Dual Interaction of a Geminivirus Replication Accessory Factor with a Viral Replication Protein and a Plant Cell Cycle Regulator

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Received August 14, 2000; returned to author for revision October 9, 2000; accepted October 23, 2000

Geminiviruses replicate their small, single-stranded DNA genomes through double-stranded DNA intermediates in plant nuclei using host replication machinery. Like most dicot-infecting geminiviruses, tomato golden mosaic virus encodes a protein, AL3 or C3, that greatly enhances viral DNA accumulation through an unknown mechanism. Earlier studies showed that AL3 forms oligomers and interacts with the viral replication initiator AL1. Experiments reported here established that AL3 also interacts with a plant homolog of the mammalian tumor suppressor protein, retinoblastoma (pRb). Analysis of truncated AL3 proteins indicated that pRb and AL1 bind to similar regions of AL3, whereas AL3 oligomerization is dependent on a different region of the protein. Analysis of truncated AL1 proteins located the AL3-binding domain between AL1 amino acids 101 and 180 to a region that also includes the AL1 oligomerization domain and the catalytic site for initiation of viral DNA replication. Interestingly, the AL3-binding domain was fully contiguous with the domain that mediates AL1/pRb interactions. The potential significance of AL3/pRb binding and the coincidence of the domains responsible for AL3, AL1, and pRb interactions are discussed.

INTRODUCTION

Tomato golden mosaic virus (TGMV) is a single-stranded DNA virus that multiplies by rolling circle replication in nuclei of infected plant cells (Laufs et al., 1995). TGMV belongs to the Begomovirus genus, which includes many important crop pathogens that are transmitted by whiteflies and infect only dicotyledenous plants (Brown and Bird, 1992). The TGMV genome consists of two circular DNA components of about 2.5 kb in size, which together encode for at least six proteins. Two of these proteins, AL1 and AL3, are essential for high levels of viral DNA accumulation. Like other geminiviruses, TGMV recruits the remainder of its replication machinery from its plant host. Despite this high degree of dependency on host enzymes, TGMV replication occurs in differentiated cells, which have exited the cell cycle and do not contain detectable levels of plant replicative polymerases. Thus, an early step in the geminivirus infection process is the reprogramming of plant cell cycle controls to induce the synthesis of host replication machinery. As a consequence, geminiviruses are excellent models for studying plant DNA replication and cell cycle regulation (for review, see Hanley-Bowdoin et al., 1999).

The roles of AL1 as a rolling circle initiator and a modifier of plant cell cycle controls have been studied extensively (Ach et al., 1997; Fontes et al., 1994; Kong et al., 2000; Nagar et al., 1995; Orozco and Hanley-Bowdoin, 1998; Orozco et al., 2000). In contrast, little is known about the function of AL3 (or C3), which enhances viral infection and symptoms (Hormuzdi and Bisaro, 1995; Morris et al., 1991; Sung and Coutts, 1995), possibly through its capacity to increase viral DNA accumulation as much as 50-fold (Elmer et al., 1988; Sunter et al., 1990). Many eukaryotic DNA viruses encode proteins that are not required for replication but strongly impact efficiency (DePamphilis, 1988; Guo and Depamphilis, 1992). These accessory factors influence replication directly as components of the replication apparatus (Boehmer et al., 1993; Li and Botchan, 1994; Liptak et al., 1996) and/or indirectly as host modulators that alter the cellular environment to favor viral DNA replication (Jansen-Durr, 1996). In both cases, their activities frequently depend on interactions with other viral or host proteins (Frattini and Laimins, 1994; Nevins, 1992). In an earlier report, we showed that TGMV AL3 interacts with AL1 and forms oligomers (Settlage et al., 1996). In this study, we demonstrated that AL3 also interacts with the host cell cycle regulator, retinoblastoma (pRb). We also found that the protein domains that mediate the interactions between AL3, AL1, and pRb overlap. Together, these results suggested that AL3 serves a dual role in enhancing gemi-

