Changes in phosphorylation of 50 and 53 kDa soluble proteins in graviresponding oat (Avena sativa) shoots

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Received 12 April 2002; Accepted 6 November 2002

Abstract
The present work indicates that phosphorylation of a 50 kDa soluble protein is involved in the gravitropic response in graviresponsive pulvini of oat (Avena sativa) stems. This 50 kDa protein shows a differential pattern of phosphorylation between lower and upper halves of pulvini both in vivo and in vitro. The differential phosphorylation of this protein is detected only when stem segments are gravistimulated for short and long time periods. The differential phosphorylation of the 50 kDa protein occurs as early as 5 min after the initiation of gravistimulation. This corresponds closely to the presentation time of 5.2 min. This differential phosphorylation pattern was changed by treatments with cycloheximide, implying that a newly-synthesized protein is involved in the differential phosphorylation during the gravitropic response. An autophosphorylation experiment shows that the 50 kDa protein has kinase activity. The phosphorylation patterns of a 53 kDa protein were similar to those of the 50 kDa protein, but were only expressed in vitro. These findings indicate that the differential phosphorylation of the 50 (and 53 kDa) soluble proteins in graviresponding oat shoots may be an important component of the gravity signal transduction pathway.

Key words: Avena sativa, gravitropism, protein phosphorylation, pulvini.

Introduction
It has been well-established that protein phosphorylation reactions are important components of signal transduction cascades (Hirt, 1997) and that the regulation of such reactions is central to an understanding of cellular control mechanisms in micro-organisms, animals, and plants (Cohen, 1992; Hardie, 1999). In plants, such reactions appear to be important functional components of sucrose synthesis (Huber et al., 1996; Toroser and Huber, 1997), stomatal guard cell opening and closing (Hwang and Lee, 2001; Mori and Muto, 1997), cell expansion (Wagner and Kohorn, 2001), phototropism in shoots (Short et al., 1992; Salomon et al., 1997), seed germination (Anil et al., 2000; Ritchie and Gilroy, 1998), reactions of plants to osmotic stress (Hoyos and Zhang, 2000), salt stress (Guo et al., 2001; Kiegerl et al., 2000), low temperature stress (Martin and Busconi, 2001), fungal elicitor action (Adam et al., 1997; Romeis et al., 2000; Zhang et al., 1998), wounding reactions (Chico et al., 2001), and plant hormone actions (e.g. gibberellins) (Gómez-Cadenas et al., 2001). Several reports also indicate that protein phosphorylation plays a central role in the gravitropic response mechanism in plants. In this connection, Lu et al. (1996) demonstrated that calcium/calcmodulin-dependent protein kinase might be involved in the gravitropism of maize roots in response to a light signal, and recently, Rashotte et al. (2001) showed that a protein phosphatase was involved in the regulation of auxin transport in graviresponding Arabidopsis roots.

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Abbreviations: CHI, cycloheximide; OA, okadaic acid; STA, staurosporine.
In previous work, Chang and Kaufman (2000) showed that the patterns and levels of expression of several phosphoproteins change in response to gravistimulation of oat (Avena sativa) shoot pulvini. The results showed that two soluble proteins (38 and 50 kDa) and two membrane proteins (63 and 70 kDa) were differentially phosphorylated in lower as compared with upper halves of gravistimulated pulvini. The 50 kDa phosphoprotein exhibits a clear pattern of differential phosphorylation upon both short- and long-term gravistimulation as compared with the 38 kDa and two membrane proteins, which do not. Inhibitors of protein phosphorylation and dephosphorylation also affect the expression of these proteins. NaF, a non-specific inhibitor of phosphoprotein phosphatase, affects the phosphorylation patterns of all four proteins. In addition, the differential phosphorylation of the two soluble proteins was changed both in vivo and in vitro following treatment with okadaic acid, a specific inhibitor of protein phosphatase type 1 and type 2A, and with staurosporine, a protein kinase inhibitor. Taken together, it was concluded that the four phosphoproteins are likely to be involved in the gravitropic response signal transduction cascade in oat shoot pulvini.

