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Characterization of novel proteins affected by the *o2* mutation and expressed during maize endosperm development

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Abstract The effect of the *o2* mutation on protein expression during grain development was examined by two-dimensional electrophoresis (2-D PAGE) in seven different pairs of near-isogenic maize lines. The aim was to identify a set of proteins that are consistently affected in mutants, and which could be the products of new genes that are direct or indirect targets of the transcriptional activator O2. The abundance of 36 polypeptides was found to be modified in the seven backgrounds. Seventeen polypeptides were present in greater amounts in wild types than in mutants, and most of these were affected early. The remaining polypeptides were expressed at higher levels in mutants than in the wild types and were generally affected later in development, suggesting that they might be products of indirect targets of O2. Products of known direct target genes such as zeins, b-32 protein and a pyruvate orthophospate dikinase were included in the first set of polypeptides. Microsequencing of internal stretches of 15 amino acids was performed for thirteen polypeptides and homologies with sequences stored in databases were found for nine of them. Enzymes belonging to various metabolic pathways were tentatively identified, most of which were not previously known to be affected by the *o2* mutation. These results confirm that the *O2* gene could act as a connecting regulatory gene for different pathways of grain metabolism.

Key words *opaque-2* · Maize · Two-dimensional electrophoresis · *O2* target proteins

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Introduction

The recessive *opaque-2* (*o2*) mutation of maize gives an opaque character to the usually translucent mature kernel, but also has many pleiotropic effects. It produces a very marked decrease in alcohol-soluble storage proteins (50–70% of wild type, depending on the background), while amounts of lysine and tryptophan are increased, resulting in an improvement in the nutritional quality of seeds (Misra and Mertz 1975; Delhaye and Landry 1986; Habben et al. 1993). Various aspects of endosperm metabolism also appear to be modified: RNase activity is higher in mutants than in wild types (Dalby and Davies 1967), amino acid metabolism – particularly aspartate metabolism – seems to be altered (Yunès et al. 1994), as well as expression of genes involved in starch biosynthesis (Giroux et al. 1994). In some backgrounds, lower levels of carbohydrates have been found (Murphy and Dalby 1971; Di Fonzo et al. 1979). Mutant kernels are also more susceptible to plant pathogens than wild types (Loesch et al. 1976). Finally, reduction in protandry in mutants (Gupta 1979) and differences in photosynthetic activity of young seedlings (Morot-Gaudry et al. 1979) have been reported.

The *O2* gene has been isolated by transposon tagging and was found to encode a transcriptional activator of the basic leucine-zipper family (Hartings et al. 1989; Schmidt et al. 1990). An acidic activation domain was recently identified at the N-terminus of the protein (Schmitz et al. 1997). The gene seems to be specifically expressed in the endosperm, as early as 10 days after pollination (DAP) (Gallusci et al. 1994). The functional O2 protein is a dimer that binds to two different recognition sequences (Lohmer et al. 1991; Schmidt et al. 1992; Yunès et al. 1994). One of these sequences regulates the transcription of 22-kDa α-zein genes, and the other sequence is involved in the transcription of the *b-32* gene, which encodes a protein with homology to ribosome-inactivating proteins (Bass et al. 1992). Two sites that are very similar to the sequence bound by O2

in the *b-32* promoter were found in the promoter of the *cyPPDK1* gene, which encodes a cytoplasmic pyruvate orthophosphate dikinase, and were shown to be protected by O2 in DNAse I footprinting experiments (Maddaloni et al. 1996).

The range of pleiotropic effects of the *o2* mutation and the function of the *O2* gene suggest that O2 might act to ensure coordinated expression of genes expressed in different metabolic pathways that cooperate during endosperm development. The *zein*, *b-32* and *cyPPDK1* genes were identified as target genes for the activator based on the effects of *o2* mutation on their expression. In order to improve our understanding of the effects of O2, it would be interesting to characterize as many target genes as possible. In this respect, comparisons of gene expression patterns in near-isogenic *O2*/*o2* lines has already resulted in the detection of numerous differences at the mRNA level (Habben et al. 1993), as well as at the protein level (Damerval and de Vienne 1993; Habben et al. 1995).

