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Protein Changes in Response to Progressive Water Deficit in Maize[1]

Quantitative Variation and Polypeptide Identification

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Three-week-old plants of two unrelated lines of maize (*Zea mays* L.) and their hybrid were submitted to progressive water stress for 10 d. Changes induced in leaf proteins were studied by two-dimensional electrophoresis and quantitatively analyzed using image analysis. Seventy-eight proteins out of a total of 413 showed a significant quantitative variation (increase or decrease), with 38 of them exhibiting a different expression in the two genotypes. Eleven proteins that increased by a factor of 1.3 to 5 in stressed plants and 8 proteins detected only in stressed plants were selected for internal amino acid microsequencing, and by similarity search 16 were found to be closely related to previously reported proteins. In addition to proteins already known to be involved in the response to water stress (e.g. RAB17 [Responsive to ABA]), several enzymes involved in basic metabolic cellular pathways such as glycolysis and the Krebs cycle (e.g. enolase and triose phosphate isomerase) were identified, as well as several others, including caffeate *O*-methyltransferase, the induction of which could be related to lignification.

Water availability is a major limiting factor for plant growth. Limited water availability leads to reduced growth of aerial parts and, to a lesser extent, of the root system. Several other responses have been described, such as stomatal closure and synthesis of osmolytes (e.g. betaine and Pro). These responses are at least partly controlled by ABA, a phytohormone that increases in concentration in plants subjected to water deficit (Zeevaart and Creelman, 1988).

Numerous genes expressed in response to water deficit and/or ABA in different species encode RAB (Responsive to ABA) proteins or dehydrins that exhibit high hydrophilicity and contain repeated domains. Most of these genes are also highly expressed during late embryogenesis (*lea* genes). Sequence features allowed their classification into different groups (Close et al., 1989; Dure et al., 1989), and tentative functions were proposed, according to the predicted protein structure (Dure, 1993; Lisse et al., 1996), to be sequestration of ions or water or preservation of membrane or protein structure (chaperone function). Other proteins with functions related to water deprivation were found to be induced by water deficit or saline stress: e.g. proteins showing domain or sequence similarities to transmembrane channel proteins (Guerrero et al., 1990; Yamaguchi-Shinozaki et al., 1992; Fray et al., 1994; Ruiter et al., 1997) and betaine aldehyde dehydrogenase, which catalyzes the last step of betaine synthesis (Weretilnyk and Hanson, 1990; Ishitani et al., 1995).

Water deficit also induces the expression of proteins not specifically related to this stress, but rather to reactions against cell damage. These include different classes of heat-shock protein genes or cognates (Heikkila et al., 1984; Almoguera and Jordano, 1992; Kiyosue et al., 1994), thiol proteases (Guerrero et al., 1990; Williams et al., 1994), proteinase inhibitors (Downing et al., 1992; Reviron et al., 1992), and osmotin (Kononowicz et al., 1993). In maize (*Zea mays* L.) a ferritin gene induced by iron stress is also induced by drought and ABA (Fobis-Loisy et al., 1995).

Finally, several genes encoding proteins with functions not directly related to stress were shown to be expressed at greater levels in response to drought or salinity stress: several enzymes involved in glycolysis (Umeda et al., 1994; Velasco et al., 1994) and in the synthesis of Met (Glaser et al., 1993), SAM (Espartero et al., 1994; Chang et al., 1995), peroxidases (Botella et al., 1994), nonspecific lipid transferases (Torres-Schumann et al., 1992; Ouvrard et al., 1996), and early light-induced proteins (Bartels et al., 1992; Ouvrard et al., 1996).

Although the simultaneous changes in gene expression and physiological responses strongly suggest that induced proteins play a role in these responses, the correlation between their expression and the level of stress tolerance in the different genotypes has been rarely studied (Ramagopal, 1987; Hurkman et al., 1989; Moons et al., 1995). As a first step in such a study, we have undertaken the characterization of protein responses of two maize lines and their F_1 hybrid. These lines, which display contrasting behavior in response to water stress in the field, are the parents of a

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Abbreviations: 2DE, two-dimensional electrophoresis; ASR, ABA-water stress-ripening-induced protein; COMT, caffeate *O*-methyltransferase; SAM, *S*-adenosyl-L-Met.

population of recombinant inbred lines, which will be used for analyzing the relationship between protein induction and stress tolerance.

In the vast majority of studies reporting the induction of gene expression upon water deprivation, osmotic stress, or ABA treatment, young seedlings were submitted to abrupt stress. However, in the field plants are submitted to more gradual stress because water availability in the soil does not change abruptly, and, therefore, responses might be different. For example, Leone et al. (1994) showed that different sets of polypeptides were synthesized in potato cells submitted to abrupt or gradual osmotic stress. In the present study we submitted autotrophic plants (approximately 3 weeks old) to a gradual dehydration. The protein response of the two maize lines and their hybrid was studied by 2DE, and computer-assisted quantitative analysis allowed the detection of proteins with accumulation altered by drought. Nineteen induced proteins were microsequenced.