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protein–protein interactions, we generated a set of truncated AL3 proteins that differed with respect to their predicted $\alpha$-helical content (Fig. 2A). The truncated protein $\Delta83–132$ has only the first helix, whereas $\Delta36–86$ includes only the third helix. In $\Delta37–54$ and $\Delta113–132$, all three helices are predicted to be intact. The truncated AL3 proteins were coexpressed with GST-pRBR1 in insect cells and analyzed by copurification on glutathione resin. In this experiment, full-length AL3 bound GST-pRBR1 (Fig. 2B, lanes 1 and 6) but not GST (Fig. 2B, lanes 5 and 10). The truncated AL3 proteins, $\Delta113–132$ (Fig. 2B, lanes 2 and 7), $\Delta83–132$ (Fig. 2B, lanes 3 and 8), and $\Delta36–86$ (Fig. 2B, lanes 4 and 9) also bound GST-pRBR1 in amounts equivalent to or greater than those of full-length AL3. These results indicated that AL3 amino acids 36 to 132 are not required for pRBR1 binding, and suggested that the first 35 amino acids of AL3 interact with pRBR1.

The region of AL3 that interacts with AL1 was characterized in coimmunoprecipitation assays using an anti-AL3 polyclonal antiserum and recombinant proteins produced in insect cells. As published previously (Settlage et al., 1996), AL1 was detected in the AL3 immunoprecipitates in the presence (Fig. 2C, lanes 2 and 7) but not the absence (Fig. 2C, lanes 1 and 6) of full-length AL3. AL1 also precipitated with $\Delta113–132$ (Fig. 2C, lanes 3 and 8), $\Delta83–132$ (Fig. 2C, lanes 4 and 9), and $\Delta36–86$ (Fig. 2C, lanes 5 and 10). These results, which showed that AL3 amino acids 36 to 132 are dispensable for AL1 binding and implicated the AL3 N-terminus in the interaction, were identical to those observed for AL3/pRBR1 interactions in Fig. 2B.

We also examined the oligomerization properties of the truncated AL3 proteins using a histidine-tagged version of full-length AL3 (his-AL3) produced in insect cells and nickel affinity chromatography. Full-length AL3 was detected in the affinity-purified fractions in the absence (Fig. 2D, lanes 1 and 7) but not the absence of his-AL3 (Fig. 2D, lanes 6 and 12), establishing that the histidine tag does not interfere with AL3 oligomerization. The C-terminally truncated protein $\Delta113–132$ also copurified with his-AL3 at a level comparable to that of the full-length protein (Fig. 2D, lanes 2 and 8). In contrast, $\Delta83–132$ (Fig. 2B, lanes 3 and 9), $\Delta36–86$ (Fig. 2D, lanes 4 and 10), and $\Delta37–54$ (Fig. 2B, lanes 5 and 11) were unable to bind his-AL3. These results showed that AL3 amino acids 37 to 112 are necessary for AL3 oligomerization. This region is not required for interactions with either pRBR1 or AL1, establishing that AL3 oligomerization is through a different region of the protein.

Even though AL3 is a small protein of only 132 amino acids, it is involved in a number of protein interactions. Pairwise comparison of the AL3/C3 sequences from 104 begomoviruses (sequences are available at http://iltab.danforthcenter.org/quantaxmenu.html) revealed three regions of strong conservation between TGMV AL3 amino acids 1 and 31, 47 and 72, and 97 and 109 that may

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**RESULTS AND DISCUSSION**

**AL3 interacts with a maize retinoblastoma homolog**

Mammalian DNA tumor viruses encode proteins that bind to the cell cycle regulator pRb and induce quiescent cells to reenter the cell cycle and synthesize the host replication enzymes required for viral DNA replication (Nevins, 1992). Because some of these animal viruses encode multiple pRb-binding proteins, we asked whether TGMV AL3 binds to the maize pRb homolog pRBR1 (retinoblastoma related), as shown previously for AL1 (Ach et al., 1997; Kong et al., 2000). In Fig. 1, a truncated version of maize pRBR1 containing the pocket domain and the C-terminus was expressed as a GST fusion in insect cells and analyzed for protein interactions using glutathione affinity chromatography. When AL3 was coexpressed with GST-pRBR1, it was detected in both the input (Fig. 1, lane 2) and bound (Fig. 1, lane 5) fractions. In contrast, AL3 was not detected in the bound fraction when it was expressed with GST alone (Fig. 1, lanes 3 and 6). Similarly, the control protein, chloramphenicol transacetylase (CAT), did not copurify with GST-pRBR1 (Fig. 1, lanes 1 and 4). Together, these results demonstrated that AL3 interacts specifically with the maize pRb homolog.

