

Cloning of a Gene Encoding an Alt a 1 Isoallergen Differentially Expressed by the Necrotrophic Fungus *Alternaria brassicicola* during *Arabidopsis* Infection

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Alternaria species are considered some of the most important fungi responsible for allergenic morbidity in humans. The *Alternaria* protein that elicits the most intense allergic reaction in humans is Alt a 1, yet no biological function has been identified for this protein. In this study, suppression subtractive hybridization and virtual Northern blots were used to identify and characterize an Alt a 1 homolog in the phytopathogenic fungus *Alternaria brassicicola*. RNA was extracted from *A. brassicicola* spores germinated in water and on leaf surfaces of the *Arabidopsis* ecotype Landsberg for 24 h and used to create cDNA by PCR. Double-stranded cDNA was then used in suppression subtractive hybridization to identify differentially expressed genes. mRNA transcript levels were assessed by virtual Northern blotting. A sequence with significant homology (90% amino acid, 92% cDNA) to the Alt a 1 subunit from *Alternaria alternata* was identified. Virtual Northern blots demonstrated that this homolog, designated Alt b 1 precursor, was highly up-regulated during the infection process of *A. brassicicola* on *Arabidopsis*. The full-length cDNA sequence of Alt b 1 was 815 bp, with an open reading frame of 477 bp. In this preliminary study, we identified a homolog of the major *Alternaria* allergen precursor, Alt a 1, in *A. brassicicola*, designated Alt b 1. This isoallergen is differentially expressed during fungal pathogenesis on *Arabidopsis*, suggesting a possible biological role in pathogenesis.

The genus *Alternaria* is considered one of the most important producers of fungal allergens (2) in the world. In particular, *Alternaria* allergens have been shown to be associated with asthma (9), and recently, sensitivity to fungal allergens was shown to be a risk factor for life-threatening asthma (5). Furthermore, the genus *Alternaria* is responsible for some of the world's most devastating plant diseases (13). Taken together, the morbidity caused by *Alternaria* allergens and the *Alternaria*-caused damage to our food supply clearly demonstrate the importance of identifying and functionally characterizing genes required for these processes.

In order to study the biological functions of *Alternaria* allergens, molecular biology techniques such as cloning have been used. The best known of these allergens is Alt a 1, produced by *Alternaria alternata*. To date, the N-terminal amino acid sequence of this protein (16) has been obtained, and subunits of Alt a 1 have also been cloned (4, 6). The major Alt a 1 protein is a dimer of about 29 kDa that when exposed to reducing agents dissociates into 14.5- and 16-kDa subunits (6, 16). A common epitope that can serve as a binding site for a novel two-site monoclonal antibody exists on both Alt a 1 monomers (1). No biological function has been assigned to Alt a 1 to date, and surprisingly, no known homologs in other *Alternaria* species have been found.

Recently, germination of *A. alternata* spores was shown to increase allergen release (11). Allergen release was measured by immunostaining and indicated a marked increase in aller-

gen release in germinating spores compared with ungerminated spores. The authors hypothesized that Alt a 1 is secreted or released from growing germ tubes, as has been reported for *Aspergillus* (15). Thus, environmental conditions that trigger *Alternaria* spore germination may also trigger allergen production. In particular, since *Alternaria* spores are too large to reach the alveoli of the lung, a high probability exists that sensitization to *Alternaria* is due to inhalation of dried mycelia or spores. Identifying when and where *Alternaria* allergens are produced may lead to a further understanding of how patients are sensitized to *Alternaria* allergens and thus help develop novel control strategies for their mitigation.

In this study, we report the identification of an Alt a 1 homolog in the necrotrophic fungus *Alternaria brassicicola* (Schwein.) Wiltshire, the causal agent of black spot disease of many cruciferous plant species. *A. brassicicola* is also known to produce a host-specific peptide toxin called AB toxin, which appears to be responsible, in part, for its pathogenesis on cruciferous plants (12). This is the first report of an Alt a 1 homolog in another species of *Alternaria*. This homolog, which we have designated Alt b 1, is preferentially expressed in spores germinating on the leaf surface of *Arabidopsis* and not in spores germinating in water. These results may indicate that Alt b 1 expression in *A. brassicicola* is controlled by specific environmental factors and may play a role in necrotrophic fungal pathogenesis of plants. Understanding the production regulation and biological function of this major fungal allergen may lead to novel control strategies and a further understanding of the major *Alternaria* allergen, Alt a 1.

