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# Relationship between gene expression and hybrid vigor in primary root tips of young maize (*Zea mays* L.) plantlets

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**Summary.** To provide an insight into the molecular basis of heterosis, we investigated gene expression in primary root tips of a heterotic maize hybrid (B73 × Mo17) and its parental lines (B73 and Mo17). This analysis was carried out (i) by differential plaque hybridization of a recombinant cDNA library made to poly(A) RNA isolated from B73 × Mo17 primary root tips, and (ii) by comparing with two-dimensional gel electrophoresis proteins synthesized in vitro in the rabbit reticulocyte system by poly(A) RNA isolated, at different stages of development, from the three genotypes. The results showed that there are sets of proteins and mRNAs that are differentially synthesized and expressed in the $F_1$ primary root tips in comparison to the parental lines. Moreover, results from the survey of 21 major in-vitro-synthesized polypeptide variants, from mRNAs of primary root tips of the parental lines and their $F_1$ hybrid, indicated that in seven instances hybrid proteins translated in vitro were more abundant or possibly new. In most of the remaining cases, hybrid spots were similar in intensity to the same protein produced by one of the two parental lines.

**Key words:** *Zea mays* L. – Hybrid vigor – Gene expression – Protein synthesis – mRNAs

## Introduction

In crop plants, hybrid vigor has been recognized from many years (cf. Shull 1952), but its genetic and biochemical backgrounds are not yet understood. In maize, hybrid vigor is particularly evident (Hallauer and Miranda 1981), and the species is a model system for the investigation of the physiological and biochemical nature of this phenomenon. Attempts to account for hybrid vigor at the physiological level have previously been focused on demonstrating that maize hybrids possess biochemical properties that exceed those of the parental lines, such as mitochondrial oxidation and phosphorylation, nucleic acid synthesis, nitrogen metabolism, plant hormone levels, and relative enzyme activities (for a review, see McDaniel 1986).

$F_1$ hybrid seeds of maize have a superior germination capacity in comparison with that of the parental lines (Sarkissian et al. 1964; Sinha and Khanna 1975; Mino 1980). Studies have also indicated that vigorous growth of the embryonic axis in germinating $F_1$ seed is related to a higher rate of RNA and protein synthesis (Cherry et al. 1961; Mino and Inoue 1980). Similarly, Nebiolo et al. (1983), in a study on RNA metabolism in protoplasts isolated from maize seedlings of a heterotic hybrid and its parents, suggested that the hybrid nucleus may have advantages in the rate of DNA and RNA synthesis.

Because hybrid vigor in maize is detectable at early stages of germination (Sarkissian et al. 1964), the molecular analyses presented here focus on the hybrid and its parental inbreds during early seedling growth. The objective was to gain an insight into the expression of genes involved in hybrid vigor by comparative analysis of mRNAs and proteins produced during this early stage of development.

## Materials and methods

### *Plant materials*

Two commonly used inbred lines of maize (B73 and Mo17) and their heterotic $F_1$ hybrid (B73 × Mo17) were included in this study. Self-pollinations and B73 (female) × Mo17 (male) crosses were made in the summer of 1986 at the Maize Section of the Experimental Institute of Cereal Crops, Bergamo, Italy. The

ears were harvested and dried to 10–12% moisture at 35 °C in a forced air dryer. Seeds were hand-shelled and stored at 5 °C until planting.

For each genotype, batches of 100 seeds were surface-sterilized with 70% ethanol for 90 s, then with 3% sodium hypochlorite for 10 min, rinsed three times in sterile, distilled water, and placed on moistened filter papers in a 150-mm petri dish containing 14 seeds. Germination took place at 25 °C in a dark incubator, and seeds were kept moistened throughout the experiments using sterile water.

Plant tissues were collected at various developmental stages of seedling growth, i.e., 72 h after germination and when the primary roots were 2–4, 8–10, 15–20, and 25–30 mm in length, respectively. At these stages, seedlings were collected, washed three times in distilled water, and tips of the primary roots were aseptically cut off and frozen in liquid nitrogen. Frozen root tips were stored at −80 °C until used for RNA extraction.

