Nuclear Localization Signal(s) Required for Nuclear Targeting of the Maize Regulatory Protein Opaque-2

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The maize regulatory protein Opaque-2 (O2) localizes to the nucleus in both maize and tobacco cells. Here we show that in-frame carboxy- and amino-terminal fusions of O2 to reporter protein β-glucuronidase (GUS) were sufficient to direct GUS to the nucleus in transgenic tobacco plants and in transiently transformed onion cells. Two independent regions of O2 containing 135 and 149 amino acids were identified that were able to redirect GUS to the nucleus in both systems. A quantitative biochemical analysis of GUS in nuclei isolated from transgenic tobacco plants revealed that the second region was more efficient than the first one. The precise location of nuclear localization signals (NLSs) was determined using an onion transformation system. The first NLS was located between residues 101 and 135 and had the structure of a simian virus 40 NLS. The second NLS was located in the basic, DNA binding domain (between residues 223 and 254) and had a bipartite structure. The presence of one of the O2 NLSs in the basic domain is in complete agreement with similar findings of NLSs in the basic domain of three other basic/leucine zipper proteins, suggesting that this domain may be bifunctional. The effect of amino- versus carboxy-terminal GUS fusions is discussed.

INTRODUCTION

Proteins synthesized in the cytoplasm function in many different compartments in the cell. Studies on protein targeting have determined the signals and machinery involved in the movement of proteins to the correct compartments. In plants, most studies on targeting of cytoplasmically synthesized proteins have focused on the movement of proteins to the chloroplast (for review, see Keegstra, 1989) and mitochondria (for review, see Hartl and Neupert, 1990). Protein targeting to the plant cell nucleus has only recently been examined. Results obtained from yeast, mammalian, and amphibian systems have led investigators to suggest some basic rules for nuclear targeting (for review, see Garcia-Bustos et al., 1991). Proteins smaller than 40 to 60 kD are thought to diffuse through the nuclear pore. However, larger proteins require ATP and at least one nuclear localization signal (NLS) to traverse the pore. Unlike targeting signals for secretory proteins (for review, see Chrispeels and Raikhel, 1992) and other organellar proteins, NLSs are located within the body of nuclear proteins and are not cleaved. Presumably, this allows nuclear proteins to enter the nucleus again after cell division.

The NLSs identified to date may be classified into three categories: (1) simian virus 40 (SV40)-like NLSs contain short tandem stretches of basic amino acids with either a proline or glycine (PKKKRKV; Kalderon et al., 1984a,1984b), (2) mating type α2–like NLSs consist of short hydrophobic regions that contain one or more basic amino acids (KIPIK; Hall et al., 1984), and (3) bipartite NLSs are usually a combination of two regions of basic amino acids separated by approximately 10 amino acids (reviewed in Dingwall and Laskey, 1991). Dingwall and Laskey (1991) suggest that the bipartite signal may be the canonical NLS.

Studies on protein targeting to the nucleus in plants have identified NLSs that are competent to redirect the β-glucuronidase (GUS) reporter protein to tobacco nuclei. Most of these reports have concentrated on proteins from viral and bacterial phytopathogens. Carrington et al. (1991) identify a bipartite NLS in the potyviral protein NIa. Bipartite NLSs are also present in the Agrobacterium proteins VirD2 (Howard et al., 1992) and VirE2 (Citovsky et al., 1992). All of these proteins from phytopathogens are thought to be involved in the movement of nucleic acids into the host plant cell nucleus.

van der Krol and Chua (1991) examined three proteins from dicot plants for basic regions that would redirect GUS to the nucleus. For the tobacco DNA binding proteins TGA-1A and TGA-1B, they propose that an SV40-like NLS, found in the basic domain of TGA-1B and in the basic/leucine zipper (bZIP) domain of TGA-1A, serves as the NLS. The one basic region examined from the Arabidopsis 37-kD TFIID could not redirect GUS. It has also been shown that the SV40 NLS can function in dicot plant cells (Lassner et al., 1991; van der Krol and Chua, 1991). However, no previous studies have identified an NLS in a monocot protein.

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We have been examining plant nuclear protein targeting of the maize regulatory protein Opaque-2 (O2) (Varagona et al., 1991). The 47-kD O2 protein has a bZIP structure (Hartings et al., 1989; Schmidt et al., 1990) and is capable of binding to a specific sequence located in the promoter of 22-kD α-zein genes (Schmidt et al., 1992). The recognition of this zein promoter element by O2 promotes transcription in maize endosperm suspension cells (Ueda et al., 1992). These results are consistent with the effects on zein gene expression of mutations in the o2 locus (reviewed by Kodrzycki et al., 1989; Motto et al., 1989; Aukerman et al., 1991). We have previously shown that O2 is localized in the nucleus of endosperm cells in wild-type maize kernels and in transgenic tobacco plants transformed with O2 cDNA under the control of the constitutive cauliflower mosaic virus 35S promoter (Varagona et al., 1991). It was concluded that the machinery necessary for nuclear transport of this monocot protein is present in the dicot tobacco plants.

The 47-kD O2 protein may be small enough to diffuse through the nuclear pore. Therefore, we examined this protein to determine if it contains NLS(s). Experiments were initiated using the transgenic tobacco system developed in our previous study (Varagona et al., 1991). Constructs encoding in-frame fusion proteins of O2 fused to both the amino and carboxy termini of the GUS reporter protein were made and used for transformation of plants. Two independent regions of O2, containing 135 and 149 amino acids, could redirect GUS to the nucleus. Biochemical analysis was performed to quantitate the efficiency of targeting for these two signals. To study nuclear targeting of the O2 protein in a monocot system, we developed an efficient transient expression system using onion epidermal cells transformed with the Helium Biolistic gene transfer system (Du Pont). The onion transformation system not only confirmed the results from the tobacco system, but was also used to define small regions of 32 and 36 amino acids that were capable of redirecting the GUS protein to the nucleus.

RESULTS

To test for the presence of an NLS in the O2 protein, DNA sequences from the O2 cDNA, shown in Figure 1, were ligated to the GUS cDNA, shown in Figure 2A, to yield translational fusions. The 68-kD GUS protein has been shown to be located in the cytoplasm (Restrepo et al., 1990; van der Krol and Chua, 1991) and is too large to enter the nucleus without an NLS. To ensure that an NLS did not escape detection due to masking caused by the folding of fusion proteins, initial fusions were made to both the amino and carboxy termini of GUS.

The GUS gene and O2/GUS fusion genes were then moved into plant expression vectors (Figure 2B). As in our previous study (Varagona et al., 1991), nuclear transport of fusion constructs was first tested in transgenic tobacco plants using the binary vector pGA643 (An et al., 1988). Regenerated plants were selected for kanamycin resistance and screened
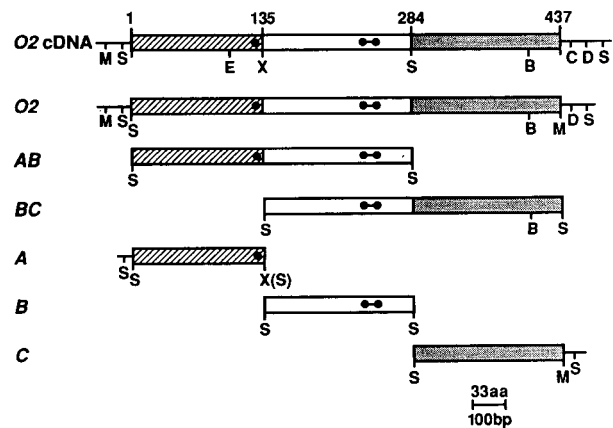

Figure 1. Strategy for Constructing O2/GUS Fusions.

The upper construct represents the coding sequence of the O2 cDNA (striped, open, and stippled boxes), showing a partial restriction map of the sites found in the original clone (Hartings et al., 1989; Schmidt et al., 1990). Positions of amino acids are indicated above the construct. Beneath are partial restriction maps of clones of individual fusion constructs. Restriction sites used to make amino- and carboxy-terminal fusions to GUS are indicated. Locations of flanking restriction sites are not drawn to scale. Restriction sites added or changed from the cDNA were done using site-directed mutagenesis (see Methods). The striped box denotes the amino-terminal 135 amino acids or A region of O2; the open box denotes amino acids 136 to 284 or B region; the stippled box denotes amino acids 285 through the carboxy terminus of O2. Filled circles represent the two putative NLSs in the A and B regions. Abbreviations of restriction enzymes are B, BamHI; C, ClaI; D, HindIII; E, EagI; M, SmaI; S, SalI; X, XhoI. aa, amino acid.

histochemically, using the X-glucuronide (X-gluc) assay, for the presence of GUS activity. Protein extracts from transgenic plants were then analyzed on immunoblots for the presence of fusion proteins using both O2- and GUS-specific antisera. To localize the fusion proteins, plants expressing GUS were first screened histochemically using the X-gluc assay, and then biochemically using the 4-methylumbelliferyl β-D-glucuronide (MUG) assay (Jefferson, 1987).

To examine nuclear localization in a monocot system, fusion constructs (Figure 1) were also introduced into the maize vector pMF6 (Figure 2B; Goff et al., 1990). Initially, localization of fusion constructs was attempted using transient expression in maize Black Mexican Sweet cultured cells. However, the low efficiency of transformation and difficulty in visualizing nuclear localization in the small Black Mexican Sweet cells led us to develop a system that was more amenable for nuclear localization studies. The Biolistic particle delivery system was used to transiently transform onion epidermal cells (Klein et al., 1987), and these cells were histochemically assayed for localization of the fusion proteins. By working with both dicot (tobacco) and monocot (onion) systems, we were able to compare, in detail, nuclear transport in these two classes of plants. Thus, in addition to allowing analysis

of nuclear targeting in a monocot system, the onion transformation system facilitated our analysis by allowing examination of several constructs in a short period of time.