These results led to the current investigation being undertaken in order to characterize further the nature of the changes in the 50 kDa phosphoproteins elicited by gravistimulation treatment of oat shoot pulvini. For this purpose, the relationships between several gravitropic-curvature kinetic parameters and the differential phosphorylation were explored and the patterns of the differential phosphorylation were examined more closely in the presence or absence of the gravistimulation. It is shown here that the in vivo and in vitro differential phosphorylations of this protein are detected only when stem segments are gravistimulated, either for short and long time periods. The differential phosphorylation occurs as early as 5 min after the initiation of gravistimulation, which corresponds closely to the presentation time of 5.2 min. Taken together, the results, including previous works, raise the possibility that the 50 kDa phosphoprotein is involved in the gravitropic response.

**Materials and methods**

**Chemicals**

[$\gamma^3$P] ATP (specific activity: 185 TBq mmol$^{-1}$) and okadaic acid were obtained from Amersham Life Science Inc. (Cleveland, OH 44128, USA) and Calbiochem-Novabiochem Co. (San Diego, CA 92121, USA), respectively. [$^{32}$P] phosphoric acid was obtained from New England Nuclear Co. (Boston, MA 02118, USA), and other fine chemicals from Sigma Chemical Co. (St Louis, MO 63178, USA).

**Plant materials**

Seeds of oat (Avena sativa cv. ‘victory’) were obtained from Svalöf, International, A. B. (Swedish Seed Association), Svalöf, Sweden. Greenhouse-grown plants were illuminated with 400 W high pressure sodium vapour lamps (PE Lighting Systems, Grimsby, Ontario, Canada) giving a light intensity of 1000 µmol m$^{-2}$ s$^{-1}$ at the tops of the plants. The photoperiod regime was 18/6 h light/dark for each 24 h cycle and the greenhouse temperature was set at 27/23 °C day/night (Brock et al., 1989). At 45 d, shoots were harvested by cutting them off just above the roots and placing the shoots vertically in a bucket containing c. 10 cm of water. The same day, 9 cm stem segments were excised from top portions of the shoots. Each stem segment consisted of 3 cm stem tissue, a graviresponsive leaf-sheath pulvinus (designated as the ‘p-1 pulvinus’), the next-to-last formed pulvinus, located just below the last-formed peduncular pulvinus (designated as the ‘p pulvinus’) subtending the panicle inflorescence, and 6 cm of leaf sheath/stem tissue above the pulvinus. As they were cut, stem segments were kept in a vertical position in a 150 ml beaker containing 20 ml distilled water. When the beaker was packed with segments, it was placed in a refrigerator at 4 °C. Under these conditions, the gravitropic responsiveness of the segments did not decrease for up to 1 week.

**Inhibitor pretreatments, gravistimulation treatment protocol and measurement of gravitropic curvature**

Pulvini of oat stem segments were gently abraded with an aqueous paste of silicic acid by rotating them three or four times between thumb and forefinger. Using a mild vacuum, abraded stem segments were infiltrated for 2 min with a solution containing 0.1 M sucrose and 50 mM HEPES-NaOH buffer (pH 7.5) in the presence or absence of solutions of cycloheximide (CHI) or LaCl$_3$. The segments were then kept in a vertical position for 10 min or 2 h in the solution containing CHI or LaCl$_3$, respectively, in the dark at room temperature.

For gravistimulation treatment, 80 stem segments were placed side by side between paper towels saturated with 0.1 M sucrose and 50 mM HEPES-NaOH (pH 7.5) and held in a horizontal position between two glass plates. The upper side of each segment was marked with a dot, using a blue Sharpie$^{\text{TM}}$ pen. The p-1 pulvini were placed just outside the edges of the plates so that upward bending was unimpeded. The glass plate ‘sandwich’ was then placed in a Plexiglas$^{\text{TM}}$ box. The Plexiglas box was covered by placing an equal-sized Plexiglas box in an inverted position on top of the lower box. 100% relative humidity was maintained by the addition of 1 cm water to the bottom of the lower tray. After various times of incubation in the dark at 25±1 °C, the gravitropic response of p-1 pulvini of the stem segments was determined by measuring degrees of negative gravitropic curvature with a protractor.