*RNA preparation and dot blots*

The root tips were ground to a fine powder in liquid nitrogen using a mortar and pestle and the resulting powder was dispersed in 10 ml of RNA extraction buffer (50 m*M* TRIS-HCl, pH 9.0, 10 m*M* EDTA, 2.0% SDS, 200 m*M* NaCl, 10 m*M* B-mercaptoethanol, and 0.2 mg/ml proteinase K). Total RNA was isolated essentially as described by Dean et al. (1985) and subjected to two cycles of oligo(dT) cellulose chromatography to isolate poly(A) RNA (Aviv and Leder 1972). RNA was stored in 70% ethanol at −80 °C.

*RNA dot blots*

The RNA dot blot analyses were carried out by the procedure described in Cullimore et al. (1984), and hybridizations were done as described (Maniatis et al. 1982), using $^{32}$P-labeled probes (Rigby et al. 1977) derived from inserts selected from phages showing differential hybridization.

*Construction and screening of a cDNA library*

Total RNA, isolated 72 h after germination at 25 °C, from primary root tips of the B73 × Mo17 hybrid seeds, was used for preparing the cDNA library. Double-stranded DNA copies of the total poly(A) RNA were synthesized according to Maniatis et al. (1982) and cloned after linker addition into the EcoRI site of bacteriophage lambda NM1149.

The cDNA library (60,000 recombinants) was grown on *Escherichia coli* strain POP 13. Replica filters were differentially screened using radioactive ($10^8$ cpm/μg) single-stranded cDNA, synthesized on poly(A) RNAs from primary root tips of B73 and Mo17 inbreds and their $F_1$ hybrid, respectively, or from poly(A) RNA labeled at the 5′-end (up to $1.5 \times 10^8$ cpm/μg), using $^{32}$P-ATP and T4 polynucleotide kinase essentially as described by Maniatis et al. (1982). The procedure for plaque and filter hybridization followed the methods outlined in Maniatis et al. (1982).

*DNA preparation and Southern analysis*

Phage and plasmid DNA purification and recombinant DNA work followed the procedure outlined in Maniatis et al. (1982). Southern blotting (Southern 1975) and hybridizations were performed according to published protocols (Motto et al. 1988).

*Hybrid-selected translation*

The method used was as previously described by Di Fonzo et al. (1988).

*In vitro translation of poly(A) RNA*

The poly(A) RNAs for B73, Mo17, and B73 × Mo17, extracted at two different stages of primary root growth (2–4 and 25–30 mm primary root length) were translated in vitro in a rabbit reticulocyte lysate system, which was prepared and used according to Jackson and Hunt (1983). For 20-μl translation assays, saturating amounts of poly(A) RNA (0.5–1 μg) were incubated for 2 h at 30 °C in the presence of [$^{35}$S]-methionine. The samples were precipitated with cold acetone and kept in ice for 1 h, then centrifuged for 5 min at 10,000 *g*. The pellet, after drying in a vacuum, was dissolved in lysis buffer for two-dimensional electrophoretic analysis.

*Two-dimensional electrophoresis*

The procedure was essentially as described by Bartels et al. (1988). The isoelectric focusing gels contained a mixture of 1/5 Ampholine pH 5–10, 2/5 Ampholine pH4–6, 1/5 Ampholine pH 5–8, 1/5 Ampholine pH 7–9. In the second dimension the focused proteins were separated on a gradient (7.5–15%) polyacrylamide gel overlaid with a 4% polyacrylamide stacking gel (Laemmli 1970). The gels were fixed in 6% (w/v) TCA, 5% (v/v) ethanol, and then prepared for fluorography according to Bonner and Laskey (1974). Methylated $^{14}$C-proteins were used as molecular-weight markers for SDS gel electrophoresis.

*Enzymes and chemicals*

DNA restriction endonucleases, DNA polymerase I klenow fragment, reverse transcriptase, RNAse A, and T4-polynucleotide kinase were purchased from Bethesda Research Laboratories and Boehringer. Acrylamide, bisacrylamide, and SDS were obtained from Bio-Rad Laboratories, ampholines from LKB. All other reagents were of the highest purity. Radiochemicals were obtained from Amersham.