MATERIALS AND METHODS

Plant Material

Two genetically distant lines of maize (*Zea mays* L.) and their hybrid were used: F_2, a flint line from the Institut National de la Recherche Agronomique (France) referred to as Lc, and Io, an American dent line from the Iodent group. The parental lines were chosen for their differential response to water deprivation in the fields: the percentage of yield decrease in nonirrigated conditions was greater for Lc than for Io.

For the quantitative analysis the two parental lines and their hybrid were grown in four successive batches in a growth cabinet under controlled conditions (450 μmol m^{-2} s^{-1} irradiance during the 16 h-photoperiod, 25°C day and 20°C night temperatures, and 60% RH). Plants were grown in perlite (one plant per 14-cm-diameter pot) and watered with nutrient solution.

The plant material required for microsequencing was obtained from approximately 500 plants of the 2 parental lines in 5 successive batches. They were grown in a greenhouse (1 plant per 14-cm-diameter pot containing soil) at uncontrolled temperature and RH.

Both in the cabinet and in the greenhouse, a subset of plants was stressed by withholding watering at the fifth-leaf stage, corresponding approximately to 3-week-old plants. After 10 d of stress, 2 to 3 cm of the etiolated part of the blade of the sixth leaf was harvested and immediately frozen in liquid nitrogen for further protein analysis.

ABA Content and Water Potential Measurement

ABA content was determined according to the method of Quarrie et al. (1988). Leaf water potential was measured with a portable pressure chamber (PMS Intrument, Corvallis, OR).

Protein Extraction and Gel Electrophoresis

Analytical 2DE

Denaturing protein extraction was applied as described in Damerval et al. (1986), except that 60 μL of resolubilization solution was used to resuspend 1 mg of pellet. The isoelectric and SDS-PAGE dimensions were as described by Damerval et al. (1987) with a protein load of about 45 μg. Gels were silver stained according to the method of Damerval et al. (1987) as modified by Burstin et al. (1993).

Preparative 2DE for Microsequencing

To facilitate isolation of proteins of low abundance, enriched fractions were obtained by differential precipitation with acetone. Proteins were first solubilized in a Tris-HCl buffer, pH 8.7, according to the method of Zivy et al. (1983), and different fractions were obtained by gradually increasing the acetone concentration in the solution. The fractions were compared by analytical 2DE, and those showing the highest relative concentration for the protein to be microsequenced were used for preparative 2DE. Preparative 2DE was similar to analytical 2DE except for the following modifications: about 500 μg of protein was loaded on IEF gels (3 mm diameter) and the slab gels were 1.5 mm thick, with stacking gels. The gels were stained with Amido black according to a procedure adapted from Chen et al. (1993) prior to in situ gel digestion. Depending on the abundance of the protein, spots were taken from 5 to 30 preparative gels for microsequencing.

Protein Microsequencing and Search for Similarities in Amino Acid Sequences

Internal amino acid sequences of leaf proteins were obtained from Drs. J. d'Alayer and M. Davi at the Laboratoire de Microséquençage des Protéines, Institut Pasteur (Paris, France). The procedure is described in Touzet et al. (1996b). Amino acid sequences were compared with the sequences in the Non Redundant Peptide Sequences Database of the National Center for Biotechnology Information by using the BLAST program (Altschul et al., 1990).

2DE Quantitative Analysis

Wet, silver-stained gels were scanned (model 7899, Eikonix, MA) with a spatial resolution of 1 pixel/100 μm and an optical density range from 0.0 to 1.2. Image treatment, spot detection, and quantification were done using the Kepler package (LSB Corp., Rockville, MD). Spot detection followed a method developed by M. Zivy (unpublished data). Quantification was based on the modeling of spots by two-dimensional Gaussians, with parameters fitted to the image obtained after background subtraction. To compensate for staining variation between gels, spot intensities of each gel were scaled relative to the sum of spot intensities within a rectangle defined in the same way for each gel. The upper left and bottom right angles of this rectangle were at approximately the positions of spots 21 and 714, respectively (Fig. 1). As shown by Damerval (1994), the intensity of the large majority of proteins is linearly related to the protein quantity when this method of silver staining is used. Only the spots with responses not affine to the mean response would not be correctly scaled (Burstin et al., 1993). Most proteins that do not have a linear response to