In order to determine the presentation time necessary to elicit a gravitropic response, oat stem segments were prepared as above, then gravistimulated in the dark at 25±1 °C for 5, 10, 15, and 20 min. Six to ten segments were used for each gravistimulation time. Immediately after each gravistimulation period, the segments were positioned horizontally, 5.5 cm from the centre of rotation, on a clinostat rotated at 2 rpm. This gives a gravity compensation value of 2.5×10$^{-5}$ g. This was accomplished by positioning the segments around the rim of a 250 ml beaker containing two large foam plugs saturated with 0.1 M sucrose. Angles of bending were measured after 24 h of clinorotation. A plot of gravitropic curvature versus the gravistimulation time was made and a regression line was calculated. The intercept of the extrapolated regression line and x-axis was taken to be an estimate of the presentation time. The equation for the regression line is $y=0.181x-0.943$.

**Extraction of soluble proteins**

All steps were performed on ice. About 0.2 g (fresh weight) samples of oat pulvini per treatment were powdered in liquid N$_2$ with a precooled mortar and pestle. Powdered tissue was homogenized with 1 ml of extraction buffer containing 50 mM TRIS-Mes (pH 7.7), 0.25 M sucrose, 1 mM MgCl$_2$, 3 mM EDTA, 2.5 mM DTT,
In vivo protein phosphorylation

For each treatment, 30 or more oat shoot pulvini were used. Pulvini of the oat stem segments were gently abraded with an aqueous paste of silicic acid by rotating them three or four times between the thumb and forefinger. Using a mild vacuum, abraded stem segments were infiltrated for 2 min with a solution containing 1 mM unlabelled orthophosphate, 0.2 mCi $^{32}$P labelled phosphoric acid (100 $\mu$l, 8500 Ci mmol$^{-1}$) 0.1 M sucrose, and 50 mM HEPES-NaOH buffer (pH 7.5). The segments were then placed in a vertical position in the same solution for 2 h in the dark at room temperature prior to gravistimulation treatment. To remove non-specifically bound $^{32}$P labelled phosphoric acid, the segments were washed with distilled water and 1 mM phosphoric acid in 50 mM HEPES-NaOH buffer (pH 7.5). Stem segments were gravistimulated for 0.5 and 8 h. Pulvini from these segments were cut into upper and lower halves. For the vertical control, pulvini from vertically positioned segments were cut into ‘left’ and ‘right’ halves. Total soluble proteins were isolated from the halved pulvini as described below and 100 $\mu$g proteins for each treatment were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). The gels were stained with 0.1% (w/v) Coomassie blue, destained and dried. After being exposed to Hyperfilm-MP (Amershem) films for 1 or 2 d at −80 °C, the films were developed in an X-Omat X-ray film processor unit (Eastman Kodak Co., Rochester, NY).

In vitro protein phosphorylation

After gravistimulation and/or inhibitor treatment, pulvini were cut into upper and lower halves. For the vertical control, pulvini from vertically positioned segments were cut into ‘left’ and ‘right’ halves. For each of the phosphorylation experiments, 70 $\mu$g proteins were used. Phosphorylation reactions were started by the addition of 1 $\mu$Ci ($^{32}$P) ATP (50 $\mu$U, made to a specific activity of 50 Ci mmol$^{-1}$ with unlabelled ATP) and run for 2 min in 50 mM HEPES-NaOH buffer (pH 7.5) at room temperature. The reactions were stopped by the addition of electrophoresis sample buffer. The phosphorylated proteins were separated by SDS-PAGE. Autoradiography was performed as described above. The phosphorylation levels were quantified with a Luminiscent Image Analyser (Fuji Photo Film Co., Ltd., Tokyo, Japan) and analysed with an Image Reader LAS-1,000, version 1.0 program (Fuji Photo Film Co., Ltd.) using developed X-ray films.

