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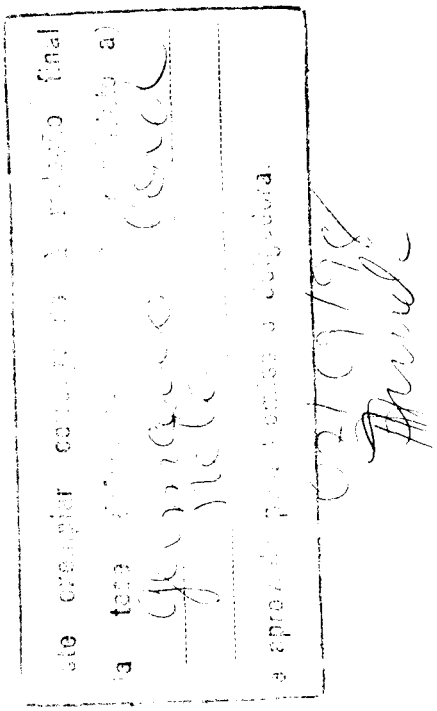
O envolvimento do ativador transcricional Opaco2 no metabolismo de lisina e na síntese de proteínas de reserva durante o desenvolvimento da semente de milho



Germano Cord Neto

Orientador: Dr. Paulo Arruda

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas para obtenção do grau de Doutor em Ciências Biológicas, área de concentração Genética de Plantas.

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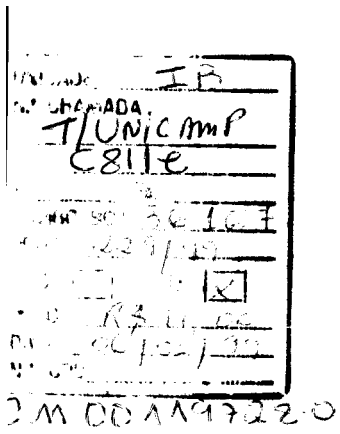

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APROVADA

A cabeça da gente é uma só, e as coisas que há e que estão para haver são demais de muitas, muito maiores diferentes, e a gente tem de necessitar de aumentar a cabeça, para o total. Todos os sucedidos acontecendo, o sentir forte da gente – o que produz os ventos. Só se pode viver perto do outro, e conhecer outra pessoa, sem perigo de ódio, se a gente tem amor. Qualquer amor já é um pouquinho de saúde, um descanso na loucura. Deus é que me sabe!

(João Guimarães Rosa, *Grande Sertão: Veredas*).

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ABREVIAÇÕES

AK	Aspartato quinase
Ask1	Aspartato quinase insensível à retroinibição por lisina
bZIP	Motivo básico-zíper de leucinas, em fatores de trancrição.
C	Carbono
cDNA	DNA complementar ao RNA
DAP	Dias após a polinização
DHDPS	Dihidrodipicolinato sintase
GCN4	Fator de transcrição de levedura, da classe das bZIP
GCN4-like	sítio de ligação ou fator protéico semelhante ao tipo GCN4.
HSD	homoserina desidrogenase
kDa	Unidade de Peso Molecular Aparente de Proteínas
LKR	Lisina-cetoglutarato redutase
LYS1	Sacaropina desidrogenase de levedura, formadora de lisina.
LYS9	Sacaropina desidrogenase de levedura, formadora de glutamato.
mRNA	RNA mensageiro
N	Nitrogênio
O2	proteína ou fator Opaco2
O2	loco *Opaco2*
o2	mutação *opaco2*
ORF	"Open Reading Frame"
pu	purina
py	pirimidina
SDH	Sacaropina desidrogenase
SDS-PAGE	Eletroforese em gel de poliacrilamida com dodecil sulfato de sódio
uORF	"Untranslated Open Reading Frame"
ZLYS1-9	Gene que codifica a LKR/SDH de milho

RESUMO

O *locus Opaco2* (*O2*) foi primeiramente identificado quando se procurava por mutantes com endosperma rico em lisina. O mutante *o2* contém o dobro de lisina em relação ao tipo selvagem, aparentemente devido tanto à elevação nos níveis de um grupo de proteínas ricas em lisina como a uma menor taxa de degradação de lisina. A mutação *o2* causa, além disso, vários efeitos pleiotrópicos no endosperma. O efeito melhor caracterizado da mutação *o2* é a drástica redução nos níveis da α-zeína de 22 kDa, a classe mais abundante de proteínas de reserva. Demonstrou-se também que os teores de várias outras proteínas sofrem redução no endosperma mutante, incluindo b-32, um tipo de albumina com propriedade de inativação de ribossomos, e cyPPDK1, a forma citosólica da enzima piruvato ortofosfato diquinase. O gene *O2* codifica um fator de transcrição da classe das bZIP que interage com os promotores destes genes ativando a sua transcrição. Neste trabalho estudamos o papel do fator O2 na expressão dos genes que codificam a β-zeína de 14 kDa e a enzima responsável pela degradação de lisina na semente, lisina-cetoglutarato redutase/sacaropina desidrogenase (LKR/SDH). No primeiro trabalho, demonstramos que o fator O2 regula transcricionalmente a β-zeína e, comparativamente, a proteína análoga em *Coix*, um cereal relacionado ao milho. Nos promotores destes genes, identificou-se um novo tipo de sítio de ligação da proteína O2. No segundo trabalho, procurando entender melhor o controle dos níveis de lisina no endosperma, isolamos o gene que corresponde à enzima LKR/SDH, denominado *ZLYS1-9*. O gene *ZLYS1-9* é expresso preferencialmente no endosperma, onde a enzima está presente no citosol das células das camadas de aleurona e sub-aleurona. O padrão de expressão temporal de *ZLYS1-9* foi examinado nos endospermas normal e mutante *o2*. A mutação *o2* afeta drasticamente a transcrição do gene *ZLYS1-9*, reduzindo os níveis de polipeptídeos LKR/SDH e a atividade enzimática. Concomitantemente, observa-se um atraso de cerca de 5 dias para o máximo acúmulo de transcritos no endosperma mutante em relação ao tipo normal. Estes resultados sugerem que os níveis de lisina no endosperma possivelmente resultam do controle da transcrição do gene responsável pela degradação deste aminoácido. Tomados em conjunto, nossos resultados indicam que a síntese de proteínas de reserva e o metabolismo de lisina operam sob um mesmo mecanismo regulador e sugerem que o fator O2 tem um papel importante no controle da expressão coordenada de vários genes durante o desenvolvimento da semente.

ABSTRACT

The *Opaque2* (*O2*) *locus* was originally identified in a screen for high-lysine endosperm mutants in maize. The *o2* mutant has double lysine levels, probably due to an increased content of lysine-rich proteins and reduced lisine degradation. However, the *o2* mutation is known to have numerous pleiotropic effects. The best characterized effect of the *o2* mutation is a drastic reduction of the 22 kDa a-zein, the major storage protein class. Subsequently, it was shown that a number of other proteins were reduced in amount in the o2 mutant, including b-32, a ribosome-inactivating protein and cyPPDK1, a cytosolic isoform of pyruvate orthophosphate dikinase. The *O2* gene encodes a bZIP tyope of transcription factor that interacts to those promoters, activating transcription. In this study, we have examined the role of the O2 factor in the expression of the genes encoding the 14 kDa β-zein, and the lysine degrading enzyme, lysine-ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH). In the first report, we demonstrate thar O2 transcriptionally regulates β-zein gene expression and, comparatively, the related prolamin from *Coix*. A new type of O2 binding site was identified in β-prolamin promoters. In the second report, to further study the influence of the *o2* mutation on lysine accumulation, we have cloned the gene encoding LKR/SDH, designated *ZLYS1-9*. *ZLYS1-9* is mainly expressed in the endosperm and the LKR/SDH enzime is located in the citoplasm of aleurone and subaleurone cell layers. The developmental expression of *ZLYS1-9* was examined in normal and *o2* mutant seeds. In the mutant endosperm, mRNA levels were severely reduced with a concomitant reduction on polypeptide levels and LKR/SDH activity. Also, a delay of about 5 days was observed for maximal transcript accumulation in the mutant endosperm. These results suggest that lysine levels in the endosperm are likely to be controlled at the transcriptional level by O2. Taking together, our results suggest that the O2 factor is involved on the coordinated control of gene expression in the developing maize endosperm.

INTRODUÇÃO

Origem, desenvolvimento e função do endosperma

A semente de milho é constituída de pericarpo, aleurona, endosperma e embrião (Figura 1). O endosperma representa a maior parte da semente dos cereais e tem sido estudado sob vários pontos de vista, em diversas áreas do conhecimento. Desde botânicos até engenheiros de alimentos têm se interessado não somente pelo seu papel no desenvolvimento da semente e na germinação mas também no seu refinamento industrial para obtenção de farinha, óleos e polímeros. Por outro lado, o avanço da biologia molecular permitiu tanto elucidar questões básicas da biologia do desenvolvimento do endosperma como desenvolver novas estratégias para o melhoramento genético vegetal com vistas à sua utilização.

A origem do endosperma ocorre no momento da fertilização. Através da dupla fertilização, evento *sui generis* que só ocorre em angiospermas, um núcleo espermático proveniente do gametófito masculino se funde à oosfera, produzindo o embrião, enquanto que um segundo núcleo espermático se funde a dois núcleos polares para formar um tecido triplóide, o endosperma. A este respeito, no entanto, surgiram duas hipóteses. Na primeira, postula-se que o endosperma deriva de um embrião gêmeo altruísta, que se desenvolveria em corpo de armazenamento. Na segunda, o endosperma resultaria de uma extensão no desenvolvimento do megagametófito, que seria promovida quando da fertilização dos núcleos polares pelo segundo núcleo espermático (revisadas em Friedman, 1994). A primeira hipótese recebe algum apoio em evidências descobertas no gênero *Ephedra*, que representa um elo evolutivo entre gimnospermas e angiospermas. A dupla fertilização nestas plantas resulta em embriões gêmeos, sugerindo que o endosperma teria evoluido a partir de um deles (Friedman, 1994).

A dupla fertilização pode ter dado ao endosperma as vantagens da heterose, e também pode ser importante na manutenção de diferenças epigenéticas que determinam o desenvolvimento do endosperma e do embrião (Lopes e Larkins, 1993). A fusão dos núcleos polares com o núcleo do gameta masculino, formando a primeira célula que origina o endosperma é claramente um pré-requisito para o início do desenvolvimento do

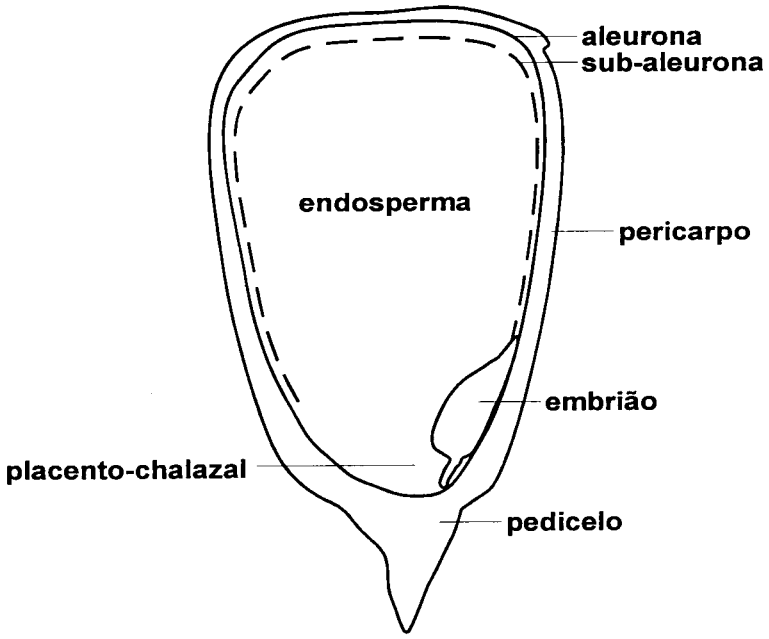

Figura 1. Representação esquemática da semente de milho e de regiões do endosperma

endosperma, mas a continuidade deste processo envolve produtos de genes do embrião e dos tecidos maternais (Olsen, 1998; Kranz et al., 1998). Estudos do desenvolvimento *in vitro* do endosperma de milho sugerem que restrições ao desenvolvimento são impostas à célula primordial. Diferentemente do que acontece com a oosfera (Kranz e Lorz, 1993), a fertilização dos núcleos polares não resulta no desenvolvimento de calos ou brotos em cultura (Kranz et al., 1998).

O milho apresenta um padrão nuclear de desenvolvimento do endosperma, que é o tipo mais comum. Segundo este padrão, logo após a fertilização dos núcleos polares pelo segundo núcleo espermático do gametófito masculino, a célula primordial do endosperma, por um período aproximado de 72 h, entra num processo seriado de divisões mitóticas sem citocinese, gerando um grande número de núcleos organizados na periferia desta célula. A partir daí, a divisão celular se inicia, progredindo em direção ao centro da célula-mãe até que o endosperma se torne inteiramente celular. (Vijayaraghavan e Prabhakar, 1984). O estágio seguinte, de 5 a 11 dias após a polinização (DAP), que precede a síntese de proteínas de reserva e de amido, envolve ainda divisões celulares e especializações em tipos particulares de células, por exemplo, aleurona, sub-aleurona e a camada de transferência de assimilados, na base do endosperma (Müller et al., 1995).

O aleurona é um tecido formado por células morfológica e funcionalmente distintas. Em milho, ele é formado por uma única camada de células. Após a maturação da semente somente as células da camada de aleurona continuam vivas e, durante a germinação, ativadas por um estímulo mediado pelo ácido giberélico produzido pelo embrião, são responsáveis pela síntese e secreção de enzimas hidrolíticas que catalisam a degradação das macromoléculas de reserva localizadas nas células do endosperma: amido, proteínas, polissacarídeos e DNA (Fincher e Stone, 1986; Fincher, 1989).

Durante o desenvolvimento do endosperma ocorre um aumento considerável no conteúdo de DNA nuclear, que se dá através de duplicações cromossômicas endonucleares sem mitose (endoreduplicação). No milho, entre 10 e 20 DAP, o conteúdo de DNA de uma célula passa de cerca de 3 vezes o genoma haplóide para até mais de 600 vezes. O papel da endoreduplicação ainda não foi estabelecido, mas é interessante notar que o processo não é seletivo para determinados genes, como os que codificam proteínas

de reserva e enzimas do metabolismo de carboidratos (Lopes e Larkins, 1993; Müller et al., 1995).

Existe claramente uma divisão de trabalho entre os diferentes tipos de células no endosperma. Primeiro, os padrões de expressão gênica nas células da base, nas regiões de sub-aleurona e aleurona e nas células centrais do endosperma são bastante distintos. Segundo, o desenvolvimento entre um determinado tipo de célula pode não estar sincronizado e refletir um efeito de posição: as células centrais do endosperma contém mais amido e menos mRNA de proteínas de reserva do que as células periféricas, das quais elas são derivadas (Müller et al., 1995).

Muitos aspectos da diferenciação do endosperma não são ainda compreendidos. Até o momento não foram identificados as moléculas ou eventos que ativam os controles bioquímicos da polaridade, divisão, formato e tipo celular, bem como aqueles controlando a poliploidização do genoma ou mesmo o acúmulo de nutrientes no endosperma.

O desenvolvimento da semente resulta das interações entre o endosperma, o embrião e os tecidos maternais. Existem ainda muitas perguntas sobre estes processos: "Que tipo de trocas bioquímicas ocorrem entre endosperma, embrião e os tecidos maternais, e como estas trocas afetam o desenvolvimento integrado destes tecidos? Existe um programa genético que coordena os processos de desenvolvimento que determinam a forma, a função, e a integração destes tecidos?" (Lopes e Larkins, 1993). Os trabalhos de Kermicle e Alleman (1990) apontam para diferenças na atividade dos genomas oriundos das partes materna e paterna, sugerindo a possibilidade de que muitos aspectos do desenvolvimento do endosperma, bem como sua interação com o embrião e os tecidos maternais, sejam definidos por *imprinting* genético diferencial. Ainda, analisando cerca de 285 diferentes *loci* em mutantes *dek* (defective kernel) de milho, Neuffer e Sheridan (1980) observaram que a maioria destes *loci* afeta o embrião e o endosperma e que apenas uma pequena parte deles podem ser específicos do endosperma. Um grande número de genes específicos da semente seriam ainda expressos no endosperma e no embrião, mesmo que estes tecidos sejam muito diferentes dos pontos de vista morfológico e fisiológico.

Outras análises genéticas sugerem que os tecidos maternais e o endosperma regulam reciprocamente seu desenvolvimento. Nos mutantes de milho com baixa atividade de invertases na região proximal do endosperma ocorre a destruição das células maternais encarregadas do transporte de sacarose (Miller e Chourey, 1992). Em cevada, a análise do efeito materno no fenótipo "shrunken endosperm" sugere que transcritos maternos possam mediar o desenvolvimento do endosperma (Felker et al., 1985).

No endosperma estão localizadas as reservas de nutrientes para a germinação da semente e o estabelecimento da nova planta. Além do seu papel como fonte de nitrogênio, enxofre, minerais e energia para o embrião durante a germinação, o endosperma também é considerado um meio que propicia a embriogênese. Desta forma, o endosperma nascente nutre o embrião desde os estágios iniciais e pode auxiliar no seu posicionamento na semente. Na maioria das dicotiledôneas, o endosperma é inteiramente assimilado durante a embriogênese e se faz necessário o desenvolvimento de folhas embrionárias (cotilédones) como meio de armazenamento de nutrientes para a germinação (Lopes e Larkins, 1993).

Nos cereais, as reservas de carboidratos do endosperma são fundamentais para prover ao embrião uma fonte de energia e carbono até que a plântula possa realizar fotossíntese. Nestas espécies, como na maioria das plantas, o amido caracteriza-se como o composto de armazenamento de carboidratos. O amido é sintetizado a partir da sacarose proveniente das folhas tão logo esta alcance o endosperma em desenvolvimento e, concomitantemente, é depositado nos grânulos de amido (revisado em Lopes e Larkins, 1993). No endosperma de milho, a transcrição dos genes que codificam enzimas chaves na síntese de amido é máxima entre 14 e 22 DAP e o padrão de expressão destes genes parece ser coordenadamente regulado com a síntese de proteínas de reserva (Giroux et al., 1994). Apesar de existirem evidências para a regulação transcricional dos genes envolvidos na via de síntese de carboidratos, até o momento não foi identificado qualquer fator regulador agindo *in trans*. Dentre as várias mutações afetanto o metabolismo de carboidratos na semente de milho, até o momento nenhuma delas foi associada a tais fatores (Lopes e Larkins, 1993). Por outro lado, é possível que esta via seja diretamente regulada pelo fluxo de fotossintatos para o endosperma através de um mecanismo de indução via sacarose. Açúcares como a glicose, frutose e sacarose podem, por exemplo,

induzir a expressão gênica da patatina em batata (Park, 1992), bem como agir como moduladores da interação fonte-dreno na folha de milho e como retro-reguladores da expressão gênica no aparelho fotossintetizador (Foyer, 1988; Sheen, 1990).

As sementes dos cereais constituem a maior fonte de proteínas para a nutrição animal e humana, apesar de suas frações protéicas constituirem apenas 15% do peso seco da semente (Payne, 1983). O endosperma contém uma variedade de proteínas. Algumas, constituem as proteínas com funções metabólicas e estruturais, tais como enzimas, proteínas nucleares e componentes da parede e membrana celulares. Outras, presentes em grandes quantidades, constituem as proteínas de reserva.

Proteínas de reserva da semente

As proteínas de reserva tem como única função armazenar nitrogênio, carbono e enxofre para a germinação. Estas proteínas caracterizam-se por possuir uma composição peculiar de aminoácidos, sendo ricas em asparagina, glutamina e arginina ou prolina mas deficientes em lisina, treonina e triptofano nos cereais, e em cisteína, metionina e triptofano, nas leguminosas. Elas são geralmente encontradas em vacúolos ou em vesículas do retículo endoplasmático rugoso.

Em geral, dois grupos principais de proteínas de reserva podem ser caracterizados. O primeiro, consiste de globulinas e albuminas que estão presentes em todas as espécies com sementes. O outro grupo, exclusivo dos cereais, constitui as prolaminas. Globulinas e prolaminas são estruturalmente muito diferentes e possuem distintos mecanismos de síntese e deposição. As globulinas são solúveis em soluções salinas, enquanto que as prolaminas, muito hidrofóbicas, somente são solúveis em soluções alcoólicas ou em presença de agentes denaturantes. Em milho e outros cereais dos gêneros *Triticum*, *Hordeum*, *Secale*, *Sorghum*, *Coix* e *Pennisetum*, as prolaminas representam 50-60% da proteína total do endosperma e as globulinas ao redor de 40%. Exceção faz-se ao arroz e à aveia, onde 80-85% são globulinas (revisado em Shotwell e Larkins, 1989).

As prolaminas são comumente agrupadas em classes de acordo com seus pesos moleculares e recebem nomes específicos que indicam o cereal de origem. Assim, por exemplo, as prolaminas do milho são chamadas zeínas (de *Zea*), as de *Coix*, coixinas, as

de cevada, hordeínas (de *Hordeum*), as do milheto, penisetinas (de *Pennisetum*) e as do sorgo, kafirinas (da variedade "kafir" desta planta).

As zeínas, quando analisadas eletrofereticamente, são subdivididas em seis classes de peso molecular aparente de 10, 14, 16, 19, 22 e 27 kDa (Wilson, 1991). No entanto, baseando-se na sua solubilidade diferencial, podem ser agrupadas em três classes de solubilidade, α-, β- e γ-zeínas (Esen, 1986). De fato, um terceiro sistema de nomenclatura vem se tornando o mais usual. Baseado na estrutura destas proteínas e não somente no seu peso molecular aparente e propriedades de solubidade, este sistema, proposto por Larkins et al. (1989), estabelece quatro classes de zeínas α-, β-, γ- e δ-zeínas. As α-zeínas, representadas pelas classes de 19 e 22 kDa, constituem aproximadamente 70% do total de prolaminas e são codificadas por famílias multigênicas (Feix e Quayle, 1993). As β- e γ-zeínas, ao contrário do que occorre com as α-zeínas, são codificadas por um ou dois genes (Kirihara et al., 1988; Gallardo et al., 1988). A β-zeína de 14 kDa constitui entre 10 a 15 % do total de zeínas e, as γ-zeínas, representadas pelos polipeptídeos de 16 e 27 kDa, constituem de 5 a 10% da fração total de zeínas. As zeínas de 10 kDa, por suas características de solubilidade, foram inicialmente classificadas como β-zeínas; porém, análises estruturais destes polipeptídeos levaram à inclusão de uma quarta classe, a das δ-zeínas (Kirihara et al., 1988; Larkins et al., 1989). As β-, γ- e δ-zeínas são caracterizadas pelo alto teor de aminoácidos sulfurados, metionina e cisteína (Shewry e Tatham, 1990).