In this paper, we chose to analyze the effect of the *o2* mutation during kernel development at the level of protein expression in different pairs of near-isogenic lines. This approach has two main advantages. First, characterization of protein expression in wild type and mutant allows any alteration in polypeptide abundance to be detected, irrespective of whether it results from transcriptional control by O2 or from indirect effects mediated by O2-controlled genes. In particular, effects that occur at the translational or post-translational level may be detected. Direct as well as indirect target gene products can thus be revealed, even though it is not initially possible to sort them into one or the other category. Second, changes in protein expression permit one to infer possible functional and metabolic roles for the *O2* gene as soon as the target proteins are identified. Differential effects of the mutation according to the backgrounds have been described (e.g. Dierks-Ventling 1981). In order to define a consensus set of target proteins, we compared the effects of *o2* alleles of different origins in several backgrounds and selected those polypeptides that were consistently affected. Amino acid sequencing allowed the identification of polypeptides involved in various metabolic pathways (amino acid synthesis, glycolysis, catabolism).

Materials and methods

Plant material

Seven pairs of near-isogenic lines were used: W64A*O2*/*o2*, F2*O2*/*o2* and F7*O2*/*o2* (kindly provided by Dr. M. Pollacsek, INRA France), and W22*O2*/*o2*, W23*O2*/*o2*, B37*O2*/*o2* and OH43*O2*/*o2* (obtained from the Maize Genetics Cooperation Stock Center, Columbia, Mo.). *o2* appeared as a spontaneous mutation in the W64A background, and the original allele studied by Mertz et al. (1964) was introduced by eight generations of backcrossing in the F2 and F7 backgrounds. No information about the degree of isogenicity was available for the other genotypes into which *o2* was introgressed.

Plants were grown and self-pollinated in the greenhouse in summer 1994 and 1995 (late flowering lines W64A, W22, W23, B37 and OH43) or in the field in summer 1994 (early flowering lines F2 and F7) at Gif-sur-Yvette, France. Kernels were harvested at 10, 12, 15, 17, 20, 22, 25, 30, 35, 45 and 60 DAP. At stages older than 12 DAP, the embryos were excised before the endosperms were frozen in liquid nitrogen.

RNA extraction and Northern analysis

Total RNAs were extracted from four to five 20-DAP kernels following the procedure of Bartels and Thompson (1983) with a lithium chloride precipitation step (De Vries et al. 1982). Ten micrograms of total RNA were run on 1% agarose-6% formaldehyde gels, then vacuum blotted onto nitrocellulose membranes. The membranes were prehybridized and hybridized overnight at 42° C in the presence of 50% formamide, essentially as described in Sambrook et al. (1989). Probe (a 1868-bp *O2* genomic fragment; Henry 1996) labeling was done according to Feinberg and Vogelstein (1983) using 40 μCi of [^{32}P]dCTP (3000 Ci/mmole). The blots were exposed to Hyperfilm-MP (Amersham, Little Chalfont, U. K.) for 8 days at −80° C with an intensifying screen.

Western analysis and immunodetection of O2 protein

Extracts enriched in nuclear proteins from four to six 20–25 DAP grains were performed following the procedure of Varagona et al. (1991) without the column desalting step. About 100 μg of total proteins were submitted to one-dimensional SDS-PAGE (Sambrook et al. 1989), then transferred to a ProBlott membrane (Applied Biosystem, Foster City, Calif.) in a TransBlot apparatus (Bio-Rad, Hercules, Calif.) overnight at a maximum current of 150 mA. The quality of the blot was checked by staining with Ponceau Red (0.05% in 3% TCA). Filters were blocked for 1 h in BLOTTO (Sambrook et al. 1989) at 37° C, incubated for 2 h at room temperature in a 1:1000 dilution of a polyclonal anti-O2 serum kindly provided by Dr. E. Gianazza (Università degli Studi di Milano, Italy), then incubated for 2 h in a 1:1000 dilution of a goat anti-rabbit IgG peroxidase conjugate. Color development was carried out as described by Damerval and de Vienne (1993).

Protein extraction and two-dimensional electrophoresis (2-D PAGE)

Total endosperm proteins were extracted following Damerval et al. (1986) except that 40 μl/mg of UKS solution were used to resuspend the pellet.

The pH gradient used in the isoelectric focusing (IEF) dimension was linear from 4.7 to 7.7 (12.5% Pharmalyte pH 3–10, 25% Pharmalyte pH 5–6, 62.5% Pharmalyte pH 5–8) in 24-cm long gels. About 50 μg of total proteins were loaded at the top of each IEF gel. Other steps in the 2-D PAGE were as in Damerval et al. (1987). Silver staining followed the method of Burstin et al. (1993), with impregnation with silver nitrate for 30 min. Three to four gels per genotype and developmental stage were analyzed, each one corresponding to an individual endosperm.