Detection of protein autophosphorylation

Autophosphorylation of proteins was carried out as described by Li and Assman (1996), based on the method of Kameshita and Fujisawa (1989). Extracted soluble proteins were electrophoresed on a 10% SDS-PAGE gel. In order to remove SDS from the gels, they were washed twice with a solution containing 50 mM TRIS-HCl (pH 8.0) and 20% (v/v) 2-propanol for 1 h each. The gels were washed twice again with a solution containing 50 mM TRIS-HCl (pH 8.0) and 5 mM 2-mercaptoethanol for 1 h each. The proteins in the gels were denatured in a solution containing 6 M guanidine-HCl, 50 mM TRIS-HCl (pH 8.0) and 5 mM 2-mercaptoethanol for 1 h. For renaturation of proteins, the gels were incubated in a solution containing 50 mM TRIS-HCl (pH 8.0), 5 mM 2-mercaptoethanol and 0.04% (v/v) Tween 20 for 18 h at 4 °C. The gels were equilibrated in a solution containing 40 mM HEPES-NaOH (pH 7.5), 1 mM EGTA, 20 mM MgCl$_2$, and 2 mM DTT for 30 min at 30 °C. The proteins were phosphorylated by incubating the gels in 10 ml solution containing 40 mM HEPES-NaOH (pH 7.5), 1 mM EGTA, 20 mM MgCl$_2$, 2 mM DTT, and 70 $\mu$Ci ($^{32}$P) ATP for 1 h at 30 °C. Non-reactive ($^{32}$P) ATP was removed with five changes of 5% (w/v) trichloroacetic acid and 1% (w/v) sodium pyrophosphate for 5 h. All steps were performed at room temperature unless otherwise mentioned.

Results

In vivo and in vitro protein phosphorylation

In order to discover proteins that show gravity-induced in vivo differential phosphorylation between upper and lower halves of pulvini, oat shoot pulvini were fed with $^{32}$P-labelled phosphoric acid and then gravistimulated. Since proteins from ‘right’ and ‘left’ halves of vertically positioned segments showed the same patterns of phosphorylation in most experiments, only one vertical control per treatment is shown here. An 89 kDa protein showed similar levels of phosphorylation between gravistimulated and vertical control pulvinus halves (Fig. 1A; Table 1). In the case of a 50 kDa and a 57 kDa protein, the phosphorylation level of these proteins in the vertical control was almost the same as that in the lower halves of gravistimulated pulvini. The figure also shows that a greater amount of phosphorylation of a 50 and a 57 kDa protein occurs in upper halves than in lower halves of gravistimulated pulvini at both 0.5 and 8 h after the initiation of gravistimulation treatment. Thus, the differential phosphorylation of these two proteins was observed in vivo and at both short (0.5 h) and long (8 h) times after the start of gravistimulation treatment.

In light of the results of the in vivo protein phosphorylation experiments, in vitro protein phosphorylation experiments were performed, using pulvinus halves gravistimulated for 0.5 and 8 h. According to a report by Short et al. (1992), it was assumed that a protein would be less phosphorylated in vitro if the protein was phosphorylated to a greater extent in vivo or vice versa. As shown in Fig. 1B (see also Table 1), no phosphorylation was found in the 89 and 57 kDa proteins that was clearly shown for in vivo protein phosphorylation (Fig. 1A). Instead, phosphorylation of a 65 kDa protein was evident, although no difference in phosphorylation level was found between pulvinus halves that were gravistimulated up and down and those of the vertical control. Only a 50 kDa protein showed differential phosphorylation under both in vivo and in vitro conditions. More phosphorylation of the 50 kDa protein was found in lower pulvinus halves than in upper halves or in vertical control halves. The differential phosphorylation of the protein between upper and lower pulvinus halves was exactly opposite that which occurred in vivo. This supports the assumption described above. However, the...
The phosphorylation pattern of the protein from the vertical control pulvini seems to be controversial since the phosphorylation level of this protein is similar to that of lower pulvinus halves in vivo, while the level is similar to that of upper ones in vitro. A possible explanation is that phosphorylation sites of the proteins are mostly occupied (or saturated) with phosphate meioties in vertical control pulvinus halves than in lower halves of pulvini gravistimulated during in vivo phosphorylation. It was also found that the phosphorylation pattern of a 53 kDa protein was almost the same as that of the 50 kDa protein. Although the level of phosphorylation was very weak, and differential phosphorylation was not found in vivo, a 38 kDa protein showed more phosphorylation in the upper halves of pulvini only at 0.5 h after the start of gravistimulation treatment. Taken together, these results show that the differential phosphorylation of the 50 kDa protein occurs both in vitro and in vivo upon gravistimulation. This raises the possibility that phosphorylation of the 50 kDa protein is related to the gravitropic response. Based on these results, more work was undertaken in order to investigate this possibility further. Since the patterns of in vitro phosphorylation of the 50 kDa protein reflect those which occurred in vivo, and were consistently observed, further work on in vitro protein phosphorylation was undertaken.