As zeínas são sintetizadas por polissomos ligados à membrana do retículo endoplasmático rugoso e transportadas para seu lúmem, que incha e se fissiona para formar os corpúsculos protéicos (Larkins e Dalby, 1975; Larkins e Hurkman, 1978). O acúmulo de zeínas começa logo após a queda do índice mitótico do endosperma, quando suas células iniciam o processo de poliploidização (Feix e Quayle, 1993), e a taxa máxima de biossíntese acontece geralmente ao final da terceira semana após a polinização, coincidindo com a fase de expansão das células do endosperma (Pernollet, 1985). Isto parece refletir o acúmulo dos transcritos dos genes de zeínas, que cresce exponencialmente a partir de 10 DAP para alcançar um máximo em torno dos 20 DAP, decrescendo até a maturação da semente (Marks et al., 1985).

O acúmulo das diferentes classes de zeínas caracteriza-se por taxas diferenciais de síntese (Pernollet, 1985), e pelo padrão temporal de deposição e localização dentro do corpúsculo protéico (Lending e Larkins, 1989). Inicialmente verifica-se a deposição de β- e γ-zeínas; em seguida, com o amadurecimento do endosperma, dá-se o acúmulo de α-zeínas no interior do corpúsculo protéico, limitando as β- e γ-zeínas a uma distribuição periférica (Lending e Larkins, 1989). As δ-zeínas estão localizadas no centro do corpúsculo protéico (Thompson e Larkins, 1993). Estas diferenças no tempo de deposição e na distribuição espacial das zeínas no corpúsculo protéico sugerem que as classes ricas em cisteínas, β- e γ-zeínas, tem um papel na organização das zeínas dentro do corpúsculo protéico (Lending e Larkins, 1989), mas não há ainda evidências experimentais a este respeito.

Para a nutrição do embrião ou da plântula em crescimento, é necessária a mobilização das proteínas de reserva. Nos cereais, essa mobilização é mediada pela ação de endo- e exopeptidases (Rastogi e Oaks, 1986). Agindo em conjunto, estas peptidases, secretadas no endosperma, realizam a hidrólise das proteínas de reserva gerando uma mistura de aminoácidos e pequenos peptídeos. As carboxipeptidases ácidas são as exopeptidases mais abundantes em cevada. Estas carboxipeptidases tem ação muito rápida sobre grandes peptídeos, mas atuam lentamente sobre di ou tri-peptídeos. Estes pequenos peptídeos, no entanto, são rápida e ativamente transportados para o escutelo, onde são transformados em aminoácidos livres (Fincher, 1989), capazes então de serem utilizados na nutrição da plântula.

Regulação da expressão dos genes de zeínas

O acúmulo de grandes quantidades de zeínas reflete basicamente as suas taxas de síntese, seguindo uma cinética sigmoidal relacionada com o ganho de peso seco do endosperma (Higgins, 1984). A biossíntese destas proteínas parece ser controlada principalmente a nível de transcrição, observando-se, nos diferentes estágios de desenvolvimento do endosperma, uma estreita correlação entre a taxa de síntese de cada classe e os níveis do seu respectivo RNA mensageiro (Higgins, 1984; Kodrzycki et al., 1989; Feix e Quayle, 1993). Em decorrência disto, as pesquisas enfocando os

mecanismos que regulam a expressão das zeínas estão direcionadas principalmente para a identificação de sequências de DNA atuando *in cis* e fatores nucleares atuando *in trans* no controle da transcrição dos genes de proteínas de reserva.

O estudo do acúmulo das proteínas de reserva em diferentes mutantes fenotípicos de semente permitiram o descobrimento de vários genes que regulam a biossíntese destes polipeptídeos. Mutações nestes genes reguladores têm drásticos efeitos quantitativos e qualitativos no perfil das prolaminas da semente e, frequentemente, são também acompanhadas por significativas mudanças em algumas das outras frações protéicas (não-prolaminas). Nos cereais, este último fato geralmente acarreta um vantajoso aumento no conteúdo de lisina no endosperma, às custas de um decréscimo no conteúdo protéico total e queda de produtividade.

Várias mutações que afetam a síntese de zeínas podem estar associadas a *loci* reguladores. Nos mutantes *opaco2* (*o2*) e *De-B30* (*defective endosperm B30*) ocorre preferencialmente uma redução nas zeínas de 22 kDa. Em *opaco7* (*o7*) há redução nos níveis das zeínas de 19 kDa, enquanto que nos mutantes *floury2* (*fl2*), *mucronate* (*Mc*) e *opaco6* (*o6*) ocorre supressão da síntese de todas as classes com a mesma intensidade. Foi constatado que a menor produção de polipeptídeos de zeínas nestes mutantes é resultante de uma menor população de seus mRNA nos endospermas *o2*, *o7* e *fl2*, ou de um nível menor de mRNA traduzível (fração polissomal) em *o6*, *Mc* e *De-B30* (Soave e Salamini, 1984; Motto et al., 1989). A mutação *o2* afeta significativamente a transcrição da β-zeína, mas a um nível mais brando que as α-zeínas de 22 kDa (Kodrzycki et al., 1989).

Recentemente descobriu-se que os efeitos fenotípicos da mutação *fl2* são devido a uma mutação no gene estrutural da zeína de 22 kDa e não a um *locus* regulador. A mutação afeta o processamento do peptídeo sinal, criando uma zeína de 24 kDa que permanece ancorada no retículo endoplasmático, causando formação irregular do corpúsculo protéico (Lopes et al., 1994). Este defeito na deposição, no entanto, perturba todo o mecanismo de síntese de zeínas, causando os efeitos pleiotrópicos citados anteriormente.

Exceto pelo *locus Opaco2*, não existem maiores informações sobre a natureza dos fatores reguladores que os outros *loci* possam codificar.

O gene *O2* codifica uma proteína regulatória do tipo bZIP

A mutação homozigota recessiva *o2* confere um aspecto opaco a semente madura, que normalmente seria translúcida, mas apresenta vários outros efeitos pleiotrópicos. Ela causa um decréscimo muito significativo na fração de zeínas, entre 50 e 70% de redução na síntese, e um acréscimo no teor de lisina e triptofano, resultando na melhoria das qualidades nutritivas da semente (Mertz et al., 1964; Delhaye e Landry, 1986). Nas sementes *o2*, a taxa de degradação de lisina é menor que a encontrada nas sementes normais (Sodek e Wilson, 1970). Vários outros aspectos do metabolismo da semente são também modificados: ocorre um acréscimo na atividade de RNAse no endosperma mutante em comparação com o normal (Wilson e Alexander, 1967) e os conteúdos de várias proteínas e enzimas relacionadas ao metabolismo de nitrogênio e açúcares são alterados (Habben et al., 1993; Giroux et al., 1994; Galusci et al, 1996; Damerval e Guilloux, 1998). No mutante, foram ainda encontradas alterações nos níveis de carboidratos (Murphy e Dalby, 1971), maior susceptibilidade a patógenos (Loesch et al., 1976), redução na protandria (Gupta, 1979), alterações na atividade fotossintetizante das plântulas (Morot-Gaudry et al., 1979) e aumento na concentração de amônia (Misra e Oaks, 1981). A baixa resistência ao ataque de insetos e patógenos (Loesch et al., 1976; Gupta et al., 1970) pode estar associada, nas sementes *o2*, a um decréscimo significativo nos níveis da albumina b-32, que tem atividade de inativação de ribossomos (Bass et al., 1992). A mutação *o2* afeta ainda os níveis da proteína b-70, análoga à "heat shock protein" 70, que acumula-se na superfície do corpúsculo protéico e pode atuar como chaperonina durante a formação deste (Marocco et al. 1991).

O gene *O2* codifica uma proteína pertencente à classe "basic domain/leucine zipper" (bZIP; Figura 2) dos fatores reguladores da transcrição (Hartings et al., 1989; Schmidt et al., 1990), cujo aparecimento se inicia especificamente no endosperma em torno de 11 DAP, estando presente até 30 DAP (Gallusci et al., 1994). A partir do isolamento e caracterização do fator Opaco2 (O2), alguns aspectos da regulação transcricional de genes afetados pela mutação *o2* puderam ser detalhados através do análise da interação da proteína O2 e seus alvos nos promotores dos genes por ela regulados.

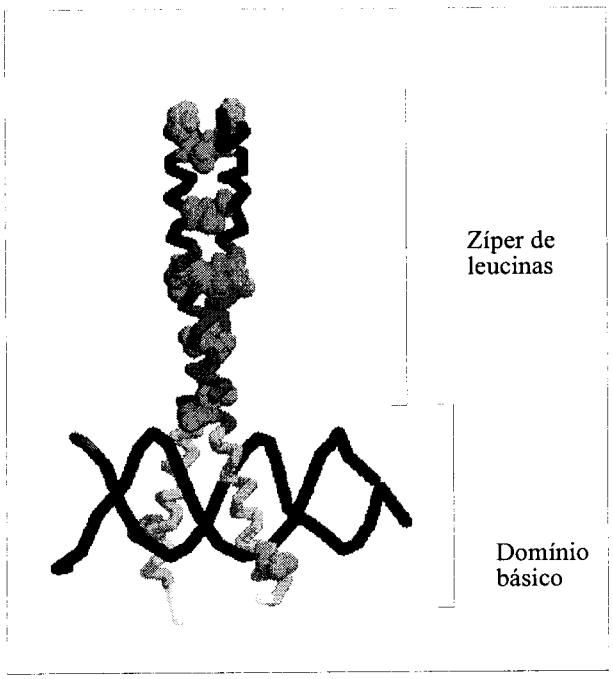

Figura 2. Representação esquemática de um fator de transcrição do tipo bZIP interagindo com o DNA. Complexo GCN4/sítio AP1. Ellenberger et al. (1992).

A proteína O2 é capaz de ligar-se a diferentes sequências de DNA. O primeiro gene em cujo promotor foi identificado um sítio de ligação da proteína O2 foi o gene b-32 (Lohmer et al., 1991). Neste estudo, através de experimentos de "footprint" *in vitro*, foram identificados cinco sítios de ligação da proteína O2 no promotor b-32, os quais apresentam a sequência consenso 5'-GATGApypuTGpu-3', onde py representa um nucleotídeo pirimidínico e pu, um purínico. Em ensaios de transformação transitória de protoplastos de tabaco, verificou-se um aumento de cerca de 80 vezes na expressão de um gene indicador sob controle do promotor b-32 quando transferido conjuntamente com o gene O2 sob controle de um promotor constitutivo, corroborando assim a transativação do gene b-32 pela proteína O2.

O gene *O2* foi o primeiro gene regulador para o qual demonstrou-se uma função no controle da expressão dos genes de proteínas de reserva. No gene da zeína de 22 kDa, a proteína O2 liga-se à sequência 5'-TCCACGTAGA-3' (Schmidt et al., 1992). Posteriormente, através de ensaios de transativação de protoplastos de endospema, concluiu-se que uma única alteração na região central do sítio, ACGT, é suficiente para suprimir a ligação da proteína O2 ao DNA *in vitro*, bem como a transativação *in vivo* (Ueda et al., 1992).

A partir de então, parece ter havido certa controvérsia em torno dos sítios de interação da proteína O2 nos diferentes promotores analisados. Os trabalhos de Ueda et al. (1992) e Izawa et al. (1993) indicavam que a região central ACGT seria essencial para a ligação de alta afinidade da proteína O2. Por outro lado, analisando a interação da proteína O2 de milho com o promotor de um gene de α-coixina, encontrou-se um sítio de ligação de O2 sem o motivo ACGT que apresentava maior similaridade com os sítios encontrados no promotor b-32 do que com aquele descrito para o promotor da α-zeína (Yunes et al., 1994). Estudando a interação da proteína O2 com o promotor do gene *psl*, que codifica uma lectina da semente de ervilha, de Pater et al. (1994) corroboraram os resultados obtidos com a α-coixina, mostrando que a proteína O2 reconhece um sítio sem motivo ACGT que se enquadra na sequência consenso proposta para o gene b-32. É interessante notar que este sítio é idêntico ao sítio de ligação da proteína GCN4 (Hill et al., 1986), uma bZIP de leveduras que controla vários genes do metabolismo de aminoácidos.

Recentemente, determinou-se que o fator O2 controla a expressão da forma citosólica da enzima piruvato ortofosfato diquinase-1 (cyPPDK-1) no endosperma do milho através da interação com dois sítios de ligação muito semelhantes aos do promotor b-32 (Maddaloni et al., 1996). Por outro lado, nos promotores dos genes da β-zeína e da β-coixina, foram encontrados sítios de ligação da proteína O2 apresentando o motivo ACGT. As análises de mRNA e do perfil de zeínas em endospermas *o2* e normal, juntamente com experimentos de transativação de um gene indicador sob o controle destes promotores, revelaram que as β-prolaminas estão sob controle do fator O2 (Cord Neto et al., 1995; Trabalho 1).

Em face de tais descobertas, acredita-se que a proteína O2 seja responsável pelo controle da expressão de vários genes distintos, de tal modo que um certo relaxamento na especificidade de ligação ao DNA seria vantajoso ou até mesmo necessário (Yunes et al., 1998).

O endosperma *opaco2* apresenta alto conteúdo de lisina

Os cereais são excelentes fontes de carboidratos, principalmente pelo alto teor de amido, porém são relativamente deficientes em proteínas, que representam 10 a 15% do peso seco da semente (Payne, 1983). Além disso, o valor nutricional destas proteínas é muito baixo devido à deficiência em aminoácidos essenciais, principalmente lisina e triptofano (Mertz et al., 1964).

O milho *opaco2* foi originalmente identificado quando se procurava selecionar genótipos ricos em lisina (Mertz et al., 1964). As vantagens nutritivas deste tipo de semente logo foram notadas. Ratos alimentados com uma dieta de milho *opaco2*, minerais e vitaminas ganhavam peso quase quatro vezes mais rápido do que aqueles que recebiam apenas milho normal na dieta (Mertz et al., 1965). Crianças sofrendo de deficiência crônica de proteínas devido a sua dieta a base de milho comum foram curadas com a substituição por milho *opaco2* (Harpstead, 1971). Até os dias de hoje o milho *opaco2* permanece no topo da relação, entre os mutantes viáveis simples e cultivares do tipo normal, em termos de conteúdo de lisina e triptofano, apesar de outros genótipos ricos em lisina terem sido encontrados (Mertz, 1986).

A percentagem de lisina no endosperma é influenciada por três componentes: o teor de aminoácidos livres, o conteúdo de zeínas, e o conteúdo de outras frações protéicas (não-zeínas). No endosperma *o2*, o teor de aminoácidos livres aumenta de duas a cinco vezes em relação ao normal (Misra et al., 1975); no entanto, apenas 5% do conteúdo total de lisina no endosperma é lisina não-protéica (Mehta et al., 1979). Assim, este componente contribui relativamente pouco para os níveis elevados deste aminoácido na semente. É característico das zeínas a quase total ausência de resíduos de lisina (Dalby e Tsai, 1975). Assim, a redução na síntese destas proteínas, proporcionalmente às outras frações, também contribui para o aumento no teor percentual de lisina. A maior parte da lisina origina-se da fração de não-zeínas, as albuminas, globulinas e glutelinas (Murphy e Dalby, 1971). Pressupõe-se que estas proteínas tenham funções metabólicas e estruturais. De fato, a caracterização de vários clones de cDNA isolados a partir desta fração através de imuno-seleção, identificaram mRNAs envolvidos no metabolismo de carboidratos, biossíntese de amino-ácidos e síntese de proteínas (Habben et al., 1993).

No endosperma *o2*, o aumento na síntese de várias proteínas não-zeínas corresponde a um aumento específico na transcrição da expressão dos seus respectivos genes (Habben et al., 1993). Isto não pode ser explicado pelo que se conhece até o momento sobre a propriedades transativadoras do fator O2, mas é possível que tais efeitos pleiotrópicos da mutação *o2* resultem não diretamente de interações entre O2 e o promotor dos genes com expressão modificada, mas de alterações no metabolismo de nitrogênio. Aparentemente, a planta de milho transporta muito mais nitrogênio, sob a forma de aminoácidos translocáveis, para o grão em desenvolvimento do que é requerido para o seu máximo desenvolvimento (Singletary et al., 1990). Se assim for, a assimilação de nitrogênio no endosperma *o2* pode estar alterada devido a reduzida capacidade para sintetizar zeínas. Tais alterações parecem de fato ocorrer, uma vez que no endosperma *o2* ocorre um aumento na concentração de amônia (Misra e Oaks, 1981) e no teor de aminoácidos livres (Misra et al., 1975), em relação ao endosperma normal.

Por outro lado, postula-se que o aumento na síntese de proteínas ricas em lisina seja uma consequência direta do aumento na disponibilidade de lisina livre no endosperma *o2* (Arruda et al., 1994). Em outras palavras, lisina pode ser um substrato limitante para a síntese protéica no endosperma. O aumento na concentração de lisina

pode estar associado com a baixa atividade de degradação deste aminoácido no endosperma mutante (Sodek e Wilson, 1970; Arruda e Silva, 1983). Analisando as frações protéicas e a composição de aminoácidos de endospermas do tipo normal e de mutantes simples (*o2o2*) e duplo (*o2o2Ask1Ask1*), Azevedo et al. (1990) observaram um aumento na síntese de proteínas ricas em lisina no mutante duplo. Este aumento foi atribuído a uma maior disponibilidade de lisina decorrente tanto da presença de uma aspartato quinase, codificada pelo gene *Ask1*, menos sensível a retroinibição por lisina, como da reduzida atividade de degradação, determinada por *o2*. Além disso, estes resultados, juntamente com as observações feitas por Habben et al. (1993), sugerem que a lisina, ou um metabólito dela derivado, possa atuar como um sinal na regulação da expressão gênica no endosperma.

Regulação da síntese e degradação de lisina

O acúmulo de lisina nos tecidos vegetais é regulado por processos complexos envolvendo síntese, deposição e degradação.

A lisina é sintetizada nas plantas superiores pela via do diaminopimelato, um ramo específico da via biosintética dos aminoácidos derivados do aspartato (Bryan, 1980; Azevedo et al., 1997). Nesta via, o aspartato é o precursor inicial para a produção de lisina, além de metionina e treonina, através de outras ramificações (Figura 3). A treonina, por sua vez, é precursora da isoleucina que, através da via de síntese dos aminoácidos de cadeia ramificada, também origina leucina e valina (Singh e Shaner, 1995; Azevedo et al., 1997). Uma série de enzimas são alostericamente retroinibidas pelos aminoácidos produzidos pelas respectivas ramificações da via. A atividade da aspartato quinase (AK; n° 1, Figura 3), a primeira enzima da via, é inibida por lisina e treonina. Adicionalmente, a lisina também inibe a atividade da dihidrodipicolinato sintase (DHDPS; n° 3, Figura 3), enquanto que a treonina inibe a homoserina desidrogenase (HSD; n° 10, Figura 3). Ou seja, lisina e treonina regulam sua própria síntese por inibição da atividade da primeira enzima do ramo que leva exclusivamente a sua produção e também por inibição da atividade da primeira enzima da via comum (Figura 3). A DHDPS é muito mais sensível a inibição pela lisina em comparação com a AK (Galili, 1995), o que a torna particularmente importante na regulação fina da síntese de lisina.

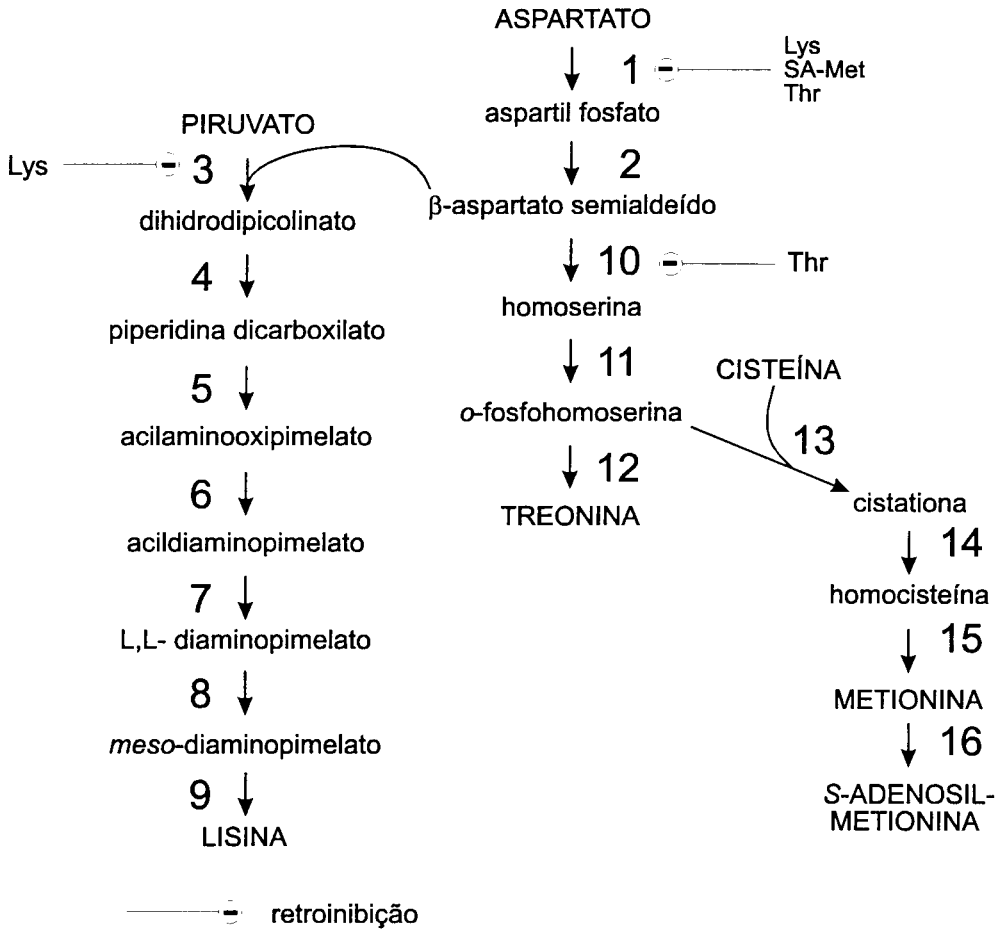

Figura 3. Via de síntese dos aminoácidos da família do aspartato. Os números especificam as atividades enzimáticas: **1**, aspartato quinase; **2**, aspartato semialdeído desidrogenase; **3**, dihidrodipicolinato sintase; **4**, dihidrodipicolinato redutase; **5**, piperidina dicarboxilato acilase; **6**, acildiaminopimelato aminotransferase; **7**, acildiaminopimelato deacilase, **8**, diaminopimelato epimerase; **9**, diaminopimelato decarboxilase; **10**, homoserina desidrogenase, **11**, homoserina quinase, **12**, treonina sintase, **13**; cistationa γ-sintase; **14**, cistationa β-liase; **15**, metionina sintase; **16**, *S*-adenosil-metionina sintetase (adaptado a partir de Azevedo *et al.*, 1997). Lys, lisina; Thr, treonina; SA-Met, *S*-adenosil-metionina.