**pRb and AL1 bind to a similar region of AL3**

Figure 2A depicts a secondary structure prediction of TGMV AL3 with three $\alpha$-helices in the C-terminal two-thirds of the protein. The helices, which are predicted at greater than 80% probability (Rost and Sander, 1993), are between AL3 amino acids 56 and 63, 78 and 92, and 101 and 112. Because $\alpha$-helices are frequently important for protein–protein interactions, we generated a set of truncated AL3 proteins that differed with respect to their predicted $\alpha$-helical content (Fig. 2A). The truncated protein $\Delta83–132$ has only the first helix, whereas $\Delta36–86$ includes only the third helix. In $\Delta37–54$ and $\Delta113–132$, all three helices are predicted to be intact. The truncated AL3 proteins were coexpressed with GST-pRBR1 in insect cells and analyzed by copurification on glutathione resin. In this experiment, full-length AL3 bound GST-pRBR1 (Fig. 2B, lanes 1 and 6) but not GST (Fig. 2B, lanes 5 and 10). The truncated AL3 proteins, $\Delta113–132$ (Fig. 2B, lanes 2 and 7), $\Delta83–132$ (Fig. 2B, lanes 3 and 8), and $\Delta36–86$ (Fig. 2B, lanes 4 and 9) also bound GST-pRBR1 in amounts equivalent to or greater than those of full-length AL3. These results indicated that AL3 amino acids 36 to 132 are not required for pRBR1 binding, and suggested that the first 35 amino acids of AL3 interact with pRBR1.

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mediate these interactions. Analysis of deleted proteins suggested AL1 and pRBR1 interact with AL3 similarly, most likely through its N-terminus. This region contains six conserved, charged residues interspersed among clusters of bulky hydrophobic and aromatic amino acids, an arrangement that is typical of many protein interfaces (Rost and Sanders, 1994; Bogan and Thorn, 1998). AL3 oligomerization is dependent on amino acids 37–112, which includes the other two conserved sequences and the highly predicted \( \alpha \)-helices (Fig. 2A). However, we were unable to precisely map these domains in AL3 because of technical limitations. A peptide corresponding to the AL3 N-terminus could not be produced stably in insect cells (unpublished data). It was also not possible to assay for loss of AL1 or pRb binding using a C-terminal AL3 peptide because it could not be detected by our AL3 polyclonal antisera (unpublished data). Neither problem could be overcome by using protein fusions because of interference by heterologous domains with AL3 protein interactions (Settlage et al., 1996). However, because truncated AL1 proteins that differed by only 10 amino acids differentially bound AL3 (see Fig. 3), it is clear that AL1/AL3 interactions are specific. The specificity of AL3/pRBR1 interactions was established using GST and CAT as negative controls in parallel assays.