**Differential phosphorylation is shown to occur for two soluble proteins (50 and 53 kDa in size) during the gravitropic response**

In order to test the hypothesis posed in the introduction, it was necessary to determine the kinetics of gravitropic curvature of excised oat shoots. This would give an insight into the most critical times to collect pulvini for in vitro protein phosphorylation analyses. Figure 2A indicates the long-term kinetics of gravitropic curvature for p-1 pulvini in excised oat shoots. Upward bending is first evident after 60 min of gravistimulation. The fastest rate of bending occurs between 1 and 8 h.

In order to learn whether or not gravistimulation of oat shoot pulvini elicits changes in the amounts of phosphorylation of the 50 and 53 kDa soluble proteins, phosphorylation patterns were compared for these proteins in gravistimulated pulvini. Figure 3A shows that differences of phosphorylation levels of 50 and 53 kDa proteins occur between upper and lower halves of pulvini during the gravitropic response, where there is significantly more phosphorylation of these proteins in lower halves than in upper halves. The phosphorylation level of the vertical control was greater than (0–3 h upon gravistimulation) or similar (from 8–48 h upon gravistimulation) to that of lower halves and is consistent with slight changes over the...
period. It can also be seen that the differences in amounts of phosphorylation start to appear fairly early during gravitropic bending, namely, 1 h after the initiation of gravistimulation treatment. The result shown in Fig. 3B indicates that differences in phosphorylation levels of the 50 and 53 kDa proteins between the upper and lower halves of pulvini persist as long as a gravistimulus is present.

The 50 and 53 kDa phosphoproteins are early responsive and specific to gravistimulation treatment

Determination of the presentation time needed to elicit a gravitropic curvature response is also germane to these studies. It is possible that these differential phosphorylation events will occur before this time, and others after it, up to the time and following initiation of upward bending. Figure 2B is a plot of curvature for stem segments gravistimulated for different lengths of time up to 30 min. From this plot, the presentation time for the gravitropic response in p-1 pulvini of excised oat stem segments is estimated to be c. 5.2 min (0.943/0.181). This means that the least possible length of time needed for gravistimulation treatment to elicit an upward bending response is about 5.2 min.

In order to analyse the kinetics of early phosphorylation changes for the 50 and 53 kDa proteins more precisely, stem segments were gravistimulated for different lengths of time up to 30 min. In Fig. 4A and B, the 50 and 53 kDa proteins show that greater amounts of phosphorylation occurred in lower halves of pulvini than in upper halves from 10 min after the initiation of gravistimulation treatment. Such differential phosphorylation was also observed at 5 min after the start of gravistimulaiton treatment, although the differences in the amount of phosphorylation between the proteins from upper and lower halves were not so large. In addition, at that time, the phosphorylation level is very low in both halves of pulvini when compared with that at 10 and 30 min after the initiation of gravistimulation treatment (Fig. 4A). The phosphorylation level of vertical control halves was consistent over the gravistimulation period and was greater (before 30 min) than or similar (at 30 min) to that of lower halves. It is apparent from these results that differential phosphorylation of the 50 and 53 kDa proteins starts