The O2 Protein Contains NLSs

The size of the O2 protein is 47 kD; thus, it is in the size range of proteins able to diffuse through the nuclear pore. To determine if the O2 protein contains an NLS, we made a series of fusion constructs encoding proteins containing the entire O2 protein, the first (AB), and last (BC) two thirds of O2 fused to GUS (Figure 1). For reasons that are unclear, we were able to regenerate GUS-expressing plants only from those transformed with a construct containing the AB region of O2 fused to the carboxy terminus of GUS (GUS::AB). The plants that expressed the GUS::AB fusion protein had low levels of GUS activity detected histochemically after ~20 hr, and no fusion protein could be detected on immunoblots (data not shown). However, as indicated in Table 1, histochemical analysis of epidermal layers and roots from transgenic plants showed that the fusion proteins were localized to the nucleus. This could

Table 1. Summary of Histochemical Localization of O2/GUS Proteins in Tobacco

Fusion[a]	Fusion::GUS		GUS::Fusion	
	No. Plants[b]	LOC[c]	No. Plants[b]	LOC[c]
AB	NA[d]		2	N/C
A	3	N/C	2	N
B	2	N	3	N
C	3	C	3	C

[a] Refer to Figure 1 for names of fusion proteins.
[b] Number of plants analyzed.
[c] Localization of proteins: N, nuclear; C, cytoplasmic; N/C, both nuclear and cytoplasmic.
[d] NA, no transformed plants were obtained.

occur only if the AB region of the O2 protein contained an NLS. Therefore, we concluded that although the O2 protein is small enough to diffuse through the nuclear pore, it contains at least one NLS that is competent to redirect a reporter protein to the nucleus.

To examine the entire O2 protein for the location of NLSs, the O2 protein was divided into thirds and each third was translationally fused to GUS (Figure 1). The A region consists of amino acids 1 through 135 (Figure 1, striped box). The B region consists of amino acids 136 through 284 (Figure 1, open box) and forms the putative bZIP domain (Hartings et al., 1989; Schmidt et al., 1990). The C region consists of amino acids 285 through 437 (Figure 1, stippled box) and is the carboxy terminus of the O2 protein.

Plants expressing fusion proteins containing these smaller regions of O2 were more readily obtained. Most plants expressed the fusion proteins at levels high enough to detect histochemically within a few minutes to a few hours and also showed proteins of the predicted size on immunoblots (data not shown). However, the plants containing the B::GUS fusion contained low levels of fusion protein that required overnight incubation to detect the blue precipitate. Also, no protein from B::GUS plants could be detected on immunoblots.

Localization of fusion proteins was initially performed histochemically using the X-gluc assay. After the blue staining was visible, tissues were simultaneously stained with the nuclear-specific stain 4′,6-diamidino-2-phenylindole (DAPI). Several tissues were examined including roots, epidermal layers, and trichomes. Roots were usually the easiest tissue in which to visualize localization because of the lack of chloroplasts and the presence of large nuclei in the root hairs. Data from tobacco plants containing fusion proteins made with the three independent domains of O2 are shown in Figure 3 and summarized in Table 1. Plants expressing fusion proteins containing the C region of O2 showed localization to the cytoplasm (Table 1 and Figures 3c and 3c′) similar to the localization of GUS indicated in Table 2.

Other fusion proteins localized to the nucleus. The nuclear localization was most evident in plants expressing fusion
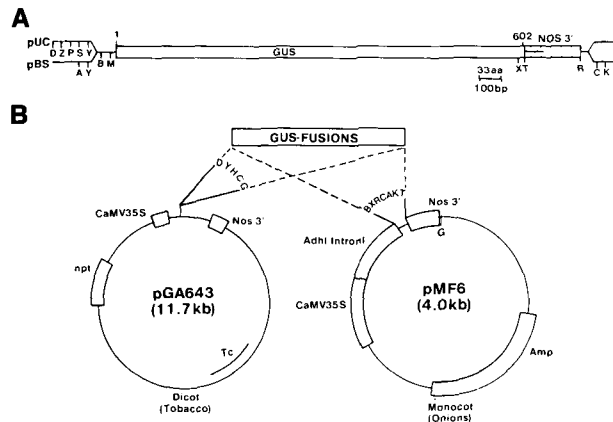

Figure 2. Cloning Strategy for Inserting O2/GUS Fusions into Plant Transformation Vectors.

(A) Partial restriction map of the GUS gene used for making fusion proteins (Jefferson, 1987). Forked ends (not drawn to scale) indicate restriction sites used to make fusions and to insert fusions into plant expression vectors.

(B) Vectors used for plant transformations (not drawn to scale). Both vectors express insertions from the 35S promoter from CaMV and terminate expression using the 3′ end of the nos gene. In addition, pGA643 confers kanamycin resistance (NPT; neomycin phosphotransferase) and tetracycline resistance (Tc). pMF6 confers ampicillin resistance (Amp) and incorporates the first intron from the maize Adh1 gene into inserted genes. This intron confers higher levels of gene expression in monocot cells. Abbreviations of restriction enzymes are A, SacII; B, BamHI; C, ClaI; D, HindIII; G, BglII; H, HpaI; K, KpnI; M, SmaI; P, PstI; R, EcoRI; S, SalI; T, SstI; X, XhoI; Y, XbaI; Z, SphI. aa, amino acid.
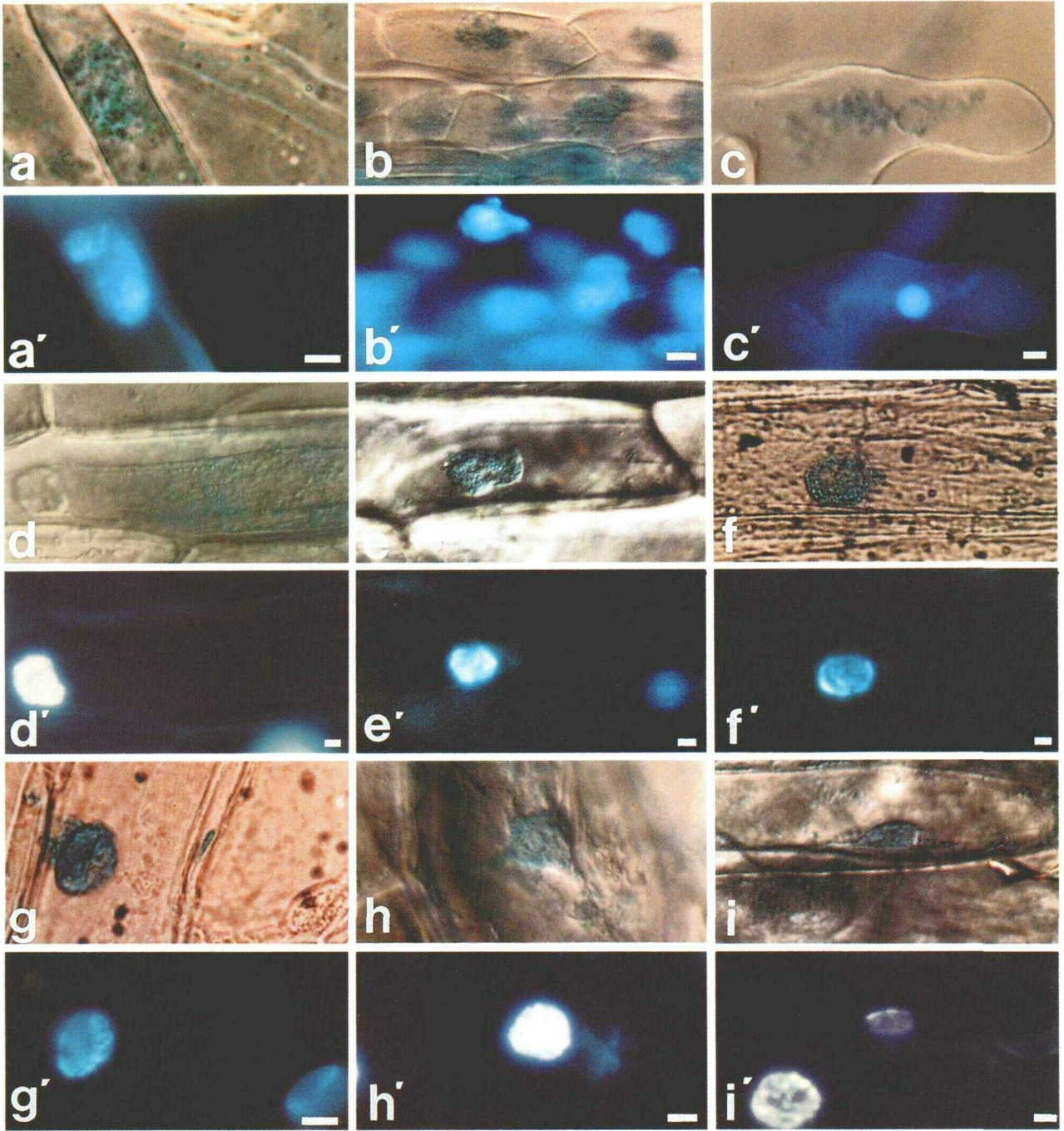
Figure 3. Histochemical Localization of GUS and O2/GUS Fusion Proteins in Transformed Tobacco and Onion Tissues.