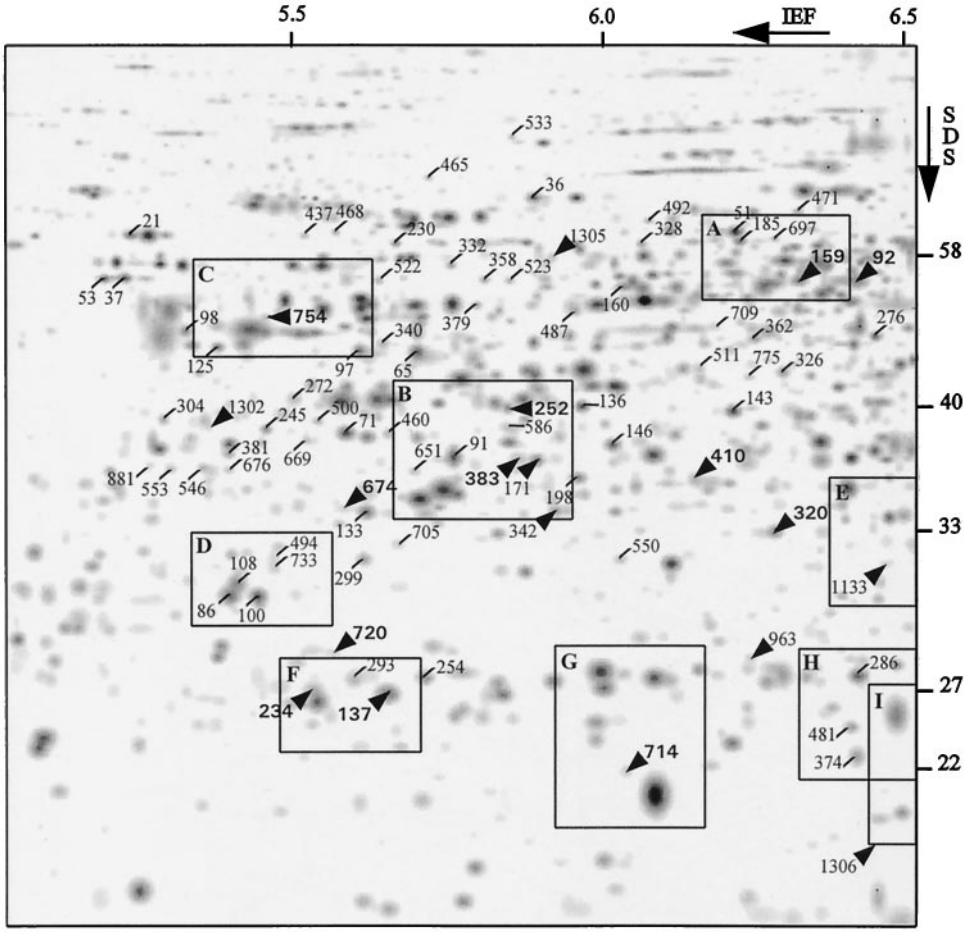

Figure 1. Computer image of a silver-stained 2DE gel of leaf proteins from the hybrid Io × Lc subjected to water stress in a growth cabinet. Numbered spots correspond to proteins in which quantity was altered during water stress (increased or decreased). Arrows indicate sequenced proteins induced in both growing conditions (bold type) or only in the greenhouse (normal type). Boxes correspond to gel regions enlarged in Figure 2. Molecular masses (in kilodaltons) are indicated.

silver stain show a plateau; for these proteins, the computed induction ratio is a minimum value of induction.

One two-dimensional gel per plant was run. Considering the three genotypes (Io, Lc, and the hybrid) and the two treatments (irrigated and nonirrigated), six genotype × treatment combinations were studied. Fifty-six two-dimensional gels were used, approximately 10 gels per genotype × treatment combination. The four batches of cultivation counted 10, 16, 15, and 15 plants, and each genotype × treatment combination was represented in each batch. To take into account possible genotype × batch interactions, two models of analysis of variance were used:

$$\text{Spot intensity} = \mu + T + G + B + (G \times T) + (G \times B) \quad (1)$$

where T, G, B, G × T, and G × B stand for treatment, genotype, batch effect, genotype × treatment, and genotype × batch interactions, respectively. As only two spots (s143 and s254) showed a significant G × B interaction, a more simple model was used for the other spots:

$$\text{Spot intensity} = \mu + T + G + + (G \times T) \quad (2)$$

Spots were retained in the analysis when the treatment effect was significant at $P < 0.01$ or when the G × T interaction was significant at $P < 0.05$.

RESULTS

Plant Responses to Water Stress

Plants Grown in the Growth Cabinet

Watering was stopped when the fifth leaf was emerging. At this stage, plants from the two unrelated lines and their hybrid had three ligulated leaves. The height of the last ligule was on average 10.8 cm, although Io was 3 cm shorter than Lc and the hybrid ($P < 0.0001$). During the following 10 d, growth was substantially lower in stressed plants than in controls ($P < 0.0001$): height increase of the last ligule was 4.0 and 10.0 cm, respectively, for stressed and control plants. In the same time, stressed and control plants produced, respectively, 1.2 and 2.8 emerged leaves ($P < 0.0001$). In both control and stress conditions, the height increase was significantly less in Io than in the

hybrid and Lc (difference of approximately 2 cm), whereas Lc plants produced 0.8 fewer new leaves than Io and the hybrid. A significant genotype × treatment interaction was found for the number of ligulated leaves: Lc plants produced 0.7 fewer ligulated leaves than Io or the hybrid in control conditions, but the same number in stress conditions.

The percentage of dry leaves was significantly smaller in stressed Lc plants (34%) than in the two other genotypes (46%), which is consistent with data from studies conducted in the field. ABA content in the 6th leaf was significantly higher in stressed plants (715 ng/g of dry matter) than in controls (227 ng/g). Water content ([fresh weight − dry weight]/[dry weight]) was also significantly lower in stressed plants (6.0) than in controls (12.4). ABA and water content showed no significant genotype effect.

Plants Grown in the Greenhouse

After 10 d of water deprivation, the average measures in the sixth leaf of stressed plants and controls were, respectively, −1.5 and −0.3 MPa for leaf water potential, 73.9% and 94.6% for relative water content, 7.0 and 14.0 for water content, and 1321 and 244 ng/g of dry matter for leaf ABA content. No significant genotype effect was observed.