Alguns trabalhos mostraram que mutantes contendo AK insensível a retroinibição por lisina superproduziam treonina mas tinham muito pouco efeito no acúmulo de lisina em cevada e milho (Bright et al., 1982; Diedrick, 1990). A partir deste resultado, procurou-se isolar mutantes contendo DHDPS insensível a lisina, mas estes também não mostraram aumentos significativos no conteúdo de lisina (Negrutiu et al., 1994). Mesmo mutantes duplos AK/DHDPS insensíveis a lisina não mostraram superprodução de lisina (Frankard et al., 1991). Plantas transgênicas de tabaco e canola expressando AK ou DHDPS ou ambas, mostraram superprodução de treonina e significativos aumentos no acúmulo de lisina em folhas jovens e sementes (Karchi et al., 1993; Falco et al., 1995). Estas plantas, no entanto, apresentavam alterações fenotípicas e alta atividade de degradação de lisina foi observada concomitante ao acúmulo de lisina (Karchi et al., 1994; Falco et al., 1995).

Embora haja síntese de lisina através da via do diaminopimelato no endosperma de milho em desenvolvimento (Sodek, 1976), lisina é translocada de outros tecidos para o endosperma em quantidades relativamente altas (Arruda e Silva, 1979), constituindo cerca de 5% do total de amino ácidos translocados. Comparativamente, a glutamina é a forma predominante (de 18 a 30%), enquanto que metionina é pouco translocada (de 0.8 a 1.7%) e prolina e cisteína não são detectadas, o que implica na obrigatoriedade de sua síntese *in situ* (Arruda e Silva, 1979).

O catabolismo de lisina desempenha um papel importante no mecanismo de controle dos níveis de lisina livre no endosperma de milho (Arruda e Silva, 1979; Silva e Arruda, 1979). Ao longo do desenvolvimento da semente de milho, a quantidade de lisina encontrada na seiva do pedúnculo da espiga e na região pedicelo-planto-chalazal é superior à encontrada no endosperma (Arruda e Silva, 1979; Arruda e da Silva, 1983; Lyznik et al., 1985). A quantidade de lisina translocada é aproximadamente duas a três vezes maior que a necessária para a síntese de proteínas (Arruda e da Silva, 1983). Dessa maneira, esperar-se-ia um acúmulo de lisina livre no endosperma, mas este não é o caso. O conteúdo total de lisina é cerca de 1.5% do "pool" de aminoácidos, e a concentração de lisina livre é mantida baixa durante o desenvolvimento (Arruda e da Silva, 1983). Ademais, a quantidade de lisina livre no endosperma deve ser mantida baixa porque este aminoácido inibe a via do aspartato, conforme dito anteriormente. A inibição da AK na

presença de níveis elevados de lisina resultaria na falta de substratos precursores para a síntese de metionina (Figura 3). Considerando que a metionina, comparativamente aos outros aminoácidos, é muito pouco translocada para o endosperma, isto poderia limitar a síntese protéica e ter efeitos adversos para o desenvolvimento do endosperma. Assim, para manter baixos os níveis de lisina livre, as células do endosperma precisariam degradar o excesso deste aminoácido.

Neste tecido, a lisina é degradada através da via da sacaropina. Altamente ativa no endosperma, a enzima bifuncional lisina-cetoglutarato redutase/sacaropina desidrogenase (LKR/SDH) catalisa os dois primeiros passos da degradação de lisina (Figura 4), condensando lisina e α-cetoglutarado em sacaropina e subsequencialmente hidroliosando esta em ácido α-aminoadípico e glutamato (Gonçalves-Butruille et al., 1996).

Regulação coordenada do metabolismo de lisina e da síntese de proteínas de reserva

O catabolismo de lisina também é afetado pela mutação *o2*. Sodek e Wilson (1970) demonstraram através do monitoramente de ^{14}C-lisina que no endosperma *o2* ocorre menor degradação deste aminoácido quando comparado ao endosperma normal. Posteriormente verificou-se que o padrão de atividade da enzima LKR é coordenado com o aporte de nitrogênio e com a síntese de zeínas no endosperma em desenvolvimento (Arruda e da Silva., 1983) e que a atividade de LKR no endosperma mutante é de 2 a 3 vezes menor que no endosperma normal (Brochetto-Braga et al., 1992). Esta diminuição na atividade está correlacionada com um menor número de moléculas da enzima, o que nos leva a postular que o controle do catabolismo de lisina e da síntese de zeínas opera sob um mecanismo comum, envolvendo o regulador transcricional Opaco2.

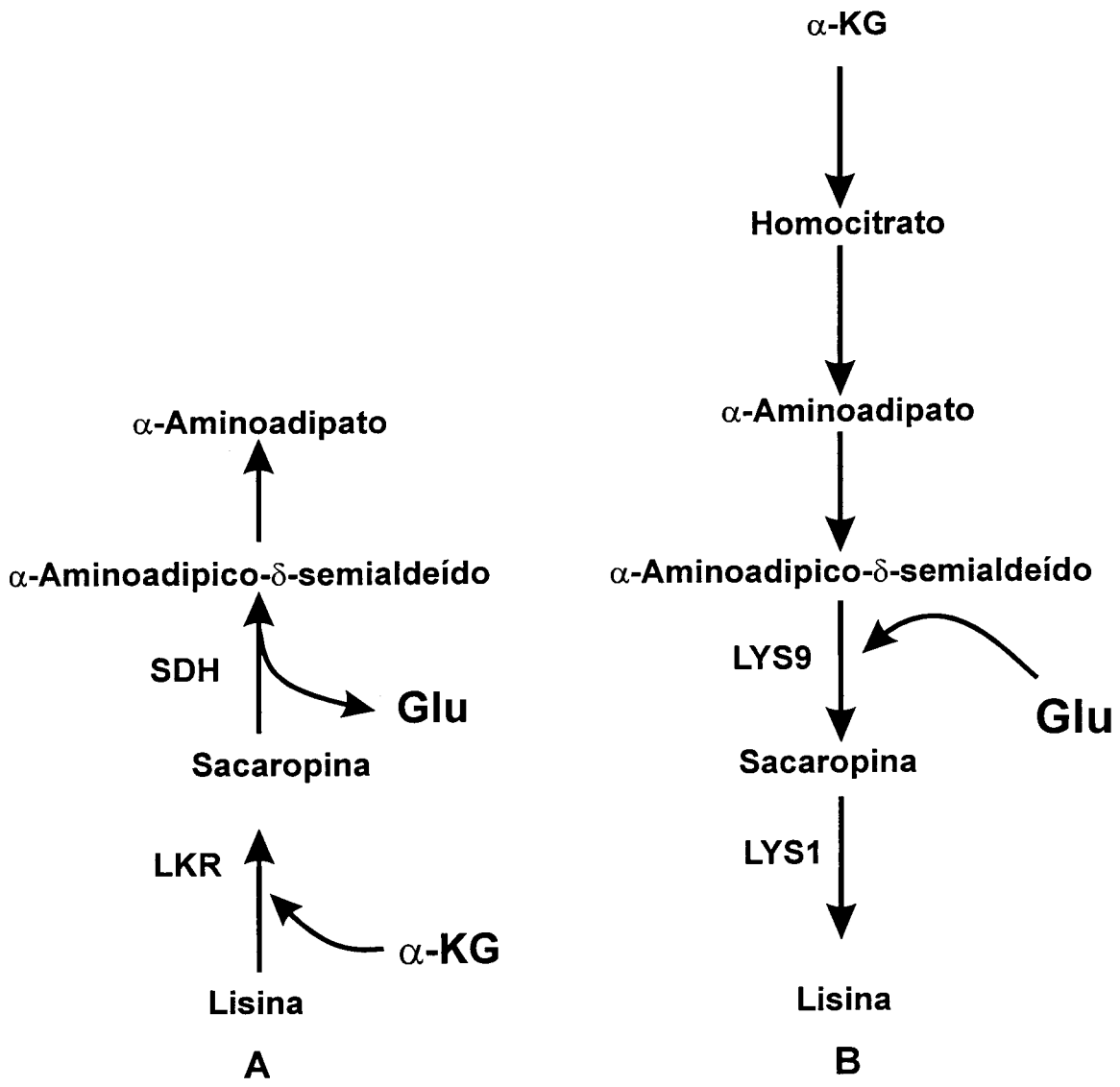

Figura 4. Via da sacaropina para degradação (A) de lisina em plantas e para síntese (B) em leveduras (Ramos et al., 1988; Brochetto-Braga et al., 1992.). Glu, glutamato; α-KG, α-cetoglutarato LYS9, sacaropina desidrogenase; LYS1, sacaropina desidrogenase, formadora de lisina LKR, lisina-cetoglutarato redutase; SDH, sacaropina desidrogenase

APRESENTAÇÃO DOS TRABALHOS E OBJETIVOS

Esta tese é constituída de dois trabalhos, um deles já publicado e o outro submetido à publicação. Os trabalhos versam sobre o papel do ativador transcricional Opaco2 no controle da síntese de proteínas de reserva e no metabolismo de lisina no endosperma de milho em desenvolvimento. O primeiro trabalho trata da regulação transcricional de uma classe de proteínas de reserva e o segundo descreve aspectos da regulação transcricional do gene responsável pelas atividades de degradação da lisina. Ambos podem ser considerados como evidências do papel do fator Opaco2 no metabolismo de aminoácidos na semente.

Entretanto, estes trabalhos basearam-se em outros estudos de caracterização de genes e do mecanismo de regulação da expressão gênica no endosperma em desenvolvimento.

Arruda, P., Cord Neto, G., e Gonçalves-Butruille, M (1994) Lysine catabolism and the regulation of lysine accumulation in maize endosperm. *In* Larkins, B.A., e Mertz, E. eds. Quality Protein Maize: 1964-1994. Proceedings of the International Symposium on Quality Protein Maize, Sete Lagoas, Brasil. Sasakawa Global 2000-Mozambique. pp. 175-184.

Yunes, J.A., Cord Neto, G., Silva, M.J., Leite, A., Ottoboni, L.M.M., e Arruda, P. (1994). The transcriptional activator Opaque-2 recognize two different target sequences in the 22-kD-like a-prolamin genes. Plant Cell **6,** 237-250.

Yunes, J.A., Cord Neto, G., Leite, A., Ottoboni, L.M.M., e Arruda, P. (1994). The role of the Opaque2 transcriptional factor in the regulation of protein accumulation and amino acid metabolismo in maize seeds. An. Acad. Bras. Ci. **66,** 227-237.

Dante, R.A., Cord Neto, G., Leite, A., e Arruda, P. (1998). Cloning, characterization and expression of the *DapA* gene encoding the lysine biosynthetic enzyme dihydrodipicolinate synthase from *Coix lacryma-jobi L.* Em preparação.

Vettore, A.L., Yunes, J.A., Cord Neto, G., Silva, M.J. da, Arruda, P., e Leite, A. (1998). The molecular and functional characterization of an Opaque2 homologue gene

from *Coix* and a new classification of plant bZIP proteins. Plant Mol. Biol. **36,** 249-263.

Kemper, E.L., Cord Neto, G., Capella, A.N., Gonçalves-Butruille, M., Azevedo, R.A., e Arruda, P. (1998). Structure and regulation of the bifunctional enzyme lysine-oxoglutarate reductase-saccharopine dehidrogenase in maize. Eur.J. Biochem. **253,** 720-729.

Leite, A., Cord Neto, G., Vettore, A.L., Yunes, J.A., e Arruda, P. (1998). Prolamins of Sorghum, *Coix* and Millets. *In* Shewry, P.R. ed. Seed Proteins. Em preparação.

Trabalho 1: **The involvement of Opaque-2 on β-prolamin gene regulation in maize and *Coix* suggests a more general role for this transcriptional activator.**

Germano Cord Neto, José A. Yunes, Márcio J. da Silva, André L. Vettore, Paulo Arruda e Adilson Leite. Plant Mol. Biol **27,** 1015-1029. (1995).

Objetivos:

Estudos anteriores mostravam que a mutação *opaco2*, que afeta primariamente as α-zeínas de 22 kDa, causava uma redução na expressão da β-zeína. Nesta ocasião, o pensamento corrente ditava que o fator Opaco2, recentemente isolado, seria o ativador transcricional específico das α-zeínas. Assim, decidimos verificar a sua contribuição para a expressão das β-zeínas, com os seguintes objetivos:

1. Verificar em que grau a transcrição do gene da β-zeína era afetada pela mutação *o2*.
2. Determinar o possível sítio de ligação da proteína O2 no promotor da β-zeína.
3. Isolar o clone genômico da β-coixina e realizar um estudo comparativo da expressão das β-prolaminas de milho e *Coix*.
4. Determinar se a proteína O2 era capaz de ativar a transcrição dos promotores de β-prolaminas *in vivo*.

Trabalho 2: **The role of Opaque2 on the control of lysine degrading activities in developing maize endosperm.**

Germano Cord Neto, Edson L. Kemper, Fabio Papes, Karen C. M. Moraes, Adilson Leite e Paulo Arruda. Submetido. (1998).

Além dos seus efeitos marcantes na síntese de proteínas de reserva, a mutação *o2* causa também um acréscimo significativo no teor de lisina na semente. Dentre os vários efeitos pleiotrópicos desta mutação, sabe-se que a taxa de degradação da lisina no endosperma *o2* é menor em comparação com o endosperma normal, e que a atividade da enzima que realiza a catálise sofre uma redução de 2 a 3 vezes no endosperma *o2*. Desta forma, o catabolismo de lisina e a síntese de proteínas de reserva parecem operar sob a coordenação de um mecanismo comum de regulação gênica, possivelmente envolvendo o fator O2. Os objetivos deste trabalho foram:

1. Isolar e caracterizar o gene que codifica a enzima responsável pela degradação de lisina no endosperma de milho, lisina-cetoglutarato redutase/sacaropina desidrogenase (LKR/SDH).
2. Determinar o padrão de expressão do mRNA e da deposição e atividade da enzima na semente em desenvolvimento.
3. Determinar o efeito da mutação *o2* na transcrição do gene LKR/SDH.

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The involvement of Opaque 2 on β-prolamin gene regulation in maize and *Coix* suggests a more general role for this transcriptional activator

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Key words: β-prolamin, *Coix lacryma-jobi*, different *O2*-binding sites, *Opaque 2*, transcriptional regulation, *Zea mays*

Abstract

The maize *opaque 2* (*o2*) mutation is known to have numerous pleiotropic effects. Some polypeptides have their expression depressed while others are enhanced. The best characterized effects of the *o2* mutation are those exerted on endosperm genes encoding the storage protein class of the 22 kDa α-zeins and the ribosome inactivating protein b-32. The *Opaque 2* (*O2*) locus encodes a basic domain-leucine zipper DNA-binding factor, O2, which transcriptionally regulates these genes. In the maize-related grass *Coix lacryma-jobi*, an O2-homologous protein regulates the 25 kDa α-coixin gene family. We show in this paper that O2 transcriptionally regulates the structurally and developmentally different class of the β-prolamins. A new O2-binding box was identified in β-prolamin genes from maize and *Coix* that, together with the boxes previously identified in other endosperm expressed genes, forms a curious collection of O2 *cis* elements. This may have regulatory implications on the role of O2 in the mechanism that controls coordinated gene expression in the developing endosperm. Considering that the *O2* locus controls at least three distinct classes of genes in maize endosperm, we propose that the O2 protein may play a more general role in maize endosperm development than previously conceived.

Introduction

The major class of seed storage proteins in maize and *Coix lacryma-jobi* is a group of prolamins called zeins and coixins, respectively. These proteins can be separated by SDS-PAGE into polypeptide classes of 27, 22, 19, 16, 14 and 10 kDa in maize [9] and 27, 25, 22, 17 and 15 kDa in *Coix* [37]. Based on differential solubility zeins can be separated into three fractions, α-, β- and γ-prolamins [9], while coixins are separated into α- and γ-prolamin fractions [26]. In maize as well

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X79885.

as in *Coix*, the α-prolamins account for around 70% of the total prolamins, being encoded by large multigene families of the 19 and 22 kDa polypeptide classes in maize and 25 and 27 kDa in *Coix* [38, 53]. The characterization and sequencing of a 17 kDa coixin cDNA clone [27] revealed a sulfur-rich protein showing high similarity to the 14 kDa β-zein [40], thus this coixin size class was named β-coixin.

The prolamin genes are coordinately expressed, being under tissue-specific and developmental control [25, 28, 47, 51].

Regulation of zein gene expression is controlled by several different loci. The *Opaque 2* (*O2*) controls the levels of transcription of the 22 kDa α-zein class [3, 21, 45, 49]. A homozygous loss-of-function mutation at *O2* results in an overall reduction of 50 to 70% in zein content in maize endosperm. Another endosperm protein, a 32 kDa albumin referred as b-32, whose expression is temporally and quantitatively coordinated with the deposition of zeins, is greatly affected by the homozygous *o2* mutation [7].

The O2 protein is a member of the basic domain/leucine zipper (bZIP) class of transcriptional activators [16, 44]. It has been shown to bind to specific sequences in the promoter of the genes encoding b-32, 22 kDa α-zein, and 25 kDa α-coixin [30, 45, 56].

In contrast to the 22 kDa α-zein class, little is known about the mechanism controlling the regulation of the other zein classes. A mutation at the regulatory locus *Opaque 7* significantly affects expression of the 19 kDa class [6], while the *floury 2* mutation generally decreases the expression of 19 and 22 kDa α-zeins [20]. There is no additional information about the genes these loci may represent.

Among the minor zein classes, the methionine-rich 10 kDa δ-zein is regulated at the post-transcriptional or translational level by a *trans*-acting factor encoded by the locus *Zpr10/(22)* [5]. With respect to the γ-zeins, no regulatory loci identified so far have influence on its synthesis, although *opaque 2 modifier* genes have been shown to increase the levels of the 27 kDa polypeptide in modified *o2* endosperm [15].

Concerning the regulation of the 14 kDa β-zein gene, it has been found that an endosperm-specific and coordinately expressed nuclear protein binds to the β-zein promoter [48]. In addition, nuclear run-on transcription assays indicated that the *o2* mutation may affect the transcription of the β-zein, but to a lesser extent than the 22 kDa α-zein [21].

In this paper we report that the *o2* mutation contributes for about 60% decrease in the 14 kDa β-zein synthesis. Moreover, the O2 protein binds to and transactivates β-prolamin gene promoters from maize and *Coix* suggesting that the decrease in protein synthesis is a direct effect of reduced transcription rates. The core sequence of the O2 target site in these genes (TCCACGTCAT), identical in both species, is similar to the 22 kDa α-zein O2 target site.

Considering that O2 interacts with the promoters of at least three distinct classes of genes (22 kDa α-zein, 14 kDa β-zein and *b-32*), we discuss its role on the mechanisms that controls coordinated gene expression in maize endosperm. This may be achieved through its capacity to recognize different binding sites in such promoters.

Materials and methods

Plant materials

The wild-type and *opaque 2* W64A and Cat100-1 inbred lines were grown at the experimental field of University of Campinas (Campinas, São Paulo, Brazil). Mature and immature seeds from wild type and *o2* Oh43 were kindly provided by Dr Robert J. Schmidt (University of California at San Diego, USA). The seeds of the commercial hybrid F-352 were purchased from Sementes Agroceres (São Paulo, Brazil).

Extraction of zeins and SDS-PAGE

Total zeins were isolated from mature kernels according to Wallace *et al.* [54]. Protein samples equivalent to 0.5 mg of flour were separated elec-

trophoretically in 10 to 20% (w/v) continuous gradient SDS-polyacrylamide gels and stained with Coomassie Brilliant Blue R250 [24]. Protein extractions were repeated three times and the average values for protein content were determined by scanning with a laser densitometer (LKB UltroScan XL, Bromma, Sweden).

RNA gel blot analysis

Total RNA was isolated from 17 DAP wild-type and *opaque 2* maize seeds. About 5 g of liquid nitrogen-frozen seeds were ground with a mortar and pestle and total RNA was extracted according to the procedure described by Precott and Martin [42]. The RNA blot analysis was performed according to the procedure described by Fourney *et al.* [12]. A 12 μg portion of total RNA was analyzed for the presence of 14 kDa β-zein, 22 kDa α-zein, 19 kDa α-zein, 27 kDa γ-zein and *Opaque-2* transcripts using ^{32}P-labelled-cDNAs as probes (the respective cDNA clones are listed in the legend to Fig. 2). Relative amounts of hybridized mRNA were analyzed through scanning with a laser densitometer (LKB).

Isolation and sequencing of the β-coixin genomic clone

Total DNA was extracted from *Coix* seedlings according to Rivin *et al.* [43]. The DNA was partially digested with *Mbo* I and separated on a sucrose gradient. Fractions containing fragments of 9–20 kb were pooled and cloned in the λ Dash vector (Stratagene, La Jolla, CA). About 1×10^6 phage were screened with the labelled *Eco* RI-*Xho* I fragment of the β-coixin cDNA clone pBCX17.9 [27] and four positive clones were isolated. One of them, the β-55.1 clone, was further characterized and sequenced. The recombinant phage was sub-cloned into the pBluescript KS$^+$ vector (Stratagene) and sequenced on both strands using the T7 DNA sequencing kit (Pharmacia/LKB Biotechnology). The sequence data were analyzed using the DNASIS sequence analysis software (Pharmacia/LKB). The alignments were primarily performed on CLUSTAL V software [17] and the best alignment was further arranged manually.

Construction of plasmids

All sequence position numbers refer to the ATG initiation codon. The plasmids pRT101-O2, Pα-CxGUS, Pα-Z4GUS, Pα-Cx103 and Pα-Z4103 have been described previously by Yunes *et al.* [56].

pβ-λ55.1EE is a 0.89 kb *Eco* RI clone spanning from −97 to −985 of the β-coixin promoter that was excised from a ca. 14 kb β-coixin genomic clone (β55.1) and inserted into *Eco* RI-digested pBluescript KS$^+$.

Pβ-ZEP is the entire *Eco* RI-*Pst* I β-zein promoter fragment clone spanning from the −233 region to +81, excised from the 5.6 kb fragment of pgZ15A [40] and cloned into pBluescript KS$^+$.

Pβ-ZGUS is a reporter construct where the GUS-coding region is under the control of a 0.22 kb β-zein promoter fragment. This fragment was excised from the pβ-ZEP clone by digestion with *Taq* I-*Eco* RI and inserted into the *Cla* I-*Eco* RI sites of pBluescript KS$^+$. Subsequently, this β-zein promoter subclone was recovered by *Bam* HI-*Sal* I digestion and inserted into the *Bgl* II and *Sal* I sites of pDMC200 (Cambia TG0025; formerly called pS275R, kindly provided by Dr R. A. Jefferson, Cambia, Canberra, Australia), which is a vector composed of a polylinker site, the GUS coding sequence and *rbcS* terminator.