## Results

*Isolation of cDNAs differentially expressed in primary root tips of the $F_1$ and parental lines*

To ask whether hybrid vigor may be related to the expression of a specific gene product(s), a cDNA library was prepared in phage vector lambda NM1149, with RNA extracted from B73 × Mo17 primary root tips dissected 72 h after germination. We used a direct screening procedure to identify and isolate clones for genes with differential expression in the $F_1$ hybrid relative to expression in the parental lines. cDNA clones from the library were plated at low density and triplicate nitrocellulose lifts were made from each plate. One filter was hybridized with $^{32}$P-labeled single-stranded cDNA; this was synthesized from the RNA extracted from the primary root tips of the hybrid that had been used to construct the library. The second and third filters were hybridized with a similar probe made from primary root tips dissected 72 h after germination from the parental lines. The plaques showing differential hybridization were picked and plated at 200 pfu/plate. The putative differential clones were plaque-purified and retested in two more rounds of screening until all plaques scored positively.

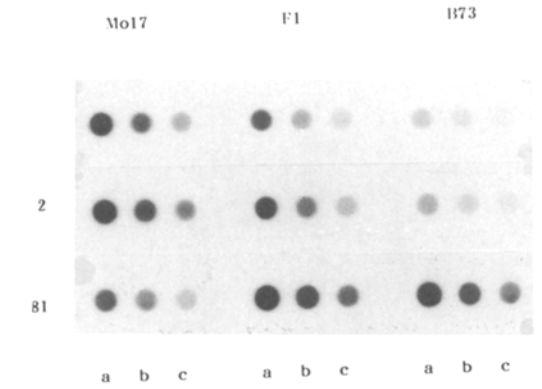


**Fig. 1.** Dot-blot analysis of total RNA from the $F_1$ hybrid (B73 × Mo17) and parental lines (B73 and Mo17) hybridized to $^{32}$P-labeled cDNA inserts that are differentially expressed among the three genotypes. Several dilutions of each RNA sample were applied; *a* 4.0 µg; **b** 1.0 µg; **c** 0.25 µg

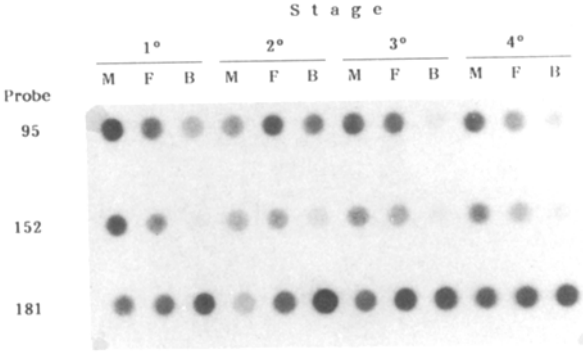


**Fig. 2.** Dot-blot analysis of poly(A) RNA from primary root tips isolated at different stages of development of the $F_1$ hybrid (*F*) and parental lines Mo17 (*M*) and B73 (*B*); 2.0 µg each of mRNA was dotted onto nitrocellulose and hybridized with the indicated $^{32}$P-labeled DNA probe

Final screening was performed using DNA dot blots of phage DNAs isolated from the putative differential clones. For each clone, 100 µg of DNA was applied to nitrocellulose membranes through the wells of a Hybri-Dot manifold (Bethesda Research Laboratories). Triplicate DNA dot blots were hybridized with similar $^{32}$P-labeled probes used in the original screening steps. Only 3 out the 72 putative differential clones were identified that hybridized more intensively to the parental or to the hybrid $^{32}$P-labeled single-stranded cDNA. The differential hybridization of these three clones was further confirmed (i) by repeating the hybridization of a similar experiment with 5′-labeled mRNA (data not shown), and (ii) by dot-blot analysis of varying amounts of poly(A) RNA for each of three genotypes hybridized to the $^{32}$P-labeled cloned probes (Fig. 1). It was also evident from this last analysis that clone 95 displayed a hybridization intensity intermediate between the parental ones, while clones 152 and 181 showed hybridization intensities of

