J Mol Evol (1995) 41:231–237

JOURNAL OF MOLECULAR EVOLUTION

Genetic Polymorphism in Maritime Pine (*Pinus pinaster* Ait.) Assessed by Two-Dimensional Gel Electrophoresis of Needle, Bud, and Pollen Proteins

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Received: 12 October 1993 / Accepted: 13 December 1993

Abstract. Using two-dimensional polyacrylamide gel electrophoresis, the genetic variation of proteins was examined in three organs (needle, bud, and pollen) from 18 trees of maritime pine. Three types of variation were noted: presence/absence, staining intensity, and position variation of the spots. Of the 902 polypeptides scored in the three organs, 245 (27.2%) were polymorphic. Moreover, among these variable spots, 117 were found in a single organ, demonstrating an increased polymorphism of the organ-specific polypeptides (56.0% vs 18.4% for the organ-unspecific polypeptides). Finally, a positive correlation was found between variability level and subunit molecular weight for spots showing position variation but not for spots showing presence/absence or staining intensity variations. Possible explanations for this observation are discussed.

Key words: Maritime pine — 2D electrophoresis — Genetic variability — Diversity — Organ-specific polypeptides — Mobility variants — Molecular weight

Introduction

Since the study of O'Farrell in 1975, two-dimensional gel electrophoresis of denatured proteins has been used as a powerful tool in biological investigations. It is an appropriate technique for genetic investigations, since up to approximately 700 gene products may be revealed on a single gel (Bahrman et al. 1988a,b; Thiellement et al. 1989).

The genetic variability of proteins can be detected qualitatively as well as quantitatively with this technique (Anderson et al. 1985; Jungblut and Klose 1985; Leonardi et al. 1988; de Vienne et al. 1988; Bahrman and Damerval 1989). Differential genome expression in tissues and organs according to developmental or physiological stage has been studied (Klose 1982; Zivy et al. 1984; Leonardi et al. 1988). The modification of this expression by environmental stresses (e.g., heat shock and water deficit) has also been investigated (Baszczynski and Walden 1982; Zivy 1987; Damerval et al. 1988).

From numerous studies using isozyme loci, gymnosperms have been shown to have a higher percent of polymorphic loci and maintain more diversity within populations as compared to angiosperms. This is not a consequence of their taxonomic status per se, but rather of the life-history characteristics typical of these plants: they are long-lived woody species with large geographic ranges and outcrossing breeding systems (Hamrick et al. 1992). However, at the level of the nature or localization of the protein themselves, little is known of the determinants of the observed polymorphism in these trees. In this paper, we analyze the genetic polymorphism of 18 trees of maritime pine (*Pinus pinaster* Ait., $2n = 2x = 24$) from the Landes (France) by two-dimensional polyacrylamide gel electrophoresis. The genetic variability of needles, buds, and pollen is compared. An attempt has been made to assess the genetic variability and differential genome expression in the three organs.

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Materials and Methods

Plant Material. A total of 18 clones of the same age (25 years) which corresponded to 18 different, unrelated individuals of maritime pine from the Landes (France) provenance were analyzed. They were therefore equivalent to samples from a natural population, with the advantage of being grown within a common environment. Young needles and dormant vegetative buds of each tree were sampled from upper lateral branches and frozen in liquid nitrogen. Pollen samples were collected from each tree separately and stored at – 80°C until extraction.

Protein Extraction and Electrophoresis. The needle and bud proteins were extracted according to Damerval et al. (1986). The protein pellets from needles and buds were solubilized in UKS buffer (9.5 M urea, 5 mM K_2CO_3, 1.25% SDS, 0.5% dithiothreitol, 2% Pharmalyte pH 3–10, and 6% Triton X – 100). One hundred milligrams of each pollen sample were directly crushed in UKS buffer. After a 5 min centrifugation at 13,000 *g*, the supernatants were stored at –80°C until isoelectrofocusing. The isoelectrofocusing (IEF) rod gels were 24 cm long and 1.5 mm inner diameter. The mixture was 4% acrylamide, 9.2 M urea, 2% Triton X – 100, and 4% carrier ampholytes (¾ pharmalyte pH 5–8, ¼ pharmalyte pH 5–6). The IEF was performed for 40,000 Vh (Bahrman and Thiellement 1987). The second dimension was realized on slab gels (200 × 240 × 1.5 mm) bound to Gelbound PAG (Marine colloids) in a Dalt tank. Uniform gel composition was 11% acrylamide, 0.5 M Tris - Cl^-, 0.15% SDS, and 1% sucrose (Bahrman and Damerval 1989). Then 20 gels were simultaneously run and silver stained according to Damerval et al. (1987) in the apparatus described by Granier and de Vienne (1986). Two gels were made per genotype and for each organ (a total of 2 × 18 × 3 = 108 gels). An electrophoresis calibration kit (Pharmacia) was used to determine the molecular weight of polypeptide subunits.