Pβ-CxGUS is a reporter construct where the GUS-coding region is under the control of a 0.89 kb β-coixin promoter fragment. The β-coixin promoter fragment spanning from −97 to −985 with respect to the ATG was excised from the pβ-λ55.1EE clone by digestion with *Bam* HI and *Hin*d III and inserted into the same sites of pDMC200.

pRT101-O2NA is an *opaque 2* effector plasmid identical to pRT101-O2 except that the O2 basic domain region contains a substitution at the in-

variant asparagine to alanine [1]. To create the pRT101-O2NA, the O2 cDNA fragment of pRT101-O2 [56] from the *Pst* I site to the *Sal* I, site (nucleotides 566 to 855) was replaced by the same fragment excised from the O2 mutagenized pURN-A clone [1]. The pURN-A clone corresponds to the 1.4 kb *Hin*d III fragment of the O2 cDNA mutagenized at the invariant asparagine of the basic domain and subcloned into pUR288 [1].

Transient expression assays in maize coleoptiles

Seeds of the commercial hybrid F-352 were surface sterilized for 15 min with 5% commercial bleach and rinsed four times in sterile distilled water. Seeds were germinated in water-saturated germination paper at 28 °C in the dark. Three-day old coleoptiles were detached at the junction with the seed, surface-sterilized for 3 min in 5% commercial bleach, rinsed four times in sterile distilled water and placed, 9 per plate, on 100 mm diameter Petri dishes containing 20 ml of MS medium [36]. A 5 μg portion of CsCl-purified DNA of the reporter construct was coprecipitated with 5 μg of pRT101 [52] or with pRT101-O2 or pRT101-O2NA and used to coat 3 mg of 1 to 3 μm diameter gold particles according to the method of Ye *et al.* [55]. The coleoptiles were bombarded twice with 1 μg DNA using a home-made helium particle delivery device [55]. After bombardment, the samples were incubated for 48 h in the dark at 28 °C. The coleoptiles were then stained for GUS activity according to the method of Jefferson [19] and the number of blue spots appearing per plate were counted after 12 h staining.

DNA binding assay

The DNA binding assay was performed with a maize or *Coix* β-galactosidase-O2 (β-gal::O2) fusion protein according to the method described by Schmidt *et al.* [45]. The plasmid expressing the *Coix* β-gal::O2 fusion protein was obtained by inserting a 886 bp *Hin*d III-*Bam* HI fragment from the O2 cDNA clone pCO2-1 (EMBL, GenBank and DDBJ accession number X78286) into pUR289 expression vector (Souza Filho *et al.*, unpublished results). About 40 ng of ^{32}P-labelled digests of plasmids pβ-λ55.1EE, pβ-ZEP, Pα-Cx103 and Pα-Z4103, and pBluescript KS$^+$ (used as negative control) was incubated with 15 μl of protein A-Sepharose beads containing the β-gal::O2 immunocomplex for 90 min at room temperature with intermittent agitation. Washes, processing, and agarose gel electrophoresis of the retained fragment were performed as described by Schmidt *et al.* [44].

DNase I footprint

In order to subclone the promoter fragment bound by the Opaque 2 protein in the β-coixin promoter, the blunt-ended 340 bp *Hinf* I fragment which contains the putative O2 target sites was digested with *Pst* I and cloned into the *Sma* I-*Pst* I sites of pBluescript KS$^+$. In the case of the β-zein promoter, the plasmid harboring the 320 bp *Eco* RI-*Pst* I fragment which contains the region through the prolamin box (−233) to the beginning of the coding sequence (+87) was digested with *Eco* RI-*Taq* I and subcloned into the *Eco* RI-*Cla* I sites of pBluescript KS$^+$ to produce a subclone containing only promoter sequences (from −233 to −13).

The subclone containing the fragment of interest from β-zein was digested with *Eco* RI-*Xho* I. The subclone containing the β-coixin promoter fragment was digested with *Eco* RI-*Not* I. The fragments containing promoter sequences plus pBluescript KS$^+$ polylinker sites were purified in agarose gel. In order to label the bottom strand of the β-zein DNA fragment and the top strand in the β-coixin one, the fragments were 3′ end-labelled by fill-in with α-^{32}P-dATP, dTTP, and the Klenow fragment of DNA polymerase I. The labelled fragments were then purified from a 7.5% polyacrylamide gel, and for each reaction, 60000 cpm of DNA was incubated with 0, 10, 20, and 30 μl of the protein A-Sepharose beads containing the immunoprecipitated β-gal::O2 fusion

plus 30, 20, 10, and 0 μl of the protein A-Sepharose beads, respectively, in a total volume of 50 μl of binding buffer containing 12.5 mM Hepes pH 7.5, 50 mM KCl, 10% glycerol, 0.05% Nonidet P-40, 0.5 mM DTT, and 0.7 μg poly(dI-dC). Reaction mixtures were incubated for 20 min at 4 °C and then digested with 0.3 units of DNase I according to recommendations of the Sure Track Footprinting Kit (No. 27-9101-01; Pharmacia LKB Biotechnology). The resulting fragments were separated by electrophoresis on a 6% sequencing gel. The same end-labelled DNA was treated with the G + A cleavage reaction, according to the Sure Track Footprinting Kit manufacturer's recommendations, and run on lanes adjacent to the footprinting reactions.

Results

14 kDa β-zein mRNA and protein levels are affected by the opaque 2 *mutation*

The effects of the *o2* mutation on 22 kDa α-zein synthesis have been extensively studied in the past few years. Although the *o2* mutation is generally assumed to dramatically reduce the amounts of the 22 kDa size class, effects on transcription levels of the 19 and 14 kDa classes are also observed [21].

Through SDS-PAGE analysis of zein composition from normal and *opaque 2* endosperm in a South American flint maize inbred line, Cat100-1 and two other inbred lines, W64A and Oh43, we have noticed, in addition to the well known effect on the 22 kDa class, a significant decrease in protein levels of the 14 kDa class in the *o2* endosperm. The SDS-PAGE profile of total zein extracted from normal and *o2* W64A, Oh43 and Cat100-1 inbred lines is presented in Fig. 1A. Densitometric scanning of the gels, shown in Fig. 1B, indicate that the relative contents of the 14 kDa β-zein class are reduced by ca. 60% in the *o2* endosperm of W64A and by ca. 70% in Oh43 and Cat100-1 (Fig. 1B).

To confirm that the *o2* mutation affects β-zein transcription, we performed RNA blot analysis of

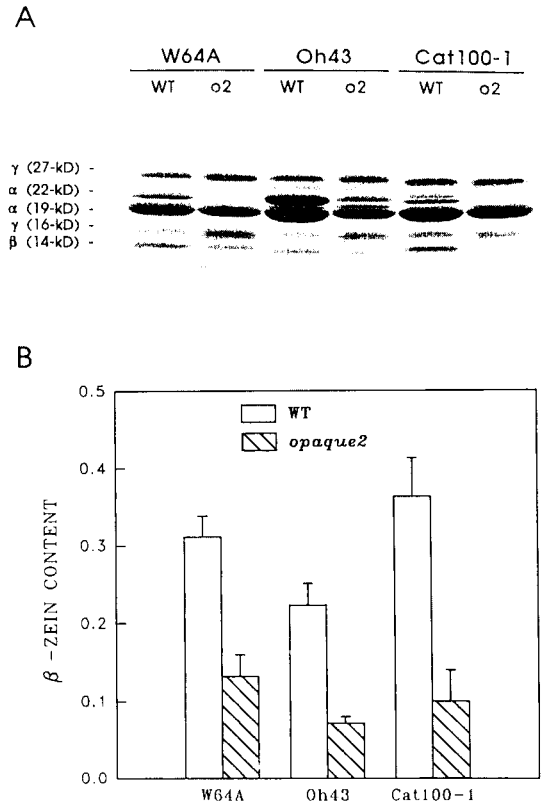

Fig. 1. SDS-PAGE analysis of zein from mature wild-type and *opaque 2* endosperm of W64A, Oh43 and Cat100-1 maize inbred lines. A. Total zeins were extracted as described in Materials and methods from wild-type (WT) and *opaque 2* (*o2*) endosperms and separated by gradient gel SDS-PAGE. Protein samples were extracted from equal amounts of endosperm, and protein extracts corresponding to 0.5 mg of flour were loaded on the gel. B. The β-zein contents of mature seeds from W64A, Oh43 and Cat100-1 wild type and *opaque 2* maize inbred lines. Coomassie Blue-stained gels were scanned with a laser densitometer, and absorbance values were used to estimate the amount of β-zein. Values are the average of three independent extractions and measurements. Standard deviation is represented by an error bar.

normal and *opaque 2* endosperm from W64A and Oh43. The *o2* allele in W64Ao2 encodes a truncated protein while Oh43o2 was shown to be a null transcript mutant [2]. Cat100-1 was not used in this experiment because the nature of the *o2* mutant allele has not been characterized.

As shown in Fig. 2, the level of the 14 kDa β-zein mRNA in 17 days after pollination (DAP) endosperm is reduced by about 80% in *opaque 2*

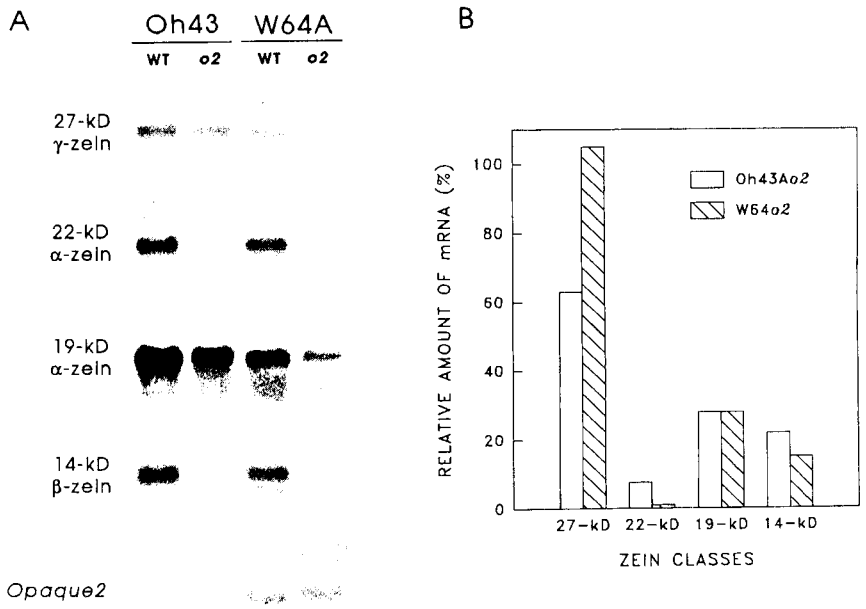

Fig. 2. RNA blot analysis of total RNA from immature wild-type and *opaque 2* maize endosperms. A. Total RNA was extracted from 17 DAP wild-type (WT) and *opaque 2* (*o2*) endosperms of Oh43 and W64A. Twelve micrograms of total RNA were fractionated in 1% agarose gel containing 0.66 M formaldehyde, transferred to Nylon membranes and hybridized with α-^{32}P-labelled cDNA 27 kDa γ-zein pME119 [41], 22 kDa α-zein pZ22.3 [32], 19 kDa α-zein pZ19.1 [32], β-zein clone pZ15.1 [34] and O2 cDNA 1–4 [44]. B. Relative amounts of hybridized zein mRNAs in 17 DAP W64A and Oh43 *opaque 2* endosperms. After 24 h exposure, the autoradiograms from the hybridized filters described in A were scanned with a laser densitometer, and absorbance values were used to estimate the decrease in hybridized mRNA levels for each zein class in the *opaque 2* endosperm relative to the wild-type control. In the wild-type endosperm the estimated absorbance value for each class was set to 100%.

relative to the normal endosperm. Among the α-zeins, the mRNA level of the 22 kDa class was severely affected and the 19 kDa class level decreased by about 70% in the mutant endosperm. The 27 kDa γ-zein class was differently affected in W64A and Oh43 *opaque 2* endosperms. In W64Ao2, the level of γ-zein mRNA was not affected while in the Oh43o2 inbred line showed a decrease of ca. 40%.

Isolation and characterization of a genomic clone encoding β-coixin

To isolate the β-coixin gene, a *Coix* genomic library was screened with the β-coixin cDNA clone pBCX17.9 [27]. One of the hybridizing phage (β-55.1), containing a DNA insert of ca. 14 kb, was characterized. The sequenced region spans 585 bp of the coding unit, 985 bp of the 5′-and 73 bp of the 3′-flanking region.

The alignment of the proximal β-prolamin promoter regions from *Coix* and maize is presented in Fig. 3. Besides the high similarity, the β-zein promoter presents large deleted regions in comparison to the same region in the β-coixin promoter. In the β-coixin promoter, the putative TATA box is located at −146 with respect to the ATG, and two copies of the prolamin box were found at −310 and −431. Due to the deletions observed in the β-zein promoter, the relative positions of these elements showed a significant displacement towards the ATG in this promoter. The search for homologous O2-like-boxes detected two related sequences in each promoter, which we have termed O2a and O2b, as indicated in Fig. 3.

```
Cx GTGTATATATACCTACAATGAACTGATGGTGATGATGAGTCATGCTGAAG -432
Mz GTGTATATGTGCCTACAATAA---------GGTGATGAGTCATGGTGATG -272
   ******** * ******** *          * ************ *** *
   |<PB1>|
Cx TGTCAAGCAATACTGCTCAGCATATATAGCCCAATTTATCCCAACAAAAA -382
Mz TGTAAAG------------------------------------------- -265
   *** ***

Cx ACAAACATACACACAAGCCATTACAAAAAGATAGCTTCACAAGCATACGA -332
Mz ---------------AGGCATTACAA--AGTTAGCTTCACAAGCGTATGA -232
                  ** ********  ** ************ ** **
                        |<PB2>|
Cx GTTCATTGACAATCCTTGACATGTAAAGTTGGTTCATATATGTGCTGAAA -282
Mz ATTCATTGACAACCCTTGACATGTAAAGTTGATTCATATGTAT-----AA -187
   *********** ****************** ******* * *     **
                                        |<O2a>|
Cx CTGAAAGCAGGAAGCTCGATGATGTACATTCCAAATCCACGTAAAAAGGC -232
Mz --GAAAGCTTAATGATCTATC-TGTACAT-CCAAATCCATGTA------- -148
     ******   * * ** **  ******* ********* ***
                      Beta-1
                |<----------->|
           |<O2b>|
Cx ACTAGTATTTCCACGTCATGCAGTGCAGTATTCCAAAATCATCAGTATTT -182
Mz -CTA-TGTTTCCACGTCATGCAACGCAACATTCCAAAA------------ -112
   *** * ***************  ***  *********
                                  |<TATA>|
Cx GCAGGTGCTGCAGAGAATGCAAGCCATGGATCATCTATAAATAGCT---- -136
Mz ----------------------CCATGGATCATCTATAAATGGCTAGCT  -85
                          ******************* ***

Cx CCCACATATGCACTA--CTCTATCATCAGACTCGCATTCAGAATTCTAGA  -88
Mz CCCACATATGAACTAGTCTCTATCATCC----------AATCC-AGA    -46
   ********** ****  ***********  *          *** * ***

Cx TCAGAGACAACATTTATTAGTGCAGCAAATTAAAGC-------AGTAGAG  -45
Mz TCAGC--------------------------AAAGCGGCAGTGCGTAGAG  -22
   ****                           *****        ******

Cx AGCTAGGATCGTCTAACAGTAACAGTAACAGAACAGCTAATAGCATG +3
Mz ----AGGATCGTC------------GAACAGAAC-------AGCATG +3
       *********             ********       ******
```

Fig. 3. Sequence comparison of β-prolamin promoters from maize and *Coix*. Promoter fragments of ca. 400 bp extending from the ATG to the prolamin-box region of 14 kDa β-zein [8] and 17 kDa β-coixin are aligned. Dashes indicate deletions introduced to maximize the homology comparison. Identical nucleotides are marked by asterisks. The locations of the putative promoter regulatory sequences are indicated above the sequence data: prolamin-box, PB1 and PB2; O2 target sites O2a and O2b; the putative binding site of the beta-1 factor and the TATA box. The translation start initiation codon (+3) is indicated at the 3′ end of the sequence.

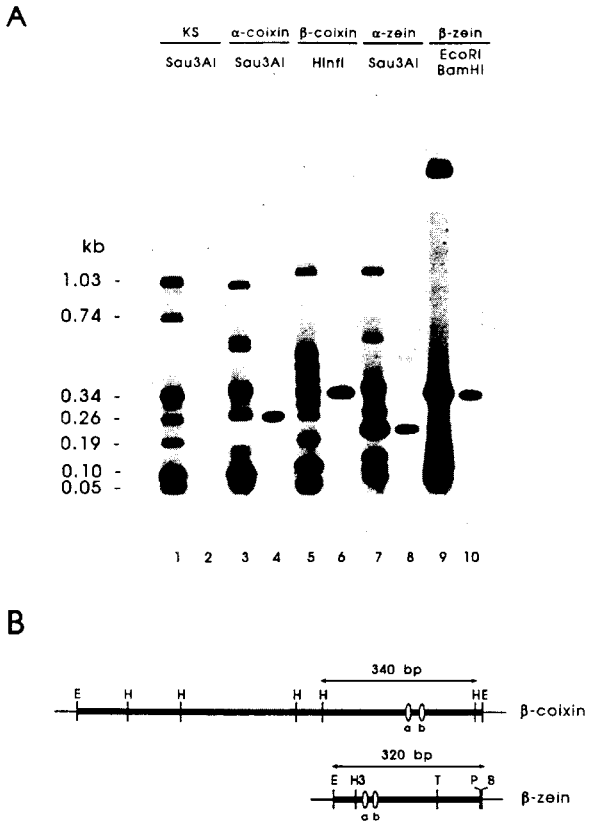

Fig. 4. Selective binding of the maize O2 protein to restriction fragments from maize and *Coix* α- and β-prolamins promoter sequences. A. Plasmids containing promoter sequences from α-coixin, β-coixin, α-zein, β-zein and pBluescript KS$^+$ (KS) were digested with *Sau* 3AI, *Hinf* I, or *Eco* RI-*Bam* HI, α-^{32}P-end-labelled and incubated with the immunoselected β-gal::O2 fusion protein. Odd-numbered lanes show the restriction fragments that were incubated with the fusion protein. Even-numbered lanes show the fragments that were specifically retained by the β-gal::O2 fusion. The lengths of the pBluescript KS$^+$ *Sau*3AI digestion fragments are given at left in kilobases. The α-prolamin promoter fragments were included as a positive control, and further information about them has been reported by Yunes *et al.* [56]. B. Partial restriction map of the promoter region from β-coixin (pβ-λ55.1EE) and β-zein (pβ-ZEP) genes cloned in *Eco* RI and *Eco* RI-*Pst* I pBluescript KS$^+$, respectively. The putative O2 target sites O2a and O2b are represented by ovals. The solid lines above the map indicate the restriction fragment that was bound by the β-gal::O2 fusion. Abbreviations for restriction enzyme sites are as follows: E, *Eco* RI; H, *Hinf* I; P, *Pst* I; B, *Bam* HI; H3, *Hind* III; T, *Taq* I.

The transcriptional activator O2 binds to promoter sequences of β-prolamin genes from maize and Coix

Selective DNA-binding assays using the maize O2 protein in a β-galactosidase fusion (β-gal::O2) were performed in order to verify whether the effect of the *opaque2* mutation on β-zein synthesis is a direct consequence of the interaction between the *opaque2* protein and the β-zein gene promoter. This approach was also used to analyze the β-coixin promoter. As shown in Fig. 4A (lanes 6 and 10), a 340 bp *Hinf* I restriction fragment of the β-coixin promoter and a 320 bp *Eco* RI-*Bam* HI restriction fragment of the β-zein promoter, containing the prolamin-box, the O2a

and O2b O2-like boxes and TATA box, were bound by the O2 protein. *Sau*3AI digestions of the 25 kDa α-coixin and 22 kDa α-zein promoters, included as positive controls in the binding assay, were bound by the O2 protein (Fig. 4A, lanes 4 and 8), as was determined previously [45, 56]. No binding was observed to the restriction fragments from the pBluescript KS^+ vector included as a negative control (Fig. 4A, lane 2). The same results were obtained using either maize or *Coix* β-gal::O2 fusion proteins. For this reason, we present here only the results obtained with the maize β-gal::O2 protein.

Maize and Coix *O2 protein recognizes the same G/C-like-box TCCACGTCAT sequence in the 14 kDa β-zein and 17 kDa β-coixin promoters*

The sites of interaction of β-gal::O2 on the 320 bp β-zein and the 340 bp β-coixin promoter fragments were mapped using DNase I footprinting analysis, as shown in Fig. 5. The assay was performed by using protein A-Sepharose beads containing the immunoselected maize or *Coix* β-gal::O2 fusion, incubated with the β-zein or the β-coixin promoter fragment, respectively. The O2 protein from both species strongly protected the same 5′-TTTCCACGTCATGCA-3′ core sequence, corresponding to the O2b sequence, on the β-prolamin promoter fragments. The underlined sequence comprises a G-box/C-box hybrid ACGT element, which is very similar to the hex-1 element previously reported to interact with O2 on mobility shift assays [18]. Therefore, we refer to the O2-binding site in β-prolamin genes according to this classification.

As shown on Table 1, the O2 target sites in β-prolamin promoters are homologous to the O2 site in the 22 kDa α-zein [45].

Transcriptional activation of the β-prolamin promoters mediated by the O2 protein

To determine whether the binding of the O2 protein to β-prolamin promoters characteristically confers transcription enhancement or represents another type of interaction, we conducted a microprojectile bombardment transient gene expression assay in 3-day old maize coleoptiles using a set of α- and β-prolamin promoter constructs. Such constructs, shown in Fig. 6, consist of reporter plasmids expressing the β-glucuronidase (*GUS*) gene under the control of α-zein (Pα-Z4GUS), α-coixin (Pα-CxGUS), β-zein (Pβ-ZGUS) and β-coixin (Pβ-CxGUS) promoter sequences. The α-prolamin constructs have been analyzed by Yunes *et al.* [56] and were included in this work as positive controls for O2 transactivation. All constructs, except for the β-zein one, harbor ca. 900 bp promoter fragments, which

Table 1. Alignment of the different groups of O2 target sites and flanking sequences.

