Assessment of Pleiotropic Effects of a Gene Substitution in Pea by Two-Dimensional Polyacrylamide Gel Electrophoresis

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ABSTRACT

We examined, by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), near-isogenic lines of the r-gene in pea (Pisum sativum) which determines round (RR) vs. wrinkled (rr) seed. The study was undertaken to assess the number of protein changes resulting from a single gene substitution as a means of quantifying pleiotropic effects. A total of 636 to 770 resolvable polypeptides were identical in all respects between RR and rr for roots, shoots, leaflets, stipules, young ovaries, and young embryos. A single difference between the lines became evident about 21–25 days after anthesis in the embryos. Mature seeds of the two lines showed 62 spot differences in addition to differences in four clusters of spots, representing about 10% of the total number of spots visible on the gels. The protein differences are presumably involved in the many known physiological differences of the two seed types. 2-D PAGE analyses of near-isogenic lines are likely to be valuable in a number of quantitative and developmental genetic contexts.

"PLEIOTROPY" refers to multiple phenotypic consequences of a single mutation. For example, in Drosophila melanogaster, vestigial causes the formation of short wings and influences the position of bristles on the scutellum; numerous other examples are described in Caspari (1952). It is now understood in principle that such multiple effects are all related as consequences of complex interactions among biochemical and developmental processes. In some cases, such as phenylketonuria and sickle cell anemia, the physiological relationships among the diverse effects are becoming understood. At the same time, it has become evident that pleiotropy is "virtually universal" (Wright 1968, p. 61), that is, essentially all gene substitutions have multiple phenotypic consequences when the phenotype is examined in sufficient detail.

The total number of morphological and physiological consequences of a particular gene substitution cannot be counted because no attempt to delimit distinct "effects" is arbitrary. Effects appear distinct only if we do not understand the connection between them. Yet, quantification would be a valuable aid to understanding the ramifications of a given mutation and assessing its overall impact on the organism.

In this paper we have compared near-isogenic lines by two-dimensional gel electrophoresis (2D-PAGE) of denatured proteins. The example we use is the r-gene studied by Mendel, which determines round (RR) vs wrinkled (rr) seed in pea (Pisum sativum). This technique allows us to quantify the effects of a single gene substitution on the class of relatively abundantly expressed proteins.

MATERIALS AND METHODS

Plants: Seeds of round and wrinkled pea were kindly provided to us by C. L. Hedley (John Innes Institute, Norwich NR4 7UH, United Kingdom). The RR and rr lines resulted from six generations of backcrossing from an initial F1 between a round and a wrinkled line. This was followed by seven generations of selfing and reselection from single heterozygous plants (Rr), making a total of 13 generations from the original cross. In the final generation a single heterozygous individual was selfed and the resulting round (RR) and wrinkled (rr) segregants were used as the near-isolines. The breeding program is described in detail in Hedley et al. (1986). The process of reselecting from a single heterozygous plant at each generation increases homozygosity without incurring genetic drift between the round and wrinkled lines. RR and Rr were distinguished by examining segregations of selfed progenies from plants exhibiting round seeds.

Pea seeds were allowed to germinate in the dark in Petri dishes on water-imbibed filter paper at 15°. After 8 days the seedlings were transferred to small pots filled with sand and supplied every 5 days with Hoagland's nutrient solution. They were grown in a growth chamber under 16-hr photoperiod at 22° and 8-hr dark periods at 15°.

For each line, we analyzed 8-day-old etiolated shoots and roots, leaflets and stipules of the fully expanded fourth leaf (on node 6) on plants having only four fully expanded leaves. In addition, we examined young ovaries (≤14 mm), very young developing seeds with their coat (weights ≤20 mg), young embryos without their coat at various developmental stages (from 25 to 600 mg), and mature seeds after 24 or 48 hr of imbibition. For every organ (except embryos) two to four individuals per genotype were separately analyzed. For each genotype of the embryos, 19 different developmental stages were examined among six individuals. In all, 78 2D gels were analyzed.
Protein extraction and electrophoresis: The protein extraction procedure, avoiding protease action, was as described in Damerval et al. (1986) except that 80 µl of resolubilization solution was used to resuspend 1 mg of protein pellet. The isoelectric focusing (IEF) was as described in Leonardi, Damerval and de Vienne (1987) except that 20 µl of solubilized extract were layered on the top of each 24 cm-long IEF gel and the run was performed for 35,000 Vh. Some embryo extracts were also analyzed by a nonequilibrium pH gel electrophoresis (NEPHGE) (O’Farrell, Goodman and O’Farrell 1977). In that procedure we used the ampholytes Pharmalyte (pH 3–10) and performed the run for 18,000 Vh with electrodes and electrode buffers reversed. The second (SDS) dimension was performed as in Damerval et al. (1987) with 2D gels bound to GelBond PAG films. This made it possible to stain eight gels simultaneously and increased reproducibility (Granier and de Vienne 1986). The silver staining was as described by Damerval et al. (1987) except that only one developer bath was applied and the temperature was 24–25°C. The comparisons of the 2D gels were made directly on the 2D gels dried on GelBond PAG films.