**The AL3- and pRb-binding domains of AL1 are contiguous**

The domains of AL1 that mediate DNA cleavage/ligation, DNA binding, oligomerization, and pRBR interactions were previously mapped to discrete regions using truncated proteins (Kong et al., 2000; Orozco and Hanley-Bowdoin, 1988; Orozco et al., 1997, 2000). In Fig. 3, we used the same strategy to identify the AL3-binding domain by monitoring coimmunoprecipitation of truncated AL1 proteins with AL3 (Fig. 3A). Initially, the domain was broadly mapped using naturally occurring restriction sites in the AL1 coding region to generate proteins truncated at the N- (AL1 \( \Delta \)113–352 and AL1 \( \Delta \)181–352) and C-termini (AL1 \( \Delta \)1–213, AL1 \( \Delta \)1–180, and AL1 \( \Delta \)1–120). The experiment in Fig. 3B showed that full-length AL1 (lanes 1 and 7), AL1 \( \Delta \)113–213 (lanes 2 and 8), and AL1 \( \Delta \)1–180 (lanes 3 and 9) coimmunoprecipitated with AL3, while AL1 \( \Delta \)1–120 (lanes 4 and 10), AL1 \( \Delta \)119–352 (lanes 5 and 11), and AL1 \( \Delta \)181–352 (lanes 6 and 12) did not. Based on these data, we concluded that AL1 amino acids 1–180 are necessary and sufficient for AL3 interactions with pRBR1, AL1, and itself. (A) A schematic of AL3 showing the three conserved \( \alpha \)-helices (filled ovals). Amino acid numbers correspond to positions used in construction of the truncated proteins shown below. Internal deletions are marked by dashed lines. The numbers to the left designate the deleted regions and the protein interaction properties are on the right. (B) GST-RBR1/AL3 interactions. Total insect cell extracts (lanes 1–5) and fractions that bound to glutathione resin (lanes 6–10) were visualized on immunoblots using anti-AL3 (top) or anti-GST (bottom) antibodies. The fractions contained GST-pRBR1 and full-length AL3 (lanes 1 and 6), AL3 \( \Delta \)113–132 (lanes 2 and 7), AL3 \( \Delta \)83–132 (lanes 3 and 8), or AL3 \( \Delta \)36–86 (lanes 4 and 9), as indicated above the blots. Lanes 5 and 10 are the GST plus AL3 controls. (C) AL3–AL1 interactions. Input fractions (lanes 1–5) and anti-AL3 immunoprecipitates (lanes 6–10) were analyzed on immunoblots using anti-AL3 and anti-AL1 antibodies (Settlage et al., 1996). The fractions contained full-length AL1, either alone (lanes 1 and 6) or coexpressed with full-length AL3 (lanes 2 and 7), AL3 \( \Delta \)113–132 (lanes 3 and 8), AL3 \( \Delta \)83–132 (lanes 4 and 9), or AL3 \( \Delta \)36–86 (lanes 5 and 10), as indicated above the blots. The IgG heavy chain is marked as Ab. (D) AL3 oligomerization. Input (lanes 1–6) and fractions that bound nickel resin (lanes 7–12) were visualized on immunoblots using an antiserum against AL3. The fractions contained his-AL3 coexpressed with full-length AL3 (lanes 1 and 7), AL3 \( \Delta \)113–132 (lanes 2 and 8), AL3 \( \Delta \)83–132 (lanes 3 and 9), AL3 \( \Delta \)36–86 (lanes 4 and 10), or AL3 \( \Delta \)37–54 (lanes 5 and 11), as indicated above the blots. In lanes 6 and 12, AL3 alone was expressed.
binding and that the AL1 C-terminus is not required for the interaction. In Fig. 3C, the N-terminal limit of the domain was further defined by AL1101–352, which interacted with AL3 (Fig. 3C, lanes 2 and 8), and by AL1110–352, which did not (lanes 3 and 9). The C-terminal limit of the AL3-binding domain was identified by AL111–180, which failed to bind AL3 (Fig. 3C, lanes 5 and 11), and by AL11168–352, which was positive for the interaction (Fig. 3C, lanes 4 and 10). Together, these results demonstrated that the AL3-binding domain is between AL1 amino acids 101 and 180 and is fully contiguous with the pRBR-binding domain (Fig. 3A) (Kong et al., 2000).

Protein oligomerization can differentially influence protein interactions (Marston et al., 1995). Our data showed that AL3 oligomerization is not required for its interactions with either AL1 or pRBR1 because deletions that did not oligomerize maintained these interactions. In contrast, any deletion that impinged on the AL1 oligomerization domain located between amino acids 134 and 180 also negatively affected AL1/AL3 interaction. Thus, AL1 oligomerization may be required for AL3 binding, as has been demonstrated for its pRBR1-binding activity (Kong et al., 2000). If AL1 multimerization is a prerequisite for AL3 binding, the actual region that contacts AL3 is likely to be a subset of AL1 amino acids 101–180.

TGMV infects mature plant cells and must induce the synthesis of DNA replication machinery in its target cells (Nagar et al., 1995). There is strong evidence implicating TGMV AL1 in the host induction process, including its abilities to induce PCNA accumulation in the absence of other viral proteins (Nagar et al., 1995) and to bind pRBR and determine tissue specificity of infection (Ach et al., 1997; Kong et al., 2000). However, these data do not preclude the involvement of other viral proteins in host induction. Our demonstration that TGMV AL3 interacts with a maize pRBR1 strongly suggested that it also plays a role in modifying plant cells, to create a replication-competent environment. In animals, virus-mediated cell cycle control generally requires several viral–host protein interactions. For example, SV40 large T antigen binds both tumor suppressors, pRb and p53 (reviewed in Levine, 1997). The papillomavirus E7 protein binds pRb (Chellappan et al., 1992), while the E6 protein binds p53 (Lechner and Laimins, 1994). Human cytomegalovirus encodes at least two proteins that bind to pRb and the related pocket protein p107 (Johnson et al., 1999). The CLEO virus encodes two proteins that bind different regions within pRb (Lehrmann and Cotten, 1999).