Box	Gene	DNA sequence	Reference
Group I			
		ACGT	
O2	*22Z-4* (α-zein)	GTCATTCCACGTAGATGAAAA	[45]
O2b	*gZ15A* (β-zein)	ATGTTTCCACGTCATGCAACG	[40]
O2b	*β-55.1* (β-xoixin)	GTATTTCCACGTCATGCAGTG	This study
Group II			
		CATG	
O2c	*α-3B* (α-coixin)	TGACTAGGAGACATGTCATCT	[56]
O2c	*α-3A* (α-coixin)	CGACTAGGAGCCATGTCATCT	[56]
B1*	*b-32*	TCTATATTAGCCATGTCATCA	[30]
B4*	*b-32*	CTACTAATAGTCATGTCATCC	[30]

* The complementary sequence of the reported O2-binding site is shown in order to maximize homology.

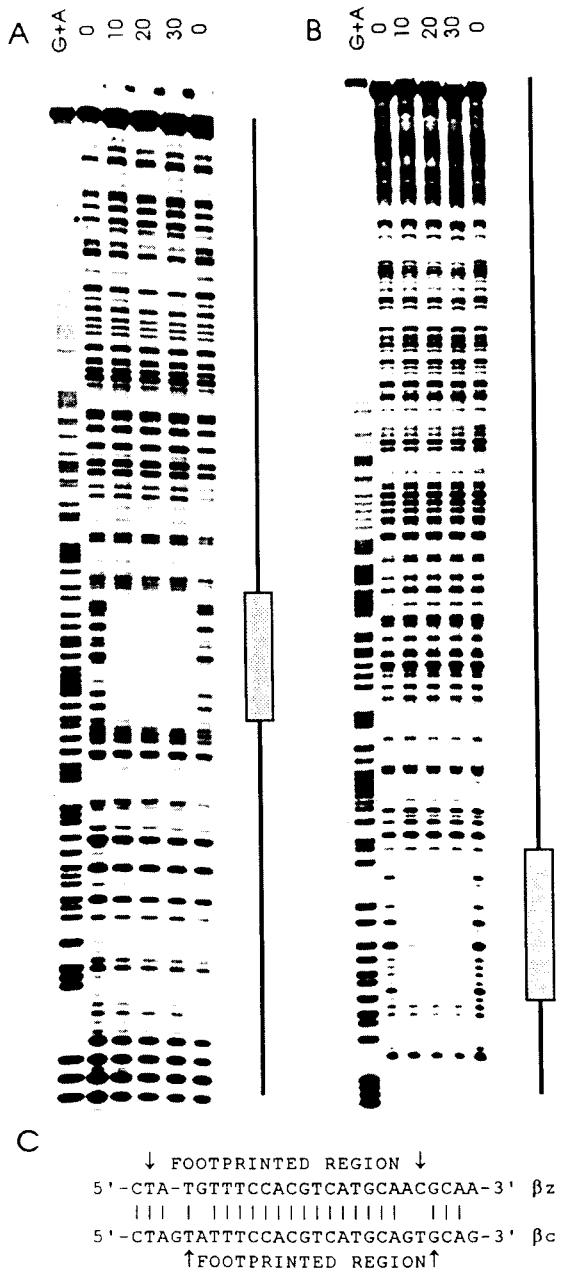

Fig. 5. Footprint *in vitro* of the O2 fusion protein on the β-zein and β-coixin promoters. The 320 bp and 340 bp restriction fragments, from β-zein and β-coixin promoters, respectively, that were retained by the β-gal::O2 fusion in the DNA binding assay were used for the footprint analysis. The lane G + A in A and B is the Maxam and Gilbert sequencing ladders. The labelled β-zein and β-coixin promoter fragments were incubated with DNase I without prior incubation with β-gal::O2 (lane 0) or with increasing amounts of the fusion protein (lanes 10, 20 and 30). A. Footprint analysis performed on the bottom strand of the β-zein promoter fragment using a maize O2::β-gal fusion protein. B. Footprint analysis performed on the top strand of the β-coixin promoter fragment using a *Coix* O2::β-gal fusion protein. The region of footprint is represented by the boxes beside each panel. C. A portion of the sequences spanning the O2 box in β-zein (βZ) and β-coixin (βC) promoters is shown. The protected nucleotides are indicated by the arrows. Vertical bars represent the homology between β-prolamin promoters in this region.

contain all sequence requirements for high expression levels [56]. The β-zein construct harbors a ca. 220 bp promoter fragment spanning from the prolamin-box PB2 to the ATG (Fig. 6). Such shorter promoter versions are sufficient to drive GUS expression and O2 transactivation, as observed for α-zein and α-coixin truncated promoters [56].

As shown in Fig. 7, the GUS activity driven by the β-zein and β-coixin promoters was greatly enhanced upon cotransfection with the pRT101-O2 effector plasmid. Cotransfection with a DNA binding-defective O2 protein, expressed by pRT101-O2NA, did not result in any significant increase in GUS activity. The mutant O2 protein contains a substitution at the invariant asparagine of the DNA-binding domain to alanine that completely abolishes the DNA-binding capacity [1]. This clearly indicates that the expression enhancement depends on the O2 protein binding to the promoter, avoiding the possible occurrence of repressor tritation by dimerization or even a titering effect of the O2 with other protein bound to the promoter.

Discussion

Previous studies have shown that the zein storage proteins genes are developmentally and transcriptionally regulated. Transcriptional regulation seems to be one of the major mechanisms that control seed storage protein deposition, as recently reviewed by Feix and Quayle [10], Gatehouse and Shirsat [14] and Schmidt [47].

The nature of the coordinated expression of the zein genetic system remains unknown. Promoter sequence comparison of the various zein gene

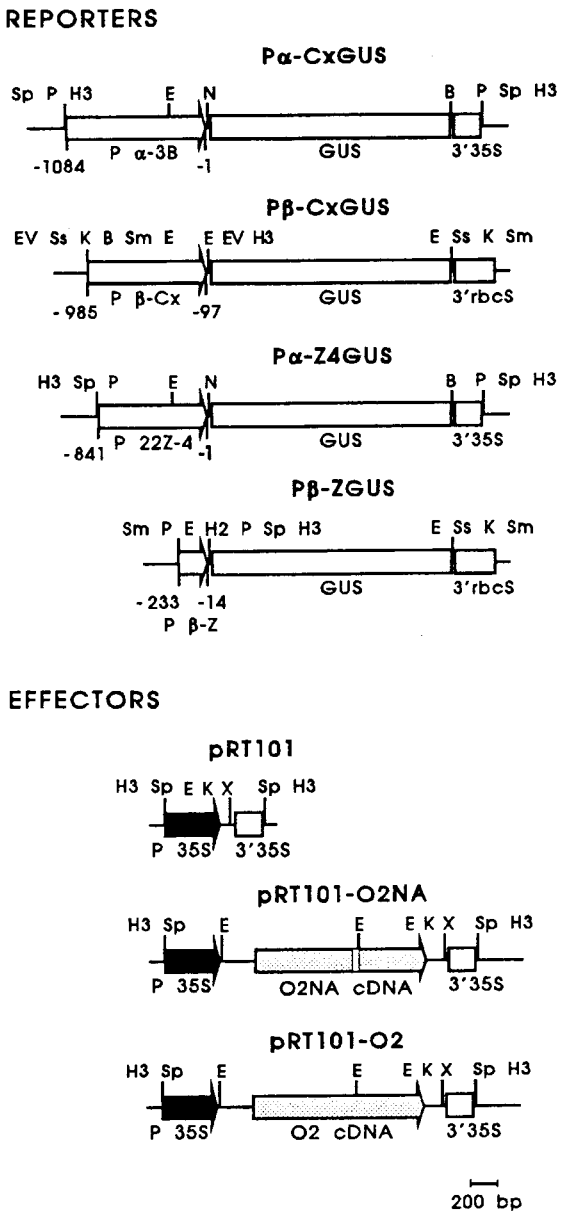

Fig. 6. Schematic representation of chimeric GUS reporter constructs and O2 effector plasmids used to analyze the *in vivo* transactivation of β-prolamin promoters by the maize O2 protein. Distances are relative to the A residue of the initiator ATG codon. Unique restriction site enzymes are: B, *Bam* HI; E, *Eco* RI; E5, *Eco* RV; H2, *Hind* II; H3, *Hind* III; K, *Kpn* I; N, *Nco* I; P, *Pst* I; Sm, *Sma* I; Sp, *Sph* I; Ss, *Sst* I; X, *Xba* I. Pα-CxGUS, Pα-Z4GUS and pRT101-O2 are as described by Yunes *et al.* [56]. The pRT101 cassette has been described by Töpfer *et al.* [52]. Pβ-CxGUS is the GUS + *rbcS* poly(A) cassette (pDMC200) under the control of a 0.9 kb β-coixin promoter. Pβ-ZGUS is the GUS + *rbcS* poly(A) cassette under the control of a 0.2 kb β-zein promoter. The effector plasmid

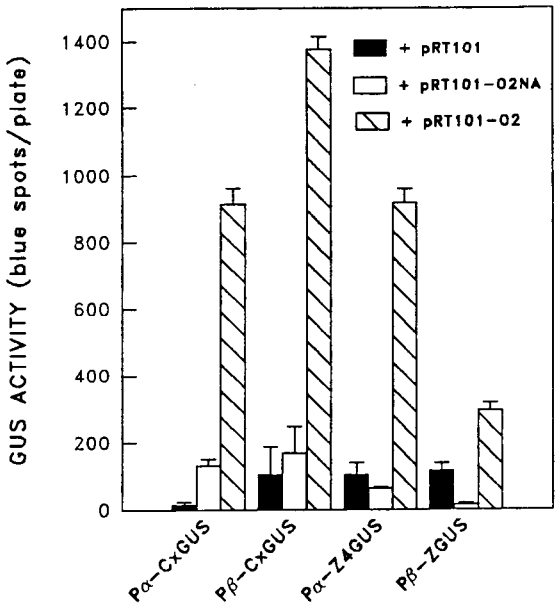

Fig. 7. Transactivation of the 14 kDa β-zein and 17 kDa β-coixin promoters by the O2 protein upon microprojectile bombardment in maize coleoptiles. The bars correspond to activity of the reporter construct upon cotransfection with the plant expression vector pRT101 or its Opaque 2 expressing versions: pRT101-O2NA, expressing a DNA-binding defective O2 protein, and pRT101-O2, expressing the O2 protein from the *O2cDNA1-4* [44], as given in Fig. 6. GUS activity was determined by counting the number of blue spots per bombarded plate. The values represent averages and standard deviations (represented by an error bar) of two independent experiments. Statistical differences between *trans*-activated and non-*trans*-activated samples were significant as determined by analysis of variance and SNK multiple range tests.

classes reveals almost no recognizable homology, except for the common TATA, CATC and the prolamin box (TGTAAAG) [4, 10, 29, 40]. Although present in many other cereal storage protein genes [11, 13, 22, 23, 29, 38], little is known about the role of the prolamin box and the CATC box in the regulation of gene expression.

No common transcriptional activator has been identified for the different zein gene families.

pRT101-O2NA consists of *O2cDNA1-4* mutagenized at the DNA-binding domain under the control of the CaMV 35S promoter and 35S poly(A) signal. The translated sequence of the *O2* cDNA is represented as a shaded arrowhead.

However, some of the several loci affecting zein synthesis may play a coordinated action. In fact, there is genetic and biochemical data indicating that *Opaque 2*, *Opaque 7* and *Floury 2* may interact in the regulation of 19 and 22 kDa α-zein genes [reviewed in 35, 46].

Most of the work involving transcriptional regulation of zein expression has been done on the control of the 22 kDa α-zein class by the O2 protein. On the other hand, specific interactions of other nuclear proteins with promoter DNA fragments of 22, 19 and 14 kDa zein genes have been demonstrated using mobility shift assays [31, 45, 48]. Despite the efforts of several groups to clone such factors, no further information about them has been reported.

The data presented here demonstrate that the transcription factor O2, which primarily regulates the 22 kDa zein class, also interacts with the β-prolamins, to enhance their expression. A new O2-binding box was identified in β-prolamin genes from maize and *Coix* that, together with other boxes previously identified in other endosperm expressed genes, forms a curious collection of O2 *cis* elements. This suggests that the O2 protein participates in the mechanism which controls the coordinated expression of some endosperm genes through its capability to recognize significantly different *cis*-acting elements in the promoters of such genes.

The levels of 14 kDa β-zein are transcriptionally regulated by the O2 protein

The *opaque 2* mutation differentially reduces zein gene transcription. Kodrzycki *et al.* [21] showed that the 22 kDa α-zein class is severely affected, whereas the 19 kDa α-zein and 14 kDa β-zein classes are reduced to a lesser extent. The effects on the α-zein classes have been discussed elsewhere [21, 45, 46], however little attention has been given to the noticeable effects on the β-zein class.

Looking closer, the *o2* mutation causes a reduction of about 80% on 14 kDa β-zein mRNA level in 17 DAP seeds and around 60% in the protein level of mature seeds. This effect was observed in three genetically distinct *o2* inbred lines, W64Ao2, Oh43o2 and Cat100-1o2. In addition, when the *o2* allele from Oh43 was introgressed into the commercial hybrid Agroceres F-352, the same effect on the β-zein class was observed (data not shown).

Unlike the 22 kDa α-zein, we found that the *o2* mutation partially affects the transcription rates and protein level of β-zein (Figs. 1 and 2), indicating that there may be other β-zein-specific transcription factors involved. This partial effect of the *o2* mutation was also observed in the 19 kDa α-zein (Figs. 1 and 2), as it has been previously reported by Marks *et al.* [33] and Kodrzycki *et al.* [21]. Although there is no evidence for O2-binding activity on the 19 kDa α-zein promoters analyzed so far [45], it will be helpful to search for O2-binding activity on as large a number as possible of promoters from this class to clarify this point. Perhaps the idea of a mere pleiotropic effect of the *o2* mutation on the transcription of this class should be redefined based on a model by which O2 controls α- and β-prolamins.

The results presented here clearly show that the transcriptional activator O2 binds to and transactivates β-prolamin promoters from maize and *Coix* (Figs. 4, 5 and 7). The fact that this occurs in both species suggests that this regulation plays an important role in endosperm development.

Interestingly, the O2-binding site in the β-zein promoter is located just upstream from the putative interaction site of the beta-1 protein factor identified by So and Larkins [48]. The last five nucleotides of the O2-binding site core (Figs. 3 and 5) overlap with the 5′ portion of the region represented by the 22 bp oligonucleotide that was used in gel retardation assays and specifically shifted by the beta-1 factor [48]. Since beta-1 is coordinately expressed with β-zein accumulation, it is likely that this factor may regulate transcription of the β-zein gene. The proximity of this putative beta-1 *cis* element to the O2-binding site suggests that these two proteins may interact and cooperatively regulate β-zein expression. The nature of such an interaction remains unknown. The

presence of a beta-1-like factor in *Coix* is also likely, as indicated by the homology found in this region in the β-coixin promoter compared to the β-zein one. In addition, the GCAAC direct repeats carried by the putative beta-1 target site in the β-zein promoter, as pointed out by So and Larkins [48], have their GCAGT counterpart in the β-coixin promoter (Fig. 3).

The transcriptional activation of the β-prolamin promoters mediated by O2 was further confirmed *in vivo* by transactivation assays using the GUS system. GUS expression driven from reporter plasmids under the control of β-prolamin promoter sequences was greatly enhanced upon cotransformation with an O2 effector plasmid (Figs. 6 and 7). The dependence upon the binding of O2 to the promoter to mediate transactivation was further confirmed by the use of a DNA binding defective O2 protein (pRT101-O2NA in Figs. 6 and 7). In addition, the β-coixin promoter is also transactivated by the O2 protein in tobacco mesophyll protoplasts (data not shown).

Finally, the participation of the O2 protein in the regulation of different zein classes suggest that this transcriptional activator plays a more general role on the coordinated expression of zein genes.

Adding a new sequence to the collection of O2 cis *elements*

The first report on O2-binding sites has been presented by Lohmer *et al.* [30]. These authors have shown that the O2 protein is capable of *trans*-activating *b*-32 gene transcription *in vivo* by interacting with five putative binding sites containing the consensus sequence GATGAPyPuTGPu. Later on, Schmidt *et al.* [45] found that O2 interacts with a quite different target sequence (TCCACGTAGA) in the 22 kDa α-zein promoter. At the time, the discrepancies between the results obtained by the two different groups were attributed to the experimental conditions used. Recently, using the same experimental procedures and conditions described by Schmidt *et al.* [45], we have reported that O2 binds to and *trans*-activates the 22 kDa-like α-coixin gene through interaction with another different target site (GACATGTC) [56]. This sequence shares more homology with the B1 and B4 O2-binding sites of the *b*-32 gene than with that of the 22 kDa α-zein gene.

As shown in Table 1, the O2 target sites (TCCACGTCAT) identified in β-prolamin genes are identical in maize and *Coix* and very similar to that found in the 22 kDa α-zein gene [45]. Except for the ACGT core, the protected region does not encompass any palindromic sequence, similar to the sites described by Lohmer *et al.* [30]. Such asymmetric elements definitively do not represent any obstacle for the O2-binding activity [18].

We found it difficult to draw a consensus sequence through the alignment of all O2 target sites identified so far. In order to systematize these completely different sequences, they were grouped according to the central ACGT or CATG symmetric core (Table 1). The O2-boxes in β-prolamin genes also contain a CATG sequence 3′-adjacent to the ACGT core. However, we have grouped them among the ACGT elements for two reasons: (1) the presence of the perfect hybrid G/C box and (2) the CATG sequence is not in the same context as the CATG core from the other O2 boxes.

Group I of O2 target sites, bearing the ACGT core, clearly represents *cis*-acting sequences that can be classified according to the degree of affinity for interaction with plant bZIP proteins [18]. According to this classification, the β-prolamin O2 target sites comprise 5′ G-box and 3′ C-box half-sites (hybrid G/C box). The 22 kDa α-zein O2 target site has been classified as a hybrid G/A box. Both types of boxes represent high-affinity O2 interaction sites [18]. On the other hand, the Group II of O2 target sites, bearing the CATG core, remains uncharacterized. These sites, however, are undoubtedly bound by O2 and have functional relevance [30, 56].

The β-gal::O2 fusion protein did not bind to the 5′-TCCACGTAAA-3′ O2a sequence located 20 bp upstream from the O2 target site (O2b) in the β-coixin promoter (Figs. 3 and 5). This sequence differs from the 22 kDa α-zein O2 target site by only one G-to-A substitution at the second

nucleotide of the 3′ end. This observation seems controversial, since the sequence can be classified as a hybrid G/A-box ACGT element, the same group of the 22 kDa α-zein TCCACGTAGA O2 box, according to Izawa *et al.* [18]. It should be pointed out that the neighboring sequences around this β-coixin hybrid G/A-box element do not bear any similarity with the weakly protected nucleotides around the core sequence strongly protected by O2 (Fig. 3). This suggests that the flanking sequences may have some importance in a sense that they provide the context in which the O2 target site is inserted.

The great variability and the lack of a conserved sequence pattern of the target sites in the two groups suggest that the binding specificity of the O2 protein cannot be explained just through the hydrogen bonding and van der Waals interactions of DNA base pairs with the protein side chains. Thus, the ability of a particular sequence to undergo an 'Opaque 2 recognizable' structural conformation may reflect (1) a sequence-dependent capacity of the DNA to distort upon protein binding or (2) a pre-organization of the DNA into a distorted structure recognized by the O2 protein, as it is currently in discussion for the recent models on DNA-protein interaction [39, 50].

Taking together, these variable O2-binding sites found in several different genes (Table 1) represent a clear evidence that the O2 protein supports a relatively high degree of degeneracy at the nucleotides flanking the ACGT core, as discussed previously by Izawa *et al.* [18], or, even this core sequence can be replaced by the CATG one [56]. This may present the means by which a promoter selectively interacts with homo- or heterodimers of the O2 protein to drive specific expression patterns. The above lead us to propose that the O2 protein participates in a more general mechanism that controls the coordinated expression of different classes of genes, as shown for *b*-32, 22 kDa α-zein and 14 kDa β-zein.

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The Role of Opaque2 on the Control of Lysine Degrading Activities in Developing Maize Endosperm

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ABSTRACT

We have isolated a cDNA clone, designated *ZLYS1-9*, encoding the bifunctional enzyme lysine-ketoglutarate reductase-saccharopine dehydrogenase (LKR/SDH). The predicted polypeptide has an N-terminal LKR domain and a C-terminal SDH domain which are similar to the yeast LYS1 and LYS9 monofunctional proteins, respectively. The developmental expression of *ZLYS1-9* was examined in normal and *opaque2* (*o2*) mutant seeds. The maize LKR/SDH is located in the cytoplasm of aleurone and subaleurone endosperm cell layers. Transcripts, polypeptides, as well as, enzyme activities showed an up and down regulation during endosperm development. In the mutant endosperm, mRNA levels were reduced in more than 90% with a concomitant reduction on polypeptide levels and LKR/SDH activity. These results suggest that lysine levels in the endosperm are likely to be controlled at the transcriptional level by the Opaque2 transcription factor.

INTRODUCTION

Since the discovery of the high lysine *opaque2* (*o2*) maize mutant (Mertz et al., 1964), much attention has been given toward the understanding of the biochemical and molecular mechanisms leading to the increase in lysine content in the endosperm. Studies carried out during the past 30 years revealed that the homozygous *o2* mutation causes a ca. 70% reduction in zein content, also affecting the contents of a number of proteins and enzymes related to nitrogen and sugar metabolism in maize endosperm (Habben et al., 1993; Giroux et al., 1994; Gallusci et al., 1996). The cloning of the *O2* gene revealed that it encodes a bZIP transcription factor involved in the transcriptional control of the zein genes, the b-32 ribosome-inactivating protein and the cytoplasmic pyruvate orthophosphate dikinase (cyPPDK), an enzyme involved in carbon partitioning (Lohmer et al., 1991; Schmidt et al., 1992; Bass et al., 1992; Cord Neto et al., 1995; Gallusci et al., 1996). Lysine catabolism is also affected in the *o2* mutant. ^{14}C-lysine feeding experiments revealed that lysine was converted to glutamic acid and proline to a much lesser extent in *o2* than in normal endosperm (Sodek and Wilson, 1970).