Tissues were simultaneously analyzed using both X-gluc histochemical staining (**[a]** through **[i]**) and nuclei-specific DAPI staining (**[a′]** through **[i′]**). **(a)** through **(c)** Photomicrographs made from tissues of the following tobacco plants.

(a) and **(a′)** Root hair from a GUS::A plant.

(b) and **(b′)** Root from a GUS::B plant.

(c) and **(c′)** Root hair from a C::GUS plant.

(d) through **(i)** Photomicrographs are from onion cells transformed with **(d)** and **(d′)** GUS, **(e)** and **(e′)** GUS::AB, **(f)** and **(f′)** A::GUS, **(g)** and **(g′)** B::GUS, **(h)** and **(h′)** $A_{101-135}$::GUS, and **(i)** and **(i′)** $B_{223-254}$::GUS.

Photomicrographs **(a)**, **(f)**, and **(g)** were made using bright-field optics. Photomicrographs **(b)**, **(c)**, **(d)**, **(e)**, **(h)**, and **(i)** were made using differential-interference optics. Bars = 10 μm.

proteins containing the B region. Regardless of the orientation of the fusion, B region fusion proteins localized to the nucleus (Figures 3b and 3b'). However, localization of fusion proteins containing the A region was not as clear. In some tissues, the fusion protein localized to the nucleus (Figures 3a and 3a'); in others, the blue color formation occurred in both the nucleus and the cytoplasm (Table 1). From this study, we concluded that the O2 protein contains two NLSs: one NLS, located within the B region, appears to be more efficient in nuclear targeting; the other NLS, in the A region, appears to be less efficient.

The NLS in the B Region Is More Efficient than the NLS in the A Region

Histochemical analysis indicated that the NLS in the B region may be more efficient in redirecting the GUS protein than the NLS in the A region. To quantitatively address the efficiency of these two NLSs, transgenic tobacco plants expressing the GUS protein and O2/GUS fusion proteins were analyzed biochemically. Nuclei were isolated from leaf protoplasts and cytoplasmic fractions were retained. The fluorometric MUG assay was used to compare the amount of GUS enzyme activity in these two fractions to the amount of GUS enzyme activity in a total extract. These activities were determined from the slope of a line showing linear accumulation of 4-methylumbelliferone (MU) product over time. Conditions for linear kinetics were found when 30,000 cells or nuclei were assayed over 1.5 to 2 hr. The amount of cytoplasmic extract assayed was standardized to equivalent amounts of cytoplasmic marker enzyme activity found in total fractions (see Methods). Formally, efficiency should refer to the rate of protein accumulation;

however, we define efficiency as the steady state percentage of total GUS activity accumulated in the nuclear fraction. Kinetic data of samples from two transgenic plants are shown in Figure 4.

Results of the biochemical analysis are summarized in Table 2. Plants transformed with GUS showed all the GUS activity in the cytoplasm (Figure 4A and Table 2). As predicted by histochemical analysis, the plants expressing the fusion proteins containing the C region also showed all the GUS activity in the cytoplasmic fraction (Table 2).

Analysis of plants expressing fusion proteins containing the A and B regions would allow us to quantitate the efficiency of each of the identified NLSs. Plants expressing the B::GUS fusion showed the highest efficiency of nuclear targeting with all of the GUS activity in the total fraction accounted for in the nuclear fraction (Figure 4B and Table 2). However, nuclear targeting of the GUS::B fusion protein was not as efficient. For each of three plants expressing the GUS::B fusion protein, an average of 30% of the GUS protein was found in the nucleus (Table 2). This result was unexpected from the histochemical analysis because no difference was observed in localization of B region fusion protein for amino- and carboxy-terminal B::GUS fusions.

Results of the biochemical analysis of plants containing the A region fusion protein were also not as predicted by histochemical analysis. Only A::GUS plants were available for biochemical localization studies. In the histochemical analysis, these two plants showed GUS activity partitioned between the nucleus and the cytoplasm (Table 1). In the biochemical analysis, these two plants showed almost all of the GUS activity in the cytoplasmic fraction (Table 2). However, statistical analysis showed that the amount of GUS activity in the cytoplasm could not account for the activity in the total extract, and thus average GUS activity measured in the nuclear fraction, 4.3%, was considered significant. We concluded that the NLS in the B region is very efficient for nuclear targeting and that the NLS in the A region is much less so.

A and B Regions Redirect the GUS Protein in Transiently Transformed Onion Epidermal Cells

Results presented above indicate that the nuclear import machinery in dicot (tobacco) cells was able to recognize NLSs in the A and B regions of the O2 protein. To examine nuclear transport of the O2 protein in a monocot system, a set of constructs encoding the fusion proteins containing regions of O2 shown in Figure 1 was made using the monocot vector pMF6 (Figure 2B; Goff et al., 1990). Onion epidermal layers were transiently transformed using Biolistic particle bombardment. Histochemical X-gluc assays were performed to determine localization of the GUS protein and the O2/GUS fusion proteins. When color development was evident, epidermal layers were mounted in the presence of the nucleus-specific stain DAPI. Results from the histochemical analysis of transformed onion cells are shown in Figure 3 and Table 3. As was observed in

Table 2. Summary of Biochemical Localization of O2/GUS Fusion Proteins in Tobacco

Construct	% Nuclear	% Cytoplasmic
GUS-1[a]	0.52 ± 0.01	103 ± 5[b]
GUS-1[a]	0.36 ± 0.08	108 ± 8[b]
GUS-2	3.0 ± 0.1	99 ± 5
A::GUS-1	5.8 ± 0.4	97 ± 6[c]
A::GUS-2	2.8 ± 0.2	96 ± 8[c]
B::GUS-1	109 ± 9[b]	16 ± 1
B::GUS-2	103 ± 4[b]	11 ± 2
GUS::B-1	38 ± 1	45 ± 2
GUS::B-2	23 ± 1	54 ± 4
GUS::B-3	29.5 ± 0.4	79 ± 6
C::GUS-1	1.0 ± 0.1	100 ± 5[b]
C::GUS-2	1.9 ± 0.1	89 ± 3[b]
GUS::C-1	1.9 ± 0.2	90 ± 3
GUS::C-2	1.1 ± 0.2	100 ± 11[b]

[a] Data from two independent nuclei isolations.
[b] Compared lines not significantly different at the 95% level.
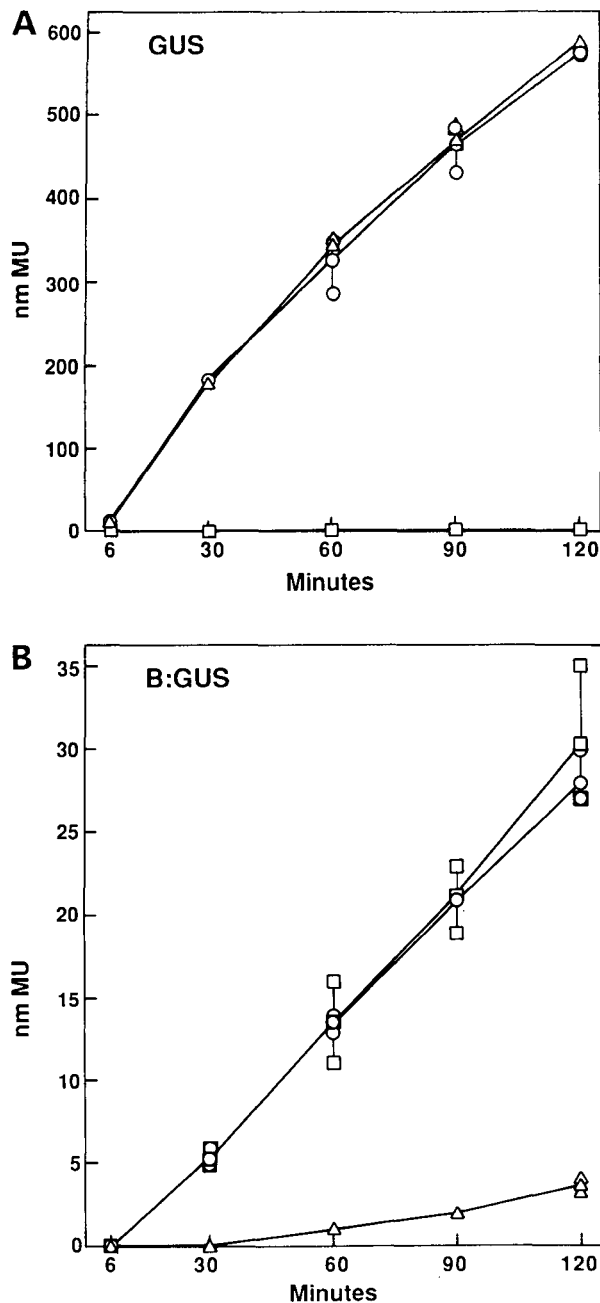[c] Compared lines significantly different at the 95% level.

Figure 4. Biochemical Localization of GUS Activity in Two Transgenic Plants. .

(A) Analysis of GUS activity in nuclear, cytoplasmic, and total extracts of the GUS-1 plant.
(B) Analysis of GUS activity in nuclear, cytoplasmic, and total extracts of the B::GUS-1 plant.
MUG accumulation given in: O, total extract; △, cytoplasmic fraction; and □, nuclear fraction. Range of values in triplicate samples is indicated.

tobacco, the GUS protein expressed in onion cells was localized in the cytoplasm (Figures 3d and 3d').

