulating *de novo* syn-

Fig. 2. Autoradiography of 2D-gel electrophoresis of phosphorylated cytoplasmic proteins induced by auxin, insulin or PA on *Zea mays* coleoptiles. The experiment was independently reproduced at least three times with similar results. The arrow heads indicate phosphorylated cytoplasmic proteins induced by auxin; the arrows with closed heads those phosphorylated by insulin, and the arrows represent phosphorylated proteins by PA.

Fig. 2. Autoradiografía de electroforesis en geles 2D de proteínas citoplásmicas fosforiladas inducidas por auxinas, insulina o PA en coleoptilos de *Zea mays*. El experimento se reprodujo al menos tres veces en forma independiente con resultados similares. Las puntas de flecha indican proteínas citoplásmicas fosforiladas inducidas por auxinas; las flechas con puntas negras, indican las fosforiladas por insulina y las flechas representan las proteínas fosforiladas por PA.

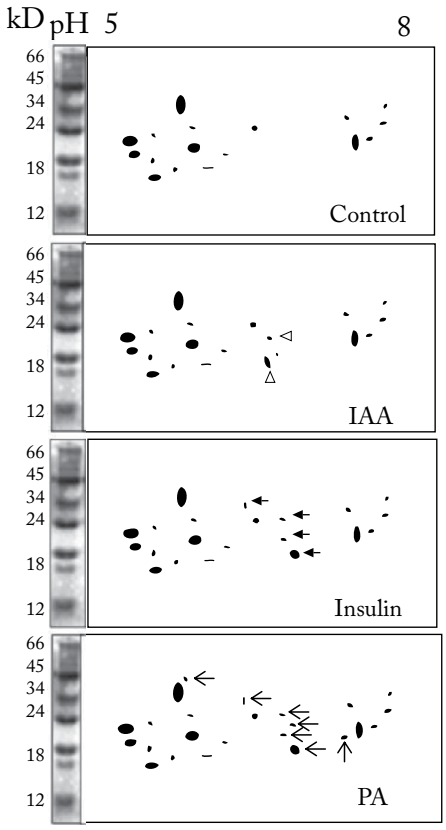
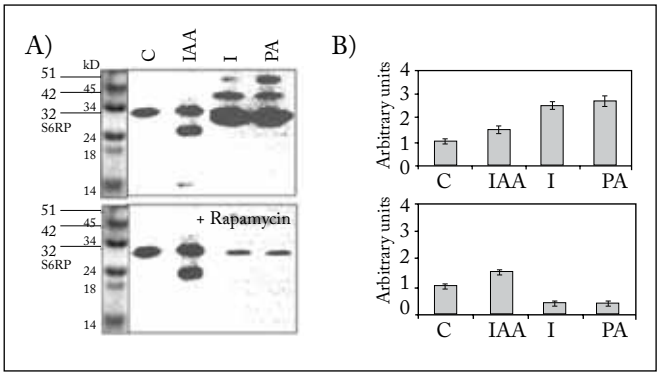


Fig.3. Analysis of phosphorylated ribosomal proteins, induced by auxin, insulin, or PA on *Zea mays* coleoptiles. A) Autoradiography of phosphorylated ribosomal proteins resolved by SDS-PAGE. B) Densitometric analysis of phosphorylated S6rp. The experiments were reproduced independently at least three times with similar results.

Fig. 3. Análisis de las proteínas ribosomales fosforiladas, inducidas por auxinas, insulina o PA en coleoptilos de *Zea mays*. A) Autoradiografía de las proteínas ribosomales fosforiladas resueltas por SDS-PAGE. B) Análisis densitométrico de prS6 fosforilada. El experimento se reprodujo al menos tres veces en forma independiente con resultados similares.