Despite the observation of extensive lysine degradation in maize seeds, there were several attempts to establish positive selection strategies in order to isolate mutants having aspartate kinase (AK) insensitive to feedback inhibition caused by lysine. AK is the first regulatory enzyme of the aspartate pathway that leads to the biosynthesis of lysine, methionine, threonine and isoleucine. Thus, by derepressing AK one would expect an overproduction of these amino acids (for review see Azevedo et al., 1997). Several AK insensitive mutants overproduced threonine but had only marginal effects on lysine accumulation in barley (Bright et al., 1982), maize (Diedrick et al., 1990), and tobacco (Frankard et al., 1992). The absence of lysine overproduction in these mutants was attributed to the second regulatory enzyme, dihydrodipicolinate synthase (DHDPS), which is the first enzyme of the lysine branch of the aspartate pathway and it is much more sensitive to lysine feedback inhibition than the AK (for review see Azevedo et al., 1997). Few experiments succeeded in isolating DHDPS insensitive mutants, but they did not show significant lysine increase in the seeds (Negrutiu et al., 1984). Also, AK/DHDPS insensitive double mutants did not result in a significant increased lysine content (Frankard et al., 1991). Recently, transgenic tobacco and canola plants expressing lysine insensitive AK or DHDPS or both enzymes were produced. These transgenic plants showed threonine overproduction and appreciable free lysine increments in young leaves and seeds. These plants, however, displayed severe phenotypic alterations (Shaul and Galili, 1993; Karchi et al., 1993; Falco et al., 1995; Pederson et al., 1996) and lysine overproduction was followed by increased lysine degradation (Karchi et al., 1994; Falco et al., 1995).

We have postulated that lysine catabolism is an important mechanism for the control of free

lysine levels in maize endosperm cells (Arruda and Silva, 1979; Silva and Arruda,1979). Lysine degradation in maize endosperm is catalyzed by lysine-ketoglutarate reductase (LKR) (Arruda et al., 1982). During kernel development, LKR activity is coordinated with nitrogen input and zein synthesis, and it is probably involved in maintaining free lysine at low levels (Arruda and Silva, 1983). Further analysis of the developmental pattern of LKR activity in normal and *o2* endosperms showed that the enzyme activity is decreased 2- to 3-fold in the mutant, suggesting that the reduced lysine degradation in the *o2* endosperm could be due to a lower content of LKR polypeptides (Brochetto-Braga et al. 1992).

The maize LKR was purified to homogeneity and demonstrated to be associated with saccharopine dehidrogenase (SDH) in a bifunctional 125 kD polypeptide (Gonçalves-Butruille et al., 1996), similarly to the previously isolated mammalian enzyme (Markovitz and Chuang, 1987). Recently, complete genomic and cDNA sequences have been reported for the Arabidopsis LKR/SDH gene (Epelbaum et al., 1997; Tang et al., 1997).

To further study the influence of the *o2* mutation on lysine accumulation in the developing maize endosperm, we have cloned the maize bifunctional LKR/SDH cDNA and examined its spatial and temporal patterns of expression. We found that the maize LKR/SDH is a cytosolic enzyme encoded by a single gene which is highly expressed in the aleurone and subaleurone cell layers of the distal part of the developing endosperm, presenting a temporal up and down regulatory pattern. In the *o2* mutant, LKR/SDH mRNA and protein levels are severely reduced and the expression pattern during kernel development is markedly modified.

RESULTS

Isolation of a cDNA Encoding the Maize LKR/SDH Bifunctional Enzyme

LKR/SDH was purified to homogeneity from 17 days after pollination (DAP) maize endosperms and the amino-terminal sequences of four tryptic peptides were used to design a set of 21-23-mer degenerate oligonucleotides. These primers and the cDNA resulting from reverse transcribed 17 DAP total endosperm RNA were used in RT- PCR reactions. A 1.2 kb DNA fragment was amplified and confirmed to be part of the LKR/SDH gene through sequence comparison to the yeast *Lys1* (LKR) and *Lys9* (SDH) genes. This fragment was used as a probe to screen an immature endosperm cDNA library (Aukerman et al., 1991). The nucleotide sequences from twenty positive clones were analyzed, but they either contained mostly the SDH domain or were chimeric at their 5′-end. In order to obtain a full-length clone, the 5′-end of the cDNA was isolated using RACE (rapid amplification of cDNA ends) and a 3.5 kb cDNA was further amplified using primers annealing to the 5' and 3' untranslated regions. The maize gene was designated

ZLYS1-9 for *Zea mays Lys1* and *Lys9* homologs.

As shown in Figure 1A, the amplified cDNA encodes an open reading frame (ORF) of 1056 amino acids, which predicts a protein of 116.5 kD, close to the maize 125 kD polypeptide observed in SDS-PAGE (Gonçalves-Butruille et al., 1996). The 5' untranslated region is 178 bp long and presents two in frame ATG codons that are 33 nucleotides apart, but only the downstream one is in a favorable context for translation initiation (Luehrsen and Walbot, 1994). The 3' untranslated region is 148 bp long and has two putative polyadenilation signals (AATAAA) located 105 and 114 bp downstream from the TAG stop codon (data not shown).

Figure 2 shows a schematic representation of similarities between the predicted maize polypeptide and other known mono- and bifunctional LKR/SDH enzymes. The bifunctional maize enzyme is constituted by a SDH C-terminal domain sharing 42% similarity to the yeast LYS9 protein, and an N-terminal domain sharing 27% similarity to the yeast LYS1 protein. Similarities to the related mouse (Papes, F., Cord Neto, G., Kemper, E.L. and Arruda, P., unpublished results), *C. elegans* and Arabidopsis proteins are significantly higher (44%; 46% and 72%, respectively). The interdomain region (Figure 1A), which is about 106 residues long in maize, is 57% identical to the corresponding Arabidopsis sequence. Interestingly, this region is very short (10-15 residues long) in *C. elegans* and mouse (Figure 2).

Comparisons of primary protein structures also reveal several conserved motifs (Figure 2). The initiation ATG and stop codons, as well as some motifs with high degree of similarity, appear at comparable sites in the open reading frames. Residues H-110, K-113 and R-146 of the maize LKR domain (Figure 1B) are conserved in all sequences. These residues were shown to be essential for substrate binding in the LYS1 protein (Ogawa et al., 1979; Fujioka et al., 1980; Ogawa and Fujioka, 1980; Fujioka and Takata; 1981).

ZLYS1-9 is Present in Maize and Related Species

We investigated the presence of the *ZLYS1-9* gene in maize and related cereals. The 1.2 kb RT-PCR DNA fragment, comprising part of the LKR domain, the inter-domain region and part of the SDH domain (residues 444 through 849, Figure 1), was hybridized to genomic DNA from maize, Coix and sorghum. As shown in Figure 3, both Bam HI and Eco RV digests gave single strong hybridizing bands, suggesting that *ZLYS1-9* is likely to be present as a single copy gene in the *Andropogoneae* species.

Analysis of *ZLYS1-9* mRNA Levels in Different Maize Tissues

Total RNA extracted from roots, leaves, coleoptiles, embryos and endosperms were hybridized to either LKR or SDH specific probes. As shown in Figure 4, *ZLYS1-9* mRNA was mainly detected in the endosperm. No signal was detected in embryos and only marginal levels of expression were detected in roots, leaves and coleoptiles. Five different transcripts were detected in 20 DAP developing endosperm with both probes. Along with the major 3.5 kb band, which corresponds to more than 90% of the total hybridizing mRNA, alternate transcripts with 4.1, 2.6, 2.4 and 1.5 kb were detected. The 4.1 and 3.5 kb transcripts were equally detected with both probes. The 2.4 and 2.6 kb species seem to contain mainly SDH sequences (Figure 4B), as they weakly hybridized to the LKR probe (Figure 4A), whereas the 1.5 kb transcript seems to contain only LKR sequences (Figure 4A).

Developmental Expression of *ZLYS1-9* in Normal and *opaque2* Mutant Endosperm

We showed previously that the LKR activity during endosperm development is reduced 2-3-fold in the *o2* endosperm (Brochetto-Braga et al., 1992). To test whether this lower enzyme activity was due to a reduced expression of the *ZLYS1-9* gene, normal and *opaque2* seeds were harvested at 10, 15, 20, 25, 30 and 35 DAP, and assayed for enzyme activities and analyzed by RNA gel blot and immunoblotting using anti-LKR/SDH polyclonal antibodies (Kemper et al., 1998).

As shown in Figure 5, transcripts, polypeptides, as well as enzyme activities showed an up and down regulation during endosperm development. In normal endosperm, LKR and SDH activities, *ZLYS1-9* mRNA and LKR/SDH protein levels reached a maximum at 20 DAP, whereas, in the mutant endosperm, maximum transcript, polypeptide levels and enzyme activities were observed at 25 DAP.

At 20 DAP, LKR and SDH activities in the mutant were reduced, respectively, to 15% and 30% of the normal seeds (Figure 5A). This was accompanied by a 90% reduction on the transcript and polypeptide levels (Figures 5B and 5C). At 25 DAP, the levels of mRNA and polypeptide in the mutant were similar to the wild-type, however, a concomitant increase in enzyme activities was not observed.

LKR/SDH is Localized in the Cytosol of the Aleurone and Subaleurone Cell Layers

To localize the LKR/SDH activity within the endosperm, protein extracts from different endosperm regions, embryo and pedicel (Figure 6A) were analyzed by western blot using an anti-maize LKR/SDH polyclonal antibodies. The 125 kD LKR/SDH polypeptide was only detected in the peripheral distal parts of the endosperm, comprising the aleurone and sub-aleurone cell layers (Figure 6B). The localization of LKR/SDH activity in these regions was further confirmed by

histochemical staining for SDH activity (Figure 7).

Subcellular fractionation was used to determine the cell compartmentalization of LKR/SDH. As shown in Figure 8, LKR and SDH activities were only detected in the cytosol, while the activities of the marker enzymes, cytochrome C oxidase and cytochrome C reductase, were observed in the endoplasmatic reticulum and mitochondria, respectively.

DISCUSSION

The biochemical and molecular mechanisms by which the *o2* mutation increases lysine content in the endosperm have been investigated during the past 30 years. Both lysine synthesis and degradation, as well as its incorporation into lysine-rich proteins, should contribute for the final content of this amino acid in the seed (Sodek, 1976; Arruda and Silva, 1979; Habben et al., 1993; Azevedo et al., 1997). This report describes the cloning of a maize cDNA encoding the lysine degrading enzyme LKR/SDH and presents evidences on how lysine levels may be controlled in the endosperm. A conspicuous correlation between lysine degrading activities and transcriptional regulation by the O2 factor was observed.

The Bifunctional Maize LKR/SDH Enzyme

A cDNA encoding the bifunctional enzyme LKR/SDH was isolated from the immature maize endosperm mRNA pool. The cDNA predicts a 117 kD protein (Figure 1) bearing distinct N- and C-terminal domains identified respectively as LKR and SDH owing to similarities to yeast monofunctional enzymes (saccharopine dehydrogenase lysine- and glutamate-forming enzymes, encoded by the *Lys1* and *Lys9* genes, respectively). Further confirmation of cDNA identity was obtained by comparison to similar genes recently isolated from Arabidopsis (Epelbaum et al., 1997; Tang et al., 1997) and mouse (Papes, F., Cord Neto, G., Kemper, E.L. and Arruda, P., unpublished results).

In plants and mammals, the saccharopine pathway is utilized for lysine catabolism whereas in yeast and other fungi this pathway is used in the reverse order for lysine biosynthesis. Either bifunctional (maize, soybean, Arabidopsis, bovine, murine and human) or monofunctional (yeast, rat and Arabidopsis) polypeptides operate in this pathway. As illustrated in Figure 2, the amino acid sequence from these enzymes share a high degree of similarity. Nevertheless, there are marked differences between bifunctional LKR/SDH polypeptides from plants and animals.

First, the enzyme seems to be expressed in different cell compartments. The rat LKR and SDH enzymes have been located in the mitochondrial matrix (Blemings et al., 1994) and other mammalian enzymes have been extracted from mitochondrial fractions (Markovitz et al., 1984; Ameen et al., 1987). On the other hand, the maize enzyme is located in the cytosol (Figure 8), what is consistent with the observation that neither the predicted maize LKR/SDH (Figure 1) nor the Arabidopsis protein (Epelbaum et al., 1997) contain putative mitochondrial or chloroplast targeting sequences.

Second, the interdomain region is present in plants but absent from the animal enzymes (Figure 2). This region is 57 % identical in maize and Arabidopsis, but its functional role is still unknown. Limited proteolysis studies with the maize LKR/SDH suggest that it might be involved in the modulation of the LKR activity (Kemper et al, 1998). Other evidences for the role of the interdomain region on the modulation of LKR came from studies of the effect of Ca^{2+} and ionic strength on the maize and bovine enzymes. The maize LKR is activated by Ca^{2+} and high salt concentration, while the bovine is insensitive to these modulators (Kemper et al, 1998).

Tissue Specific and Developmental Expression of *ZLYS1-9*

The RNA gel blot analysis of total RNA 20 DAP endosperm revealed multiple hibridizing bands ranging in size from 1.5 to 4,1 kb (Figure 4). The 3.5 kb transcript, which accounts for more than 90% of total *ZLYS1-9* transcribed mRNA, has the expected size of the isolated cDNA shown in Figure 1. The alternate transcripts may originate from a single gene and whether they have any functional relevance is still unknown.

Tang et al. (1997) have shown that Arabidopsis cells contain mRNA species encoding bifunctional LKR/SDH and monofunctional SDH, likely to be transcribed from a single gene. In maize, however, the sole protein product ever detected corresponds to a single protein band of 125 kD exhibiting both LKR and SDH activities (Brochetto-Braga et al., 1992; Gonçalves-Butruille et al., 1996; Kemper et al., 1998). In addition, our immunoblot results (Figures 5, 6 and 9) clearly show the presence of a single 125 kD polypeptide in the maize endosperm cells. The antibodies raised against the bifunctional LKR/SDH protein are capable to detect SDH, as the antiserum recognizes proteolytic separated LKR and SDH domains with similar efficacy, as shown in Figure 9.

Detection of LKR and SDH activities in plants was reported only in developing seeds (Arruda and da Silva, 1983; Karchi et al., 1994, Gaziola et al., 1997) and Arabidopsis cell suspension cultures (Tang et al., 1997). Although the LKR/SDH transcripts were detected in several organs in Arabidopsis, they were most abundant in the ovary and the embryo (Tang et al., 1997). In maize LKR/SDH mRNA is abundantly found in the endosperm but is completely absent in the embryo and scarcely detected in roots, leaves and coleoptiles (Figure 4). Enzymatic activity, indeed, was

not detected in those organs. Furthermore, endosperm activity is about 30-fold higher in maize than in soybean and common bean (data not shown), suggesting that LKR/SDH may play an important physiological role in maize endosperm.

Histochemical and immunological assays revealed that LKR/SDH is expressed in the outer endosperm cell layers (Figures 6 and 7). This region comprises cells that are actively dividing and expressing zein genes at high rates (Dolfini et al., 1992). These cells probably have a low lysine requirement because zeins, which represent more than 70% of total endosperm protein, are devoid of lysine residues. Thus, we suggest that LKR/SDH activity is important to regulate lysine levels in this part of the endosperm. This is in accordance with the idea of a coordinated gene expression of zeins and *ZLYS1-9* genes. On the other hand, some product rising from lysine degradation could regulate cellular and developmental processes operating in these peripheral endosperm cells. Interestingly, it have been suggested that lysine degradation may have influence on the growth of mammalian brain, since LKR is highly active during embryonic rat brain development (Rao et al., 1992).

Effects of the *o2* Mutation on *ZLYS1-9* mRNA Levels

Altered lysine catabolism (Sodek and Wilson, 1970; Arruda and Silva, 1979) may be one of the mechanisms by which the *opaque2* mutation creates a high lysine maize phenotype. Most of the lysine in the endosperm seems to originate from lysine-rich proteins, which are more abundant in *o2* than in normal endosperm (Habben et al., 1993). Synthesis of lysine-rich proteins precludes the existence of increased availability of free lysine to be incorporated. We observed increased contents of lysine-rich proteins in the double mutant *o2o2Ask1Ask1*. This was attributed to increased lysine availability due to the aspartate kinase less sensitive to feedback inhibition by lysine encoded by Ask1 and to reduced lysine degradation determined by *o2* (Azevedo et al., 1990).

We have previously reported that the level of LKR activity in the *o2* endosperm is reduced in comparison to the wild type (Brochetto-Braga et al., 1992). In the present work, by calculating LKR and SDH activities per amount of endosperm we found a 6-fold reduction of LKR and 4-fold reduction of SDH activities in the mutant endosperm (Figure 5A). Between 15 and 20 DAP, this decrease correlates well with reduced levels of both LKR/SDH polypeptide (Fig. 5B) and *ZLYS1-9* transcribed mRNA (Fig. 5C). Otherwise, at 25 DAP, mRNA and protein accumulate to levels comparable to the normal endosperm. LKR and SDH activities in *o2* endosperm, however, do not follow this increment. We were not able to readly explain the more pronounced decrease in LKR in relation to the SDH activity, and why these activities did not reach the wild-type levels at 25 DAP in

the *o2* endosperm. It is possible that *o2* presents a differential LKR modulation in relation to the normal endosperm. In soybean, lysine modulates the response of LKR to dephosphorilation *in vitro* (Miron et al., 1997). We hypotesize whether high lysine levels in the *o2* endosperm operate likewise, changing the phosphorilation/dephosphorilation status of the LKR domain.

At 20 DAP, the *o2* mutation causes a 90% reduction in *ZLYS1-9* steady state mRNA levels. This effect was observed in two near isogenic mutant lines derived from alleles *Oh43o2* (Figure 5) and *W64Ao2* (data not shown). The severe reduction caused by the *o2* mutation on *ZLYS1-9* mRNA accumulation is comparable to the reduction observed for α-zein in the same genetic background (Cord Neto et al., 1995). The O2 protein is a transcription activator of the basic domain/leucine-zipper family that is specifically expressed in the endosperm (Lohmer et al., 1991; Gallusci et al., 1994). The O2 recognizes different binding sites in the promoters of several endosperm-specific genes (Lohmer et al., 1991; Schmidt et al., 1992; Cord Neto et al., 1995; Gallusci et al., 1996). Recent evidences suggest that O2 is involved in the coordinated regulation of protein synthesis, nitrogen and sugar metabolisms during seed development (Giroux et al., 1994; Gallusci et al., 1996). Therefore, the involvement of O2 on the transcriptional regulation of lysine catabolism reinforce its role on the control of nitrogen metabolism in the seed.

In yeast, the GCN4 factor is one of the major determinants of the regulatory system termed general control of nitrogen (GCN). In this system, the synthesis of lysine and many other amino acids is coordinately regulated by nutritional conditions (Hinnebusch, 1988). In Arabidopsis there are evidences for cross-pathway regulation of metabolic gene expression leading to amino acid biosynthesis activation or repression (Guyer et al., 1995). In addition, it has been demonstrated that the GCN4-like box may be a key element in regulating the response of a barley storage protein to nitrogen (Muller and Knudsen, 1993). Also, the prolamin composition of maize seeds is dictated by nitrogen status (Singletary et al., 1990). The effects of the *o2* mutation on enzymes participating in amino acid metabolism and carbon partitioning, coupled with structural (Lohmer et al., 1993) and functional (Mauri et al., 1993) similarities between O2 and the yeast GCN4 factor, suggest the O2 protein may be involved in this general amino acid control in the maize endosperm.

METHODS

Plant Material

The commercial maize (Zea Mays L.) hybrid F-352 from Agroceres S.A (Brazil) or near isogenic lines for the *Oh43o2* and *W64Ao2* alleles introgressed into F-352, were used. Oh43*o2* is a null *o2* transcript mutant and W64A*o2* encodes a truncated form of the O2 protein (Bernard et al., 1994). Plants were grown in the field, self-pollinated and the ears were harvested at either 17 DAP or distinct developmental stages and stored frozen at -70°C. Four-day old seedlings, growing in vermiculite under controlled conditions, were used to collect roots, leaves and coleoptiles for the isolation of total RNA.

Enzyme Purification

LKR/SDH was purified from 17 DAP F-352 endosperms as described by Gonçalves-Butruille et al. (1996), with minor modifications. All steps were performed at 4°C. Tissues were homogenized in buffer A (25 mM sodium phosphate, pH 7.4, 1 mM DTT, 1 mM EDTA and 5 mM benzamidine), centrifuged at 20,000g for 10 min and the supernatant was brought to pH 5.5 by addition of solid NaH_2PO_4. PEG 8000 at 50% (w/v) was added to the homogenate to obtain a final concentration of 7.5%. The mixture was centrifuged at 20,000g for 10 min and the supernatant was brought to a 15% (w/v) PEG concentration and centrifuged again at 20,000g for 10 min. The pellet was resuspended in buffer B (50 mM Tris-HCl, pH 8.5, 1 mM DTT and 1 mM EDTA) and dialyzed overnight against the same buffer. The dialyzed sample was applied to a DEAE-Sepharose column (2.5 x 40 cm) previously equilibrated with buffer B. The enzyme was eluted from the column with a linear gradient from 0 to 0.5 M NaCl in buffer B. Fractions containing enzyme activity were combined, brought to 70% saturation with solid ammonium sulfate, and centrifuged at 20,000g for 10 min The pellet was resuspended in buffer B, dialyzed against the same buffer, and applied to a Protein-Pak Q 8HR (Waters) column. The enzyme was eluted from the column with the linear gradient described above. Fractions containing enzyme activity were combined, brought to 70% saturation with solid ammonium sulfate, and centrifuged at 20,000g for 10 min The pellet was resuspended in buffer B and applied to a Superdex 200 HR (Pharmacia) column previously equilibrated with buffer C (buffer B containing 0.3 M NaCl). The enzyme was eluted from the Superdex column with buffer C and stored at -70°C.

N-terminal Protein Sequencing

The purified LKR/SDH was separated in a 7% SDS-PAGE. After electrophoresis the gel was stained with Coomassie Blue and sent to the protein sequencing facility of the Weizmann Institute of Science (Rehovot, Israel). The protein band was eluted from the gel, digested with trypsin and the major peaks were sequenced. Four internal peptide sequences were obtained: (a)

GLIDFLHGL, (b) RYEGFSEIMVTLS, (c) RLTPLYEYI and (d) RELPAFALEHLPNR.

cDNA Cloning

Cloning of a full length maize LKR/SDH cDNA was completed by means of a combination of three procedures: **(A)** RT-PCR, **(B)** cDNA library screening and **(C)** 5'-RACE. **(A)** After protein sequencing, a set of degenerate oligonucleotides were synthesized based on the tryptic peptide sequences (above) and used in RT-PCR experiments. One μg of total RNA extracted from 17 DAP endosperm was used for reverse transcription reaction, according to the RT-PCR kit (Stratagene). A cDNA fragment (1.2 kb) was amplified by subsequent PCR reactions using primers derived from peptide sequence b and c (above) and confirmed to be part of the maize LKR/SDH gene by sequence comparison to the yeast *Lys1* and *Lys9* genes. This fragment was then used as a probe to **(B)** screen a maize endosperm cDNA library constructed from RNA extracted from 25 DAP seeds of the R-802 inbreed line (Aukerman et al., 1991). Twenty clones were isolated and sequenced, but all of them were incomplete. The 5' cDNA end was cloned by **(C)** RACE using the system for rapid amplification of cDNA ends (Gibco-BRL), according to the supplier's instructions using primers designed on the basis of the available cDNA sequences.