Classes of storage proteins (albumins, globulins, zeins and glutelins) were extracted from five to seven 35-DAP endosperms and four to five mature endosperms from the F2 wild type and mutant, following Landry and Moureaux (1970).

Preparative electrophoresis for microsequencing of spots used minor modifications of the analytical technique that are described in Touzet et al. (1995). Sets of four or five endosperms of F2 wild type or mutant were used at the appropriate developmental stage, and 20 μl/mg of UKS solution was used to resuspend the pellet.

Protein quantification

Wet 2-D gels from the 11 developmental stages analyzed in the F_2 background were digitized with an Eikonnix scanner using 256 gray

levels and a spatial resolution of 100 μM, which generates 2048 × 2048 pixel images. The optical density range was 0 to 1.2. The images were then processed using the spot detection method of M. Zivy (manuscript in preparation) and the KEPLER package (Large Scale Biology, Rockville, Md.) for 2-D gel analysis. For each polypeptide spot, integrated optical density was adjusted to a 2-D gaussian, allowing us to calculate a "volume", which was shown to be related to protein amount (Damerval 1994). Statistical analyses allowed polypeptides whose quantity was altered by the *o2* mutation at any developmental stage to be identified (see below). These effects were then visually checked on dried gels from endosperms of 15, 25 and 35 DAP in the six other backgrounds.

Statistical analysis of the effects of *o2* in F2 background

The spot volumes were compared in wild type and mutant at each developmental stage in the F2 genotype. In order to compensate for global differences in staining intensity and/or total protein load, the linear scaling described by Burstin et al. (1993) was used. Student's *t*-test was performed for each spot on the corrected volumes. Spots exhibiting a significant effect ($P < 0.05$) in at least two developmental stages were selected for analysis in the six other backgrounds.

Internal microsequencing and database searches

Amido black-stained polypeptide spots from 12 to 25 preparative 2-D gels were used for microsequencing. Enzymatic cleavage, HPLC separation and internal microsequencing were done by J. d'Alayer and M. Davi (Laboratoire de Microséquençage des protéines, Institut Pasteur, Paris, France), as described in Touzet et al. (1995). Amino acid sequences were compared to the 262 309 sequences in the Nonredundant Peptide Sequence database at the National Center for Biotechnology Information (Washington DC) by the use of the BLASTp program (Altschul et al. 1990).

Results and discussion

Background-specific effects of *o2* have been reported. These may be due to (i) differential action of altered mutant proteins; (ii) effects of linked genes when the *o2* allele was introgressed through backcrossing; and/or (iii) epistatic interactions between *O2* and backgrounds. Such marginal effects, which can obscure the main action of O2 can be eliminated by focusing on gene products that are consistently affected by the mutation in several unrelated genetic backgrounds. In this study, we first analyze expression of *o2* alleles in seven backgrounds, in order to determine whether they are null mutants or can encode an altered protein. 2-D PAGE was then used to analyze extensively protein expression patterns in endosperm over the course of grain development in one background, and to select polypeptides that were consistently affected in the six other pairs of near-isogenic lines for further characterization.

o2 allele characterization

Several independent *o2* mutations have been reported in different maize lines (Bernard et al. 1994). Northern analysis of total RNA extracted from 20-DAP kernels showed one band at 1.9 kb in wild types and also in B37*o2* and W64A*o2*. No signal was detected in the other mutants. W64A*o2* exhibited an additional faint band at 2.4 kb, as reported by Bernard et al. (1994) (Fig. 1A). Western blotting with an antibody raised against the first 24 amino acids of O2 allowed detection of one or two bands at about 67 kDa in wild types. No protein expression was detected in any mutant. This was expected for the mutants lacking mRNA expression (F2*o2*, F7*o2*, W22*o2* and W23*o2*), and suggests that none of the others is able to synthesize a fully functional protein (Fig. 1B). However, the possibility that mutant proteins lacking a portion of the N-terminus exist is not excluded. Indeed, a 40-kDa protein was detected in W64A*o2* (Bernard et al. 1994), which may be modified or truncated at the N-terminus.