Scoring Procedure. The dried gels were visually scored upon an illuminated box by superimposition. A difference was retained for further study only when both gels of the same genotype and organ gave the same result. In every case, no difference could be seen between the two repetitions. In addition, co-electrophoresis of different genotypes and/or organs were realized in order to verify that spots of similar molecular weight and isoelectric points present in different organs were indeed identical. These control experiments, coupled with the level of resolution of our gels, make it unlikely that different polypeptides may be confused. Indeed, it has been observed that "when a major spot is close to a minor spot and the gel is overloaded, the minor spot is shifted rather than being engulfed by the expanded major spot" (O'Farrell 1975). Finally, we checked the stability of protein patterns for needles and vegetative buds by sampling at different levels in the crown of an individual ramet and/or different ramets of the same genotype. No difference could be detected. Similar results were found for the conifer Douglas fir (Bahrman et al. 1985 and unpublished results).

For each protein, polymorphism was inferred from the examination of the 18 genotypes. The level of variability (or polymorphism) of a class of proteins was simply measured by the fraction of polymorphic proteins in this class.

Results

Differences of Protein Patterns Among Organs

Most polypeptides weighed between 20 and 100 kilodaltons (mean = 43.7 kD). The distribution of the weights of the 902 spots present at least in one of the three organs is given in Fig. 1. The total number of reproducible spots found in the 18 trees was equivalent in needle and bud (762 vs 744) but lower in pollen (702). There were 613 polypeptides common to all three organs, though they often differed in staining intensity. Hence, most spots found in a given organ are common to all organs: from 80.4% for needle to 87.3% for pollen. We detected 209 organ-specific spots (Table 1), 81 needle-specific (10.6% of the spots found in this organ), 54 (7.3%) bud-specific, and 74 (10.5%) pollen-specific spots. Needle and pollen shared 72.6% of their spots, and bud and pollen 76.1%. The greatest similarity was found between bud and needle with 81.9% of common spots.

Nature of Variation of the Protein Characters

Among the 902 spots found in the three organs, 245 (27%) were variable. For a given organ, three kinds of genetic variation were detected in 2D protein patterns (Figs. 2–4): (1) presence/absence variations, (2) position variations, and (3) staining intensity variation (quantitative variations).

1. Presence/absence variations were scored for 166 spots (Fig. 2 and Table 1).
2. Position variations were conservatively defined as isoelectric differences (amino acid substitutions causing isoelectric changes of the polypeptide). Spots were considered as allelic products of a structural gene when they differed in their isoelectric point though had similar molecular weights, aspects, and quantities. All the loci found were encoded by co-dominant alleles, as illustrated in Fig. 3. Moreover, when two of these spots co-occur, they usually displayed a reduced staining intensity (heterozygous state). We scored 52 such spots which belong to 26 structural genes (Fig. 3 and Table 1). All of them were diallelic. We found 155 heterozygotes and 313 homozygotes among the 18 × 26 combinations exam-

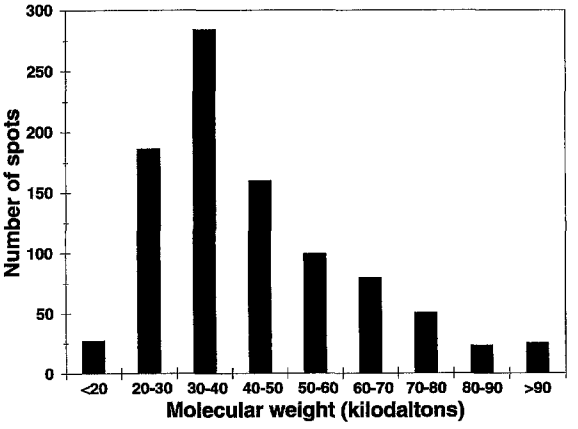

Fig. 1. Distribution of molecular weight of protein subunits found in three organs of maritime pine. All variable and unvariable spots scored in 2D PAGE were included.