Although it was difficult to obtain tobacco plants that would express constructs encoding the full-length O2 protein fused to GUS, it was possible to examine all of the generated constructs in the transient onion system. Fusion proteins containing the entire O2 protein fused to GUS localized to the nucleus (Table 3). As seen in tobacco, fusion proteins containing the AB region also localized to the nucleus (Table 3 and Figures 3e and 3e'). Transformed onion cells containing the GUS::BC fusion protein showed localization to the nucleus and the cytoplasm just outside of the nucleus (Table 3).

Constructs containing the A, B, and C regions of O2 (Figure 1) showed similar results in the monocot system (Figures 3f, 3f', 3g, and 3g' and Table 3), as observed in the tobacco histochemical localization experiments (Figures 3a, 3a', 3b, 3b', 3c, and 3c' and Table 1). Fusion proteins containing the C region localized to the cytoplasm (Table 3), fusion proteins containing the B region localized to the nucleus (Figures 3g and 3g'), and fusion proteins containing the A region localized to the nucleus (Figures 3f and 3f'). However, the A region, when presented as a carboxy-terminal fusion (GUS::A), showed GUS activity partitioned between the nucleus and the cytoplasm (Table 3). These results demonstrated that nuclear protein targeting in the onion transformation system is identical to nuclear protein targeting in the transgenic tobacco plants. Thus, the onion system was used to further define the NLSs in the A and B regions of the O2 protein.

The A Region Contains an SV40-like NLS

Localization of fusion proteins containing the A region of the O2 protein showed that these 135 amino acids contain an NLS that is recognized in cells of both tobacco and onion. Analysis of the amino acid sequence of the A region revealed only one small area that resembles SV40-like NLSs, an RRK sequence found at amino acids 128 to 130, indicated by the filled circles in the A region of Figures 1 and 5. This area containing the

Table 3. Summary of Histochemical Localization of O2/GUS Fusion Proteins in Onion

Fusion[a]	Fusion::GUS[b]	GUS::Fusion[b]
O2	N	N
AB	N	N
BC	—[c]	N,C near
A	N	N > C
B	N	N
C	C	C

[a] Refer to Figure 1 for names of fusion proteins.
[b] Localization of proteins: N, nuclear; C, cytoplasmic; C near, in the cytoplasm just outside of the nucleus; N > C, more protein in the nucleus than in the cytoplasm.
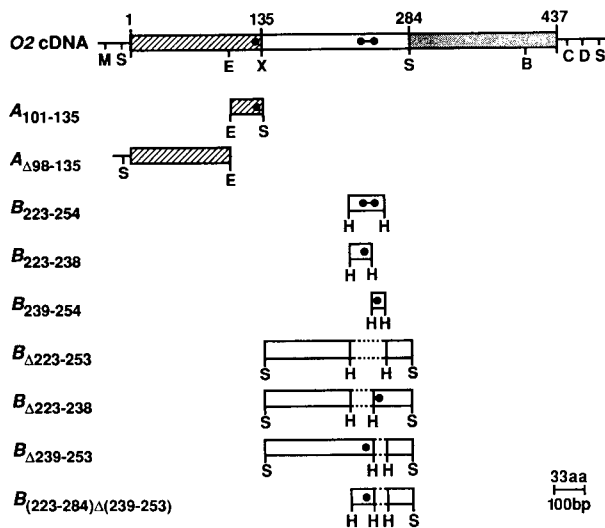[c] Construct not available.

Figure 5. Constructs Used to Define NLSs in the A and B Regions of O2 Protein.

Partial restriction map of the original O2 cDNA clone with positions of amino acids indicated. Partial restriction maps of constructs used to define the A and B region NLSs (filled circles). These constructs were prepared from initial constructs used to generate fusion proteins A::GUS, B::GUS, and GUS::B (see Methods). Abbreviations of restriction enzymes: B, BamHI; C, ClaI; D, HindIII; E, EagI; H, HpaI; M, SmaI; S, SalI; X, XhoI. aa, amino acid. Box designations are the same as in Figure 1.

putative NLS could be isolated from the rest of the A domain using an EagI restriction digest of the A::GUS fusion construct (Figure 5; see Methods for details). We examined the localization of fusion proteins containing 36 amino acids, including the putative NLS fused to GUS (Figure 5, A$_{101-135}$::GUS), and those in which these amino acids had been deleted (Figure 5, A$_{\Delta 98-135}$::GUS). Data from these experiments are shown in Table 4 and Figure 3. In transformed onion cells, the fusion protein containing the putative NLS (A$_{101-135}$::GUS) localized to the nucleus and to the cytoplasm surrounding the nucleus (Figures 3h and 3h' and Table 4). These results confirmed that the NLS in the A region is a relatively inefficient targeting signal. The fusion protein without the NLS (A$_{\Delta 98-135}$::GUS) remained in the cytoplasm (Table 4). Thus, the SV40-like signal, found at the carboxy-terminal end of the A region, is the only NLS in the A region.

The B Region Contains a Bipartite NLS

Analysis of the 149 amino acids in the B region indicated that the putative NLS might be a bipartite signal located within the basic DNA binding domain (Figures 1 and 5, filled circles in the B region). This putative signal consists of four amino acids (RKRK, beginning at amino acid 230), followed by seven amino acids and then a group of seven amino acids where five of

the seven are basic (RRSRYRK). Site-directed mutagenesis was used to make a series of constructs to analyze the putative bipartite NLS and its individual halves (Figure 5). Results from the onion transformation experiments are shown in Table 4.

Fusion proteins consisting of 32 amino acids, including the entire putative bipartite NLS (Figure 5, B$_{223-254}$::GUS), were localized to the nucleus (Figures 3i and 3i' and Table 4). However, when the individual halves of the NLS were fused separately to GUS (Figure 5, B$_{223-238}$::GUS and B$_{239-254}$::GUS), neither half was able to redirect GUS to the nucleus (Table 4). Although, the first half did appear to have some affinity for the nuclear envelope. We concluded from these experiments that the B region contains a bipartite NLS that is able to redirect the GUS protein to the nucleus.

Deletion constructs were made to examine the remaining amino acids in the B region and to determine the targeting ability of the halves of the bipartite signal when presented in the context of the whole B region. In one construct (Figure 5, B$_{\Delta 223-253}$::GUS), the entire bipartite NLS was deleted from the B region. Fusion protein from this construct remained in the cytoplasm (Table 4), and thus proved that no additional NLSs are present in the B region.

Two constructs were made that contained the B region with either half of the bipartite NLS deleted (Figure 5, B$_{\Delta 223-238}$::GUS and B$_{\Delta 239-253}$::GUS). When these were fused to the amino terminus of GUS, the fusion protein from the construct missing the first half of the bipartite NLS (B$_{\Delta 223-238}$::GUS) was localized to the cytoplasm; however, the fusion protein from the construct missing the second half (B$_{\Delta 239-253}$::GUS) was localized to the nucleus (Table 4). These results, taken together with the analysis of the B$_{223-238}$::GUS construct, led us to suggest that the first half of the NLS may be partially recognized by the import machinery.

Several new constructs were tested to determine why the first half of the bipartite NLS appears sufficient for localization in the context of the B$_{\Delta 239-253}$::GUS construct but not

Table 4. Histochemical Localization Analysis of the A and B Regions

Fusion[a]	Fusion::GUS[b]	GUS::Fusion[b]
A$_{101-135}$	N,C near	—[c]
A$_{\Delta 98-135}$	C	—
B$_{223-254}$	N	—
B$_{223-238}$	C near	—
B$_{239-254}$	C	—
B$_{\Delta 223-253}$	C	C
B$_{\Delta 223-238}$	C	C
B$_{\Delta 239-253}$	N	C
B$_{(223-284)\Delta(239-253)}$	C	—

[a] Refer to Figure 5 for names of fusion proteins.
[b] Localization of proteins: N, nuclear; C, cytoplasmic; C near, in the cytoplasm just outside of the nucleus.
[c] Construct not available.

when present separately in the $B_{223-238}$::*GUS* construct. The first of the new constructs tested contained a deletion of the second half of the bipartite NLS but otherwise retained the carboxy-terminal amino acids in the B region (Figure 5, $B_{(223-284)\Delta(239-253)}$::*GUS*). This region could either serve as the second half of the bipartite NLS or enhance the exposure of amino acids 223 to 238. Fusion protein from this construct localized to the cytoplasm (Table 4), showing that the carboxy-terminal amino acids could not substitute for the second half of the bipartite signal and confirming that amino acids 223 to 238 are not sufficient for targeting. Preliminary results from a similar construct containing only the amino-terminal amino acids from the B region through the first half of the NLS ($B_{136-238}$::*GUS*) showed a localization pattern similar to the construct containing only the first half of the bipartite signal ($B_{223-238}$::*GUS*), with localization just outside of the nucleus (data not shown).

The other new constructs that were tested were designed to address the question of whether the conformational context of the entire B region contributes to the recognition of amino acids 223 to 238. For these experiments, constructs were made in which the deleted B regions were fused to the carboxy-terminal end of GUS (Figure 5, *GUS*::$B_{\Delta223-238}$ and *GUS*::$B_{\Delta239-253}$); proteins from these constructs localized to the cytoplasm (Table 4). These data suggest that nuclear targeting of the $B_{\Delta239-253}$::GUS fusion protein is dependent on the presentation of the entire B region (see Discussion).