Quantitative analysis of protein expression in F2 background

When all eleven developmental stages were taken into account, 1062 protein spots were detected by 2-D PAGE and automated quantitative analysis. Protein expression appeared to change significantly over the course of endosperm development in both wild type and mutant (Fig. 2). Protein b-32 and α-zein polypeptides were visible from 15 DAP on in the wild type; b-32 appeared as a faint spot in the mutant from 22 DAP on. The amount of α-zein was strongly reduced in the mutant, with a stronger effect on the 22-kDa than on the 19-kDa class.

Statistical analysis of automatically quantified spot intensities allowed the detection of 136 polypeptides whose amounts varied between wild type and mutant in at least two developmental stages. Visual inspection of polypeptides in crowded areas of the gel profiles, where

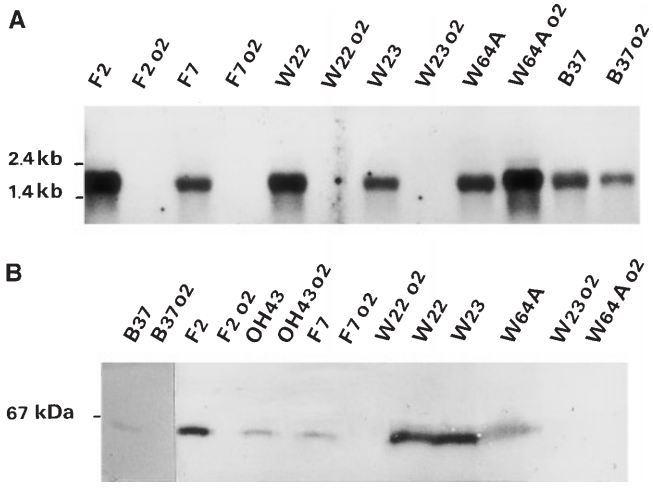

Fig. 1 A, B Analysis of *O2* and *o2* mRNAs and proteins in the pairs of near-isogenic lines. A Northern analysis of total RNA probed with a 1868-bp genomic *O2* probe (3′ end of exon 1 to 5′ end of exon 6). B Western analysis with a polyclonal antibody directed against the first 24 amino acids of O2. The signal was faint for B37, OH43 and F7

