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# Destiny of a 57 Kilodalton Protein in Maize Genotypes Responding to Heat Shock and Pathogenic Infections

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**ABSTRACT** : In an effort to elucidate the mechanism of redistribution of a 57 KDa protein from chloroplasts to cytoplasmic polysomes when maize seedlings encountered a stress, maize genotypes resistant or susceptible to *Bipolaris maydis* were used for evaluation under inoculated or heat-shocked conditions. Western blot analysis indicated that the redistributed 57 KDa protein caused by a treatment with pathogens or heat shock was identical. When maize seedlings were resistant to pathogens, little amount of this protein could be detected in the cytoplasmic polysome fraction; presumably, the 57 KDa protein remained localized in chloroplasts. Association of this protein with cytoplasmic polysomes in response to stresses is not unique to maize. A preliminary study employing a compatible *Phytophthora megasperma*-soybean interaction system indicated that soybean also responded to pathogenic stress in that the 57 KDa protein was detected in the preparation of cytoplasmic polysomes.

**KEY WORDS** : **Heat shock, Maize (*Zea mays* L), Pathogen, Polysome-associated-protein, Stress.**

## INTRODUCTION

A protein with molecular weight of 57 KDa was found to associate with cytoplasmic polysomes for inhibiting translation when maize seedlings were inoculated with pathogens or treated with heat shock (Wu *et al.*, 1988). This protein formed an aggregate of 92 KDa when a polysome sample prepared from inoculated leaves was mixed with sample buffer and briefly heated in a boiling water bath, a heating condition customarily employed before electrophoresis. A heating period of longer than 20 seconds was necessary to break up the aggregate to give a protein band of 57 KDa on SDS polyacrylamide gels (Wu *et al.*, 1991). This protein was not *de novo* synthesized, instead it was localized in chloroplasts and redistributed to cytoplasmic polysomes in response to a stress (Wu *et al.*, 1991). However, the mechanism of redistribution remains unclear. In an effort to elucidate this mechanism, maize genotypes representing different degree of resistance to *Bipolaris maydis* were used for evaluation.

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*Bipolaris maydis* causes southern leaf blight of maize, and the resistance toward this pathogen is usually determined by polygenic inheritance (Payne and Yoder, 1978; Counter and Gracen, 1979), with *rhm* being a single recessive gene for nuclear resistance (Smith and Hooker, 1973). The sensitivity or insensitivity of maize toward T toxin produced by *B. maydis* race T is determined by a cytoplasmic gene which also determines male sterility (Hooker *et al.*, 1970; Gengenbach *et al.*, 1977). The other race of *B. maydis*, race O, shows no specificity to plant cytoplasms and is primarily a leaf pathogen. In this study, maize inbreds with normal cytoplasm were used to study interaction with *B. maydis* race O.

## MATERIALS AND METHODS

### Plant Materials

Seeds of maize (*Zea mays* L.) inbreds W64A, Mo17, B73, and B73 *rhm* mutant with normal cytoplasm were grown in moist vermiculite at 29±1 ℃ with a 14-h photo period. Seven-day-old seedlings were inoculated with conidial suspensions of *Bipolaris maydis* race O and *B. zeicola*, respectively, or heat-shocked at 40℃ for 2 h. *B. zeicola* was included in this study to compare its polysome profile with that of *B. maydis* because development of necrotic lesions on maize leaves usually takes a longer period of time inoculating with *B. zeicola.* For inoculation, conidial suspensions were adjusted to a concentration of $5 \times 10^5$ conidia $ml^{-1}$. Tween 80 (0.1% v/v) was added as a surfactant. The conidial suspensions (0.2 ml per plant) were sprayed onto the foliage evenly with a Devilbis atomizer. After inoculation, the seedlings were placed in a moist chamber at 29±1 ℃. Leaf tissues were harvested at 3, 6, 12, 24, and 48 h after inoculation, frozen in liquid nitrogen, and stored at -70℃ before polysome isolation.