DISCUSSION

The O2 Protein Contains Two Regions That Are Involved in Nuclear Targeting

Two regions of the O2 protein are competent to redirect the GUS protein to the nucleus; these are shown in Figure 6A. NLS A (Figure 6A) has a structure similar to the SV40 NLS (Kalderon et al., 1984a,1984b). Biochemical analysis of nuclei isolated from transgenic plants indicated that NLS A is not efficient in targeting GUS to the nucleus (Table 2). Histochemical analysis of A::GUS proteins in tobacco and GUS::A and $A_{101-135}$::GUS proteins in onions showed partitioning of GUS activity between the cytoplasmic and nuclear compartments (Tables 3 and 4). We concluded that the NLS A is a weak targeting signal.

As shown in Figure 6A, NLS B is found in the basic domain of O2 and has a bipartite structure. Biochemical analysis of B::GUS fusion proteins suggests that NLS B is efficient in redirecting GUS to the nucleus (Table 2). Histochemical analysis has shown that the entire bipartite signal is sufficient to redirect GUS to the nucleus. However, in some constructs, the first half may be recognized by the import machinery (Table 4). Other studies show that the first half of bipartite NLSs may be a more efficient targeting signal than the second half (Carrington et al., 1991; Howard et al., 1992; Xia et al., 1992).

The basic domain of all plant bZIP proteins is highly conserved (Figure 6B); thus, NLSs may be present in the basic domain of other plant bZIP proteins. van der Krol and Chua (1991) identify an NLS in the basic domain and bZIP domain of two tobacco bZIP proteins, TGA-1B and TGA-1A, respectively. These authors propose that the NLSs in the TGA proteins are SV40-like in structure; however, the smallest fusion that was tested contained 24 amino acids, and thus includes an entire bipartite NLS. Other plant bZIP proteins do not have exactly the same basic amino acids in the same positions as O2 and the TGA proteins; however, they do have basic amino acids nearby that will fit the model consensus for bipartite NLSs (Dingwall and Laskey, 1991).

Nonplant bZIP proteins have been studied extensively for their role in DNA binding (Struhl, 1989; Vinson et al., 1989); however, only preliminary studies on nuclear targeting have been reported. Roux et al. (1990) examined nuclear targeting of c-Fos, but did not identify an NLS. However, it is probable that the NLS in c-Fos and v-Fos is located in a conserved region in the center of the two proteins which contains the bZIP domain (Jenuwein and Müller, 1987; Roux et al., 1990). Recently, Chida and Vogt (1992) identified a complex NLS in the basic domain of c-Jun. Although the basic domains of nonplant proteins are not as highly related to the basic domains of plant bZIP proteins, it is possible that the basic domains of all bZIP proteins may contain NLSs and thus may serve a dual function as both the DNA binding domain and as an NLS.

Because we have proposed a dual role for the basic domain in O2 and other bZIP proteins, it is important to distinguish between the DNA binding and NLS functions of this domain. The O2 mutant *o2-676* has an R to K mutation at amino acid 244 in the basic domain and will no longer bind DNA (Aukerman et al., 1991). Preliminary studies using immunolocalization of the mutant protein in immature maize kernels harboring the *o2-676* allele show localization of the o2-676 protein to the nucleus. These data indicate that DNA binding of the O2 protein is independent of nuclear localization.

There are a few examples of proteins that arrive in the nucleus through protein–protein interactions (Tsuneoka et al., 1986; Moreland et al., 1987). In one example, a homologous leucine zipper interaction causes nuclear targeting of a reporter protein that has no NLS (Xia et al., 1992). However, it is unlikely that leucine zipper dimerization contributes to the nuclear targeting of the O2/GUS fusion proteins examined in this study. Several of the O2/GUS fusion proteins we examined contain the leucine zipper domain but were localized to the cytoplasm (Table 4, $B_{\Delta223-253}$, $B_{\Delta223-238}$, $B_{\Delta239-253}$, and $B_{(223-284)\Delta(239-253)}$).

Most Plant NLSs Identified Have a Bipartite Structure

The most efficient NLS in the O2 protein (NLS B) has a bipartite structure. As described, this NLS is located in the conserved, basic domain found in all bZIP proteins, and this region contains the NLSs in the two tobacco proteins TGA-1A and TGA-1B (van der Krol and Chua, 1991). Most likely, the

A   **NLS A**   MEEAVTMAPAAVSSAVVGDPMEYNAILRRKLEEDLE(*SRGSPGGQSL*)

     NLS B   MPTEERVRKRKESNRESARRSRYRKAAHLKEL(*GGQSL*)

B

Protein	Basic Domain	Reference
O2 (maize)	RVRKRKESNRESARRSRYRKA	Hartings et al. (1989) and Schmidt et al. (1990)
TGA-1B (tobacco)	KK-A-LVR-----QL--Q--K	VanDerKrol and Chua. (1991)
TGA-1A (tobacco)	K-LR-LAQ---A--K--L--K	VanDerKrol and Chua. (1991)
CPRF-1 (parsley)	KRER--Q----------L--Q	Weisshaar et al. (1991)
CPRF-2 (parsley)	KRVR-ML----------R--Q	Weisshaar et al. (1991)
CPRF-3 (parsley)	KRQR--Q----------L--Q	Weisshaar et al. (1991)
EMBP-1 (wheat)	KRER--Q----------L--Q	Guiltinan et al. (1990)
HBP-1A (wheat)	KKQ---L----------L--Q	Tabata et al. (1989)
HBP-1B (wheat)	KTMR-LAQ---A--K--L--K	Tabata et al. (1991)
OCSBF-1 (maize)	-RE--RL----------L--Q	Singh et al. (1990)
OCSBF-2 (maize)	KKKM-QIR--D--MK--E--K	Singh et al. (1990)
TAF-1 (tobacco)	KRE---Q----------L--Q	Oeda et al. (1991)
GBF1 (arabidopsis)	KRQ---QS---------L--Q	Schindler et al. (1992)
GBF2 (arabidopsis)	KRE---QS---------L--Q	Schindler et al. (1992)
GBF3 (arabidopsis)	KRER--QS---------L--Q	Schindler et al. (1992)
GCN4 (yeast)	PAALKRAR-T-A-----A--L	Hinnebusch. (1984)
CPC1 (neurospora)	V-AMKRAR-TLA--K--E---	Paluh et al. (1988)
C/EBP (rat)	EY-V-RER-NIAV-K--DKAK	Landschulz et al. (1988)
C-JUN (human)	KAERKRMR--IA-SKC-K--L	Angel et al. (1988)
C-FOS (human)	KR-I-R-R-KMA-AKC-N-RR	VanStraaten et al. (1983)

C

NIa   GKKNQKHKLKM-32aa-KRKGTTRGMGAKSRKFINMYGFDPTDFSYI   Carrington et al. (1991)

VirD2   VLSKRPREDDDGEPSERKRERDERSKDGRGGNRR   Howard et al. (1992)

VirE2   NSE1   KLDRNYKLRPEDRYIQTEKYGRREIQKRYEH   Citovsky et al. (1992)

VirE2   NSE2   FEEFERAIKTKYGSDTEIKLKSKSGIMHDSK   Citovsky et al. (1992)

Figure 6. NLSs and Putative NLSs in O2 and Other Plant Proteins.

Proposed amino acids critical for nuclear targeting are underlined.
(A) Amino acid sequences of O2 NLSs shown to redirect GUS to the nucleus. Amino acids contributed by cloning procedures are italicized and in parentheses.
(B) Table of basic domains in bZIP proteins. Amino acids conserved between O2 and other bZIP proteins are indicated by dashes.
(C) NLSs identified in other proteins targeted to plant cell nuclei. aa, amino acid.

TGA NLSs also have a bipartite structure. Bipartite NLSs have also been identified in proteins from plant viral and bacterial pathogens. Figure 6C shows the amino acid sequences of NLSs that have redirected GUS fusion proteins to the plant cell nucleus. These bipartite NLSs are found in the NIa protein from potyvirus (Carrington et al., 1991) and in the VirD2 and VirE2 encoded on the Agrobacterium Ti plasmid (Howard et al., 1992; Citovsky et al., 1992, respectively). Dingwall and Laskey (1991) suggest that the bipartite NLS is the most prevalent NLS in nonplant proteins. From this small data base of plant proteins, it appears that the bipartite NLS may be the most prevalent NLS in all nuclear proteins.

Many proteins contain multiple NLSs (for review, see Garcia-Bustos et al., 1991). We identified two NLSs in the O2 protein; Citovsky et al. (1992) identify two bipartite NLSs in VirE2 (Figure 6C). It is thought that multiple NLSs increase the efficiency of nuclear transport of very large proteins (Dingwall et al., 1982; Dworetzky et al., 1988). Further analysis will determine if both of the NLSs that we have identified in O2 are required for localization of the intact protein.

Small Nuclear Proteins and NLSs

Historically, it has been assumed that small nuclear proteins, 40 to 60 kD, could diffuse through the nuclear pore (Bonner, 1975). However, no physiologically relevant macromolecule has been shown to diffuse to the nucleus. In most cases, when determining if NLSs are present in small nuclear proteins, NLSs have been found. In this report, we show that the 47-kD O2 protein contains NLSs that are capable of redirecting a 68-kD GUS protein to the nucleus of both dicot and monocot cells.

In addition, the 13.8-kD histone 2B protein in yeast (Moreland et al., 1987) and the 28-kD high-mobility group 1 protein from calf thymus (Tsuneoka et al., 1986) contain NLSs. Although no NLS was identified in the 21-kD histone H1 from calf thymus, nuclear import of this small nuclear protein is arrested by chilling and energy depletion, which are characteristics associated with NLS-mediated transport (Breeuwer and Goldfarb, 1990). It is possible that all small nuclear proteins are targeted to the nucleus using a signal-mediated process.