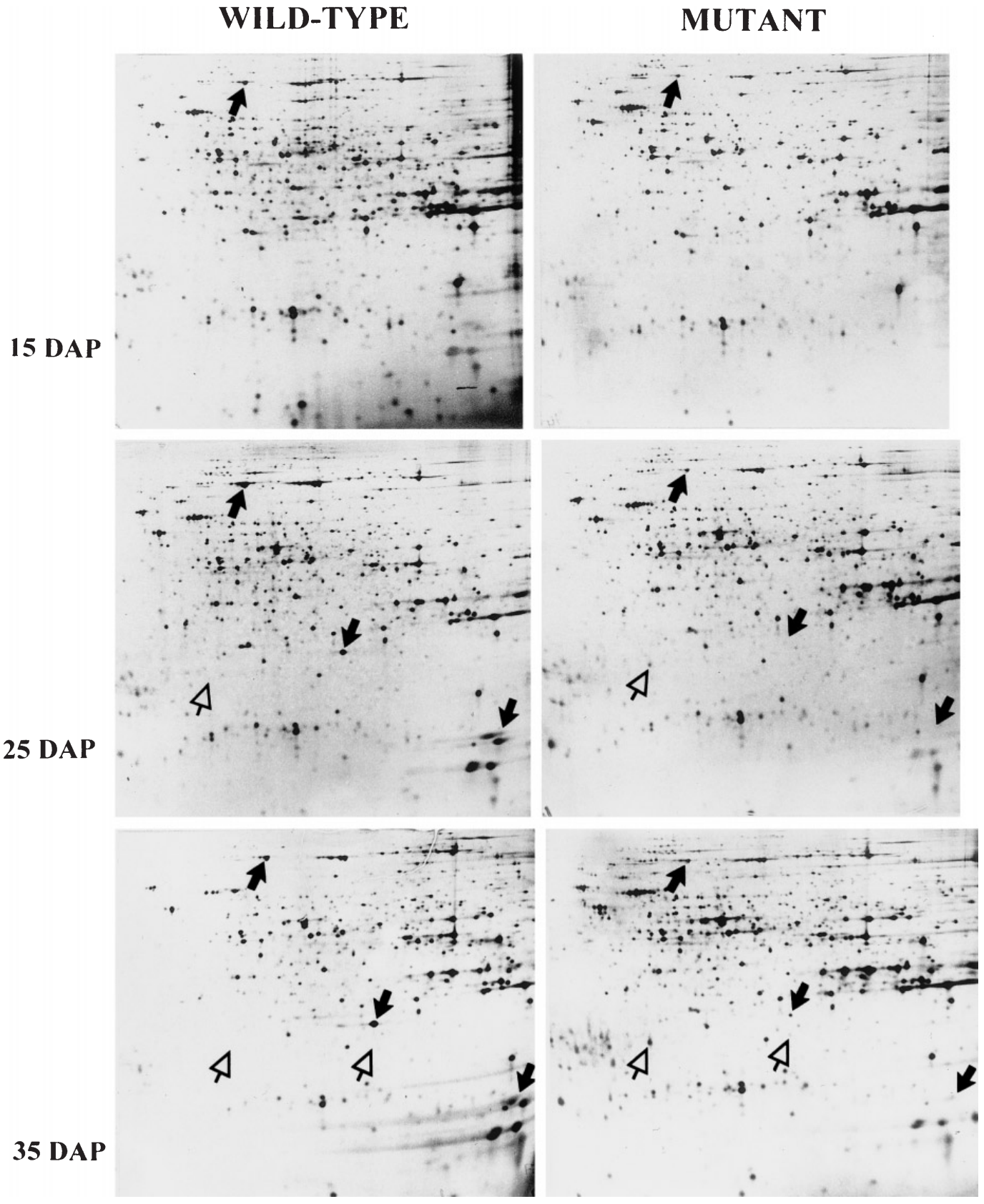

Fig. 2 Changes in protein patterns in F2*O2* (first column) and F2*o2* (second column) over the course of grain development. The three stages analyzed in all the backgrounds (15, 25 and 35 DAP) are shown: *Black arrows* point to examples of polypeptides present at higher levels in wild types than in mutants (PPDK: #2, b-32: #187, zeins: #236, #296, see Fig. 3). *White arrows* point to examples of polypeptides presents in larger amounts in mutants than in wild types (#950, #708)

automatic analysis failed to give good spot detection, allowed 24 additional polypeptides to be found.

During development, the number of polypeptides affected by the mutation increased from 14 at 10 DAP to more than 70 at 35 and 45 DAP. Proteins present at higher levels in wild type than in mutant were the most numerous from 15 to 25 DAP. Conversely, proteins expressed at higher levels in mutant than in wild type were the most numerous at late stages, beginning at 35 DAP.

Consistency of *o2* effects in the six other genetic backgrounds

The expression of the 160 proteins retained following F2 analysis was analyzed at 15, 25 and 35 DAP in W64A*O2*/*o2*, F7*O2*/*o2*, W22*O2*/*o2*, W23*O2*/*o2*, B37*O2*/*o2* and OH43*O2*/*o2*. The effect of the mutation was specific for F2 background for 45 polypeptides. For 79 other polypeptides the effect was not confirmed, and/or the spot was not visible, in all backgrounds. Thus more than 75% of the polypeptides affected in any one background would be affected by *o2*-linked genes or by epistatic actions.

The *o2* effect was consistent in all seven backgrounds for 36 polypeptides, including six zein proteins (Fig. 3). For 35 polypeptides, the difference between wild type and mutant was of the same sign in all backgrounds, albeit not always of the same magnitude. Polypeptide #137 was the only exception, being more abundant in mutants than in wild types in backgrounds F2, F7, W64A, OH43 and W22, but showing the reverse effect in W23 and B37. This suggests that #137 may be an indirect target of O2, with epistatic interactions playing a role in expression. Ten of the 36 proteins belonged to the set of polypeptides reported to be affected by the mutation in F2 and W64A mature kernels (Damerval and de Vienne 1993). All the early affected polypeptides (15 DAP), including b-32 and zeins, were present in higher amounts in wild types than in mutants, which is consistent with a transcription activator role of O2. For all but three of these polypeptides, the effect was visible all through development. Differences occurring later than 25 DAP all concerned polypeptides whose abundance was increased by the mutation, which is consistent with indirect effects of O2 (Fig. 4).