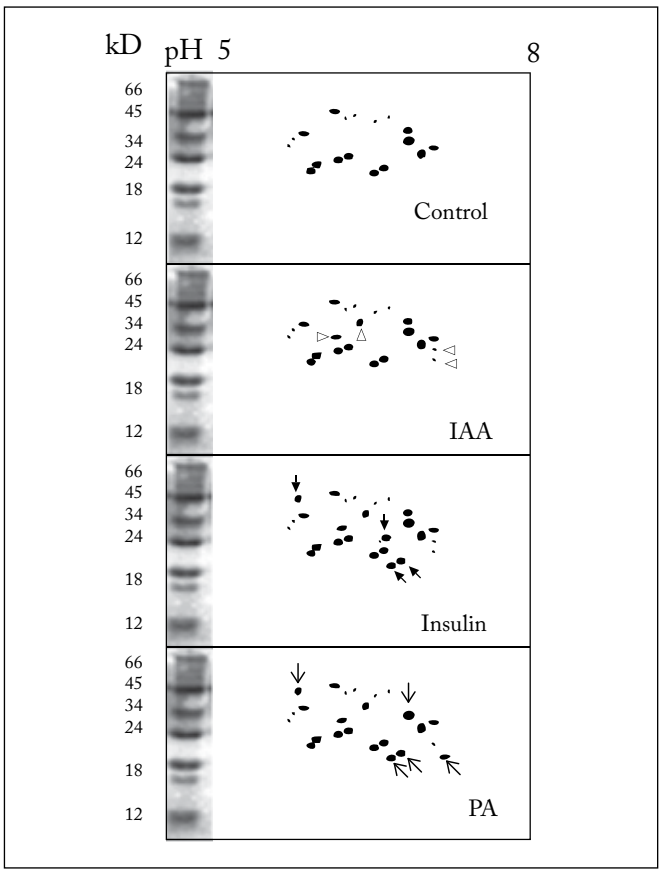


thesis of proteins in the stimulated cells. To test whether auxin and insulin differentially affected the pattern of protein synthesis in *Z. mays* coleoptiles, the next step was to analyze the proteins synthesized after coleoptile stimulation either by insulin, PA or auxin. Excised embryonic axes from 24 h germinating seeds were incubated with [^{35}S]-methionine and stimulated by either effector. The total amount of [^{35}S] incorporated into cytoplasmic proteins was determined and resolved by 2D gel electrophoresis.

Slight quantitative changes were found on the [^{35}S]-methionine incorporated in the cytoplasmic protein fraction, by either IAA, insulin or PA induction. The incorporation ranged within 25% to 31%, as compared to the control. These proteins, further resolved by two-dimensional gel electrophoresis, showed specific patterns for each effector in the correspondent autoradiographs. IAA samples showed changes in the pattern of synthesized cytoplasmic proteins compared to the control (arrow heads), and were different from those induced by insulin (arrows with closed head) or PA (arrows). It is important to emphasize that PA induced the synthesis of an extra protein with respect to insulin, and inhibited the synthesis of a protein with respect to the control (Fig. 4). These data clearly indicated that each effector (auxin, insulin or PA) caused a different biochemical output after coleoptile stimulation.

Fig. 4. Auxin, insulin and PA effect on de novo synthesis of cytoplasmic proteins from *Zea mays* coleoptiles. Autoradiography of 2D-gel electrophoretic patterns of *de novo* synthesized cytoplasmic proteins. The experiments were reproduced at least three times with similar results. The arrow heads indicate *de novo* synthesized cytoplasmic proteins induced by auxin; the arrows with closed heads those synthesized by insulin, and the arrows represent those induced by PA.

Fig. 4. Efecto de auxinas, insulina y PA en la síntesis de novo de proteínas citoplásmicas de coleoptilos de *Zea mays*. Autoradiografía de los patrones electroforéticos en geles 2D de las proteínas citoplásmicas sintetizadas *de novo*. Los experimentos se reprodujeron al menos tres veces con resultados similares. Las puntas de flecha indican proteínas citoplásmicas sintetizadas *de novo* inducidas por auxinas; las flechas con puntas negras indican aquellas sintetizadas por insulina, y las flechas representan aquellas inducidas por PA.



DISCUSSION

Plants have two different modes of growth. Plant growth follows two different modes in response to various stimuli: cell elongation and cell division. The balance between these two growth modes depends on the stimulus, the type of target tissue and its developmental stage (Eckardt, 2005). Our results seem to be consistent with this proposal, since two growth regulators produced different biochemical events after targeting the same tissue in *Z. mays*, even when these effectors have some intermediates in common. Coleoptile elongation is well known as an auxin effect (Benjamins & Scheres, 2008). On the other hand, insulin as well as insulin-like growth factors such as ZmIGF have been recognized to exert mitogenic activity in plant tissues, inducing DNA synthesis in *Z. mays* seedlings, and cell division in both *Daucus carota* and *Z. mays in vitro* cell cultures (García et al., 2001; Yamazaki et al., 2003). The literature support the proposal that auxins might be considered growth stimulators by promoting cell elongation, whereas insulin might do so by preferentially stimulating cell division.