One report that presents data suggesting passive diffusion of small nuclear proteins is that of van der Krol and Chua (1991). The authors analyze a basic region of the 23-kD TFIID protein from Arabidopsis for redirection of the GUS reporter protein and find that the tested region does not redirect GUS to the nucleus. However, the authors did not test fusions of the entire protein, and thus, an NLS in TFIID may have escaped detection.

Effect of Amino- versus Carboxy-Terminal GUS Fusions

Restrepo et al. (1990) found that plants expressing amino-terminal GUS fusions of NIa and NIb potyviral proteins have significantly less GUS enzymatic activity than plants expressing carboxy-terminal fusions. Based on this result, many studies (Carrington et al., 1991; Citovsky et al., 1992; Howard et al., 1992) have used only carboxy-terminal GUS fusions to identify NLSs. van der Krol and Chua (1991) used only amino-terminal fusions to identify NLSs in tobacco proteins, and in this report we used both amino- and carboxy-terminal GUS fusions to locate NLSs in the O2 protein. We compared total GUS enzymatic activity in transgenic plants expressing the A, B, and C region fusion proteins and found that relative levels of activity could not be predicted by the orientation of the fusion (data not shown). For A and C region fusions, plants containing amino-terminal fusions showed higher levels of GUS activity. For B region fusions, however, plants containing carboxy-terminal fusions showed higher levels of GUS activity than amino-terminal fusions.

Although we found no correlation between total GUS activity and the orientation of GUS fusions, we did find a difference in nuclear targeting of amino- and carboxy-terminal fusions. In general, amino-terminal fusions appeared to be more efficiently targeted than carboxy-terminal fusions. This difference is best seen when comparing enzymatic activity of B::GUS and GUS::B fusion proteins (Table 2). One hundred percent of the B::GUS protein was localized to the nucleus, whereas approximately 30% of the GUS::B fusion protein was localized to the nucleus. Onion histochemical data from fusions made with the A region indicated that the A::GUS fusion protein was targeted to the nucleus better than the GUS::A fusion and that the O2::GUS and AB::GUS fusion proteins were targeted to the nucleus better than the GUS::BC fusion proteins (Table 3). In addition, the B region deletion protein $B_{\Delta239-253}$::GUS localized to the nucleus, whereas the GUS::$B_{\Delta239-253}$ protein localized to the cytoplasm (Table 4).

For fusion proteins containing the B region, tertiary structure of fusion proteins may explain the apparent increased efficiency of nuclear targeting of amino-terminal fusions. Fusion proteins have been analyzed for the presence of an active GUS enzyme, which is thought to be a tetramer (Jefferson, 1987). Thus, regions of O2 may be held in close proximity as a result of tetramerization. In carboxy-terminal fusions, the basic, DNA binding domain, containing the NLS, is located between the GUS tetramer and the leucine zipper domain. It is possible that transient association of zipper domains may reduce the availability of the NLS, causing 30% localization for the GUS::B protein. However, in amino-terminal fusions, the zipper domain is adjacent to GUS with the basic domain free for recognition by the targeting machinery, yielding 100% localization for B::GUS fusion proteins. This model would explain the more efficient targeting of amino-terminal fusions in constructs that contain the B region.

Conclusions

We have identified two NLSs in the O2 protein that were capable of redirecting the GUS reporter protein to the nucleus in both monocot and dicot cells. One of the NLSs identified, NLS A, has a structure similar to an SV40-like NLS (Figure 6A) and is located in the first third of the O2 protein (Figures 1 and 5). The most efficient of these NLSs is the other NLS in O2, NLS B, that has the structure of a bipartite NLS (Figure 6A) and is located in the basic domain of the O2 protein (Figures 1 and 5). The basic domain is conserved in many bZIP proteins; thus, it is possible that this domain confers two functions to bZIP proteins: DNA binding and nuclear localization. Future studies will examine the necessity of the two NLSs in O2 for nuclear targeting of the intact protein.

METHODS

Plant Materials

Tobacco plants (*Nicotiana tabacum* var Wisconsin 38) were maintained in sterile conditions as described in Wilkins et al. (1990). White onions were purchased locally, stored at room temperature in the dark, and used within 3 weeks.

Constructs

All cloning strategies were derived from basic methods described in Sambrook et al. (1989) using restriction enzymes purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN) and other enzymes for molecular manipulations purchased from New England Biolabs (Beverly, MA). In general, regions of the *Opaque-2* (*O2*) cDNA were fused in frame to the β-glucuronidase (*GUS*) cDNA and then fragments encoding the fusion proteins were ligated into the tobacco

expression vector pGA643 (An et al., 1988) and into the maize expression vector pMF6 (Goff et al., 1990). Limited restriction maps of the constructs are shown in Figures 1, 2, and 5. Constructs have been described by vector abbreviation followed by an abbreviated name of the inserted DNA, e.g., pUC::*B*::*GUS* is pUC119 containing the B region of O2 fused to the amino terminus of GUS. Double restriction digests have been designated by the names of enzymes separated by a "/" symbol. When it was necessary to make blunt ends, the enzyme used has been designated by "-T4" or "-Klenow." All cloned inserts and vectors treated with alkaline phosphatase were isolated and purified on low-melting-point agarose gels (FMC, Rockland, ME) followed by phenol extraction and ethanol precipitation. Site-directed mutagenesis was performed using the method of Kunkel et al. (1987). Primers, listed below, have been referred to by either the designated number or by a brief description of the alteration they caused. After each round of mutagenesis, and after making translational fusions, constructs were tested for their integrity by dideoxy sequencing using Sequenase (U.S. Biochemicals, Cleveland, OH).

Primers:

(1)+SmaI CACATGGACATGTATTCCCGGGAGGCACTGCGGGT, O2, bp 1297 to 1325.

(2)−SalI GACGCTAACGTGGACAACAGGGT, O2, bp 841 to 863.

(3)+SalI GATAGTTACCTATTAGTCGACTTGGGCATGGAGCA, O2, bp −21 to 8.

(4)+XhoI CAGGGAGGCAAACAACTCGAGTGAATCAACAACTCTCC, GUS, bp 1792 to 1823.

(5)X→S CTGGAGGAGGACGTCGACGCCTTCAAAATGTG, O2, bp 391 to 422.

(6)+HpaA GAGATTCTGGGGTTCAAGTTAACGATGCCTACCGAGGAA, O2, bp 649 to 681.

(7)+HpaB GAATCCAATAGAGAGTTAACCATGGGATCAGCCAGACG, O2, bp 700 to 725.

(8)+HpaC CGCTCACCTGAAAGAGTTAACCATGGGACTGGAAGAC-CAGGT, O2, bp 744 to 773.

GUS To facilitate making fusion constructs, BamHI/EcoRI fragments containing the *GUS* coding region and *nos* terminator were cloned from the three different reading frame vectors pBI101.1, pBI101.2, and pBI101.3 (Jefferson, 1987) into both pUC119 and pBluescript KS+(pBS) (Figure 2A). These constructs were designated pUC::*GUS.1*, pUC::*GUS.2*, pUC::*GUS.3* and pBS::*GUS.1*, pBS::*GUS.2*, and pBS::*GUS.3*. To prepare pGA::*GUS*, the construct pBS::*GUS.2* was digested with ClaI/XbaI, and the fragment encoding the GUS protein and containing the *nos* terminator was ligated into the plant expression vector pGA643 (Figure 2B). To make pMF::*GUS*, the construct pBS::*GUS.2* was digested with BamHI/EcoRI, and the fragment encoding the GUS protein and containing the *nos* terminator was ligated into the maize transformation vector pMF6 (Figure 2B).

O2::GUS To fuse the entire O2 coding sequence (Figure 1) in frame to the amino terminus of *GUS*, pUC::*O2* was mutagenized with primer 1 to add a SmaI site at the 3′ end of the O2 coding region and primer 2 to remove the internal SalI site. The entire O2 reading frame was then fused in frame to *GUS* by digesting pUC::*O2*,+SmaI−SalI with SmaI and ligating the fragment encoding O2 into pBS::*GUS.2*. To make the pGA::*O2*::*GUS* construct, pBS::*O2*::*GUS* was digested with XbaI/ClaI and the fragment encoding the fusion was ligated into the

pGA643 vector. To prepare the pMF::*O2*::*GUS* construct, pBS::*O2*::*GUS* was digested with SalI/ClaI, and the fragment encoding the fusion was ligated into pMF6 digested with XhoI/ClaI.

GUS::O2 To fuse the entire O2 coding sequence (Figure 1) to the carboxy terminus of *GUS*, pUC::*O2* was mutagenized with primer 2 to remove the SalI site from the O2 coding sequence and also with primer 3 to add a SalI site directly in front of the O2 gene. A *GUS* vector was prepared for all the carboxy-terminal fusions by mutagenizing pUC::*GUS.3* with primer 4, which adds an XhoI site in front of the stop codon in the *GUS* gene. The *GUS*::*O2* fusion construct was then made by digesting pUC::*O2*,−SalI+SalI with SalI and by ligating the insert encoding the entire O2 coding sequence into pUC::*GUS.3*,+XhoI digested with XhoI. To make the pGA::*GUS*::*O2* construct, pUC::*GUS*::*O2* was digested with HindIII-T4/XbaI. The fragment encoding this fusion was cloned into pGA643 digested with XbaI/HpaI. To make the pMF::*GUS*::*O2* construct, a pMF6 vector containing the O2 coding sequence was prepared. This was done by ligating a SmaI/ClaI fragment containing the entire O2 coding sequence into the pMF6 vector digested with ClaI/SstI-T4 (pMF::*O2*). A vector for accepting the *GUS*::*O2* fragment was made by digesting the pMF::*O2* plasmid with BamHI, which removed most of the O2 sequences except the 3′ end. The fusion construct pUC::*GUS*::*O2* was then digested with BamHI, and the fragment containing most of the O2 sequence was cloned into the BamHI-digested pMF::*O2* vector, thereby reconstituting the entire intact O2 coding sequence.