Several lines of evidence support the idea that AL3 is involved in reprogramming mature plant cells for DNA replication competency. AL3 is produced early in infection from a polycistronic mRNA that is also translated to generate AL1 (Thommes and Buck, 1994; Hanley-Bowdoin et al., 1999). Like AL1 and pRb, AL3 localizes to the nucleus, thereby providing an opportunity for interactions between the three proteins (Nagar et al., 1995; Ach et al.,

**FIG. 3.** AL3-binding domain of AL1. (A) Schematic of AL1 and its functional domains. Two sets of predicted α-helices are shown as hatched ovals, the shaded box indicates an ATP-binding consensus, and the solid boxes mark motifs conserved among rolling circle replication initiator proteins. The solid and dashed lines above the protein mark the locations of the overlapping DNA-binding (solid line) and oligomerization (dashed line) domains. The DNA cleavage/ligation domain is also shown as a solid line. Truncated AL1 proteins and their activities in AL3, pRBR1, and AL1 oligomerization assays are diagrammed below. The dashed box shows the limits of the AL3- and pRBR1-binding domains. Amino acid positions are marked above the schematic and indicated for each truncated protein. (B) Immunoblots showing AL1–AL3 interactions. Input fractions (lanes 1–5) and anti-AL3 immunoprecipitates (lanes 6–10) were visualized with anti-AL1 (top) and anti-AL3 (bottom) antibodies. The fractions contained full-length AL3 coexpressed with full-length AL1 (lanes 1 and 7) or the truncated proteins, AL11–213 (lanes 2 and 8), AL11–168 (lanes 3 and 9), AL11–120 (lanes 4 and 10), AL11–110 (lanes 5 and 11), or AL11–19 (lanes 6 and 12). (C) Same as (B), except the fractions contained full-length AL3 coexpressed with full-length AL1 (lanes 1 and 7) or the truncated proteins, AL11–213 (lanes 2 and 8), AL11–168 (lanes 3 and 9), AL11–120 (lanes 4 and 10), or AL11–19 (lanes 5 and 11). In lanes 6 and 12, AL1 alone was expressed. In both panels, the identities of the AL1 proteins are indicated above the blots, and the IgG heavy chain is marked as Ab.
1997). In addition, significantly higher levels of PCNA accumulate in nuclei of infected plants versus transgenic plants that express only AL1 (Nagar et al., 1995). However, unlike AL1, functional AL3 can be stably expressed in transgenic plants and AL3 by itself is not sufficient to induce PCNA expression (unpublished data). Together, these results suggest that AL3 and AL1 serve different roles in the host induction process. Analogous to cytomegalovirus (Johnson et al., 1999), AL3 and AL1 may target different pRb family members, both of which must be inactivated for efficient PCNA accumulation. To date, only a single pRb homolog has been identified through the Arabidopsis genomic sequencing project, which is nearing completion. Thus, unlike mammalian systems (Herwig and Strauss, 1997), pRBR may not exist as a small gene family in dicotyledonous plants.

One possibility is that AL3 regulates AL1/pRBR interactions through shared-protein domains. Both AL1 and pRBR1 are predicted to bind to AL3 within its first 35 amino acids (Fig. 2). Given that pRBR1 is greater than 100 kDa in size and that AL1 probably binds AL3 as an oligomer, AL3 may not be able to interact simultaneously with both proteins. The same may also be true for AL1 interactions with AL3 and pRBR1, both of which bind between AL1 amino acids 101 and 180 (Fig. 3) (Kong et al., 2000). This region also includes the AL1 oligomerization domain (Orozco et al., 2000), key residues necessary for DNA binding and the catalytic site for DNA cleavage and ligation (Orozco et al., 1998). We propose that AL1/AL3 but not AL1/pRBR complexes are functional for initiation of geminivirus replication. According to this model, AL3 modulates the stoichiometry of different AL1 complexes, and hence AL1 activity, through its binding to both AL1 and pRBR. A role for AL3 in modulating AL1/pRBR complexes is consistent with the observation that Mastreviruses do not encode an AL3 homolog. In these geminiviruses, the Rep protein is responsible for replication initiation, whereas a separate protein, RepA, binds to pRBR (Horvath et al., 1998; Liu et al., 1999; Xie et al., 1996), thereby obviating the need for an AL3 protein to modulate protein interactions. However, AL3 is required for efficient viral replication in cultured cells, which are competent for DNA synthesis and contain low levels of pRBR (Gladfelter et al., 1997; Nakagami et al., 1999). Thus, it is likely that AL3 also plays a direct role in viral replication separate from host activation. Future biochemical experiments that characterize the various complexes formed by AL1, AL3, and pRBR in infected plants and cultured cells will address AL3 function during host activation and viral replication.