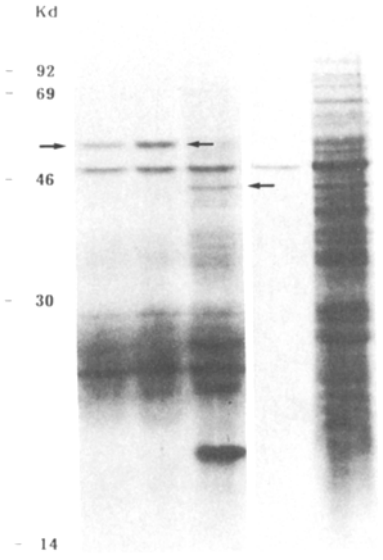


**Fig. 3.** [$^{35}$S]-methionine labeled cell-free reticulocyte system translation products of poly(A) RNA hybrid selected with plasmids 95, 152, and 181 after SDS-gel electrophoresis and autoradiography. *Lane C*, reticulocyte lysate without any added poly(A) RNA. *Lane T*, pattern of in vitro synthesis directed by total poly(A) RNA extracted from the hybrid primary root tips. Molecular weights are shown on the left side of the fluorograph

the $F_1$ root extracts that were similar to the more intense parental spots. Similar results were also obtained by hybridizing each cDNA clone with mRNA isolated at different stages of development from primary root tips dissected from each of the three genotypes (Fig. 2).

The three cDNA clones differentially expressed in the three genotypes were subcloned into pUC9 plasmid. The insert size of these clones varied from ca. 300 bp to ca. 1,300 bp. They did not show cross-hybridization and represented cDNA copies of mRNAs, as shown by hybrid selection of mRNAs and cell-free translation. Clones 95 and 152 hybridized with a specific mRNA, from primary root tips, that codes for a protein of 54 kDa, while clone 181 hybridized with an RNA that directs in vitro the synthesis of a polypeptide with an apparent molecular weight of 43 kDa (see arrows in Fig. 3).

### *Expression of proteins translated in vitro from poly(A) RNA derived from $F_1$ and parental lines in primary root tips of young plantlets*

Polyadenylated RNA was isolated after germination from each parental line and its $F_1$ hybrid at different stages of primary root growth, and was translated in vitro in a rabbit reticulocyte lysate system. The proteins synthesized were separated by two-dimensional electrophoresis. Figure 4 shows in-vitro-synthesized proteins derived from poly(A) RNA of the three genotypes con-