Protein identification

Among the 36 polypeptides affected in the seven backgrounds, eight corresponded to known proteins. The six zein spots (#236, 244, 248, 254, 257, and 296) were identified by a specific extraction procedure, and the b-32 polypeptide (#187) and a high molecular weight globulin (#489) were previously identified by Western blotting (Damerval and de Vienne 1993). Differential extraction of storage proteins revealed that most of the 28 other polypeptides belonged to the albumin-globulin

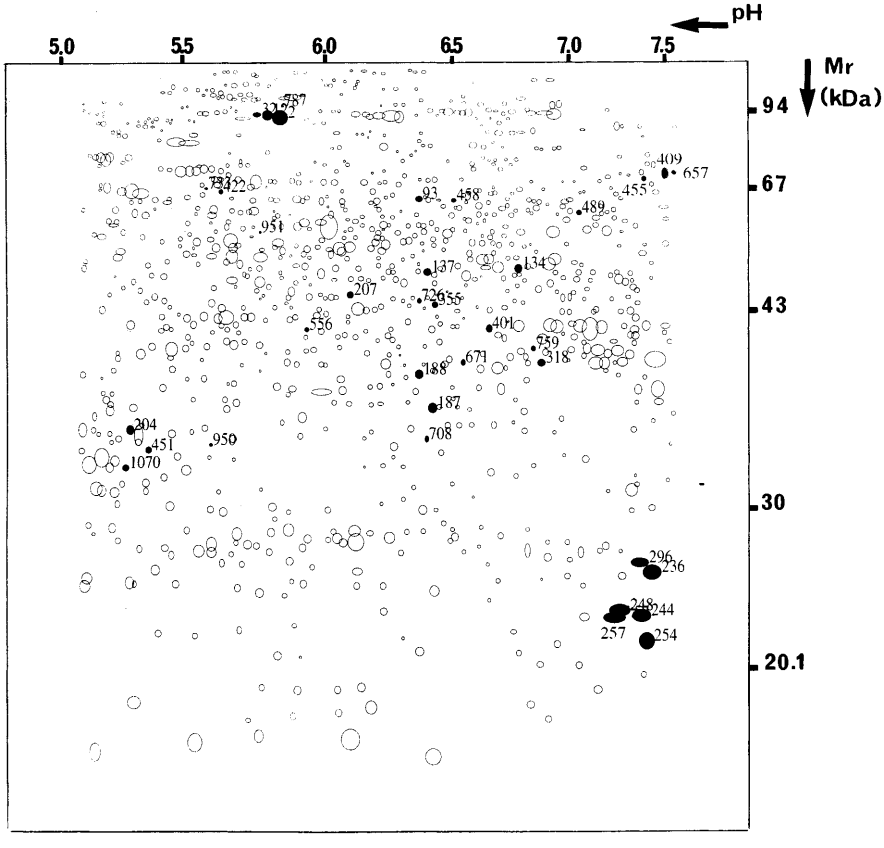

Fig. 3 Synthetic 2-D map showing the polypeptides expressed during grain development in F2. The map does not correspond to an actual 2-D pattern, but was drawn from a spot file including parameters for all the 1062 spots detected in F2 wild type and mutant 2-D gels from 10 to 60 DAP (quantitative data on spot volumes in F2 are available on request). The 36 polypeptides affected in the seven backgrounds analyzed are in *black*

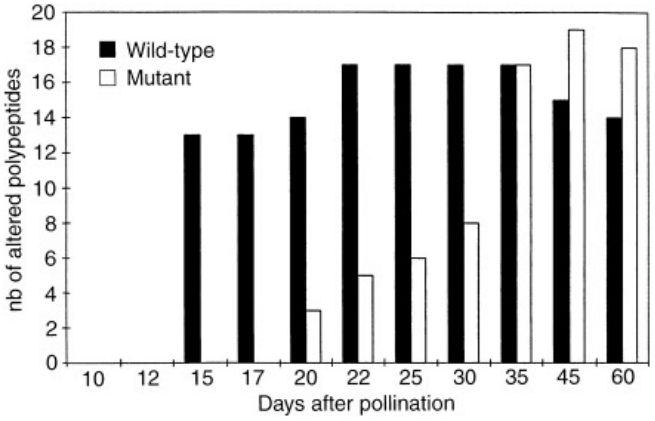

Fig. 4 Number of polypeptides whose quantity is affected by the *o2* mutation in all backgrounds. Black and white bars indicate polypeptides present at relatively higher levels in wild types and mutants, respectively

fraction (not shown). Thirteen polypeptides present in sufficient abundance were chosen for internal microsequencing.