Plants grown in the greenhouse were more stressed than those cultivated in the growth cabinet; not only was their ABA content almost doubled, but they also exhibited much more leaf rolling. Other experiments with the same genotypes cultivated in the same way confirmed that the 10 d of water stress was more severe in the greenhouse than in the growth cabinet: the water potential of stressed leaves was generally between −0.8 and −1.0 MPa in the growth cabinet compared with −1.5 MPa in the greenhouse, and plant growth was more quickly inhibited in the greenhouse than in the growth cabinet.

Quantitative Analysis of the Protein Responses

Out of 413 spots reproducibly detected, 78 were affected by drought (Table I); of these, 50 were increased, and of these, 10 were present only in stressed plants. Twenty-three other proteins were decreased, and 5 showed no treatment effect but did show a significant genotype × treatment interaction. Stress-affected proteins are shown in Figure 1 and several examples of protein induction are shown in Figure 2.

As shown in Table I, 38 proteins showed a different response according to the genotype: 5 proteins were synthesized by only 1 of the parental lines in response to water stress. The other 33 proteins showed a genotype effect in addition to the treatment effect and/or a genotype × treatment interaction. The quantities of proteins showing a significant genotype effect but no interaction were different between genotypes whatever the treatment, but showed the same increase (or decrease) in response to water stress. The existence of a significant interaction indicates that the response to the stress was not the same for the three genotypes. Tests of multiple comparisons of means (the Student-Newman-Keuls method) allowed us to study pro-

Table I. *Leaf proteins affected by 10 d of water stress*

For proteins present in stressed plants, but absent in controls (P/A), the genotype column contains the parental lines in which they were observed.

Treatment Effect[a]	Genotype Effect	Interaction	Spot No.
↑ P/A	Io and Lc		s159 s374 s383 s487 s720
↑ P/A	Io only		s705 s714 s733 s775
↑ P/A	Lc only		s494
↑ **[b]			s21 s37 s86 s97 s100 s108 s133 s137 s143 s252 s272 s293 s304 s320 s340 s410 s492 s500 s553 s676 s709 s754 s881
↑ **	*		s92 s136 s586 s674
↑ **	**		s36 s53 s234 s358 s481
↑ **		*	s91 s245 s468 s533
↑ **	**	*	s71 s198
↑ **	**	**	s254 s550
↓ **			s98 s230 s299 s328 s332 s362 s437 s460 s471 s523 s546 s669
↓ **	*		s160 s185 s465 s522
↓ **	**		s125 s697
↓ **		*	s65
↓ **	*	*	s146
↓ **	**	*	s511 s651
↓ **	**	**	s276
	*	**	s381
		*	s51 s286 s326 s379

[a] Treatment effects are as follows: ↑, increased proteins in stressed plants; ↓, decreased protein in stressed plants; and P/A, present in stressed plants but absent in controls. [b] Statistical significance, $P < 0.01$ (**) and $P < 0.05$ (*) for treatment, genotype, and interaction effects in the analysis of variance.