In a separate experiment, to study *Phytophthora megasperma*-soybean (*Glycine max* L.) interaction, 10-day-old seedlings of resistant and susceptible cultivars (kindly provided by Dr. K. L. Athow, Purdue University) grown in a greenhouse were inoculated by inserting mycelium into a longitudinal slit of hypocotyl (Lam-Sanchez *et al.*, 1968). Seedlings were harvested 48 h after inoculation and separated into hypocotyl, epicotyl, and foliage leaves, respectively, before freezing in liquid nitrogen and polysome isolation.

For the heat shock treatment, maize seedlings were kept in a growth chamber at 40℃ for 2 h, after which leaves were harvested and frozen in liquid nitrogen.

### Isolation of Polysomes

Total polysomes were isolated by a procedure described previously (Wu *et al.* 1988). Leaf tissues were ground in liquid nitrogen with a mortar and pestle, and homogenized subsequently with a polytron in 2 volumes of extraction buffer (200 mM Tris-HCl, pH 9.0, 400 mM KCl, 200 mM sucrose, 1% Triton X-100, 35 mM $MgCl_2$, 25 mM EGTA, and 1% 2-mercaptoethanol). The homogenate was strained through 4 layers of cheesecloth and centrifuged at 29,000 xg for 10 min. The total polysomes were pelleted from the supernatant (15 ml) through a 10 ml pad of 1.75 M sucrose (in 40 mM Tris-HCl, pH 9.0, 200 mM KCl, 30 mM $MgCl_2$, and 5 mM EGTA) by centrifugation at 260,000 xg for 60 min. The pellet

was resuspended in 0.1 ml of resuspension buffer (40 mM Tris-HCl, pH 8.5, 200 mM KCl, 30 mM $MgCl_2$, and 5 mM EGTA), rapidly frozen in liquid nitrogen, and stored at -70°C.

**Polyacrylamide Gel Electrophoresis of Ribosome-Associated Proteins**

Ribosome-associated proteins (RAPs) were analyzed by SDS polyacrylamide gel electrophoresis according to Laemmli (1970). Polysomes containing 1.2 $A_{260}$ unit were routinely mixed with five-fold concentrated sample buffer (the final concentration was 25 mM Tris-HCl, 190 mM glycine, pH 8.3, 2% SDS, 2% 2-mercaptoethanol, and 10% glycerol), and briefly heated in a boiling water bath before loading onto each well of a slab gel (12.5%). However, in some cases, a heating period of longer than 20 seconds was carried out in order to break up the 92 KDa component into 57 KDa protein. For some experiments, an exponential 10-16% gradient gel was used for better resolution. The protein bands were detected by staining with Coomassie brilliant blue R.

**Labeling and Extraction of Proteins Synthesized *in Vivo***

Proteins were synthesized *in vivo* by a procedure described previously (Wu *et al.*, 1988). Healthy, inoculated (24 h after inoculation with *B. maydis*) or heat-shocked (40 °C , 2h) maize seedlings were excised 1 cm below the primary leaf and placed singly into a vial containing 30 $\mu$Ci of $^3$H-leucine in water. The seedlings were incubated in a growth chamber at a temperature of 30 $\pm$ 2°C and with constant fluorescent light. After the solution had been taken up by the seedling, 5 mM phosphate buffer (pH 6.5) was added, and the seedling was incubated for a total time of 1 h. The leaves of each seedling were removed and ground in a mortar containing three volumes of extraction buffer (25 mM Tris-HCl, 200 mM glycine, pH 8.3, 2% SDS, 2% 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride). The extract was pipetted into an Eppendorf microcentrifuge tube and centrifuged at full speed on a table top centrifuge for 5 min. The supernatant was heated in a boiling water bath for 3 min, chilled, and centrifuged at 37,000 xg for 30 min. The supernatant containing proteins was stored at -70°C before analysis by gel electrophoresis.