Table 1. Number of variable and unvariable spots scored in the three organs

Kind of variation	Organ							
	Needle	Bud	Pollen	Needle/bud	Needle/pollen	Bud/pollen	Needle/bud/pollen	Total
Presence/absence variation	25	23	33	12	0	2	71	166
Position variation	4	6	10	0	0	2	30	52
Staining intensity variation	7	9	0	0	0	1	10	27
Subtotal	36	38	43	12	0	5	111	245
Unvariable	45	16	31	53	3	7	502	657
Total	81	54	74	65	3	12	613	902

ined. The number of heterozygote loci per individual genotype varied from 6 to 12 (from 23 to 46%), with a mean of 8.6 (33%) and standard deviation of 1.7 (6.5%), indicating no strong heterogeneity among individuals. The sum of the expected number of heterozygotes at each of the 26 loci (161.2) is close to the observed value (155), indicating no systematic deviation from the Hardy-Weinberg equilibrium.

3. Staining intensity variations (quantitative variations) were identified in 27 spots (Fig. 4 and Table 1). We were not able to distinguish more than two classes of intensity by visual scoring. Hence, only a subset of this class of polymorphism could be identified using this method, as compared to studies where precise quantification of spot intensities were made (cf., e.g., Damerval and de Vienne 1993). Nevertheless, we have shown elsewhere that the scored polymorphisms have typically a genetic basis, being inherited in a mendelian manner (Bahrman and Damerval 1989).

Level of Variability in the Three Organs

Considering all types of polymorphism together, the three organs showed very similar levels of variability, ranging from 20.8–22.6% of their polypeptides (Table 2). Moreover, the distribution of the types of polymorphism detected were also similar for the three organs: presence/absence variation accounted for 65.1% to 67.9% of all polymorphism, position variation from 21.3–26.4%, and quantitative variation from 6.9–12.1%. However, we did not detect pollen-specific variation in protein amounts (Table 3).

Level of Variability for Organ-Specific and Organ-Unspecific Polypeptides

The organ-specific polypeptides were characterized by a very high level of polymorphism (56.0%). This value was 44.4% for spots specific to needles, 70.4% for those of the buds, and 58.1% for the pollen polypeptides (Table 2), the proportion observed in needles being signifi-

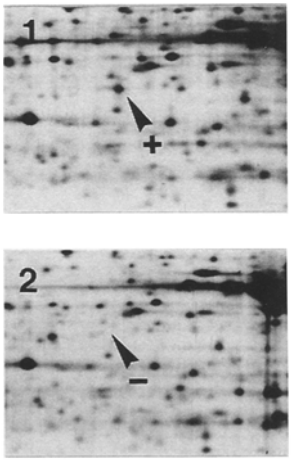

Fig. 2. Cuttings from 2D gels showing a presence/absence variation in the needles of two genotypes.

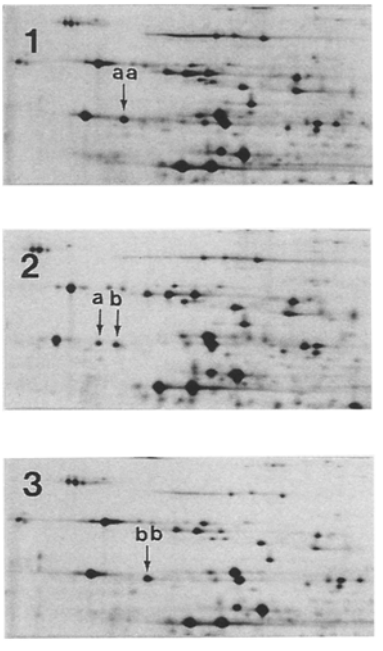

Fig. 3. Cuttings from 2D gels showing a position variation (allelism) in the needles of three genotypes. 1 and 3, homozygous genotypes; 2, heterozygous genotype.

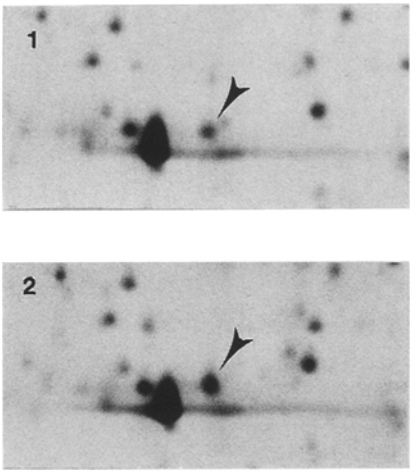

Fig. 4. Cuttings from 2D gels showing staining intensity variation in the needles of two genotypes.

cantly smaller than for the buds ($p < 0.01$). Among spots common to all three organs, only 18.4% were variable.