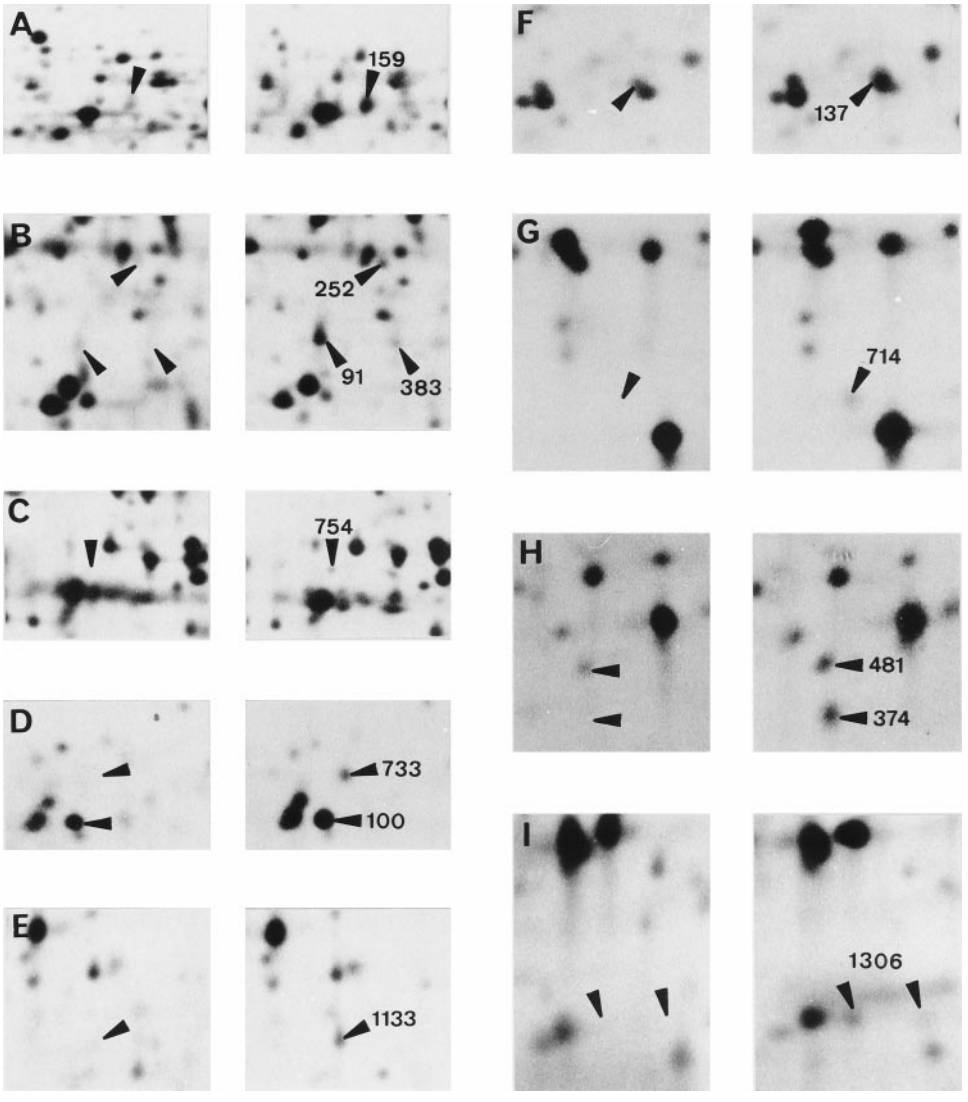

Figure 2. Examples of protein induction in leaves of the maize line Io in response to 10 d of water deprivation in a control plant (A–E) and in a stressed plant (F–I). Letters correspond to the boxes in Figure 1. All plants except that shown in I were grown in a growth cabinet. Peptides from spots 159 (A), 252 (B), 383 (B), 754 (C), 1133 (E), 137 (F), 714 (G), and 1306 (I) were microsequenced (see Table II).

teins showing significant genotype × treatment interaction. For all of them the interaction was due to a differential increase or decrease of protein by drought in the different genotypes. No protein was significantly increased in one genotype and significantly decreased in the other genotypes.

Identification of a Subset of the Induced Proteins

Two-dimensional analytical gels of the parental lines grown in the greenhouse were visually analyzed and compared with the one used for quantitative analysis. Among the proteins that reproducibly increased in quantity under water stress in both culture conditions, a subset of 12 was selected for amino acid sequencing. Four of them were detected only in stressed plants (s159, s383, s714, and s720), and in the others the induction factor (intensity in stressed plants/intensity in control plants) varied from 1.3 to 5.0 (Table II).

Seven other proteins (s171, s342, s963, s1133, s1302, s1305, and s1306) that were reproducibly increased in the greenhouse but not in the cabinet were microsequenced. Among them, s1133, s1302, s1305, and s1306 were observed only in stressed plants. Therefore, a total of 19 proteins was selected, for which 22 internal amino acid sequences were obtained, with a length of 10 to 26 residues (Table II).

Sequence comparisons showed nine similarities to proteins previously characterized in maize with 93% to 100% identity: ferritin, COMT (EC 2.1.1.6), β-glucosidase (three spots, EC 3.2.1.21), RAB17, enolase (EC 4.2.1.11), cytosolic triose phosphate isomerase (EC 5.3.1.1), and a putative cytoplasmic NAD-malate dehydrogenase (EC 1.1.1.37). An ASR initially described in tomato can be added to this

Table II. *Induction level and sequence similarity of proteins induced by water stress in leaves of maize lines Io and Lc*