Homologies with known protein sequences were detected for nine polypeptides (Table 1). The lowest identity score was between polypeptide #188 and the α-chain of cruciferin precursor from radish (Depigny-This et al. 1992). The apparent molecular mass (Mr) computed for the α-chain alone appeared lower (about 30 kDa) than the Mr of #188 (39 kDa), while the Mr of the $\alpha + \beta$ chain was much higher (about 50 kDa), which makes this identification quite dubious. In every other case, the Mr estimated from our 2-D gels corroborated the sequence homology.

Polypeptide #2, with 100% identity to a maize pyruvate orthophosphate dikinase (PPDK), is the major form in a cluster of three polypeptides shown to be affected in the mature kernel also (Damerval and de Vienne 1993). The effect of the *o2* mutation on the product of the *cyPPDK1* but not the *cyPPDK2* gene has already been noted (Gallusci et al. 1996). The two less abundant polypeptides in the cluster (#12 and #32), with slightly more acidic pI and an Mr slightly higher than the major form, might correspond to the second cyPPDK1 isoform, also controlled by O2, visualized in 1D-SDS PAGE profiles (Gallusci et al. 1996). It has been shown that the promoter of the *cyPPDK1* gene is trans-activated by O2 (Maddaloni et al. 1996). However, since the protein is observed in the mutants, O2 is not the only trans-activating factor required for *cyPPDK1* expression, but would act to enhance transcription of the gene.

Polypeptide #93 is 100% identical to maize acetohydroxyacid synthase (AHAS, Fang et al. 1992), an enzyme that catalyzes the first common step in the biosynthesis of the branched amino acids that can be derived from threonine. *O2* has already been suspected of playing an important role in the regulation of amino acid biosynthesis, especially of those amino acids derived from aspartate, i.e., lysine, methionine and threonine (Yunès et al. 1994). The activities of aspartate kinase and of the bifunctional lysine ketoglutarate reductase-homoserine dehydrogenase that is involved in lysine catabolism are altered in *o2* mutants as compared to wild type (Brochetto-Braga et al. 1992; Brennecke et al. 1996). AHAS is the third enzyme involved in amino acid metabolism whose expression can be modified by *o2*.

Table 1 Polypeptides whose expression is modified in *o2* mutants, and for which an amino acid sequence was determined

Spot	*o2* effect[a]	Mr (kDa)[b]	pI[b]	Amino acid sequence	Percentage identity[c]	Homology	Accession number
2	↓	91	5.8–5.9	AGLDYVSCSPFRVPI	100	Pyruvate orthophosphate dikinase	M58656
				QP(L)SPPAL(S)GDLGT F	100	(maize)	P11155
93	↓	65	6.38	SFDFGSWNDELDQQK	100	Acetohydroxy acid synthase (Maize)	S38034
134	↓	51	6.83	DDNPLFGSSRIEQAH	–	None	
137	↑	50	6.42	IVAWAPQREVLAHPA	80	Putative tomato ripening protein	S39507
188	↑	39	6.36	VAYVLQGVGTCGLVLPE	54	Cruciferin precursor (Plants)	Q02498
204	↑	35	5.23	FGGILGLGFQEISVG	93	Aspartic proteinase precursor (Plants)	X80067
207	↓	45	6.08	(T)VTGLELPVQPVHTL	–	None	
318	↑	39	6.91	FGIVEGLMTTVHAIT	100	Glyceraldehyde 3-phosphate	PQ0179
				EVAVFGCRNPEEIPW	100	dehydrogenase (maize)	PO8477
				GILGYVEEDLVSTDF	100		PQ0179
401	↑	42	6.7	FFATPPVHGSLANQV	73	Similar to sorbitol dehydrogenase (*C. elegans*)	Z70782
				AVGICGSDVHYLREM	78	Sorbitol dehydrogenase (*S. pombe*)	P36624
451	↑	34	5.26	VYMNAQYFGEIGVGS	92	Aspartic proteinase precursor (plants)	P42210
726	↓	45	6.37	(M)IE(H)QAVENIXAX	–	None	
				CIVTVGAWTSK	–		
759	↑	40	6.87	PVTVFGIRNPEEIPWG	100	Glyceraldehyde 3-phosphate	P26520
				VIHDNFGIIEGLMTTV	100	dehydrogenase (maize)	PQ0178
950	↑	34	5.57	DEGLVVAPELGPEGL	–	None	

[a] Effects of o2 are indicated relative to wild-type levels
[b] Mr and pI are calculated from the 2-D PAGE profiles
[c] Percentage identity is computed for the complete amino acid sequence

Two acidic polypeptides that are abundant in the mutant from 30 DAP to the mature endosperm stage exhibited a high identity score with plant aspartic proteinases. In plants, aspartic proteinase activity has been localized in seeds of a broad variety of species (*e.g.* rice, Asakura et al. 1995). A role in digestion of storage proteins at the onset of germination in cereals has been suggested (Runeberg-Roos et al. 1991).