Table 1. Comparison of phosphorylation patterns of soluble proteins in oat shoot pulvini upon gravistimulation

<table>
<thead>
<tr>
<th>Proteins</th>
<th>In vivo</th>
<th>In vitro</th>
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<tbody>
<tr>
<td>89 kDa</td>
<td>Phosphorylation detected</td>
<td>Phosphorylation detected</td>
</tr>
<tr>
<td>65 kDa</td>
<td>Phosphorylation detected</td>
<td>Differential phosphorylation</td>
</tr>
<tr>
<td>57 kDa</td>
<td>Differential phosphorylation</td>
<td>Differential phosphoryation</td>
</tr>
<tr>
<td>53 kDa</td>
<td>Differential phosphoryation</td>
<td>Differential phosphoryation</td>
</tr>
<tr>
<td>38 kDa</td>
<td>-</td>
<td>Differential phosphoryation</td>
</tr>
</tbody>
</table>

Fig. 2. Time-course (A) for gravitropic curvature and presentation time for gravitropic curvature in oat shoot pulvini (B). In (A), stem segments were gravistimulated for various periods up to 48 h, as indicated in the graph. In (B), the intercept of the regression line and the x-axis is estimated to be the presentation time (5.2 min; see arrow). Vertical bars represent standard errors and standard deviation of means from five (A) and two (B) experiments, respectively.
around, and is evident after, the presentation time of 5.2 min.

The next question one may ask is this: can the early differential phosphorylation of the 50 and 53 kDa proteins be nullified if the gravistimulus is removed? This question is based on the fact that differential phosphorylation of these proteins was found for the entire time that oat stems were gravistimulated (Fig. 3). In order to answer to this question, protein phosphorylation assays were performed by using proteins from stem pulvini that were gravistimulated for 5 min, then positioned vertically for 5–25 min (Fig. 4B). The differences in phosphorylation of the 50 and 53 kDa proteins between lower and upper halves, that might be elicited during 5 min of gravistimulation treatment, continued for 5 min (see 10 min), decreased during the next 10 min (see 15 min), and then were absent by 25 min (see 30 min) after stems were positioned vertically. Once again, the phosphorylation level of vertical control halves was consistent over the gravistimulation period; namely, it was greater than (before 30 min) or similar to (at 30 min) that of lower halves. These results indicate that differential phosphorylation of these proteins is gravi-specific at an early stage of the gravitropic response; that it continues only for a short time (c. 5 min) after a gravistimulus is given to the pulvini; and for it to persist, the gravistimulus must be provided continuously.

Effects of LaCl₃ and cycloheximide (CHI) on the gravitropic response and extent of phosphorylation of the 50 and 53 kDa soluble proteins

Since LaCl₃ decreases the gravitropic response (Table 2) and starch content in chloroplast gravisensors in graviresponding pulvini (Chang et al., 2001), it was hypothesized that LaCl₃ may also block or reduce the differential phosphorylation in the pulvini. After oat shoots were treated with 10 mM LaCl₃, they were gravistimulated for 30 min. Then, soluble proteins were extracted for determination of the phosphorylation reaction. As shown in Fig. 5B, LaCl₃ nullified gravity-induced differential phosphorylation of the 50 kDa and 53 kDa soluble proteins. Once again, the notion that the differential phosphorylation is gravi-specific is supported by this inhibitory effect elicited by LaCl₃.

It is possible that the differential phosphorylation of the 50 and 53 kDa proteins between upper and lower halves of graviresponding pulvini is due to de novo synthesis of these or other related proteins. This possibility was tested by pretreatment with cycloheximide (CHI). CHI is a well-known inhibitor of protein synthesis. It also significantly decreases the gravitropic curvature of oat shoot pulvini (Table 2). The phosphorylation of the 50 and 53 kDa protein was increased in upper halves of gravistimulated pulvini by CHI, resulting in the disappearance of the differential phosphorylation in graviresponding oat shoot pulvini. This result suggests that a protein(s) that is responsible for the differential phosphorylation of the two proteins is synthesized de novo upon gravistimulation.