Correlation Between Genetic Variability and Molecular Weight of the Polypeptides

The level of variability was assessed in nine molecular weight classes (see Fig. 1). The relation between the fraction of each type of variation (presence/absence, position, and quantitative) and the mean molecular weight of the polypeptides in the class was tested by simple regression analysis. Polypeptides of higher molecular weights appeared to show an increased level of position variation (as can be seen in Figs. 5 and 6b): a test of the deviation from zero of the slope of the regression was highly significant ($p < 0.001$). The mean molecular weight of these polypeptides was 62.2 kD (standard error = 25.5), much higher than the value observed for the 902 spots studied (43.7 kD, standard error = 18.3). Only a weak and nonsignificant positive relation was observed between molecular weight and the other types of polymorphism (Fig. 6a and c).

Table 3. Number and percentage of variable spots (organ-specific and organ-unspecific) in each organ

	Organ		
Kind of variation	Needle	Bud	Pollen
Presence/absence variation	108 (67.9%)	108 (65.1%)	106 (66.6%)
Position variation	34 (21.3%)	38 (22.9%)	42 (26.4%)
Staining intensity variation	17 (10.7%)	20 (12.1%)	11 (6.9%)
Total	159 (100.0%)	166 (100.0%)	159 (100.0%)-

Discussion

The present paper has examined the genetic variability of abundant proteins in needle, bud, and pollen of 18 mar-

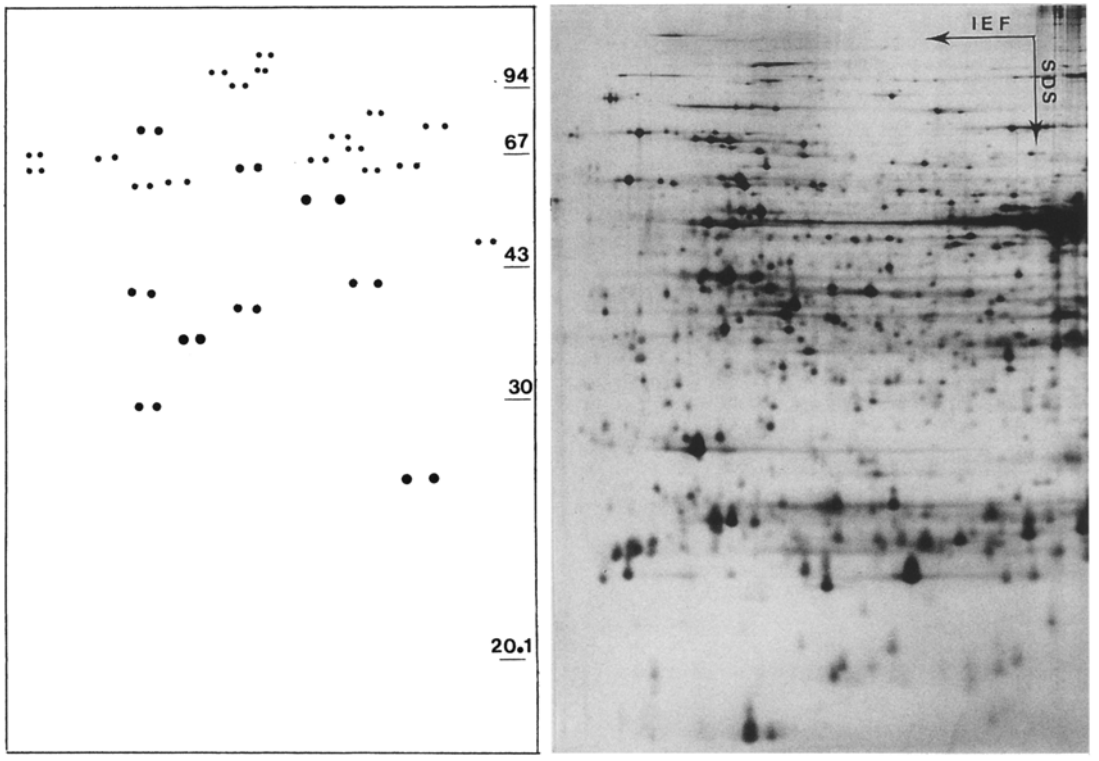

Fig. 5. Distribution of position variants in 2D gels. *Left:* map of the 26 diallelic loci found in the study of the three different organs. *Right:* an example of a 2D pattern from needles. The molecular weight (in kilodaltons) of standard proteins is indicated.