AB::GUS To fuse the first 284 amino acids from the O2 protein (Figure 1, AB) to the amino terminus of *GUS*, the pUC::*O2* construct was mutagenized with primer 3 to add a SalI site in front of the O2 protein. The resulting plasmid (pUC::*O2*,+SalI) was digested with SalI and the fragment encoding the AB region was ligated into pUC::*GUS.1*. To prepare the pGA::*AB*::*GUS* construct, pUC::*AB*::*GUS* was digested with (SphI/SacI)-T4, and the fragment encoding the fusion was ligated into pGA643 digested with HpaI. To make the pMF::*AB*::*GUS* construct, pUC::*AB*::*GUS* was digested with SphI-T4/SacI, and the fragment encoding the fusion was ligated into pMF6 digested with ClaI-T4/SacI.

GUS::AB To fuse the first 284 amino acids of O2 (Figure 1) to the carboxy terminus of GUS, pUC::*O2*,+SalI was digested with SalI, and the fragment encoding the AB region was ligated into pUC::*GUS.3*, +XhoI digested with XhoI. To make the pGA::*GUS*::*AB* construct, pUC::*GUS*::*AB* was digested with XbaI/SacI-T4, and the fragment encoding the fusion was ligated into pGA643 digested with XbaI/HpaI. To prepare the pMF::*GUS*::*AB* construct, pUC::*GUS*::*AB* was digested with BamHI/SacI, and the fragment encoding the fusion was ligated into pMF6.

GUS::BC Constructs containing O2 amino acids 136 to 437 (Figure 1, BC) were made by mutagenizing pUC::*O2* with primer 5 to change the first XhoI site in O2 to a SalI site (X→S) and with primer 2 to remove the internal SalI site. The resulting plasmid (pUC::*O2*,X→ S-SalI) was digested with SalI and the O2 BC fragment was ligated into pUC::*GUS.3*,+XhoI digested with XhoI. To make the pMF::*GUS*::*BC* construct, pUC::*GUS*::*BC* was digested with BamHI, and the fragment containing *GUS* and most of O2 was ligated into BamHI-digested pMF::*O2* (similar to the pMF::*GUS*::*O2* construct, see above). To prepare the pGA::*GUS*::*BC* construct, pMF::*GUS*::*BC* was digested with SmaI/BglII, and the fragment encoding the fusion was ligated into pGA643 digested with HpaI/BglII.

A::GUS   For amino-terminal fusions of the first 135 amino acids of O2 (Figure 1, A) to GUS, pUC::O2,X→S was digested with SalI. The fragment encoding the A region was isolated and ligated into pUC::GUS.1 digested with SalI. The fusion construct pUC::A::GUS was then digested with HindIII/SacI-T4, and the fragment encoding the fusion was ligated into pGA643 digested with HindIII/HpaI. To make the pMF::A::GUS construct, pUC::A::GUS was digested with SphI-T4/SacI, and the fragment encoding the fusion was ligated into pMF6 digested with ClaI-T4/SacI.

GUS::A   To make the pUC::GUS::A construct (Figure 1), pUC::O2,+SalI was digested with SalI/XhoI, and the fragment containing the A region was ligated into pUC::GUS.3,+XhoI digested with XhoI. To make the pGA::GUS::A construct, pUC::GUS::A was digested with XbaI/SacI-T4, and the fragment encoding the fusion was ligated into pGA643 digested with XbaI/HpaI. To make the pMF::GUS::A construct, pUC::GUS::A was digested with BamHI/SacI, and the fragment encoding the fusion was ligated into pMF6.

B::GUS   To make the pUC::B::GUS construct (Figure 1), pUC::O2,X→S was digested with SalI, and the fragment encoding the B region was ligated into pUC::GUS.1 digested with SalI. To make the pGA::B::GUS construct, pUC::B::GUS was digested with (SphI/SacI)-T4, and the fragment encoding the fusion was ligated into pGA643 digested with HpaI. To make the pMF::B::GUS construct, pUC::B::GUS was digested with SphI-T4/SacI, and the fragment encoding the fusion was ligated into pMF6 digested with ClaI-T4/SacI.

GUS::B   To make the pUC::GUS::B construct (Figure 1), pUC::O2,X→S was digested with SalI, and the fragment encoding the B region was ligated into pUC::GUS.3,+XhoI digested with XhoI. To make the pGA::GUS::B construct, pUC::GUS::B was digested with XbaI/SacI-T4, and the fragment encoding the fusion was ligated into pGA643 digested with XbaI/HpaI. To make the pMF::GUS::B construct, pUC::GUS::B was digested with BamHI/SacI, and the fragment encoding the fusion was ligated into pMF6.

C::GUS   To make the pBS::C::GUS construct (Figure 1), pUC::O2, +SmaI was digested with SalI-Klenow/SmaI, and the fragment encoding the C region was ligated into pBS::GUS.2 digested with SmaI. To make pGA::C::GUS, pBS::C::GUS was digested with XbaI/ClaI, and the fragment encoding the fusion was ligated into pGA643. To make pMF::C::GUS, pBS::C::GUS was digested with (SacII/KpnI)-T4, and the fragment encoding the fusion was ligated into pMF6 digested with KpnI-T4.

GUS::C   To make the pUC::GUS::C construct (Figure 1), pUC::O2 was digested with SalI, and the fragment encoding the C region was isolated and ligated into pUC::GUS.3,+XhoI digested with XhoI. To make pGA::GUS::C, pUC::GUS::C was digested with XbaI/EcoRI-Klenow, and the fragment encoding the fusion was ligated into pGA643 digested with XbaI/HpaI. To prepare pMF::GUS::C, pUC::GUS::C was digested with XbaI-Klenow/EcoRI, and the fragment encoding the fusion was ligated into pMF6 digested with BamHI-Klenow/EcoRI.

A$_{101-135}$::GUS   To make pMF::A$_{101-135}$::GUS (Figure 5), pUC::A::GUS.1 was digested with EagI-T4/SacI, and the fragment encoding the fusion was ligated into pMF6 digested with ClaI-T4/SacI.

A$_{\Delta98-135}$::GUS   To make the pMF::A$_{\Delta98-135}$::GUS construct (Figure 5), pUC::GUS.1 was digested with SmaI/EcoRI, and the fragment encod-

ing GUS and the *nos* terminator was cloned into pUC::A::GUS.1 digested with EagI-T4/EcoRI. The fused fragment was then ligated into the pMF6 vector by digesting pUC::A$_{\Delta98-135}$::GUS with SphI-T4/SacI and ligating into pMF6 digested with ClaI-T4/SacI.

B$_{223-254}$::GUS   To prepare the pMF::B$_{223-254}$::GUS construct (Figure 5), pUC::B::GUS was mutagenized with primer 8; the resulting plasmid (pUC::B::GUS,+HpaC) was digested with HpaI/SmaI and religated to remove the O2 DNA encoding amino acids 255 to 284. This plasmid (pUC::B$_{136-254}$::GUS) was mutagenized with primer 6, the resulting plasmid (pUC::B$_{136-254}$::GUS,+HpaA) was digested with HpaI/SacI, and the fragment encoding the fusion was ligated into pMF6 digested with ClaI-T4/SacI.

B$_{223-238}$::GUS and B$_{239-254}$::GUS   To make the pMF::B$_{223-238}$::GUS and pMF::B$_{239-254}$::GUS constructs (Figure 5), essentially the same steps as described above for pMF::B$_{223-254}$::GUS were used. For pMF::B$_{223-238}$::GUS, primer 7 was used in the first mutagenesis step to remove the region encoding O2 amino acids 239 to 284. And for pMF::B$_{239-254}$::GUS, primer 7 was used in the second mutagenesis step to allow fusion of only O2 amino acids 239 to 254.

B$_{\Delta223-253}$::GUS   To prepare the pMF::B$_{\Delta223-253}$::GUS construct (Figure 5), pUC::B::GUS was mutagenized with primers 6 and 8; the resulting plasmid (pUC::B::GUS,+HpaA+HpaC) was digested with HpaI and religated to remove the O2 sequences that encode amino acids 223 to 253. This plasmid (pUC::B$_{\Delta223-253}$::GUS) was digested with SphI-T4/SacI and the fusion fragment ligated into pMF6 digested with ClaI-T4/SacI.

B$_{\Delta223-238}$::GUS and B$_{\Delta239-253}$::GUS   Constructs pMF::B$_{\Delta223-238}$::GUS and pMF::B$_{\Delta239-253}$::GUS (Figure 5) were made essentially as described above for pMF::B$_{\Delta223-253}$::GUS except that the combination of primers used in the mutagenesis step were primers 6 and 7 for pMF::B$_{\Delta223-238}$::GUS, and primers 7 and 8 for pMF::B$_{\Delta239-253}$::GUS.

GUS::B$_{\Delta223-253}$ To make the pMF::GUS::B$_{\Delta223-253}$ construct (Figure 5), pUC::GUS::B was mutagenized with primers 6 and 8 to make pUC::GUS::B,+HpaA+HpaC, and the resulting plasmid was digested with HpaI and religated to delete the O2 sequences encoding amino acids 223 to 253. This plasmid (pUC::GUS::B$_{\Delta223-253}$) was digested with BamHI/SacI and the fusion fragment was moved into the pMF6 vector.