The 50 kDa protein autophosphorylates

Autophosphorylation experiments were performed in order to gain an insight into the function(s) of the 50 kDa protein. The result of this reaction reveals that there are at least three proteins that could phosphorylate themselves. Only the 50 kDa protein showed an increased level of phosphorylation after 5 min, with more occurring in upper halves than in lower halves of the pulvini, while the 65 kDa and 57 kDa proteins did not (Fig. 6). This pattern of
autophosphorylation is similar to that of in vivo phosphorylation (Fig. 1A). However, such evident differential autophosphorylation was not so clear when oat segments were gravistimulated for 10 and 30 min and for a longer period, namely, 8 h (data not shown). From these results, and those shown in Figs 3 and 4, the phosphorylation status and autophosphorylation activity of the 50 kDa protein is thought to be changed simultaneously at early stages of the gravitropic response.

**Discussion**

In light of previous results presented by Chang and Kaufman (2000), further characterization of the 50 kDa phosphoprotein was performed in relation to the gravitropic response. Results of a previous study show that the 50 kDa phosphoprotein exhibits a much clearer pattern of differential phosphorylation upon both short- and long-term gravistimulation as compared with the 38 kDa and two membrane proteins. These findings led to the current investigation being focused on the 50 kDa phosphoprotein by examining several gravitropic-curvature kinetic parameters, by analysis of in vitro phosphorylation, and by using additional inhibitors, for example, cycloheximide and lanthanum ion.

The results obtained in the present study and from previous work (Chang and Kaufman, 2000) raise the possibility that the 50 kDa soluble phosphoprotein is involved in gravity signal transduction. The evidence in support of this contention is as follows: first, in relation to the gravitropic response, the activity of this protein is manifested via phosphorylation, like many other proteins involved in signal-transduction pathways. Second, differential phosphorylation of the 50 (and 53) kDa proteins between upper and lower halves of pulvini is detected only when a gravistimulus is applied (Figs 3A, 4). Third, the differential phosphorylation occurs as early as around the presentation time of the gravitropic response. This means that a minimum time of 5 min of gravistimulation is needed to elicit the differential phosphorylation event (Fig. 4A). Fourth, the autophosphorylation activity of the 50 kDa protein changes differentially, also around 5 min after the initiation of gravistimulation treatment. This suggests that the protein may be a kind of protein kinase (Fig. 6).

Since the 53 kDa protein showed a pattern of in vitro differential phosphorylation similar to that of the 50 kDa protein (from Fig. 3 to Fig. 5), one may ask if the two proteins represent a doublet of a protein, or if they are different from each other. The results of in vitro phosphorylation and autophosphorylation showed that only the 50 kDa protein exhibits a phosphorylation pattern, while the 53 kDa protein did not. Further, results of isoelectric focusing showed that pI of the 50 kDa protein is near 7, while that of the 53 kDa protein is near 9 (data not shown). Taken together, these results indicate that the two proteins do not constitute a doublet of a single protein; rather, they are distinctly different from each other.

One question posed in the present study is whether or not the differential phosphorylation of the 50 and 53 kDa proteins that occurs during the early stages of the gravitropic response is due to de novo synthesis of new proteins? Results shown in Fig. 5A indicate that differential phosphorylation of the 50 and 53 kDa proteins induced by gravistimulation is changed by cycloheximide, a protein translation inhibitor. Cycloheximide is thought to inhibit the synthesis of protein kinase(s) or of protein(s) that inhibit the protein phosphatase(s) action. This raises the possibility that de novo synthesis of a protein(s) that can dephosphorylate these two proteins is required during the early stages of the gravitropic response.