Table 2. Number and level of variability for organ-specific and organ-unspecific polypeptides

Organ	Organ-specific spots	Variability	Organ-unspecific spots	Variability	Total number of spots	Total variability
Needle	81	44.4%	681	18.1%	762	20.8%
Bud	54	70.4%	690	18.5%	744	22.3%
Pollen	74	58.1%	628	18.4%	702	22.6%
all organs	209	56.0%	693	18.4%	902	27.2%

itime pines. The comparison of protein profiles in pollen samples with those of needle and bud samples is expected to be genetically relevant since a mixture of haploid pollen grains from the same tree should reflect the paternal genotype if the segregation of polypeptides at heterozygous loci is not distorted at meiosis. In a previous study of the segregation of polypeptides among megagametophytes of the same 18 trees, little meiotic distortion was indeed noted (Gerber et al. 1993). Moreover, high pollen viability was found using a fluorescence method.

Most polypeptides (from 80–87%) found in a given organ appear to belong to the class of common polypeptides. These abundant polypeptides, present in several well-differentiated organs, may be considered as "house-keeping proteins," being involved in primary metabolism. Their proportion is comparable, for example, to that found by Leonardi et al. (1988) in maize mesocotyl (80%). However, our study included both sporophytic and gametophytic phases of the life cycle and confirm the extensive overlap in the gene expression of both generations already demonstrated using a more limited number of proteins (Zamir 1983; Ottaviano et al. 1991).

The proportion of polymorphic spots is nearly the same in all three organs (about 22% of the polypeptides), though the needle-specific proteins are less variable than those of the buds. It is interesting to point out that polypeptides encoded by the chloroplast DNA molecule are known to have a slow rate of evolution (Palmer 1987). However, they represent only a small fraction of the chloroplast polypeptides, though they are implicated in the constitution of several major multimeric proteins.

In a comparison of two maize lines using similar techniques, Leonardi et al. (1988) found a significantly smaller level of variation in the blade (7.5%) than in the mesocotyl or the sheath (12.6% and 13.2%, respectively). Previous work on endosperm of maritime pine using the same 18 trees (Gerber et al. 1993) identified 150 variable spots which were classified into 84 putative loci. This compares well with the number of variable spots found in our study (from 151–159, depending on the organ). However, a higher proportion of position variants could be demonstrated in the endosperm, which is probably due to the superior power of the genetic analysis used to infer the allelic state of polypeptides, especially when the alleles are far from each other in the IEF dimension, or when the spots also differ in molecular weight and in shape. In the absence of a genetic analysis in the present study, a conservative approach had to be