GUS::B$_{\Delta223-238}$ and GUS::B$_{\Delta239-253}$ Constructs pMF::GUS::B$_{\Delta223-238}$ and pMF::GUS::B$_{\Delta239-253}$ (Figure 5) were made using essentially the same steps as described above for pMF::GUS::B$_{\Delta223-253}$. For pMF::GUS::B$_{\Delta223-238}$, primers 6 and 7 were used in the mutagenesis step to remove only O2 amino acids 223 to 238, and for pMF::GUS::B$_{\Delta239-253}$, primers 7 and 8 were used to remove only O2 amino acids 239 to 253.

B$_{(223-284)\Delta(239-253)}$::GUS   To make the pMF::B$_{(223-284)\Delta(239-253)}$::GUS construct (Figure 5), pUC::B::GUS was mutagenized with primer 6 to make pUC::B::GUS,+HpaA; this plasmid was digested with (PstI-HpaI)-T4 and religated to delete the O2 sequences encoding amino acids 136 to 222. The resulting plasmid (pUC::B$_{223-284}$::GUS) was mutagenized with primers 7 and 9 to make pUC::B$_{223-284}$::GUS,+HpaB+HpaC; this plasmid was digested with HpaI and religated to delete the O2 sequences encoding amino acids 239 to 253. This final construct pUC::B$_{(223-284)\Delta(239-253)}$::GUS was digested with SphI-T4/SacI to move the fragment encoding the fusion into pMF6 digested with ClaI-T4/SacI.

Tobacco Transformation System

Constructs for tobacco transformations were electroporated into *Agrobacterium tumefaciens* LBA4404 using a Bio-Rad Gene Pulser (Richmond, CA). The method for electroporation was essentially that described by the manufacturer for transformation of *Escherichia coli* (Dower et al., 1988). Tobacco leaf discs were transformed and transgenic plants were maintained as described in Wilkins et al. (1990). Kanamycin-resistant plants were tested for stability of expression of GUS fusion constructs by screening after each subculture using the X-glucuronide (X-gluc) assay (Jefferson, 1987).

Onion Transformation System

The Helium Biolistic gene transformation system (Du Pont) was used to transform onion epidermal cell layers with O2/GUS fusion constructs made using the maize plasmid vector pMF6 (Goff et al., 1990). Inner epidermal layers were peeled and placed inside up on Petri dishes containing Murashige-Skoog (MS; Murashige and Skoog, 1962) basal media (per liter: 4.3 g MS salts [Gibco-Bethesda Research Laboratories, Gaithersburg, MD], 1 mg thiamine, 10 mg myoinositol, 180 mg KH$_2$PO$_4$ [Miller's I], 30 g sucrose, pH 5.7) with 2.5 mg/L amphotericin B (Sigma, St. Louis, MO) and 6% agar. DNA samples were prepared as described by the manufacturer (Du Pont); 2.5 μg of column purified (Quigen, Chatsworth, CA) plasmid DNA was precipitated onto 1.25 mg of 1.6-μm gold particles using 25 μL of 2.5 M CaCl$_2$ and 10 μL of 0.1 M thiamine (free base, Sigma). DNA-coated particles were washed with 125 μL of 100% ethanol and then resuspended in 30 μL of 100% ethanol. These samples were sonicated using a cup horn probe at approximately 30% power until the sample was dispersed ($\sim$4 sec). The sample was divided evenly onto two particle delivery discs, and both discs were used to transform a single set of onion cell layers, thus allowing delivery of the whole 2.5 μg of DNA per sample. Each construct was analyzed at least three times. Pressure at 1300 p.s.i. was found to be optimal for delivery into onion layers. After particle bombardment, Petri dishes were sealed with parafilm and incubated for 18 to 24 hr at 28°C in the dark.

Histochemical Analysis

The colorimetric X-gluc assay was used to determine the location of O2::GUS fusion proteins (Jefferson, 1987). Tobacco and onion tissues were incubated at 37°C in X-gluc solution (50 mM NaPO$_4$, pH 7.0, 1 mM EDTA, 0.001% Triton X-100, 10 mM 2-mercaptoethanol, 0.05 mM sodium ferricyanide and sodium ferrocyanide, 2 mM X-gluc). Tobacco roots and epidermal layers were monitored every 15 to 30 min for 3 to 5 hr and then examined after 10 hr; onion epidermal layers were examined every hour after an initial 2-hr incubation. After detection of blue color, tissues were mounted on a clean glass slide using a solution that contained the nucleus-specific dye 4',6-diamidino-2-phenylindole (DAPI; Varagona et al., 1991). Intercellular localization of the blue precipitate was determined using a Zeiss Axiophot microscope with differential interference microscopy or bright-field optics. Location of the blue precipitate was compared with the location of DAPI-stained nuclei using fluorescence optics (Varagona et al., 1991).

Biochemical Localization

To quantitate the steady state levels of O2/GUS fusion proteins in the nucleus and cytoplasm, nuclei were isolated from leaf protoplasts from transgenic plants and were analyzed using the fluorogenic 4-methylumbelliferyl β-D-glucuronide (MUG) GUS assay. Leaf protoplasts were prepared as described by Bednarek et al. (1990), and nuclei isolation procedures were based on those described by Willmitzer and Wagner (1981), Saxena et al. (1985), and Carrington et al. (1991) with the modifications indicated below. Isolated protoplasts were resuspended in 3 mL of nuclei isolation buffer (NIB) (10 mM Mes-KOH, pH 5.6, 0.2 M sucrose, 10 mM NaCl, 10 mM KCl, 2.5 mM EDTA, 2.5 mM DTT, 0.1 mM spermine, and 0.5 mM spermidine) containing 0.3% Triton X-100. Cells were incubated on ice for 7 min and then lysed by passing five times through a 25-gauge needle. A 0.5-mL aliquot was removed to be used as a total cell extract sample. The remaining extract was placed on a two-step gradient consisting of 1.5 mL (15%) and 1 mL (50%) Percoll (Sigma) in NIB containing 0.01% Triton X-100. Gradients were centrifuged for 10 min at 1000g. The top fraction was retained as the cytoplasmic fraction, and the interface between the 15 and 50% Percoll steps was analyzed on the microscope. If the interface contained only nuclei, then the fraction was resuspended in 6 mL of nuclei resuspension buffer (NRB; NIB containing 20% glycerol), centrifuged for 10 min at 750g, and the resulting pellet was resuspended in 1 mL of NRB. However, if the nuclear fraction contained a significant amount of chloroplasts, the chloroplasts were removed by resuspending the fraction at the 15 to 50% interface in 6 mL of NIB containing 0.6% Triton X-100 and incubated for 20 min on ice. The resulting extract was loaded on a fresh 15 to 50% Percoll step gradient and processed as above.

To normalize the amounts of extracts used in the MUG assays, total, nuclear, cytoplasmic, and chloroplast fractions were analyzed for the presence of nuclei and for the activity of the cytoplasmic enzyme glucose-6-phosphate dehydrogenase (GPD). Nuclei were analyzed in each fraction by diluting 5 μL of the fraction into a mixture of 44 μL NRB and 1 μL DAPI mount (Varagona et al., 1991) and counted using a hemacytometer. GPD assays were done as described by Simcox et al. (1977). A 100-μL aliquot of each sample was analyzed spectrophotometrically for the reduction of NADPH. Nuclear and chloroplast fractions never showed a significant level of GPD activity. Amounts of extract used in the MUG assays were determined by using an equal number of nuclei (30,000) for the nuclear and total fractions, and an amount of the cytoplasmic fraction that contained an equal amount of GPD activity as the total fraction.

Normalized amounts of nuclear, cytoplasmic, and total fractions were first treated with DNase I and centrifuged to release nucleoplasmic proteins. Then MUG assays were performed in triplicate, essentially as described by Jefferson (1987). Fractions were brought to 200 μL in DNase I buffer (50 mM Tris-Cl, pH 7.5, 10 mM MgCl$_2$, 50 μg/mL BSA, 4 unit/sample DNase I [Tabor and Struhl, 1991]). Samples were incubated for 15 min at 37°C, and reactions were terminated with the addition of 5 μL of 0.5 M EDTA. Samples were then centrifuged for 3 min in a microcentrifuge at full speed, and 100 μL of GUS assay buffer (50 mM NaPO$_4$, pH 7.0, 1 mM EDTA, 0.01% Triton X-100, 10 mM 2-mercaptoethanol) was added to each sample. MUG assays began by the addition of 200 μL of GUS assay buffer containing 1 mM MUG (Clonetech, San Francisco, CA) and by immediately vortexing the sample. After 6 min, a 50-μL aliquot was removed into 950 μL of 0.2 M CaCO$_3$ stop solution. Reactions were then incubated at 37°C, and 100-μL aliquots were removed into 900 μL of 0.2 M CaCO$_3$ at 30-min intervals over a 2-hr time course. Fluorescence from the 4-methylumbelliferone (MU) product was measured using a Sequoia-Turner fluorometer (model 450) calibrated to 1000 fluorescence units for a 1 μM MU solution (Clonetech). Data were analyzed using the PlotIT computer program (Interactive Graphics and Statistics). Linear regression analysis of data values showing linear kinetics was used to

determine the rate of MU production in each sample. Rates of MU accumulation in nuclear and cytoplasmic fractions were compared to the rate of MU accumulation in the total extract. Lines that appeared to be similar were analyzed statistically at the 95% level to determine if they were statistically identical.

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