Calcium is a well-known second messenger in the signal-transduction cascade (Poovaiah and Reddy, 1993;

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**Table 2. Effects of inhibitors on the gravitropic response in oat shoots**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Gravitropic curvature (degrees)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0.5 h</td>
</tr>
<tr>
<td>Control</td>
<td>3.1±0.5</td>
</tr>
<tr>
<td>+ 10 mM CHI</td>
<td>2.6±0.4</td>
</tr>
<tr>
<td>+ 10 mM LaCl₃</td>
<td>1.7±0.5</td>
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Oat stem segments were abraded, vacuum-infiltrated for 2 min in the presence or absence of inhibitors at the concentrations indicated in the table, then placed in the dark in the same solutions (±inhibitors) for 2 h at 25±1 °C. The stem segments were then gravistimulated for 0.5 or 24 h at 25±1 °C in the dark, and simultaneously, were fed 0.1 mM sucrose. The values are expressed as averages ±SE from three trials. N=18 segments per treatment.
In this connection, it has been reported by Gehring et al. (1990) that calcium levels increase more in the lower halves of maize coleoptiles than in upper halves following gravistimulation treatment. How such gravity-elicited calcium asymmetry functions as a second messenger in gravity signal transduction in maize seedlings is still unknown. Some insights into the function of calcium in this process may come from results on oat shoot pulvini. When treated with LaCl$_3$, a calcium channel blocker, gravitropic curvature is significantly reduced (Table 1). At the same time, it also nullifies the differential phosphorylation of the 50 and 53 kDa proteins (Fig. 6B) that occurs in control gravistimulated pulvini. These results can be regarded as evidence for the possible involvement of calcium in the differential phosphorylation process. However, calcium chelators, such as EGTA and BAPTA, do not exhibit any inhibitory effect on gravitropic curvature and differential phosphorylation (data not shown), while calcium channel blockers, such as verapamil and ruthenium red, reduce gravitropic curvature (Chang et al., 2001). In addition, because of the absence of any direct measurements of calcium levels in the cytosol or in cell organelles, there can be no speculation as to the possible role of the two phosphoproteins in oat shoot pulvini in the phosphorylation. Instead of direct involvement in the process, LaCl$_3$ seems to reduce the differential phosphorylation by inhibiting settlement of starch statoliths, the gravisensors in oat shoot pulvini (Chang et al., 2001).

Only the 50 kDa soluble protein shows changes in autophosphorylation activity at 30 min after the initiation of gravistimulation treatment (Fig. 6). This result suggests that this same 50 kDa protein is differentially phosphorylated and autophosphorylated upon gravistimulation treatment. It is well known that many kinds of protein kinases have autophosphorylation activity (Stone and Walker, 1995). Using an in gel assay method, preliminary results show that less Histone III-s kinase activity of the 50 kDa soluble protein is detected in upper than in lower halves of oat shoot pulvini at 5 min after the initiation of gravistimulation (data not shown). Taken together, these results suggest that the 50 kDa soluble phosphoprotein may be a protein kinase. It is still not known what functional role autophosphorylation of the 50 kDa protein plays in the gravitropic response mechanism. One possible explanation is that the 50 kDa protein may activate itself by autophosphorylation once the protein is phosphorylated by gravistimulation treatment (compare Figs 1A, 6).

It is clear now that the graviresponsive 50 kDa phosphoprotein must be purified and sequenced. Once this information is in hand, the nature of its gene can be determined. With the use of RNA antisense inhibition and DNA microarray technology (Kaufman et al., 2003), it may then be possible to unravel its function(s) in the...
gravity signal transduction process in oat shoot pulvini. In addition, it will be important to learn which protein kinases and phosphoprotein phosphatases are involved in phosphorylation and dephosphorylation, respectively, of this protein.

Acknowledgements

The authors thank Laura Olsen, Sang Ho Jeong and David Labman from University of Michigan and Sonia Philosoph-Hadas from the Vocaci Institute, Bet Dragon, Israel for suggestions and discussions in connection with these studies. We also thank the unknown reviewer of our paper for an excellent critique and many helpful suggestions. The oat plants used for the experiments were grown by Michael Palmer at the University of Michigan Matthaei Botanical Gardens. This work is supported by Grant Nos IS-2434-96 and IS-3133-99 from BARD, the United States-Israel Binaional Agricultural Research and Development Fund (to PBK, SCC) and by R01-1999-000-00075-0 (to S-KK) and 98-0401-08-002-3 (to MCH) from KOSEF.

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