**Protein Analysis by Fluorography and Western Blot**

Proteins prepared as described above were routinely mixed with five-fold concentrated sample buffer (the final concentration was 25 mM Tris-HCl, 190 mM glycine, pH 8.3, 2% SDS, 2% 2-mercaptoethanol, and 10% glycerol), heated for 20 seconds in a boiling water bath. The dissolved proteins were loaded onto each well at about 80,000 cpm per well. After electrophoresis, gels were fixed in 10% (w/v) TCA:10% (v/v) glacial acetic acid:30% (v/v) methanol, and radioactive protein bands were detected by fluorography (Larskey and Mills, 1975) with EN$^3$HANCE (New England Nuclear). Dried gels were exposed to Kodak XAR-5 film (Eastman-Kodak) at -70 °C for 4 days. Immunoblots were prepared as described by Towbin *et al.* (1979). Proteins transferred to nitrocellulose membranes were immuno-adsorbed with the 57KDa-specific antibodies and the destection was carried out by incubating in secondary antibody conjugated with alkaline phosphatase (Bio-Rad, Hercules, CA, USA).

# RESULTS AND DISCUSSION

## Symptom Development on Inoculated Leaves

Symptom development on leaves of W64A with normal cytoplasm following inoculation with *B. maydis* is shown in Figure 1. After inoculation, the disease progressed gradually on leaves. There was no visible symptom at 3 to 6 h after inoculation, chlorotic spots appeared 12 h after inoculation which progressed to necrotic lesions at 24 h. The lesions coalesced and leaves became blighted by 48 h after inoculation. Because susceptibility to the pathogen could be clearly identified at 24 h after inoculation, leaves harvested at this stage were routinely used for preparation of polysomes. Unlike *B. maydis*, inoculation with *B. zeicola* resulted in some chlorotic spots at 24 h and development of necrotic lesions at 48 h (data not shown).

Fig. 1. Symptom development on leaves of W64A inbred following inoculation with *B. maydis* race O. From left to right: uninoculated control, 3, 6, 12, 24, and 48 h after inoculation, respectively.

## Changes of Ribosome-Associated Proteins in Maize under a Stress

A previous study showed that a protein with molecular weight of 57 KDa was found to associate with polysomes when maize seedlings were inoculated with a pathogen (Wu *et al.*, 1988). This ribosome-associated protein (RAP) formed an aggregate and produced a molecular weight of 92 KDa when the polysome preparation was briefly heated in a boiling water bath. Heating with SDS buffer in a water bath for more than 20 seconds was necessary to break up the aggregate for producing the 57 KDa protein (Wu *et al.*, 1991). Since protein samples were heated for only a few second before application to slab gels in some of our preparations; thus the 57 KDa and 92 KDa components in this paper are interchangeably presented based on heating conditions.

Figure 2 shows the RAP patterns of W64A leaves following heat shock treatment or inoculation with pathogens. At 3 h (lane 2) and 6 h (lane 3) after inoculation with *B. maydis* race O, the RAP pattern was similar to that of the 12 h control (lane 1); however, 12 h after inoculation, there was an increase in intensity of a 92 KDa RAPs (lane 4). The intensity of this RAPs was increased further at 24 and 48 h (lanes 5 and 7, respectively) after inoculation. This change of RAP pattern was not associated with age, because it did not occur in the 48 h aged control (lane 6).

Inoculation of W64A with *B. zeicola* caused a similar alteration in the RAP pattern; however, the 92 KDa was detectable only after 48 h (lane 11). This observation agrees with symptom developments and confirms a previous study indicating *B. zeicola* is a weaker pathogen than *B. maydis* (Wu *et al.*, 1988). The 92 KDa RAP was also induced by heat shock for 2h at 40°C (lane 12). These RAP patterns derived from a brief period of heating before electrophoresis were basically similar to those obtained from a long period of heating with the exception that the 92 KDa component was replaced by 57 KDa (Wu *et al.*, 1988; 1991).