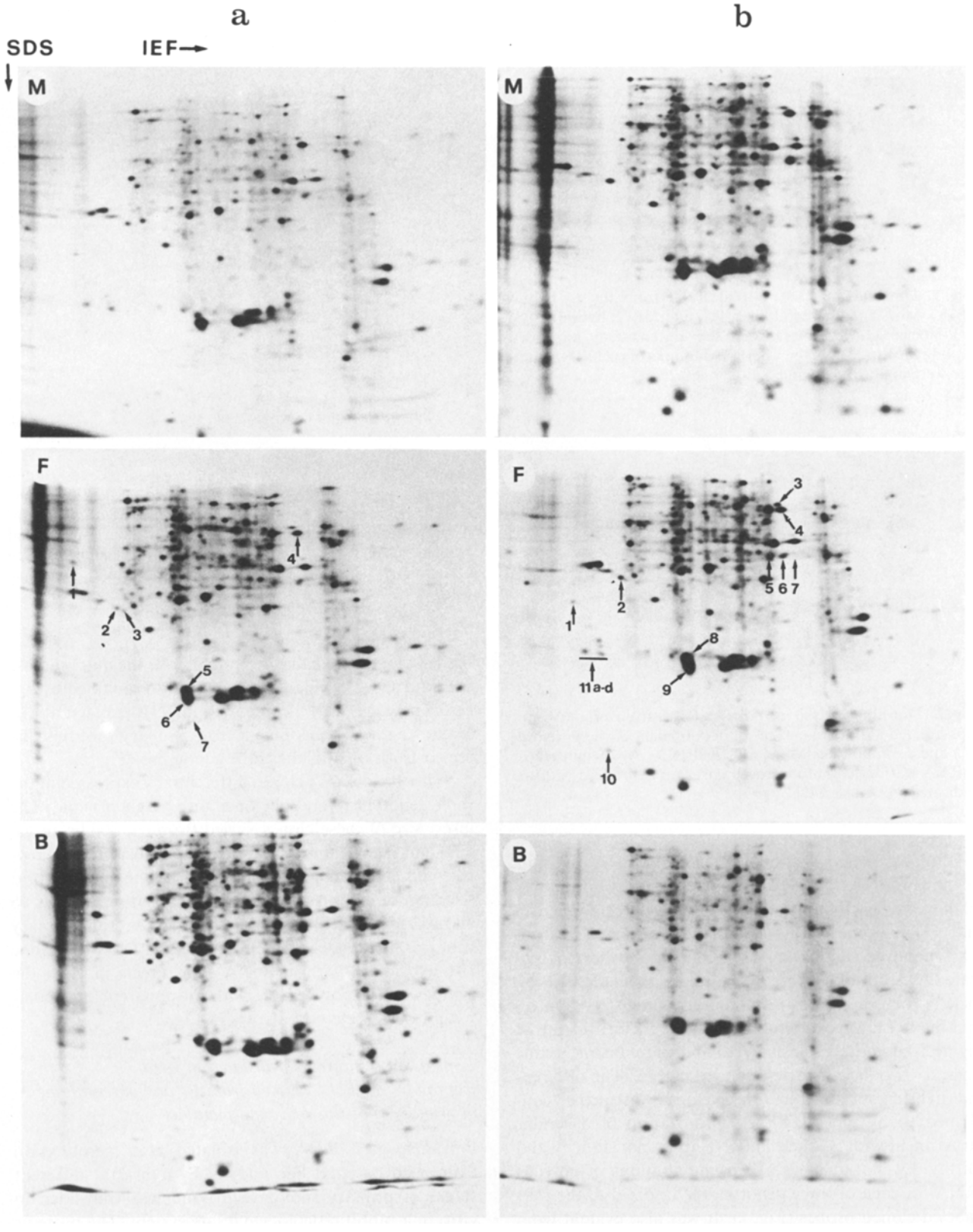


**Fig. 4a, b.** A fluorography of in-vitro-synthesized proteins derived from poly(A) RNA extracted from primary root tips, at two stages of development [2–4 mm (*panel a*) and 25–30 mm (*panel b*) in length], of an $F_1$ hybrid (*F*) and its parental lines Mo17 (*M*) and B73 (*B*). The proteins, which change in comparison with those of the translation patterns of the parental lines, are indicated by *arrows* and numbered (see also Table 1 for a summary)

**Table 1.** Survey of 21 major variant polypeptides derived from in vitro synthesis of poly(A) RNA from maize primary root tips of an $F_1$ hybrid (B73 × Mo17) and its parents

| Polypeptides in Fig. 4a[b] | First stage Comparative intensity[a] | | | Polypeptides in Fig. 4b[b] | Second stage Comparative intensity[a] | | |
|---|---|---|---|---|---|---|---|
| | Mo17 | B73 × Mo17 | B73 | | Mo17 | B73 × Mo17 | B73 |
| 1 | − | + | ++ | 1 | − | + | − |
| 2 | + | + | ± | 2 | + | + | ± |
| 3 | − | ± | ± | 3 | + | + | ± |
| 4 | ++ | ++ | + | 4 | ++ | ++ | − |
| 5 | + | +++ | +++ | 5 | − | + | + |
| 6 | +++ | +++ | + | 6 | − | + | ± |
| 7 | − | ± | + | 7 | ++ | − | − |
| | | | | 8 | + | +++ | +++ |
| | | | | 9 | +++ | +++ | ± |
| | | | | 10 | − | + | + |
| | | | | 11a–d | − | ++ | ± |

[a] Comparison of the expression of in-vitro-synthesized proteins from an $F_1$ hybrid and its parents; + and − represent presence and absence, respectively, of the spots. The number of crosses indicates the different intensities of the proteins on the films
[b] Stages of sampling of primary root growth: 2–4 mm long (a) and 25–30 mm long (b)

sidered at two different stages of primary root growth, i.e., when the primary roots were 2–4 mm long (Fig. 4a) or 25–30 mm long (Fig. 4b). For each stage of growth the majority of the polypeptides detected in the hybrid primary root tips appeared to be similary expressed in the parental lines. However, some reproducible major changes involving both qualitative (presence or absence of spots) and quantitative (spots more or less intense) variations were clearly visible in the replicates of the parental lines and their $F_1$ hybrid, and the most prominent polypeptide variations are indicated and numbered in Fig. 4 and summarized in Table 1.