The full-length maize *ZLYS1-9* cDNA clone was finally isolated from F-352 total RNA extracted from 17 DAP endosperms. Primers annealing to the 5'- and 3' cDNA untranslated regions were used to obtain a 3.5 kb complete cDNA in RT-PCR reactions catalized by pfu polymerase (Stratagene). This fragment was subsequently cloned into pBluescript KS and sequenced in an automatic DNA sequencer (ABI 377, Perkin Elmer).

Southern Blot

Genomic DNA was extracted from maize, Coix and sorghum seedlings as described by Rivin et al. (1982). Ten μg of genomic DNA were digested to completion with Bam HI or Eco RV. The digests were ethanol precipitated and loaded onto a 0.7% agarose gel, blotted onto a nylon membrane (Hybond-N, Amersham) and hybridized to the ^{32}P-labeled (Megaprime DNA labeling system, Amersham) 1.2 kb RT-PCR fragment. Hybridization was carried out at 65 °C in SSPE buffer (5X SSPE, 5X Denhardt's and 0.5% SDS) and washes were performed twice at room temperature in 2X SSPE, 0.1% SDS solution for 20 min and repeated twice at 65 °C in 0.1X SSPE, 0.1% SDS. Autoradiography was performed at -70°C for 72 hours using intensifying screens.

Total RNA Isolation and RNA Gel Blot Analysis

Total RNA used in both reverse transcription reactions or RNA gel blot analysis was extracted from developing seeds and seedling tissues according to the procedures described by Prescott and Martin (1987). For RNA gel blot experiments, 20 μg of total RNA samples were electrophoresed in 1.0 or 1.5 % agarose-formaldehyde gel (0.66M formaldehyde, 50 mM MOPS). After electrophoresis, gels were stained with ethidium bromide for loading control. The RNA was then transferred from the gel onto a nylon membrane (Hybond-N^+, Amersham). The probes utilized were either the 1.2 kb RT-PCR fragment or specific LKR or SDH domain probes. The LKR probe consisted of a 0.55 kb cDNA fragment corresponding to the region spanning residues 96 through 284 and the SDH one (0.49 kb) encompassed residues 652 to 814 (Figure 1). DNA labeling, hybridization and washing were as described above for the DNA gel blots. For quantitative experiments, the radioactive membrane was exposed to pre-flashed X-ray films. Relative amounts of hybridized mRNA were analyzed through scanning with a laser densitometer (LKB UltroScan XL, Bromma, Sweden).

Immunoblotting

Immunoblotting was performed as described by Kemper et al. (1998) with minor modifications. After electrophoresis, the gel was soaked in 25 mM Tris-base, 190 mM glycine, 20% methanol for 10 min. Proteins were electrotransferred to nylon membranes (Hybond-N, Amersham) in a semidry blotting apparatus (LKB/Pharmacia). Membranes were blocked overnight at 4°C in 20 mM Tris, pH 7.4, 137 mM NaCl, 0.1% (v/v) Tween 20, and 5% (w/v) non-fat dry milk and then incubated with anti-LKR polyclonal antibodies. After incubation with anti-rabbit IgG alkaline phosphatase conjugate, the membranes were incubated for 20 min in the dark in a developing mixture containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl and a 1:1000 solution of CSPD (Tropix). Bands were detected by exposure onto a pre-flashed chemiliminescence-sensitive film (Amersham) and quantified through laser densitometry (LKB).

Histochemical Staining

Histochemical staining of SDH activity on maize kernel sections was based on the gel staining reaction described by Gonçalves-Butruille et al. (1996). Twenty DAP whole maize kernels were longitudinally hand sectioned with a razor blade. Sections were fixed in 4% formalin (pH 7.0) for 30 min, rinsed in water to remove endogenous substrates and then incubated for 10 min at room temperature in a reaction mixture containing 2 mM saccharopine, 1 mM NAD, 0.5% nitroblue tetrazolium, 0.1 mM phenazine methasulfate, 100 mM Tris-HCl pH 8.5. Control sections were

incubated in the absence of saccharopine. Rinsing sections in double distilled water stopped the reaction.

Subcellular Localization of the LKR/SDH Enzyme

Subcellular fractionation was carried out according to Habben et al (1993) with the following modifications. Buffer A contained 10 mM HEPES, pH 7.5, 1 mM EDTA, 10 mM KCl, 200 mM sucrose, 1 mM DTT and 5 mM benzamidine. After centrifugation in a discontinuous sucrose gradient, the interface fractions were collected by lateral puncture through the centrifuge tube wall and assayed for LKR and SDH activities. The activities of marker enzymes, cytochrome c oxidase and cytochrome c reductase, were assayed for resolution of organellar components, as described by Tolbert (1974) and Larkins and Hurkman (1978).

Separation of Maize LKR and SDH Domains by Limited Proteolysis

Aliquots of LKR/SDH partially purified by chromatography on DEAE-Sepharose were incubated with elastase (Sigma). Enzyme/protease proportions, and proteolysis conditions are indicated in the legend of Figure 9. The reaction was stopped by the addition of PMSF to a final concentration of 2 mM. The digest was applied to a Protein-Pak Q 8HR column (Waters) previously equilibrated with buffer B. The column was washed with buffer B and then eluted with a linear gradient from 0 to 0.5 M NaCl in buffer B. The fractions containing separated LKR and SDH domains were used for enzyme assays and immunobloting analysis.

Enzyme Assays

LKR and SDH activities were measured spectrophotometrically by following the oxidation of NADPH to $NADP^+$ and reduction of NAD^+, respectively, at 30°C. LKR assays were performed in 0.3 ml final volume, containing 20 mM L-lysine, 10 mM α–ketoglutaric acid (neutralized to pH 7.0 with potassium hydroxide), 0.1 mM NADPH, 150 mM Tris-HCl buffer, pH 7.4, and 0.04 to 0.1 mg protein. For SDH, the reaction mixture contained 2 mM L-saccharopine, 2 mM NAD^+ and 100 mM Tris-HCl buffer, pH 8.5. Oxidation of NADPH and reduction of NAD^+ were monitored at 340 nm in a DU-65 Beckman spectrophotometer. One unit of enzyme activity is defined as 1 nmol of NADPH oxidized or NAD^+ reduced per min at 30°C. The protein concentration in the enzyme extracts was determined by the method of Bradford (1976) using the Bio-Rad protein assay dye reagent.

Computer Analysis

Amino acid sequence alignments and similarity analysis was performed using MACAW (Schuler et al., 1991) and BOXSHADE (FTP downloaded from "Vax0.biomed.uni-koeln.de") programs.

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FIGURE LEGENDS

Figure 1. Predicted Sequence of the *ZLYS1-9* cDNA Encoded Protein.

(A) The protein sequence encoded by the 3.5 kb LKR/SDH cDNA is shown. Underlined residues highlight the interdomain region. Conserved residues shown in **(B)** are boldface. GeneBank accession number is AF003551.

(B) Alignment of a LKR domain subregion that is homologous to the putative substrate-binding site previously assigned to the yeast LYS1 protein. The essential conserved residues H-110, K-113 and R-146 of the maize protein are shown in boldface characters. Identical residues are marked by asterisks. ZEA, *Zea mays*; ATH, *Arabidopsis thaliana*; CEL, *Caenorhabditis elegans*; CAL, *Candida albicans*; YLI, *Yarrowia lipolytica*; SCE, *Saccharomyces cerevisiae*. GeneBank accession numbers are listed in the Figure 2 legend.

Figure 2. Schematic Diagram of Similarities Among Mono and Bifunctional LKR/SDH Polypeptides from Yeast, Plants and Animals.

The predicted amino acid sequences for monofunctional saccharopine dehydrogenase (LYS1, lysine forming) and saccharopine dehydrogenase (LYS9, glutamate forming) from yeast were aligned to plant, nematode and mammalian bifunctional LKR/SDH polypeptides. Sequence alignments and similarity analysis were performed using the MACAW program applying the BLOSUM 62 matrix. The similarity level at a given position is designated by the following color code: **red**, > 90%; **blue**, 50 to 90%; **cyan**, 10 to 49% and **white**, < 10% of similarity. The LKR and SDH domains, corresponding respectively to the yeast LYS1 and LYS9 proteins, as well as the unique interdomain region present in plant bifunctional polypeptides, are indicated. Blank spaces indicate sequence gaps introduced to maximize homology. Nomenclature and accession numbers are: SCLYS1, *Saccharomyces cerevisiae* (P38998); CALYS1, *Candida albicans* (P43065); YLLYS1, *Yarrowia lipolytica* (P38997); ZMAYS, *Zea mays* (AF003551); ATH, *Arabidopsis thaliana* (U95759); CEL, *Caenorhabditis elegans* (AF038615); MUS, *Mus musculus* (AJ224761); SCLYS9, *S. cerevisiae* (P38999) and SZLYS9, *Schizosaccharomyces pombe* (AL022244).

Figure 3. DNA Gel Blot Analysis of *ZLYS1-9* in Maize, Coix and Sorghum

Ten μg of DNA isolated from maize, Coix and sorghum were digested with Bam HI and Eco RV restriction enzymes, separated on a 1% agarose gel, transferred onto a nylon membrane and hybridized under high-stringency conditions to the LKR/SDH 1.2 Kb RT-PCR cDNA fragment as a

probe. Restriction fragments hybridizing to the probe are present in maize (M), Coix (C) and sorghum (S). The migration of molecular length markers is indicated at left.

Figure 4. RNA Gel Blot Analysis of LKR/SDH mRNA in Different Maize Tissues

Twenty μg of total RNA from roots (R), leaves (L), coleoptiles (C), embryos (Em) and endosperms (En) were fractionated in 1% agarose gel containing 0.66M formaldehyde, transferred to a Nylon membrane and hybridized to a LKR domain probe **(A)**. After 48 hours exposure, the membrane was stripped and hybridized against a SDH domain probe (**B**). The sizes for detected LKR/SDH transcripts are shown at left. A better resolution of the 2.4 and 2.6 kb bands is shown in Figure 5C. Loading control was performed by ethidium bromide staining.

Figure 5. Developmental Expression of LKR/SDH in Wild-type and *opaque2* Endosperms.

(**A**) Seeds of wild-type (o) and *opaque2* (•) were harvested from 10 to 40 DAP at 5- day intervals. Equal amounts of endosperms were homogenized in extraction buffer and fractionated with ammonium sulfate. The fractions collected between 35-60% saturation were dialyzed against extraction buffer and assayed for LKR and SDH activities. Each point is the average of duplicate assays and the bar indicates the standard deviation.

(**B**) Aliquots of wild-type (WT) and *opaque2* (*o2*) ammonium sulfate fractionated extracts from (**A**) were separated in a 7% SDS-PAGE gel, blotted onto nylon membranes and incubated with anti-LKR/SDH polyclonal antibodies.

(**C**) Fifteen μg samples of total RNA extracted from wild-type (WT) and *opaque2* (*o2)* endosperms were fractionated in a 1.5% agarose gel containing 0.66M formaldehyde, transferred to a Nylon membrane and hybridized to the 1.2-kb RT-PCR cDNA fragment as a probe. Loading control was performed by ethidium bromide staining.

Figure 6. Analysis of LKR/SDH Expression in Maize Seeds.

(A) Schematic representation of seed parts and endosperm regions used to prepare crude extracts for immunobloting. Endosperm regions (2, 3, 4, 5, 6, 8 and 9), embryo (1) and pedicel (7) of 20 DAP seeds were carefully dissected and extracted in buffer A.

(B) Fifty μg of protein extracts from **(A)** were fractionated in a 7% SDS-PAGE, blotted onto a nylon membrane and reacted against anti-LKR/SDH polyclonal antibodies. Lanes 1-8 represent the

tissue sections in **(A)**. The migration of molecular weight markers is shown at left.

Figure 7. Histochemical Staining for SDH Activity.

Twenty DAP seeds were longitudinally sectioned and incubated in a SDH reaction mixture, as described in Methods. The left panel (**–Sac**) shows an endosperm control section incubated in the absence of saccharopine. The positive colorimetric reaction for SDH activity is indicated by an arrow in the right panel (**+Sac**).

Figure 8. Subcellular Localization of LKR/SDH.

A 20 DAP endosperm homogenate was separated by sucrose gradient centrifugation and the fractions corresponding to the cytosplasm (1), endoplasmatic reticulum (2), mitochondria (3), peroxissomes (4) and protein bodies (5) were assayed for LKR and SDH activities. Marker enzymes, cytochrome c oxidase (Cyt c oxid) and cytochrome c reductase (Cyt c red), were assayed for resolution of organellar components.

Figure 9. Delimitation of Maize Bifunctional LKR and SDH Domains.

Elution profiles for LKR and SDH activities before (**A**) and after (**B**) digestion with elastase. A partially purified preparation of LKR/SDH was digested with elastase, applied to a Protein-Pak Q 8HR column and eluted with a linear gradient of 0-5 M NaCl in buffer B at a flow rate of 1.5 ml/min. Fractions of 0.75 ml were collected and assayed for LKR and SDH activities.
(**C**) Equivalent aliquots of the most active fractions of LKR and SDH peaks were subjected to a 6-18% gradient SDS-PAGE gel. The separated proteins were blotted onto nylon membranes and incubated with anti-LKR/SDH polyclonal antibodies. Lane 1, native enzyme; lane 2, elastase digestion products; lane 3, LKR peak. Lane 4, SDH peak. Numbers at left correspond to the molecular weight markers of the 10 kD Protein Ladder (Gibco-BRL).