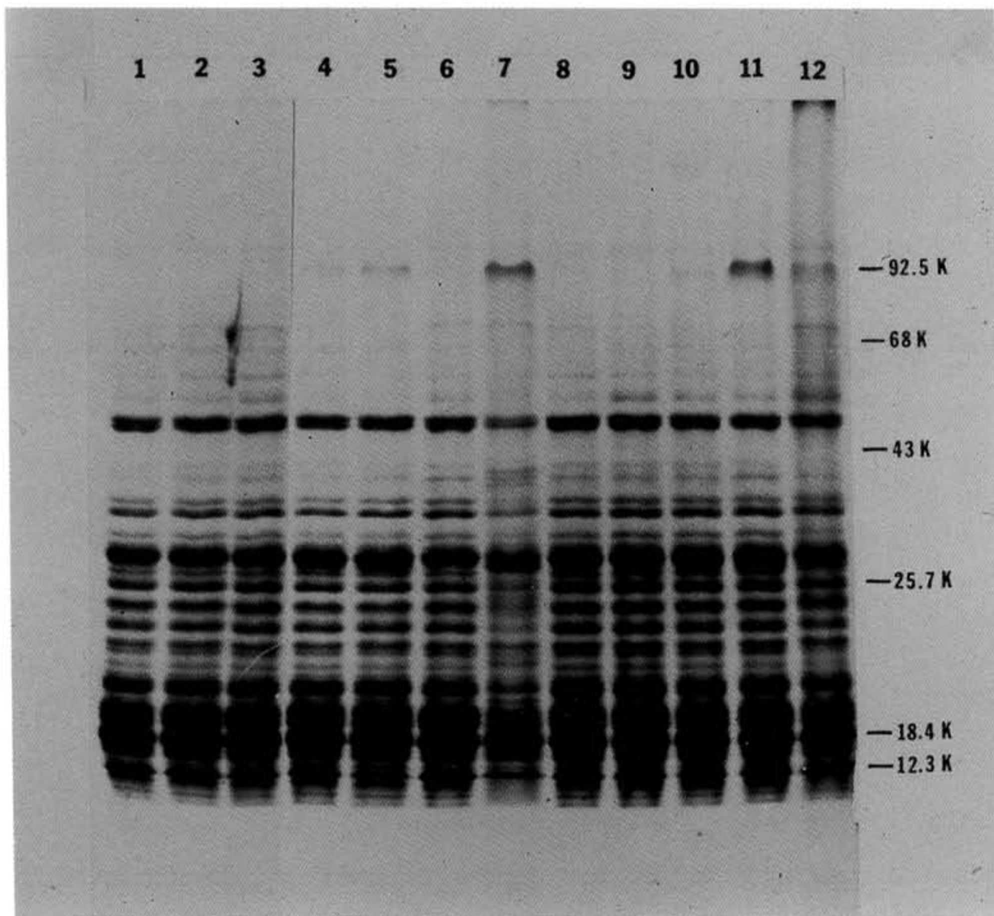


Fig. 2. SDS-polyacrylamide gel electrophoretic pattern of polysome-associated-proteins isolated from healthy, pathogen inoculated, or heat-shocked leaves. Polysome samples were heated briefly before loading onto each well of a slab gel (10-16% exponential gradient). Polysomes were isolated from healthy leaves (lanes 1 and 6 for the 12 h and 48 h controls, respectively); from leaves inoculated with *B. maydis* race 0 for 3 h (lane 2), 6 h (lane 3), 12 h (lane 4), 24 h (lane 5), and 48 h (lane 7); from leaves inoculated with *B. zeicola* for 6 h (lane 8), 12 h (lane 9), 24 h (lane 10), and 48 h (lane 11); or from leaves heat-shocked for 2h at 40°C (lane 12).

**Western Blot Analysis of The 92 KDa Component Caused by Pathogen and Heat Shock Treatments**

Our previous study indicated that the 57 KDa RAP was originally pre-existed in chloroplasts but redistributed to cytoplasmic polysomes in response to a pathogenic stress (Wu *et al.*, 1991). However, the origin of the 57 KDa RAP "induced" by heat shock was not clear, especially when several proteins have been shown to be induced by heat shock treatments (Key *et al.*, 1981; Baszczynski, *et al.* 1983). In order to study whether the 57 KDa RAP "induced" by pathogenic and heat-shock stresses are identical, extractions of total proteins (which include organelle proteins) were heated with SDS buffer in a boiling water bath for 20 seconds to break up the 92 KDa component before electrophoresis and analysis by the Western blot. This treatment was necessary otherwise the 92 KDa RAP might not be well separated from a heat shock induced polypeptide of 89 KDa (Baszczynski, *et al.* 1983).