Protein	Genotype	Induction Factor[a]	Amino Acid Sequence	Description	Species	Amino Acid Identity	Reference
						%	
s92	Io and Lc	3.3	KGENGXIGLAFDVMGR	β-Glucosidase, *glu2* gene (EC 3.2.1.21)	Maize	93	Bandaranayake and Esen (1996)
			KNWLTFNEPQTFTSFS	β-Glucosidase, *glu2* gene (EC 3.2.1.21)	Maize	100	Bandaranayake and Esen (1996)
s137	Io and Lc	2.1	KEVLSGVVFQPFEEIK	Ferritin	Maize	100	Lobreaux et al. (1992)
s159	Io and Lc	S[b]	KVCFDNFGDK	β-Glucosidase (EC 3.2.1.21)	Maize	100	Esen (1992)
s171	Lc	–	KMELVDAAFPLLK	Putative cytoplasmic malate dehydrogenase (EC 1.1.1.37)	Maize	100	Keith et al. (1993)
s234	Io and Lc	1.3	KDWSNVVLAYEPVWAI	Cytosolic triose phosphate isomerase (EC 5.3.1.1)	Maize	93	Marchionni and Gilbert (1986)
s252	Io and Lc	5.0	KSVGGQPVVFDSVK	Glu 1-semialdehyde 2,1-aminotransferase (EC 5.4.3.8)	Barley	92	Grimm (1990)
s320	Io and Lc	1.7	KHSLGQSHPVLLTRHN	ABA-induced protein	Rice	68	Moons et al. (1996)
s342	Io and Lc	–	KVLEGAEERLQLLK	NI[c]			
s383	Io and Lc	S	KWILHDWSDAHXATLL	COMT (EC 2.1.1.6)	Maize	93	Collazo et al. (1992)
s410	Io and Lc	3.7	KTIASPGRGILAMDES	Chloroplastic Fru bisphosphate aldolase (EC 4.1.2.13)	Pea	100	Razdan et al. (1992)
				Chloroplastic Fru bisphosphate aldolase (EC 4.1.2.13)	Rice	100	Tsutsumi et al. (1994)
s674	Io and Lc	1.7	KGKVXIFIGGIGTGGT	Cys synthase, chloroplastic precursor (EC 4.2.99.8)	Spinach	81	Saito et al. (1993)
s714	Io	S	KQHLGEAGAIAAGAFA	ASR 2	Tomato	60	Amitai-Zeigerson et al. (1994)
				Water-inducible protein DS2	*S. chacoense* B.	60	Silhavy et al. (1995)
				cDNA clone similar to ASR protein (Expressed sequence tag)	Maize	94	Shen et al. (1994)
s720	Io and Lc	S	KRAPKLNERILSSLSR	Soluble inorganic pyrophosphatase (EC 3.6.1.1)	Potato	81	du Jardin et al. (1995)
s754	Io and Lc	1.4	KYNQLLRIEEELGDAA	Enolase (EC 4.2.1.11)	Maize	100	Lal et al. (1991)
s963	Lc	–	KSLEGAFVLNQHQPAE	NI			
s1133	Io	S	KIATVEPVTMK	NI			
			KAVHNLVEAVSQHGVA	NI			
			KYVHVVTVDSHDFWFM	NI			
s1302	Io and Lc	S	KASIEARKPDFDAFID	Phosphoribulokinase (EC 2.7.1.19)	Wheat	100	Raines et al. (1989)
s1305	Io and Lc	–	KLAGSYNMLGLNYY	β-Glucosidase, root meristem precursor (EC 3.2.1.21)	Maize	100	Brzobohaty et al. (1993)
s1306	Io and Lc	S	KDDQHATATTGGAYGQQGHTGSAYGQ	RAB17-dehydrin DHN1	Maize	100	Vilardell et al. (1990)

[a] Average intensity in stressed plants/average intensity in control plants. Not calculated for proteins induced only in the greenhouse (visual scoring). [b] S, Protein only detected in stressed plants. [c] NI, No identification.

list because of its relationship to a maize expressed sequence tag.

Six proteins are newly described in maize in this paper, but have been described previously in other plant species: soluble inorganic pyrophosphatase (potato, EC 3.6.1.1), glutamate-1-semialdehyde 2,1-aminotransferase (barley, EC 5.4.3.8), phosphoribulokinase (wheat, EC 2.7.1.19), chloroplastic Cys synthase (spinach, EC 4.2.99.8), chloroplastic Fru 1,6-bisphosphate aldolase (pea and rice, EC 4.1.2.13), and an ABA-induced protein (rice). Although potato, spinach, and pea belong to taxonomic groups very distant from maize, 81% to 100% identity in amino acid sequences was found between the peptide fragments and pyrophosphatase, Cys synthase, and aldolase. Very good probabilities were given by the BLAST program for these identifications. For the tomato ASR protein, the probability value was only 0.85, but three other amino acid sequences obtained from maize coleoptiles confirm the relationship with this protein (Touzet et al., 1996b). Finally, the internal amino acid sequences of three other proteins did not match any sequence in the databases.

DISCUSSION

We described protein changes occurring in maize leaves after progressive dehydration of the plants. Quantitative analysis revealed 78 proteins showing a significant alteration. Forty proteins detected in the controls and in stressed plants exhibited a 1.1- to 5.0-fold increase on average in the three genotypes upon water stress, whereas 10 others were reproducibly detected only in stressed plants. However, this should not be interpreted as specific expression in stressed plants; some protein spots were present at such a low intensity in controls that they were not detected by the computer in most two-dimensional gels. The relative quantity of 23 proteins was found to decrease to 50% to 80% of the control in stressed plants: this could be due to the repression of the synthesis of some proteins, but also to differential turnover.

Comparison between protein responses of the three genotypes studied revealed different kinds of genetic variations (Table I). Several induced proteins were specific to one of the two parental lines. In two instances (s494/s733

and s37/s53), two proteins were close to each other on two-dimensional patterns, were mutually exclusive in the two parental lines, and were both present in the hybrid; therefore, these are likely to be allelic products (for review, see de Vienne et al., 1996). For the remaining genotype-specific increased proteins (e.g. s714 and s171, identified as ASR protein and malate dehydrogenase, respectively), no alternate allelic form was detected in the genotype in which they were missing: the absence of the protein could be due to a null allele or the presence/absence variation could be controlled by another locus. Also, the effect of water stress was accompanied by a genotype effect for some other proteins: although their constitutive level was different, these proteins were similarly increased or decreased by drought in the three genotypes. Finally, the response of a few other proteins exhibited a genotype × treatment interaction, i.e. the protein quantity was differentially modified by stress according to the genotype.

Microsequencing was performed to tentatively identify 19 proteins reproducibly induced upon water stress in growth cabinet and/or in greenhouse conditions. Two of these proteins, detected only in stressed plants, were already known to be induced under various conditions of water stress and/or by exogenous ABA in different species, but have only putative functions.