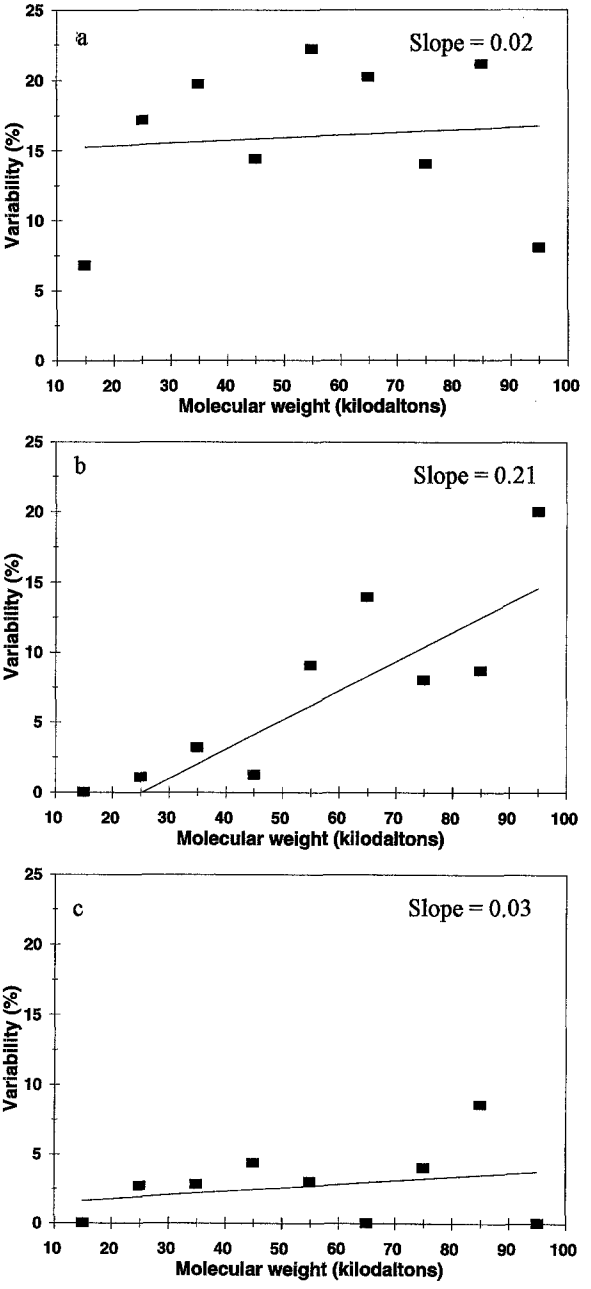

Fig. 6. Relationship between subunit molecular weight and level of (**a**) presence/absence, (**b**) position, and (**c**) staining intensity variation. The slope of the regressions are indicated in the upper right.

used to classify spots as true position variants of a polypeptide. The number of scorable quantitative variants is also similar to our study (15 in the endosperm vs 11 in the pollen, 17 in the needle, and 20 in the bud). However, we have not detected spots expressing both quantitative variation and position variation, as in the endosperm study (Gerber et al. 1993). The variability in protein amounts in these haploid tissues was shown to be under genetic control (Bahrman and Damerval 1989; Gerber et al. 1993). Though genetic variation in protein amounts may result from mutations of the affected protein, most should result from mutations in the regulatory system of the protein, as discussed by Klose (1982). In addition, the presence/absence of proteins could also correspond to a quantitative variation, as a consequence of the action of regulatory genetic elements, where the absent spot is below the level of detection by silver staining. Alternatively, the absence of a spot could correspond to a null-allele (i.e., a mutation of the gene coding for the polypeptide itself).

The much higher polymorphism in the organ-specific proteins was first reported by Klose (1982) in his study of different mouse strains. For instance, 13% of the kidney-specific proteins were polymorphic, though only 3% of the organ-unspecific proteins were variable. Our results confirm this observation, which was interpreted by Klose (1982) as a direct consequence of the dispensability of these organ-specific proteins which are not essential for the structure, maintenance, growth, and multiplication of the cell, and are therefore supposed to be submitted to less "functional constraints" (Kimura 1983).

As the "variation in the substitution rate per polypeptide is due to the difference either in the number of amino acids or in the substitution rate per site" (Nei 1987), we also expected a higher level of polymorphism in large polypeptides. This has been demonstrated by several authors using data from isoenzyme electrophoresis (Koehn and Eanes 1977; Nei et al. 1978; Chakraborty et al. 1980). However, to date, similar evidence in favor of this prediction does not appear to have been reported with the 2D PAGE technique. For example, in his study, Klose (1982) has stressed that "the genetic alteration of proteins is not correlated with the isoelectric point or the molecular weight." However, in our study, the polypeptides characterized by position variation (allelic variation) did show a strong positive correlation with molecular weight. We have also found that there are no strong correlations between presence/absence variation or quantitative variation and the subunit molecular weight. If the polymorphisms of protein amounts and in presence/absence variation are caused by regulatory elements outside of the gene coding for the polypeptide itself, as proposed above, this absence of correlation is actually expected.

In this study, we have identified several factors correlated with the level of variability of proteins: the molecular weight (in the case of position variation, as discussed above), the degree of organ-specificity of the polypeptide, and its localization in the plant (for organ-specific polypeptides). A more comprehensive study of the genetic variability of polypeptides belonging to different compartments (organs or tissues) of an individual tree could reveal additional factors or causes determining variability.

Acknowledgments. We thank Drs. H. Boucherie, C. Damerval, A. Kremer, and M. Zivy for helpful comments on the manuscript. This research was supported in part by the contract BRG of the Ministère de la Recherche et de la Technologie.

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