When total proteins were extracted from leaves and subjected to a fluorographic analysis, two unique protein bands with molecular weight greater than the 57 KDa (position indicated by the arrow on SDS gel) were induced by a heat shock treatment at 40℃ for 2 h (Fig. 3, lane 2). The 57 KDa RAP could not be identified on the gel because all visible bands represented newly synthesized radioactive proteins extracted from the whole leaf tissue and, in addition, ribosomal proteins accounted for only a small proportion of the total protein preparation. However, when this same gel was subjected to analysis by immunoblot to detect all proteins that included pre-existed molecules before labeling, the 57 KDa RAP was clearly present in the control, heat treated, and inoculated samples (Fig. 4). This study confirms our previous studies demonstrating that this protein was pre-existed in chloroplasrts. Redistribution of the 57 KDa from chloroplasts to cytoplasmic polysomes took place when leaves encountered a stress. As shown in Figure 4, the 57 KDa RAP appears to contain two polypeptides of similar sizes; a small amount of the 92 KDa component was also present presumably due to an incomplete dissociation under the boiling condition described. Western blot analysis on ribosome-associated proteins indicated that the 57 KDa band "induced" by heat shock and pathogen treatments was indeed identical (data not shown).

**Association of 92 KDa with Polysomes Took Place Only under Stress Conditions**

Association of the 92 KDa RAP with polysomes was evaluated with monogenic and polygenic resistant maize genotypes under pathogenic and heat stress conditions. As shown in Figure 5, W64A, which is susceptible to *B. maydis*, accumulated the 92 KDa RAP in response to inoculation (lane 2) and heat shock treatments (lanes 3 and 4). A relatively more pronounced band derived from inoculation with *B. maydis* than heat shock was observed for W64A. B73 inbred, on the other hand, showed a more pronounced effect due to heat shock treatment (lane 6) than inoculation (lane 5). B73 *rhm*, which is a monogenic recessive mutant resistant to *B. maydis*, responded positively to heat shock (lane 8) by showing an intensive 92 KDa band, but had only a small amount of 92 KDa protein associated with polysomes when inoculated with *B. maydis* (lane 7). Mo17 and C103 inbreds were both polygenic resistant to *B. maydis* showed little accumulation of 92 KDa (lane 9 and 11, respectively). Similarly, both inbreds accumulated a small amount of 92 KDa RAP when given a heat shock treatment although Mo17 (lane 10) appeared to have more 92 KDa than C103 (lane 12). It is not clear whether the amount of 92 KDa is related to heat tolerance;

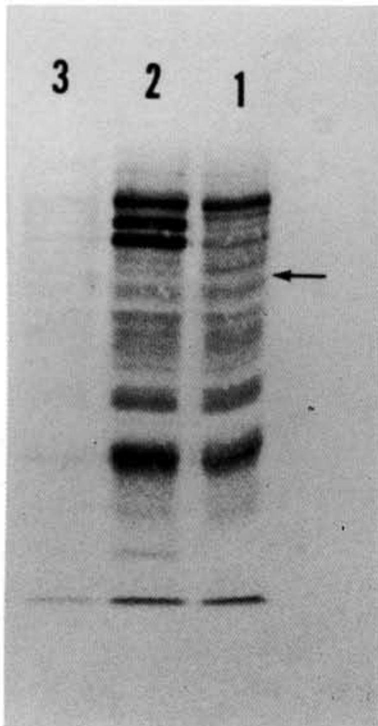


Fig. 3. Fluorograph of SDS-polyacrylamide gel of proteins synthesized *in vivo* by healthy, heat-shocked, or *B. maydis*-inoculated leaves. Proteins were synthesized *in vivo* during a 1 h pulse with $^3$H-leucine by healthy W64A leaves (lane 1), leaves that had been heat-shocked at 40 ℃ for 2h (lane 2), or leaves inoculated with *B. maydis* for 24 h (lane 3). Gel concentration was 12.5%.