RAB 17 (s1306), first described in maize embryos during the maturation phase (Vilardell et al., 1990), is induced in immature embryos and plantlet leaves by exogenous ABA and water stress (Close et al., 1989; Pla et al., 1989; Vilardell et al., 1990). This protein, located in the nucleus and in the cytosol, may play a role in nuclear protein transport through binding with nuclear-localization signal peptides (Goday et al., 1994).

ASR protein (s714), an ABA/water-stress/ripening-related protein exclusively induced in the Io genotype, was initially described in tomato (Iusem et al., 1993; Amitai-Zeigerson et al., 1994; Rossi and Iusem, 1994). It is also induced by water stress in *Solanum chacoense* (Silhavy et al., 1995) and loblolly pine (Chang et al., 1995). Subcellular fractionation (Iusem et al., 1993) and the presence of a nuclear-targeting sequence motif (Silhavy et al., 1995) led the latter authors to suggest that this basic protein (pH 7.9) may be involved in the protection of DNA structure during water loss or in gene regulation upon stress by changing DNA topology. However, s714 and the pine protein are more acidic (pH 6.1, Fig. 1). ASR protein accumulated in both growing conditions, whereas RAB17 was synthesized only in the greenhouse, i.e. in more drastic conditions of water stress.

The functions of two other induced proteins can be related to stress, although not exclusively to water stress. Ferritin (s137) is an iron-storage protein encoded in maize by at least two different genes, *fm1* and *fm2*. The expression of these two genes is induced by iron in roots and leaves, but only *fm2* was found to be induced by ABA or rapid dehydration of plantlets (Lobreaux et al., 1992, 1993; Fobis-Loisy et al., 1995). Progressive water stress increased the protein quantity by a factor of 2.1 in our conditions. The protein pair s53/s37, a supposed pair of allelic proteins, was not microsequenced but the amino acid composition is close to that of oryzain, a thiol-protease (Touzet et al., 1996a). Thiol proteases have been found to be induced by water stress (Guerrero et al., 1990; Williams et al., 1994).

In most cases the other identified proteins were enzymes involved in important metabolic pathways of higher plants. Triose phosphate isomerase (s234) and enolase (s754), which were 1.3- and 1.4 fold-induced, respectively, and NAD-malate dehydrogenase (s171) are enzymes involved in glycolysis, the Krebs cycle, and/or the oxydative pentose phosphate pathway. Several enzymes of these ATP-generating pathways, including triose phosphate isomerase, were shown to be induced upon saline and water stress in cultured cells of rice (Umeda et al., 1994). This coordinated induction is thought to be essential for activation of the entire energy-producing pathway to maintain homeostasis in stressed cells. It is notable that enolase is also involved in the response to other environmental stresses, such as anaerobic stress of maize roots (Lal et al., 1991), heat shock in yeast (Iida and Yahara, 1985), heat shock, salt stress, ABA treatment, and water stress in the common ice plant (Forsthoefel et al., 1995). Like the ASR protein, enolase seems to be induced during tomato fruit ripening (Van der Straeten et al., 1991).

Protein s383, which was detected only in stressed plants, is related to COMT, a key enzyme in the biosynthesis of lignin monomers catalyzing methylation of cinnamic acids. In normal growth conditions the gene has been shown to be expressed in elongating tissues, where active lignification of vascular system elements occurs (Collazo et al., 1992; Vignols et al., 1995). COMT genes are induced by pathogen attacks (Gowri et al., 1991; Jaeck et al., 1992; Pellegrini et al., 1993; Gregersen et al., 1994), i.e. in conditions also inducing active lignification (Lange et al., 1995). Other identified enzymes induced upon progressive dehydration are involved in general cell metabolism such as glycolysis and photosynthesis, but their function can be related to the phenylpropanoid pathway and lignin biosynthesis.

Soluble inorganic pyrophosphatase (s720), among other functions, participates in the assimilation of mineral nutrients, especially in sulfate activation (Schmidt and Jäger, 1992), and can then be connected to another induced protein, Cys synthase (s674, induced 1.7-fold). The latter is directly involved in sulfate assimilation through conversion of *O*-acetyl-Ser and sulfide into Cys and acetate. Cys is a precursor of numerous sulfur-containing compounds in the cell, especially Met and SAM. SAM is a widespread compound required in various methylation reactions, particularly for the methylation of several derivatives of the phenylpropanoid pathway, including the methylation catalyzed by COMT. Several authors have shown that the SAM-synthetase gene and/or activity are stimulated under different stress conditions: salt stress in tomato (Espartero et al., 1994), water stress in pine (Chang et al., 1995), and fungal elicitor application in parsley (Kawalleck et al., 1992). Therefore, because the increased quantity of COMT suggests the induction of a lignification process during progressive water stress, it can be hypothesized that the increase of Cys synthase (and maybe also of inorganic

pyrophosphatase) contributes to this process by providing a greater quantity of SAM precursors.