Biochemical differences between two signaling pathways. In general terms, the data presented here support a model for coleoptile growth through two different pathways induced either by auxin or by insulin. The interpretation of two different pathways induced by auxin or insulin is consistent with the different phosphorylation patterns of cytoplasmic proteins found after stimulation by each effector (Fig. 2). Furthermore, although both effectors stimulate S6rp phosphorylation, a different set of other specific phosphorylated products were identified for auxin and insulin (Fig. 3). More important and consistent with these differences are the specific rapamycin inhibition observed for insulin-induced S6rp phosphorylation, which was not observed when this phosphorylation was auxin-induced (Fig. 3). This indicates that in the first case the TOR pathway was activated (Fang et al., 2001; Hay & Sonenberg, 2004), whereas induction by auxin did not follow this path. An explanation for this difference might be the activation of two different ZmS6 kinase isoforms responsible for S6rp phosphorylation by each effector (Table 1, Fig. 4). This possibility is supported by the literature, indicating the presence of two S6K isoforms in many organ-

isms, one of which could be a nuclear enzyme (Panasyuk et al., 2006). The different S6rp phosphorylated products obtained after stimulation by either effector, as well as the different rapamycin sensitivity of S6rp phosphorylation observed in this research are consistent with this explanation (Fig. 3). In addition, there are reports indicating that S6K might also be activated by another kinase, closely related to the TOR pathway, namely PDK1, which is Ca^{2+} dependent activated (Bögre et al., 2003). Since it is known that auxin induces a cytoplasmic Ca^{2+} increase, after cell stimulation (Macdonald, 1997), these data suggest that auxin-induced S6 kinase activation in *Z. mays* coleoptile might be due to this second pathway. On the other hand, our results indicate that insulin-activated ZmS6K was mostly phosphorylated via TOR activation, since S6rp phosphorylation showed to be sensitive to rapamycin inhibition (Fig. 3). Previous results have also demonstrated that a TOR-S6K signaling pathway is functional in *Z. mays* during germination, targeting the translational apparatus through a TOR kinase that is inhibited by rapamycin (Dinkova, 2007). Interestingly, auxin, but not insulin or PA, induced phosphorylation of low molecular weight ribosomal proteins (Fig. 3A, 14 kDa approximately), which most likely correspond to acidic phosphoribosomal proteins (ARP) (Montoya et al., 2002; Santos et al., 2004). These proteins are known to play a central role in the regulation of translation due to their interaction with elongation factors (Vard et al., 1997; Santos et al., 2004). These data are consistent with the differential functional proteomics observed on the 2D-gels of *de novo* synthesized proteins induced by each effector: auxin or insulin (Fig. 4), which induced distinctive sets of synthesized proteins.

Auxin, as well as insulin induced an increase of PA levels. However, insulin stimulation was higher than that produced by auxin (Fig. 1). In addition, insulin and PA effects on cytosolic protein phosphorylation, *de novo* protein synthesis and ribosomal protein phosphorylation (among them S6rp) were more similar between them than to those produced by auxin. Moreover, rapamycin sensitivity of PA-induced S6rp phosphorylation supports the participation of PA as a second messenger of insulin action involving the TOR pathway in plants, as it was demonstrated to occur in metazoans (Fang et al., 2001). However, PA and insulin responses were not identical, possibly indicating the participation of PA as an intermediary of other pathways induced by other hormones or stresses. PA participation as an intermediary of auxin action requires further studies.

Overall, the present research contributes to the understanding of the possible mechanisms involved in plant growth regulation. The data support the proposal that auxin and insulin triggered *Z. mays* coleoptile growth by distinctive mechanisms, through two independent signal transduction pathways. Even when both effectors share some intermediates, they do not seem to share neither the same target products nor induce the same downstream biochemical events, particularly on the synthesis of proteins.

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