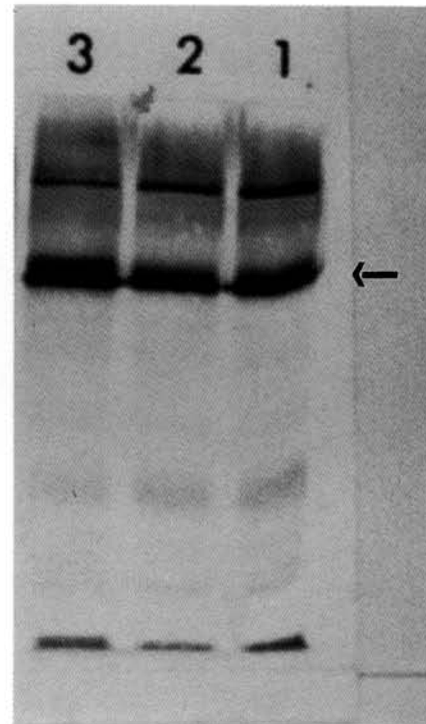


Fig. 4. Immunoblots of proteins synthesized *in vivo* by healthy, heat-shocked, or *B. maydis*-inoculated leaves. Total proteins were extracted from healthy W64A leaves (lane 1), leaves that had been heat-shocked at 40 ℃ for 2h (lane 2), or leaves inoculated with *B. maydis* for 24 h (lane 3). Gel concentration was 12.5%.

however, a differential accumulation of this protein in B73 *rhm* and other genotypes suggests that the mechanism of redistributing the 92 KDa from chloroplasts to cytoplasmic polysomes might be different between these two stress systems.

The association of the 92 KDa (or 57 KDa) protein with cytoplasmic polysomes in response to stresses is not unique to maize. A similar observation was made with soybean-*Phytophthora megasperma* interaction. When susceptible soybean seedlings were inoculated by inserting mycelium into a longitudinal slit of hypocotyl, a 57 KDa protein was also found to associate with polysomes in foliage leaves (Fig 6, lane 4). A small amount of this protein could also be detected in the hypocotyl polysomes (Fig. 6, lane 5). This 57 KDa protein in soybean also cross-reacted with the antibody prepared against maize 57 KDa (data not shown). Resistant cultivar of soybean, on the other hand, did not contain this RAP in leaves (lane 1), hypocotyl (lane 2), nor epicotyl (lane 3) when inoculated with *P. megasperma*.

This study indicates that a protein with molecular weight of 57 KDa, which was aggregated to form 92 KDa component under a mild denaturing condition, could be induced to redistribute from chloroplasts to cytoplasmic polysomes when seedlings encountered a stress. This protein induced by a biotic stress was similar to the one induced by heat shock although the mechanism of redistribution appeared to be different.