Among spots differentially expressed in the parental lines and the $F_1$, only two proteins [labeled nos. 5 and 6 at the first sampling stage (Fig. 4a), and nos. 8 and 9 at the second sampling stage (Fig. 4b)] were present at both developmental stages. In stage (a) all proteins observed in the hybrid were also expressed by at least one of the parents; however, in stage (b) there was more unique quantitative and qualitative expression of proteins in the hybrid. The detailed data presented in Fig. 4 and Table 1 can be summarized as follows.

(i) Of the 21 varying protein spots on two-dimensional gels, 6 were detected in greater abundance in the $F_1$ than in either parent (Fig. 1b; Table 1, stage b: spots 6, 10, and 11a–d). Also, one protein was present in the $F_1$, but was not detectable in either parent (Fig. 1b; Table 1, stage b: spot 1), although in the latter instance parental proteins might have been below the level of detection. These results, taken together, may be interpreted as molecular evidence for true heterosis in which it is predicted that the hybrid should have more abundant or specific gene products that may possibly underlie hybrid vigor.

(ii) The majority of the remaining specific protein spots observed in the hybrid (11 out of 21) were similar in intensity to the same protein produced by one parent only (Fig. 1a; Table 1, stage a: spots 2, 3, 4, 5, and 6; Fig. 1b; Table 1, stage b: spots 2, 3, 4, 5, and 9); this suggests a dominance of these particular parental genes that is expressed in the regulation of protein or mRNA quantity.

(iii) Hybrid spot intensity intermediate between the parental lines was observed in 2 cases (Fig. 1a; Table 1, stage a: spots 1 and 7) and may indicate codominance.

(iv) One polypeptide spot was present in a parental line but missing in the $F_1$ hybrid and the other parental line (Table 1, stage b, spot 7). It is possible that the synthesis of this polypeptide might have been inhibited in the hybrid.

## Discussion

Heterosis or hybrid vigor clearly depends upon heterozygosity, but the molecular and biochemical bases of this phenomenon remain unknown. In heterotic plants certain metabolic functions may be enhanced over those of the parents, and it should be possible to investigate this at the molecular level by studying the quantitative expression of specific gene products. Therefore, we have analyzed, after seed germination, gene expression in primary root tips of a heterotic $F_1$ hybrid and its parental lines by studying the relative abundance of tissue-specific mRNAs and in-vitro-translated proteins. The germination of seeds into young plantlets initiates a series of biochemical events reflected in a rapid increase in protein and RNA biosynthetic capacity (Bewley 1982),

and this process seems to be specifically and positively related to hybrid vigor (Woodstock and Skoog 1960; Cherry et al. 1961; Mino and Inoue 1980).

Experimental data presented in this paper demonstrate that the $F_1$ primary root tips contain mRNAs that seem to be differentially synthesized and expressed in the hybrid in comparison to the parents. However, these mRNAs may not be necessarily related to heterotic growth, i.e., their synthesis may simply result from the action of genes differentially expressed in hybrids and parental lines at different times during development. In this respect, changes in the relative abundance of mRNA species during plant cell development have been reported in different plant systems including maize (Thompson and Lane 1980; Dure et al. 1981; Aspart et al. 1984; Sanchez De Jimenez and Aguilar 1984; Sanchez-Martinez et al. 1986). Also, with the experimental procedures we have adopted, mRNAs must be present in a certain concentration in order for their translation products to be detected, and low-abundance mRNAs may not be detectable.

Previous studies of hybrid vigor have shown that dominant gene expression may be important for heterosis. For example, Leonardi et al. (1987) found, in a study on the inheritance of specific protein amount in two maize inbreds and their hybrids, nonadditive effects on spot intensities in the hybrids, and these data were interpreted as evidence of dominant gene effects. In another instance, codominant gene expression was observed in $F_1$ hybrids (Heidrich-Sobrinho and Cordeiro 1975). Also, statistical analyses of maize yield data (cf. Hallauer and Miranda 1981) and a report of hybrid-specific, elevated activities of alcohol dehydrogenase (Schwartz 1973) have provided some previous evidence in support of the concept of overdominance in hybrids.

The data herein reported add circumstantial evidence in favor of differential expression of many genes in hybrid plantlets, since approximately 33% (7 out of 21) of the major hybrid proteins translated in vitro were more abundant or possibly new. Also, despite the small relative number of differences detectable at the molecular level with the techniques we have adopted, this study further suggests that hybrid vigor may derive from simple dominant or codominant gene effects in addition to the increased expression of certain loci.

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