A

```
   1 MGSAATEGNDTLLGNGVVGILAETCNMWER
  31 RAPLTPSHCARLLLGGGKNGPRVNRIIVQP
  61 STRRIHHDAQYEDAGCEISEDLSECGLIIG
  91 IKQPKLQMILSDRAYAFFSHTHKAQKENMP
 121 LLDKILEERVSLFDYELIVGDDGKRSLAFG
 151 KFAGTAGLIDFLHGLGQRYLSLGYSTPFLS
 181 LGQSHMYPSLAAAKAAVIVVAEEIATFGLP
 211 SGICPIVFVFTGVGNVSQGAQEIFKLLPHT
 241 FVDAEKLPEIFQARNLSKQSQSTKRVFQLY
 271 GCVVTSRDMVSHKGSHQTLTSDYYAHPDTT
 301 PCFHERIAPYASVIVHCMYWEKRFPPLLNM
 331 DQLQQLMETGCPLVGVCDITCDIGGSIEFI
 361 NKSTSIERPFFRYDPSKNSYHDDMEGAGVV
 391 CLAVDILPTEFSKEASQHFGNILSRLVASL
 421 ASVKQPAELPSYLRRACIAHAGRLTPLYEY
 451 IPRMRNTMIDLAPAKTNPLPDKKYSTLVSL
 481 SGHLFDKFLINEALDIIETAGGSFHLVRCE
 511 VGQSTDDMSYSELEVGADDTATLDKIIDSL
 541 TSLANEHGEITSRARNELALKIGKVMNMND
 571 SNVIKEGQDLIFGAEESVGTTAEFLASYPD
 601 ICTYGVDDHDADQIHVIVASLYQKDAEETV
 631 DGIENTTATQLDVADIGSLSDLVSQVEVVI
 661 SLLPASFHAAIAGVCIELKKHMVTASYVDE
 691 SMSNLSQAAKDAGVTILCEMGLDPGIDHLM
 721 SMKMIDEAHARKGKIKAFTSYCGGLPSPAA
 751 ANNPLAYKFSWNPAGALRSGKNPAVYKFLG
 781 ETIHVDGHNLYESAKRLRLRELPAFALEHL
 811 PNRNSLIYGDLYGISKEASTIYRATLRYEG
 841 FSEIMVTLSKTGFFDAANHPLLQDTSRPTY
 871 KGFLDELLNNISTINTDLDIEASGGYDDDL
 901 IARLLKLGCCKNKEIAVKTVKTIKFLGLHE
 931 ETQIPTGCSSPFDVICQEWNRGWPMAYMSQ
 961 DMVLLHHEVEVDITRTGNPPKSTKRRNGVS
 991 GRVENGRSTTAMALTVGIPAAIGALLLLQN
1021 KVQTKGMIRPLQPEIYVPALEILESSGIKL
1051 VEKVKS
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B

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ZEA  FFSHTHKAQKENMPLLDKILEERVSLFDYELIVGDDGKRSLAFG
ATH  FFSHTHKAQKENMPLLDKILSERVTLCDYELIVGDHGKRLLAFG
CEL  FFSHTIKAQQDNMEMLDTILQRNIRLLDYEKICDDKGKRLVMFG
CAL  QFAHCYKDQAGWQDVLKRFPQGNGILYDLEFLENDQGRRVAAFG
YLI  QFAHCYKDQGGWKDVLSRFPAGNGTLYDLEFLEDDNGRRVAAFG
SCE  QFAHCYKDQAGWQNVLMRFIKGHGTLYDLEFLENDQGRRVAAFG
      * *  * *     *        * * *    * * *   **
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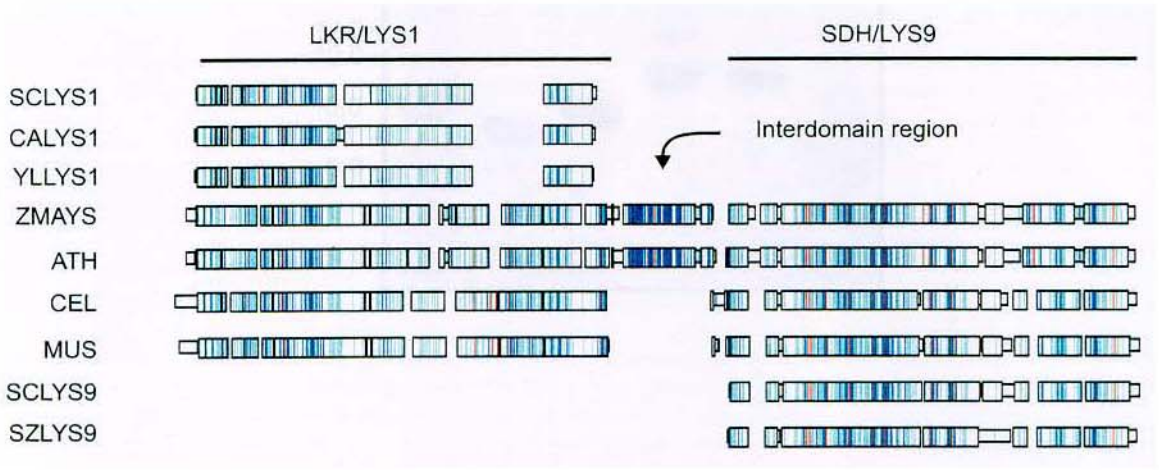
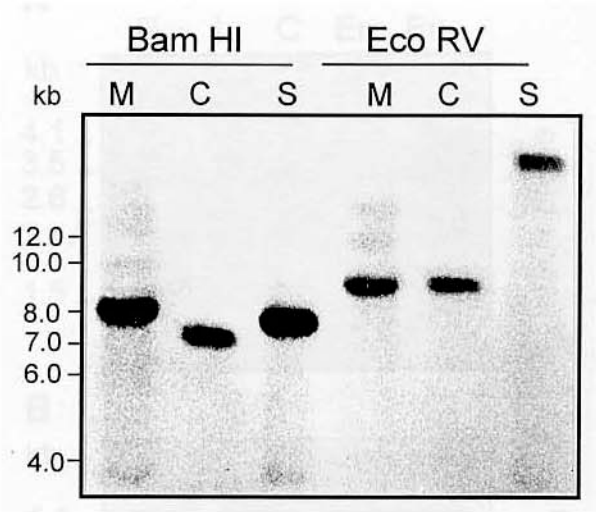
Cord Neto et al., 1998 – Figure 2

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C
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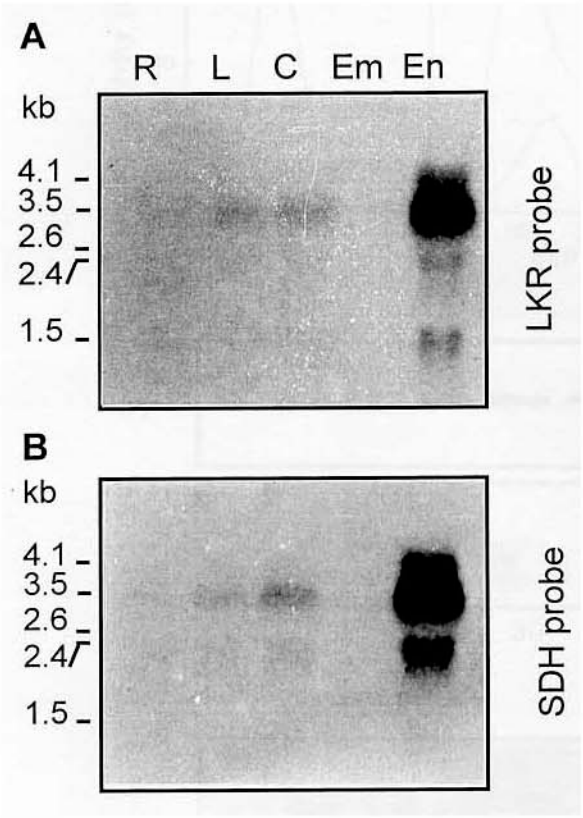
4.0


A
R
L
C
Em
En
kb
4.1
3.5
2.6
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LKR probe
B
kb
4.1
3.5
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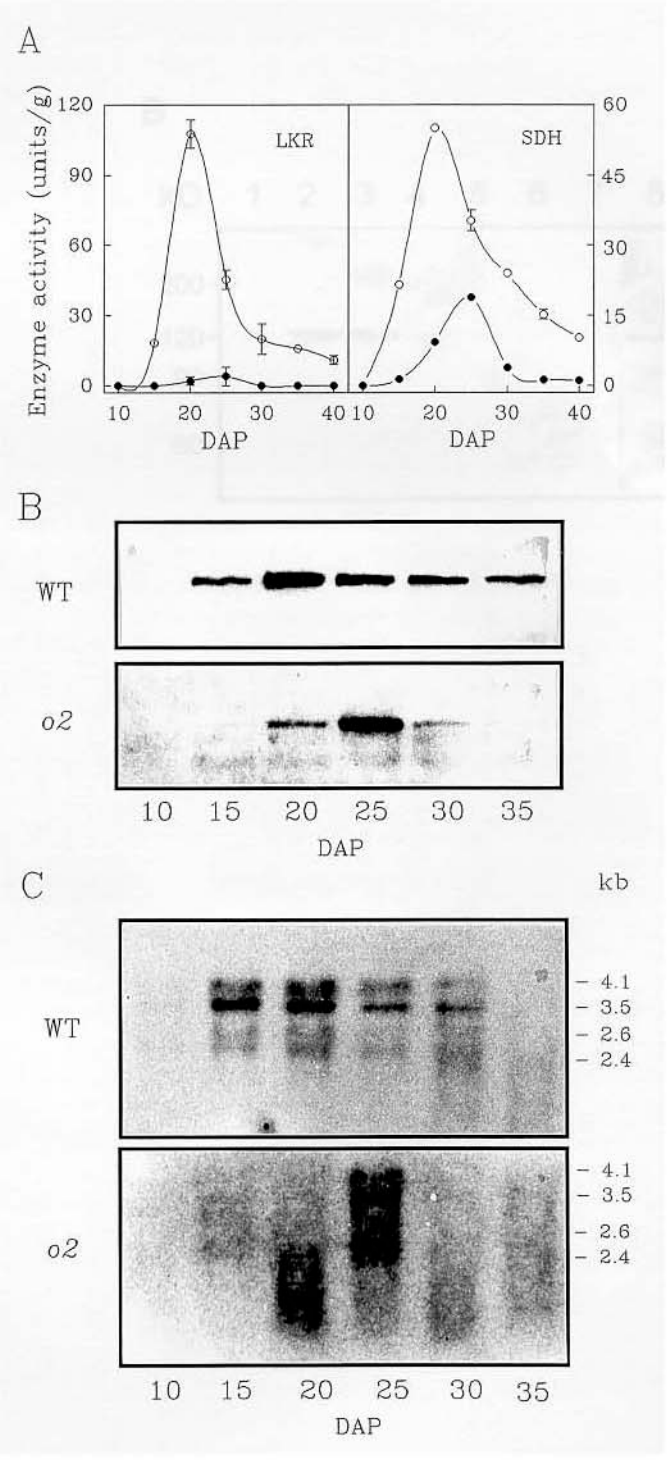
2.4
1.5
SDH probe


A
Enzyme activity (units/g)
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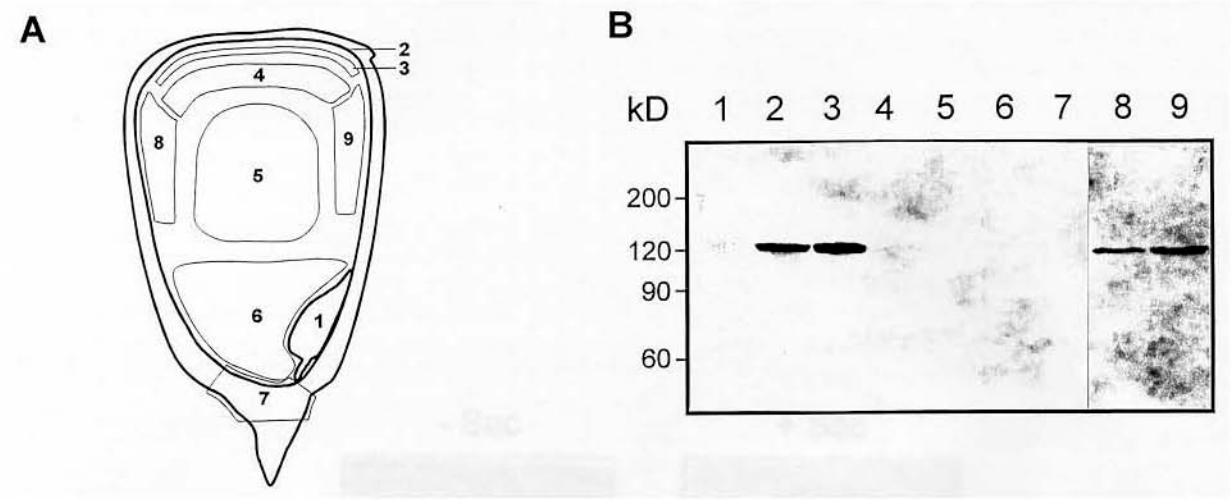
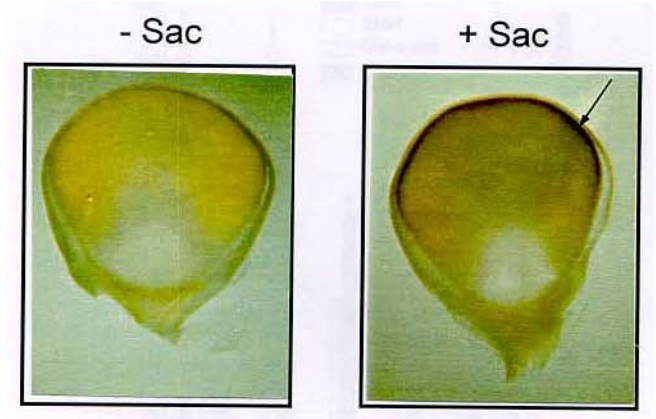
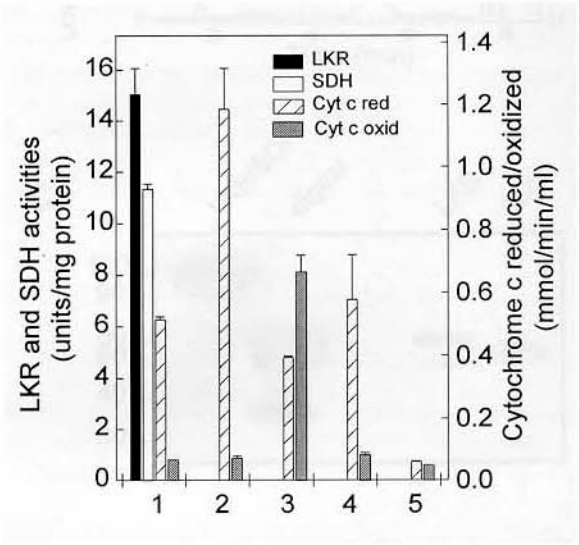
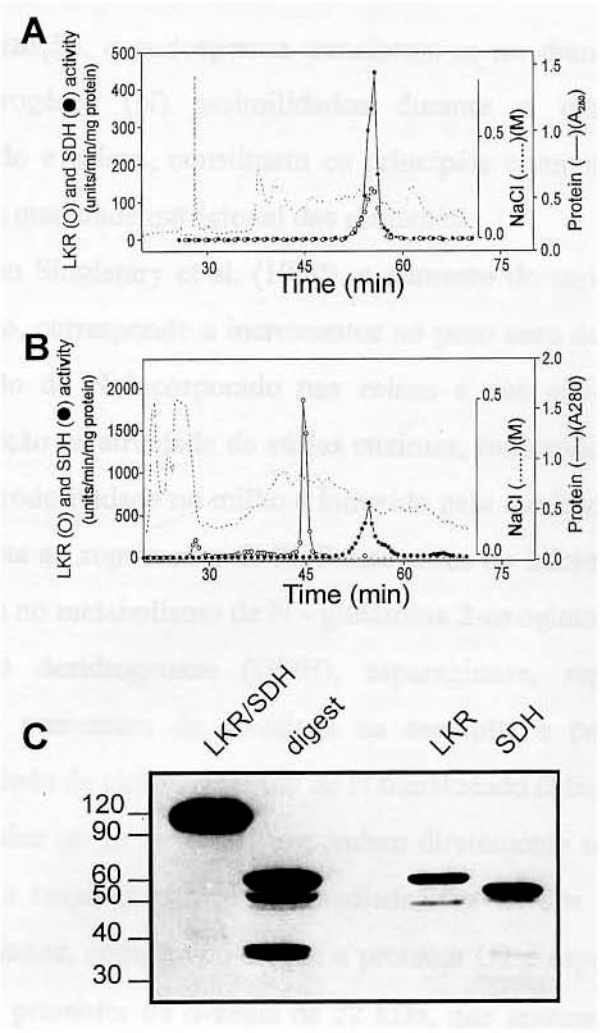
90
60
30
0
LKR
SDH
60
45
30
15
0
10 20 30 40
DAP
B
WT
o2
10 15 20 25 30 35
DAP
C
kb
WT
o2
4.1
3.5
2.6
2.4
10 15 20 25 30 35
DAP


A
2
3
4
8
9
5
6
1
7
B
kD
1
2
3
4
5
6
7
8
9
200
120
90
60


- Sac
+ Sac

Cord Neto et al 1998 - Figure 8




A
LKR (○) and SDH (●) activity
(units/min/mg protein)
500
400
300
200
100
0
30
45
60
75
Time (min)
1.5
1.0
0.5
0.0
0.5
0.0
NaCl (.....)(M)
Protein (—)(A280)
B
LKR (○) and SDH (●) activity
(units/min/mg protein)
2000
1500
1000
500
0
30
45
60
75
Time (min)
2.0
1.5
1.0
0.5
0.0
0.5
0.0
NaCl (.....)(M)
Protein (—)(A280)
C
LKR/SDH
digest
LKR
SDH
120
90
60
50
40
30

DISCUSSÃO*

Após a polinização, o endosperma transforma-se no dreno de grande parte do carbono (C) e nitrogênio (N) assimilidados durante o crescimento vegetativo. Convertidos em amido e zeínas, constituem os principais compostos armazenados que afetam a produção e a qualidade nutricional das sementes.

De acordo com Singletary et al. (1990), o aumento do suprimento de nitrogênio para a planta de milho, corresponde a incrementos no peso seco do endosperma, no teor de amido e na fração de N incorporado nas zeínas e nas não-zeínas. Estes efeitos correspondem à elevação na atividade de várias enzimas, indicando que, pelo menos em parte, o aumento de produtividade no milho é induzido pela modificação no metabolismo da semente em resposta ao suprimento de N. Pouco antes do ínicio da síntese de zeínas, as enzimas envolvidas no metabolismo de N - glutamina 2-oxoglutarato aminotransferase (GOGAT), glutamato desidrogenase (GDH), asparaginase, asparagina sintetase e glutamina sintetase - aumentam de atividade na semente, e permanecem em nível máximo durante o período de rápido acúmulo de N translocado (Misra e Oaks, 1981).

O promotores dos genes de zeínas respondem diretamente ao suprimento de N e, de certa maneira, esta resposta parece ser mediada através dos sítios de ligação da proteína O2. Recentemente, comprovou-se que a proteína O2 é capaz de reconhecer não um, mas três sítios no promotor da α-zeína de 22 kDa, que apresentam diferentes graus de afinidade por O2 (Muth et al., 1996). A interação entre estes sítios e a proteína O2 determina o grau de transativação do promotor em ensaios de expressão gênica transitória (Muth et al., 1996) e a resposta ao suprimento de N (Müller et al., 1997). A perda de qualquer um dos sítios reduz a resposta ao suprimento de nitrogênio neste promotor, provando que a presença dos três sítios é importante para a regulação apropriada. Diferentemente da α-zeína de 22 kDa, a β-zeína é relativamente insensível a alterações

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* Cada um dos artigos apresenta uma ampla discussão dos seus respectivos resultados. Assim, esta sessão constará de uma discussão mais geral onde se aborda a contribuição destes trabalhos no entendimento da regulação gênica coordenada no endosperma em desenvolvimento.

no suprimento de nitrogênio (Singletary et al., 1990), muito embora nenhum estudo detalhado deste promotor tenha sido feito neste sentido.

Mesmo que a interação da proteína O2 com seus sítios de ligação no promotor da α-zeína de 22 kDa possa mediar a resposta ao N, não se pode ainda afirmar que o fator O2 desempenhe ou não o papel de fator responsivo ao nitrogênio. De fato, existem algumas evidências que põe em dúvida este papel: primeiro, endospermas *o2* cultivados *in vitro* sob elevados níveis de N retém a capacidade de sintetizar a α-zeína de 22 kDa (Balconi et al., 1993). Segundo, tanto o promotor natural da α-zeína de 22 kDa como seus respectivos mutantes nos sítios de ligação de O2, mostraram resposta similar ao N quando transfectados, em ensaios de expressão transitória, para endospermas normais e mutantes *o2* (Müller et al., 1997). Baseado nestes resultados, Müller et al. (1997) sugerem a presença de um fator adicional responsivo ao nitrogênio, com especificidade de ligação similar ao O2 e com capacidade para compensar os efeitos da mutação *o2*. A resposta ao suprimento de N efetuada pelo "fator adicional" parece ser mediada através da interação com os sítios de ligação de O2, indicando que embora O2 não seja o fator determinante, ele faz parte do mecanismo de resposta ao N.

Não somente o nitrogênio atua como regulador metabólico da expressão gênica, mas também os compostos de carbono. No endosperma, enquanto tecido de armazenamento destes compostos, a interação entre estas vias metabólicas de C e N constitui um importante ponto de regulação da expressão dos genes que regulam o acúmulo de amido e proteínas.

As vias metabólicas de C e N consomem a maior parte da energia e do C fixado nas células fotossintetizantes. Até 55% do C da planta está comprometido com a assimilação e metabolismo de N em alguns tecidos. Os metabolismos de C e N são conectados porque necessitam compartilhar o C orgânico e a energia fornecida pelo transporte de elétrons e pela fixação do CO_2 através da fotossíntese, ou através da respiração do carbono assimilado via glicólise, ciclo do ácido tricarboxílico e da cadeia de transporte de elétrons mitocondrial (Huppe e Turpin, 1994). Nos tubérculos de batata, a inibição da síntese de amido pelo impedimento da tradução da ADP-glicose pirofosforilase (ADPGPPase) via RNA anti-sense, resulta na inibição da expressão do gene da patatina (proteína de reserva), afetando também a formação de tubérculos

(Müller-Röber et al., 1992). Nas sementes de milho, alterações genéticas nos genes de uma das vias de metabolismo de C ou de N, afeta a expressão de genes em ambas, indicando uma expressão coordenada dos genes envolvidos na síntese de amido e zeínas. Aos 30 DAP, os níveis dos transcritos que codificam diferentes subunidades da ADPGPPase, *Bt2* e *Sh2*, aumentam, respectivamente, 2.5 e 10 vezes no endosperma *o2* em relação ao tipo selvagem (Giroux et al., 1994), indicando que a alteração no balanço de N provocada pela mutação *o2* se reflete na expressão de genes da via biosintética do amido. Em plântulas e folhas de Arabidopsis, o fluxo de carbono e nitrogênio para a síntese dos aminoácidos da família do aspartato é regulado trancricionalmente através da expressão do gene de uma isoforma da enzima aspartato quinase. Neste estudo, descobriu-se que a expressão do gene da enzima bifuncional aspartato kinase/homoserina desidrogenase (AK/HSD) é regulada metabolicamente por sacarose e fosfato, compostos relacionados à fotossíntese, mas não por compostos nitrogenados (Zhu-Shimoni e Galili, 1998). Estes resultados indicam que a conversão do aspartato nos aminoácidos da via correspondente, lisina, metionina, treonina e isoleucina, ou em asparagina, com funções de armazenamento, está sujeita a um controle metabólico coordenado. Este tipo de regulação implica que a via do aspartato pode ser um ponto crucial no mecanismo de particionamente de C e N.

No endosperma, o fator O2 parece estar envolvido no mecanismo de particionamento de carbono entre amido e proteínas. A expressão do gene que codifica a forma citosólica da enzima piruvato ortofosfato diquinase (cyPPDK1), a qual catalisa a conversão de piruvato (+ ATP e Pi) em PEP, é controlada transcricionalmente pelo fator O2 (Gallusci et al., 1996; Maddaloni et al., 1996). Esta observação é particularmente interessante porque o PEP pode ser carboxilado pela PEP-carboxilase citosólica para gerar oxalacetato, que é facilmente convertido em aspartato, precursor da síntese de metionina, lisina e treonina (Azevedo et al., 1997). Por outro lado, a partir do PEP se inicia a via de biosíntese dos aminoácidos aromáticos (Herrmann, 1995). Desta forma, a célula garante o suprimento de carbono para a síntese destes aminoácidos no momento crucial que se inicia a deposição de zeínas, cujos genes também são ativados por O2. A lisina, por sua vez, não é incorporada nas zeínas e pode ser inibitória para a via do aspartato, devendo ser mantida em baixos níveis no endosperma. Esta regulação parece

ser crucial, uma vez que a LKR/SDH, responsável pelo controle dos níveis de lisina, está sob o controle transcricional do fator O2 (Trabalho 2). A primeira enzima da via do aspartato, AK, tem três isoformas distintas, uma inibida somente por lisina, outra por treonina e a terceira por lisina e S-adenosilmetionina. A forma inibida por lisina parece ser predominante nas células em rápido crescimento e representa aproximadamente 80% da atividade total de AK na maioria das plantas (revisado em Azevedo et al., 1997). Este fato parece atribuir à lisina um papel importante no controle do fluxo de carbono na via do aspartato. Desta maneira, em relação ao metabolismo de aminoácidos, o fator O2 regula a disponibilidade de C, ao controlar a cyPPDK1 e, por outro lado, ao controlar a LKR/SDH , regula o funcionamento da via do aspartato através do controle dos níveis de lisina.

A regulação por O2 da síntese de proteínas, do metabolismo de aminoácidos e do particionamento de carbono, aliada com sua similaridade funcional e estrutural com o fator GCN4 de leveduras, sugere que O2 pode estar envolvida no mecanismo geral de controle de aminoácidos no endosperma.

Mauri et al. (1993) demonstrou que o fator O2 pode complementar funcionalmente a mutação *gcn4* de leveduras. O fator GCN4 é um dos fatores determinantes do mecanismo regulador denominado "General Control of Nitrogen". Neste sistema, a síntese de aminoácidos é regulada coordenadamente pelas condições nutricionais (Hinnebusch, 1988). Estruturalmente, O2 e GCN4 pertencem a mesma família de reguladores da transcrição, e possuem um mecanismo comum de controle pós-transcricional. A tradução do mRNA de O2 é inibida por pequenas fases de leitura (uORFs) localizadas na sequência líder do RNA (Lohmer et al., 1993). Nos transcritos *GCN4*, a remoção destas uORFs por deleção ou por mutações de ponto resulta em altos níveis de expressão desregulada do fator GCN4, independentemente da disponibilidade de aminoácidos (Mueller e Hinnebusch, 1986).

Em Arabidopsis, comparativamente ao mecanismo GCN de leveduras, existem evidências de regulação cruzada da expressão gênica entre vias de biosíntese de aminoácidos, levando à ativação ou repressão da síntese destes aminoácidos (Guyer et al., 1995). A proteína O2, por sua vez, é capaz de regular diversos genes de plantas através da

interação com elementos *in cis* do tipo "GCN4-like" ($TGA^{G}/_{C}TC$). Entre estes genes constam aqueles que codificam uma α-coixina (Yunes et al., 1994), uma glutenina de baixo peso molecular em trigo (Holsworth et al., 1995), a albumina b-32 de milho (Bass et al., 1992) e a enzima cyPPDK1 (Maddaloni et al., 1996).

Segundo Müller e Knudsen (1993), um elemento "GCN4-like" é fundamental para a expressão gênica da C-hordeína em resposta ao nitrogênio em cevada. Estes autores sugeriram a existência de uma família de fatores de transcrição funcionalmente análogos que se ligariam a motivos "GCN4-like", desta forma mediando a resposta ao nitrogênio em plantas. O motivo GCN4 não está restrito a genes endosperma-específicos de cereais. Ele está presente em genes de proteínas de reserva de tecidos vegetativos, a lectina (ervilha) e a patatina (batata) e em vários outros genes (Müller e Knudsen, 1993).

O fator O2 regula os diversos genes no endosperma através da interação com diferentes sítios de ligação no seus promotores. No entanto, este tipo de regulação não depende somente da especificidade de ligação a determinado sítio no promotor, mas é decorrente de interações entre proteínas regulatórias e da ação de outros elementos moduladores, como a fosforilação. Recentemente demonstrou-se que o padrão de fosforilação da proteína O2 muda no decorrer do dia refletindo na atividade de ligação ao DNA (Ciceri et al., 1997). Por outro lado, compostos originados nas vias metabólicas podem desempenhar um papel na regulação de processos celulares e de desenvolvimento. Em leveduras, o α-aminoadipato, um intermediário na via de síntese de lisina (Figura 4), age como indutor da expressão de pelo menos quatro genes desta via - *Lys1*, *Lys9*, *Lys2* e *Lys5*. Os genes *Lys1* e *Lys9* correspondem, respectivamente, a LKR e SDH (veja Figuras 1 e 2 do Trabalho 2). O mecanismo de indução é mediado pela proteína regulatória LYS14, da classe "zinc finger" que apresenta um motivo de ligação ao DNA do tipo $Zn(II)_2Cys_6$ (Feller et al., 1994). Em plantas, no entanto, especula-se sobre o possível papel do α-aminoadipato na regulação da degradação de lisina e sobre a existência de uma proteína regulatória do tipo LYS14.

A via de degradação da lisina é altamente regulada, não somente através do controle transcricional, mas também pela modulação da atividade enzimática. A atividade LKR é modulada por cálcio (Kemper et al., 1998) e fosforilação em milho (E.L. Kemper

e P. Arruda, comunicação pessoal) e em tabaco (Karchi et al., 1995). Estas evidências sugerem que a lisina ou um metabólito dela derivado, pode estar envolvida em uma via de transdução de sinais mediada por cálcio e fosforilação. É necessário esclarecer se esta via de transdução de sinais estaria ou não relacionada à rede de regulação da expressão gênica envolvendo o fator Opaco2.

CONCLUSÕES

1. Os níveis de mRNA e proteína da β-zeína de 14 kDa são afetados pela mutação *opaco2* (*o2*).
2. O ativador transcricional Opaco2 (O2) liga-se aos promotores de β-prolaminas de milho e Coix.
3. As proteínas O2 de milho e de Coix reconhecem a mesma sequência TCCACGTCAT nos respectivos promotores de β-prolaminas.
4. Os promotores de β-zeína e β-coixina são ativados transcricionalmente pela proteína O2 em ensaios de expressão transitória.
5. Os níveis de mRNA e proteína da LKR/SDH, bem como a atividade enzimática são afetados pela mutação *o2*.
6. O padrão temporal de expressão do gene *ZLYS1-9*, que codifica LKR/SDH, sofre um atraso de aproximadamente 5 dias no endosperma mutante em relação ao normal.
7. Apesar de ser detectado em pequenas quantidades em raízes, folhas jovens e coleóptilos, o mRNA de LKR/SDH é preferencialmente transcrito no endosperma, único tecido onde se detecta a proteína. No endosperma a enzima está presente no citosol das células mais ativas das camadas de aleurona e sub-aleurona.
8. A transcrição do gene *ZLYS1-9* produz cinco espécies de transcritos, uma mais abundante, que codifica uma proteína de 117 kDa, hómologa às proteínas previamente descritas LYS1 e LYS9 de levedura e LKR/SDH de Arabidopsis; os quatro transcritos alternativos possivelmente não são traduzidos.
9. O gene *ZLYS1-9* está presente como simples cópia nos genomas do milho e dos seus cereais relacionados, coix e sorgo.

10. Tanto a síntese de β-zeína como a degradação de lisina estão sob controle do locus *O2*, o que significa que o fator por ele codificado desempenha um papel importante no controle do metabolismo de aminoácidos na semente em desenvolvimento.

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