Two polypeptides that run close to each other on 2-D gels and have basic isoelectric points are 100% identical to two isoforms of cytosolic glyceraldehyde-3-phosphate dehydrogenase encoded by two different genes, *Gpc2* (#759) and *Gpc3* (#318) (Russel and Sachs 1989). These enzymes are involved in glycolysis in the cytosol and, like other enzymes of this pathway, they can be induced by anaerobic stress. Their quantity is higher in the mutants.

Polypeptide #137 had a high identity score with a gene product deduced from the sequence of a ripening-specific cDNA clone from tomato (Picton et al. 1993). This gene product itself shows homology to the amino acid sequence of the UDP flavonol-3-O-glucosyl transferase of maize (the *Bz1* gene product) and barley, and with UDP-glucuronosyl transferases from rat and man (UDPGTs). Five of the 15 amino acids sequenced in #137 belonged to a consensus sequence defined for the UDPGTs. UDP-glucuronosyl transferases function in the detoxification of steroid metabolites in the liver. In plants, this gene product may function in detoxification of metabolites produced during the ripening process (Picton et al. 1993). Flavonol UDP-glucosyltransferase is known to play a role in the terminal steps of anthocyanin pigment formation. The maize sequences in the databases (Ufo-1 to Ufo-3) encode polypeptides with an Mr of about 48 kDa and a pI between 5.2 and 5.4, a more acidic value than the pI of #137. Polypeptide #137 is probably not be the product of the *Bz1* gene, but may belong to the same gene family and function in an as yet unknown manner in the maturing kernel.

The two sequences obtained from polypeptide #401 had some identity with sorbitol dehydrogenases of *Caenorhabditis elegans* and *Saccharomyces pombe*. Sorbitol dehydrogenase activity has been demonstrated in the maize kernel (Doehlert 1987). The enzyme catalyzes the transformation of fructose to sorbitol, which could in turn be converted to glucose by aldose reductase (Doehlert et al. 1988).

The estimated Mr and pI of the polypeptides that were not sequenced because they were present in amounts too small for analysis were compared to the corresponding values for proteins encoded by genes known to be affected by O2 (Marocco et al. 1991, Habben et al. 1993, Giroux et al. 1994). The only possible match found was between polypeptide #951 (59 kDa, pI 5.84) and the *Waxy* gene product (58 578 Da, pI 5.49). The quantity of this polypeptide was higher in mutants than in wild type, in accordance with the effects of the mutation observed at the *Wx* mRNA level (Giroux et al. 1994).

Conclusion

Comparisons of protein expression patterns between wild type and mutant in several unrelated backgrounds has proved to be powerful means of excluding marginal *o2* effects, whether these are due to residual action of mutant protein, introgressed genes or epistatic action. The 36 polypeptides consistently affected may thus represent products of genes whose expression is directly or indirectly controlled by O2. On the one hand, the polypeptides whose quantity increased in the wild types relative to the mutants from early stages in the development of endosperm on may be direct target gene products. This has been proved for 22-kDa zeins, the b-32 protein and cytosolic PPDK, and remains to be established for the others, including the newly identified AHAS. On the other hand, the polypeptides whose quantity was higher in the mutants than in the wild types may be products of indirect target genes. Actually, there is no evidence that O2 can act as a transcriptional inhibitor. Moreover, these effects are in most cases delayed as compared to those discussed above. Indirect effects could be mediated by global metabolic consequences of O2 action or through an action of the direct target gene products (e.g., modification of specific amino acid pools).

.Nineteen of the 36 polypeptides studied (including #188, #12 and #32) were tentatively or definitely identified; some are involved in metabolic functions not previously known to be affected by O2 (glyceraldehyde-3-phosphate dehydrogenases, AHAS, aspartic proteinases, UDPGTs, sorbitol dehydrogenase). These results confirm that the *O2* gene plays an important role in coordinating various pathways during kernel development.

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