Another important enzyme implicated in the general metabolism of the cell was strongly induced upon water stress (induction factor of 5.0): glutamate semialdehyde aminotransferase (s252) catalyzes 5-aminolevulinate formation via the C5 pathway. 5-Aminolevulinate is a precursor of tetrapyrrole compounds: chlorophylls, phytochrome, and porphyric proteins (cytochromes, catalases, and peroxidases). Peroxidases are known to be involved in the final step of lignin biosynthesis through the oxidative polymerization of monolignols and are induced by different stresses such as saline stress (Botella et al., 1994). Thus, induction of glutamate semialdehyde aminotransferase by water deprivation could also be linked to a stress-induced-lignification response of plants. It should be pointed out that this enzyme can also be induced by light (Grimm, 1990; Sangwan and O'Brian, 1993; Ilag et al., 1994). A few enzymes induced in our conditions are located in chloroplasts or are related to photosynthesis: phosphoribulokinase, Cys synthase, and aldolase. It is thus possible that for unknown reasons, a differentiation of chloroplasts occurred in stressed, etiolated tissues. Note that an Early Light Induced Protein (ELIP) has been shown to be induced by water stress in green leaves (Bartels et al., 1992; Ouvrard et al., 1996). It cannot be excluded, however, that some of the detected variations are not a direct response to water stress but, rather, are secondary effects of the reduction of leaf growth, cell division, and elongation.

The three β-glucosidase spots revealed by 2DE as increasing upon water stress are products of two different maize genes: s1305, exclusively present in stressed plants grown in the greenhouse, exhibited an amino acid sequence 100% identical to the *glu1* gene product, and the partial sequence of s159 was identical to the newly described protein encoded by the so-called *glu2* gene (Bandaranayake and Esen, 1996). The partial sequence of s92 is related to a region common to the two gene products s92 and s159, but according to their molecular masses (52 kD) and pI (respectively, 6.3 and 6.4) they could correspond to different products of the same *glu2* gene. However, s159 is detected only in stressed plants, whereas s92 presented a 3.3-fold induction in stressed leaves.

The *glu1* gene has been shown to be expressed in young maize tissues, where active cell division takes place (Brzobohaty et al., 1993), and it has been proposed that its activity is the release of active phytohormones (auxins or cytokinins) from glycosylated inactive storage forms (Campos et al., 1992; Brzobohaty et al., 1993; Falk and Rask, 1995). This expression pattern is close to the one observed for s1305, which is detectable in two-dimensional patterns from coleoptiles (Touzet et al., 1996b) and root tips but not in mature, green leaves (data not shown). However, it seems difficult to connect the hormone-regulating activity of β-glucosidase to its induction by water stress. Its activity has more recently been proposed to be the release of toxic hydroxamic acids as a defense response of plants against pests (Babcock and Esen, 1994). Another suggestion, which may be of particular interest in our context, is the implication of this enzyme in the lignification process. Freudenberg and Harkin (1963) hypothesized that the lignin-monomer precursors (cinnamic alcohols) are translocated toward the cell wall as β-glucoside esters, and are released in situ by a β-glucosidase to allow their polymerization into lignin. Recently, a β-glucosidase extracted from lodgepole pine xylem has been shown to hydrolyze coniferin, a β-glucoside of coniferyl alcohol and one of the major lignin monomers in pine, into the free alcohol (Dharmawardhana et al., 1995).

Considering the increased amounts of several enzymes observed upon dehydration (COMT, β-glucosidase, Cys synthase, and glutamate semialdehyde aminotransferase), activation of the lignin-biosynthesis pathway by water stress can be suggested. A cell wall reinforcement has already been reported in sorghum subjected to severe water stress: the lignosuberization occurring in roots during stress could reflect an increased resistance of the plant against a decrease in turgor and the necessity to restrict water loss from internal tissues (Cruz et al., 1992). In a study using loblolly pine treated with progressive water stress (as in the present study), Chang et al. (1995) also observed the induction of an ASR-like protein and of lignification-related proteins (SAM synthetase). COMT, Cys synthase, and SAM synthetase are related to the phenylpropanoid pathway but not specifically to lignin; their induction could also be related to the accumulation of ferulic acid, the product of the reaction catalyzed by the COMT. The quantity of ferulic acid bound to wall matrix polysaccharides is negatively correlated to cell wall extensibility (Wakabayashi et al., 1997). It has been proposed that cell wall hardening, a phenomenon occurring in maize leaves exposed to osmotic stress (Chazen and Neuman, 1994), is a response to water deficit and allows the reduction of growth rates (Neuman, 1995).

CONCLUSION

The computation of spot relative intensities and the use of two two-dimensional gels per genotype in the different conditions made it possible to use statistical tests for the detection of protein changes in response to water stress. This allowed us to quantify the variation in response, even when of low amplitude, and to compare the responses of the different genotypes. A set of proteins that increased from 1.3- to 5.0-fold upon water stress has been characterized. The main trend appears to be toward enzymes involved in basic metabolic pathways such as glycolysis and the Krebs cycle and a more specialized pathway, the phenylpropanoid pathway. Additional studies are currently in progress on COMT activity and its induction in other tissues to confirm the latter hypothesis.

Associations between protein variations and morphological and physiological traits affected by drought are being analyzed in a population of recombinant lines derived from the hybrid Io × Lc. Possible colocations of quantitative trait loci of induced proteins (Damerval et al., 1994) with those of responsive traits would be consistent with a causal relationship between the proteins and the phenotypic traits.

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