These procedures involve various improvements and simplifications of the original O’Farrell technique (1975): first dimension gels with small diameter (0.9 mm), pH gradient range optimized for our material (7/ Pharmalytes pH 5 to 8, 2/ Pharmalytes pH 6 to 8), large size 2D gels (20 cm × 18 cm), continuous running gel, and simultaneous staining of 2D gels bound to polyester films, using a simplified silver staining procedure. These modifications result in high resolution, more reproducible 2D gels and make the analyses much easier (Damerval et al. 1986, 1987; Granier and de Vienne, 1986).

The comparison of the gels was made by eye, directly by superimposition of the 2D gels dried on GelBond PAG, using a lightbox. This avoids a possible loss of precision due to the nonlinear response of photographic films. The visual analysis probably underestimated the quantitative variation. However it did not modify the assessment of spot position variation because we performed co-electrophoresis of various genotypes or organs from the two lines in order to check the coincidences of spot positions. We estimate that the sensitivity of the method is about 0.5 ng per spot.

RESULTS

For every organ, roots, shoots, leaflets, stipules, young ovaries and young embryos, the 2D protein patterns were identical in all respects between RR and rr. Figure 1 shows a representative gel of proteins extracted from RR stipules. The number of protein spots differed among the organs with about 760 resolved in both roots and shoots, about 690 in leaflets and stipules, 750 in young ovaries, and about 770 in young embryos. Apart from leaflets and stipules, many qualitative and quantitative differences were observed between organs (compare Figures 1 and 2).

A single difference between the two lines was first noted 21–23 days after anthesis in the embryos heavier than 260 mg. In contrast, many additional differences were observed in the mature seed stage. These included the appearance of proteins unique
to one or the other line as well as changes in relative spot size (intensity). Six hundred and thirty-six protein spots were resolved in mature seeds of the two lines.

In order to determine whether divergence in protein pattern during embryogenesis and seed maturation occurred gradually or all at once, the embryos were examined between 25 and 30 days (430–500 mg) in addition to the two stages mentioned above (Table 1). In the earliest stage, a single spot that was more intense in RR than in rr was observed. A number of differences became evident in the intermediate stage. Four “clusters” of spots, each containing between 20 and 30 protein spots, appeared in both lines, but the intensities of most spots were always greater in RR. Eight differences in particular protein spots also appeared in this stage (Table 1).

The mature seeds of the two lines showed 62 spot differences in addition to the four clusters of spots, about 10% of the total number of spots visible on the gels (Fig. 2). About half of the 62 spots had not been visible at the preceding stage. Seven spots were unique to rr and 11 were unique to RR. Of the shared spots, 30 were more intense in RR and 14 others were more intense in rr (Table 1). A number of the spots changed intensity between the intermediate and mature stages. Other than the four clusters mentioned above, the differences included proteins with a variety of molecular weights and isoelectric points (Figures 2 and 3). However, it was also evident, from inspection of the gels, that many proteins arranged in sets with similar molecular weight but different isoelectric points (“charge trains”) were altered in intensity between the two lines. These may represent members of the same seed storage protein families (e.g., legumin), but the matter was not studied further.

**DISCUSSION**

We have used 2D-PAGE to resolve between 636 and 770 nonallelic proteins in different organs of pea lines that were made near-isogenic for the Mendelian gene round (RR) vs. wrinkled (rr). The results show that the two lines have identical protein sets in every organ and stage tested until beginning near the middle of embryogenesis and continuing during seed maturation, when the protein patterns of the growing embryo diverge strikingly so that the mature seeds differ by at least 62 of 636 protein spots detected.