**MATERIALS AND METHODS**

Recombinant baculoviruses

Recombinant baculoviruses corresponding to AL3; AL1; the truncated AL1 proteins-AL1;1–213, AL1;1–180, AL1;1–168, AL1;1–120, AL1;101–352, AL1;110–352, AL1;119–352, and AL1;1–352; GST-pRBR1; and CAT were described elsewhere (Ach et al., 1997; Kong et al., 2000; Orozco et al., 1997, 2000; Settlage et al., 1996). Coding sequences for C-terminal truncated AL3 proteins were constructed by insertion of XbaI linkers into repaired restriction sites at TGMV A positions 1217 (Swal) and 1126 (NruI) to create stop codons. The truncated AL3 coding sequences were then cloned as Ncol–XbaI fragments into pMON27025 (Luckow et al., 1993) to give Δ33–132 (pNSB637) and Δ112–132 (pNSB579). The internally deleted Δ37–54 coding sequence (pNSB691) was made by cloning a BamHI (repaired)–HindIII fragment of the wild type AL3 baculovirus transfer vector into the wild type vector previously digested with Bsu36I, repaired with Klenow and digested with HindIII. The internally deleted Δ36–86 open reading frame (pNSB660) was constructed by inserting a 119-bp BgIII–BamHI (trimmed) fragment from pNSB46 into a pNSB46 vector with BgIII and Swal ends. The Δ36–86 fusion was then cloned as a BgIII–BamHI fragment into the BamHI site of pMON27025, to generate the baculovirus transfer vector pNSB665. The baculovirus transfer vector for his-AL3 (pNSB252) was constructed using a SacI–HindIII fragment of pNSB251, a pBluescript vector containing the his-AL3 open reading frame from pNSB195, an E. coli expression vector (Pedersen and Hanley-Bowdoin, 1994). The SacI–HindIII fragment of pNSB251 was cloned into pMON27025 also digested with SacI and HindIII.

**Protein expression, extraction, and analysis**

Recombinant proteins were produced in Spodoptera frugiperda SF9 cells using a baculovirus expression system according to published protocols (Luckow et al., 1993; Settlage et al., 1996). For immunoprecipitations and glutathione–Sepharose purifications, cells were lysed in extraction buffer [25 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Triton X-100, pepstatin (10 μg/ml), leupeptin (50 μg/ml), and 1 mM PMSF]. Immunoprecipitations and glutathione–Sepharose purifications were described previously (Settlage et al., 1996). For his-AL3 purification, cells were lysed by sonication in Tween buffer [25 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, 1% Tween 20, 0.5 M NaCl, pepstatin (10 μg/ml), leupeptin (50 μg/ml), and 1 mM PMSF] and centrifuged at 200,000 g for 1 h. Extracts were agitated with Ni–NTA resin (Qiagen, Valencia, CA) for 2 h, washed five times with 10 resin volumes of Tween buffer, and eluted with 1 resin volume of Tween buffer containing 1 M imidazole. Crude extracts and purified proteins were fractionated by SDS–PAGE, immunoblotted with anti-AL3, anti-AL1, anti-GST, or anti-CAT serum (Ach et al., 1997; Settlage et al., 1996), and visualized with the ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ). Anti-GST serum
was generated by immunization of rabbits with polyhistidine-tagged GST protein purified from *E. coli*. The anti-AL3 and anti-AL1 sera were described elsewhere (Settlage et al., 1996). The anti-CAT serum was from 5 Prime → 3 Prime (Boulder, CO).

ACKNOWLEDGMENTS

We thank Drs. Niki Robertson and Cindy Hemenway for critical reading of the manuscript. The research was supported by grants to L.H.-B. from the U.S. Department of Agriculture (96-35301-3177) and the National Science Foundation (MCB-9809953).

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