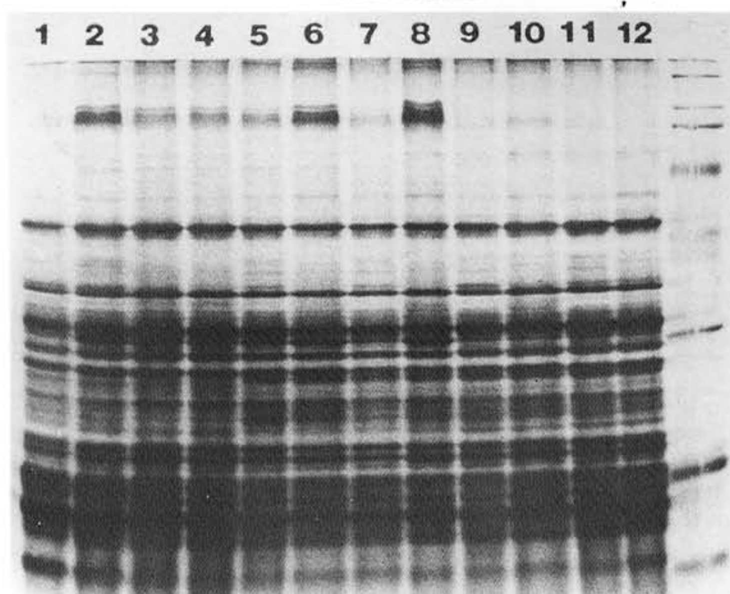


Fig. 5. SDS-polyacrylamide gel electrophoretic pattern of polysome-associated-proteins isolated from leaves of maize genotypes inoculated with *B. maydis* race O or heat-shocked for 2h at 40°C. Polysomes were isolated from healthy leaves of W64A (lanes 1), W64A leaves inoculated with *B. maydis* for 24 h (lane 2), W64A leaves heat-shocked at 40°C for 2h and 4h (lanes 3 and 4, respectively); B73 leaves inoculated with *B. maydis* for 24 h (lane 5), B73 leaves heat-shocked at 40°C for 2h (lane 6); B73 *rhm* leaves inoculated with *B. maydis* for 24 h (lane 7), B73 *rhm* leaves heat-shocked at 40°C for 2h (lane 8); Mo17 leaves inoculated with *B. maydis* for 24 h (lane 9), Mo17 leaves heat-shocked at 40°C for 2h (lane 10); and C103 leaves inoculated with *B. maydis* for 24 h (lane 11), C103 leaves heat-shocked at 40°C for 2h (lane 12). Gel concentration was 12.5%. Mol. wt standard in order of decreasing size: 116, 97.4, 92.5, 66, 45, 29 and 20.1 KDa.

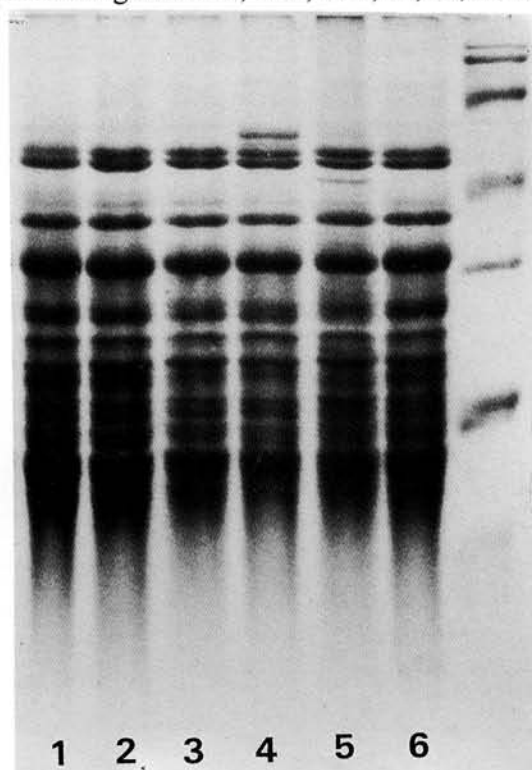


Fig. 6. SDS-polyacrylamide gel electrophoretic pattern of polysome-associated-proteins isolated from soybean inoculated with *Phytophthora megasperma*. Polysomes were isolated from foliage leaves (lane 1), hypocotyl (lane 2), and epicotyl (lane 3) of a resistant cultivar; and foliage leaves (lane 4), hypocotyl (lane 5), and epicotyl (lane 6) of a susceptible cultivar. Gel concentration was 12.5%. Mol. wt standard in order of decreasing size: 97.4, 92.5, 66, 45, 29 and 20.1 KDa.

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# 玉米基因型在接種病菌或熱休克處理情況下57KDa蛋白的分佈

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## 摘　　要

為了探討玉米幼苗在逆境下，一個 57KDa 蛋白 (Ribosome-associated-protein, RAP) 如何由葉綠體轉移至細胞質多核醣體上的機制，乃利用對於 *Bipolaris maydis* 感病和抗病的品種為材料，評估這些幼苗接種病菌及熱休克處理情況下的反應。利用 Western blot 分析的結果證明經由接種或熱休克處理後所產生的 RAP 是一樣的，而且發現當抗病型的幼苗接菌種之後，此 57KDa RAP 不會和多核醣體接合，它可能繼續停留在葉綠體中。57KDa 轉移的現象不僅發生在玉米幼苗中，大豆感染了 *Phytophthora megasperma* 後亦有同樣的現象，而且大豆的 57KDa RAP 和玉米的蛋白可能極為相似。

關鍵詞：熱休克、玉米、病菌、多核醣體蛋白、逆境。

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