That the two lines were indistinguishable in all aspects except their embryos and seeds is fully consistent with previous analyses of growth patterns and seed development (Hedley et al. 1986; Wang et al. 1987). The r-locus appears to be expressed only in embryos and seeds and is known to affect a number of physiological and biochemical attributes including sugar content, lipid content, storage protein composition, and shape and size of starch grains (reviewed in two papers cited above). During embryo development, rr seeds have higher water content than RR seeds, perhaps because the rr seeds have higher sucrose levels which produce a more negative osmotic potential and consequent greater uptake of water (Wang et al. 1987). The greater loss of water in rr seeds during drying, compared to RR seeds, is responsible for their characteristic wrinkled appearance. Thus, the numerous differences between the two lines in protein contents revealed in this study are presumably involved in these physiological differences.

Since we analyzed near-isogenic lines, all of the protein modifications (presence/absence and relative staining intensity) can be considered consequences of the R/r allelic substitution. These effects can be attributed either to changes in the level of expression of other genes or to modification of other gene products (Higgins 1984). Presumably some effects are very closely related (e.g., similar alterations in all members of a family of storage proteins), but the degrees of relatedness of different modifications represent points on a continuum. The robust feature of the present approach is that numerous distinct effects can be counted. At this level of observation, the R/r substitution affects about 10% of the proteins expressed. The 62 differences observed represent a minimum estimate of the total number of protein differences, since 2D-PAGE reveals only the more abundant proteins.

2D-PAGE of denatured proteins in isogenic lines would seem to provide a simple and general procedure to quantify pleiotropy at the level of abundant proteins. This technique would permit quantitative comparison of the pleiotropic effects of the same allele substitution on different genetic backgrounds and in different environments. Although the subset

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### TABLE 1

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<thead>
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<th>Number of protein spots in embryos and mature seeds distinguishing RR and rr near-isolines of pea</th>
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<tr>
<td>Pattern</td>
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<td>RR only</td>
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* Total does not include differences in intensities of protein spots in the four clusters of spots mentioned in text.
Figure 2.—2D gels of embryos at 21–23 days after anthesis. a, $RR$ genotype. b, $rr$ genotype. At this stage, embryos of the two lines can be distinguished only by a difference in the intensity of a single spot (identified by arrow).
Pleiotropic Effects

**FIGURE 3.**—2D gels of water-imbibed mature seeds. a, *RR* genotype, b, *rr* genotype. In addition to the four clusters (large arrows), 62 spots (small arrows) differ in intensity between the two genotypes.
of proteins assessed cannot be assumed to be representative of the total protein profile of the organism, this method of assessment provides a useful supplement to data obtained from morphology or physiology.

The present results with RR and rr near-isolines contrast sharply with the results of a similar 2D-PAGE analysis we carried out (D. de Vienne and L. D. Gottlieb, unpublished data) on near-isogenic lines of mutants of pea that modify leaf development, the afila (af) mutant that results in the replacement of leaflets with tendrils and the tendril-less (tl) mutant that replaces tendrils with leaflets (Marx 1974). In that study, we identified 686 protein spots. Both the leaflets that replace the tendrils in the tl line and the tendrils that replace leaflets in the afaf line had the same sets of protein spots as wildtype leaflets and tendrils, respectively, and the proteins of the two organs were identical to each other as well (differing only, in the relative intensity of seven spots). Thus, although the two organs are morphologically dissimilar, with distinct anatomies and modes of growth, they do not differ in detectable polypeptide profiles. This result suggests that the modification of leaf shape involves proteins of low abundance, or proteins which are expressed at critical early stages in ontogeny. The differences in anatomy and morphology are apparently not associated with major differences in physiological functions such as photosynthesis and processing of assimilates. By contrast, the morphological differences between the round and wrinkled seeds are understood to result from many differences in assimilate processing and storage.

The 2D-PAGE technique quantifies and validates the intuition that some morphological mutants are associated with far-reaching effects on the physiology of the organism, as reflected by modification of many of the abundant proteins, while others are not. Application of this technique to other near-isolines may reveal a wide spectrum of results depending on the genes examined. Information of this type should be valuable in developmental and quantitative genetic contexts.

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LITERATURE CITED