A. brassicicola strain 34622 was obtained from the American Type Culture Collection and grown on potato dextrose agar (Sigma Chemical Co., St. Louis, Mo.) for 14 days. Spores were

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collected by flooding the plate with 10 ml of sterile water and quantified with a hemacytometer. The inoculum concentration was adjusted to approximately 5×10^5 spores per ml. Aliquots (20 μ l) of the spore suspension were placed on detached leaves of the susceptible *Arabidopsis* ecotype Landsberg, and the leaves were incubated at 24°C in 100% humidity for 24 h. The remaining spores were allowed to germinate in water for 24 h under similar conditions. A third population, ungerminated spores, was also collected for comparison purposes in expression studies but not used in the subtraction.

After 24 h, germination of the spores was checked by light microscopy. Approximately 80% of the spores germinated both in water and on the leaf surface, and germinating spores were then collected and used to extract total RNA. RNA extraction was done according to the manufacturer's instructions by using an RNeasy plant minikit (Qiagen Inc., Valencia, Calif.). Following RNA extraction, approximately 75 ng of total RNA was used to create cDNA from each of the three RNA samples described above with a Smart PCR cDNA synthesis kit (Clontech, Palo Alto, Calif.). RNA extracted from the water-only-germinated spores was used to create the driver cDNA, while RNA extracted from leaf-germinated spores was used to create the tester cDNA. Double-stranded cDNA from the tester and driver populations generated from the Smart PCR cDNA synthesis kit was used in suppression subtractive hybridization (SSH) (7). SSH was performed with the PCR Select cDNA subtraction kit (Clontech) according to the manufacturer's instructions. Following SSH, amplification products enriched for sequences expressed specifically on *Arabidopsis* leaf surfaces were directly cloned with the AdvanTAGE PCR cloning kit (Clontech) and transformed into TOP-10 *Escherichia coli* cells (Invitrogen, Carlsbad, Calif.) for blue-white selection. White colonies were randomly picked and used in a differential screening to confirm up-regulation of the selected clones.

Clones with putative up-regulation were further screened with virtual Northern blots (8). Blots for virtual Northern analysis were created by using 2 μ g each of double-stranded cDNA from the tester, driver, and ungerminated spore populations. The cDNAs were electrophoresed in a 1% agarose gel and blotted onto positively charged nylon membranes (Roche Diagnostics, Mannheim, Germany). Individual cDNA clones were used as probes and labeled by using a PCR digoxigenin (DIG) probe synthesis kit (Roche Molecular Biochemicals, Mannheim, Germany). Filters were hybridized with the PCR-amplified DIG-labeled probes at a probe concentration of 25 ng/ml. Filters were washed in a final solution of $0.1 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate at 68°C.

Reverse transcription (RT)-PCRs were performed to assess transcript accumulation of Alt b 1 under different environmental conditions. Two microliters of first-strand cDNA created with a Smart PCR cDNA synthesis kit were used in a 25- μ l PCR. Primers used in the amplification to detect the presence of Alt b 1 transcript were Aller5' (5'-ATG CAG TTC ACC ACC ATC GC-3') and Aller3' (5'-GTA ACG AGG GTG ACG TAG GC-3'). Transcript levels of Alt b 1 were compared with 18S rRNA levels by using the primers 5'GCG GTA ATT CCA GCT CCA ATA GC-3' and 5'GAT GAC ACG CGC TTA CTA GGC-3'. PCR amplification conditions were 95°C

for 2 min followed by 40 cycles of 95°C for 30 s, 53°C for 45 s, and 72°C for 1 min. A final 5-min extension was done at 72°C.

For Southern analysis, *A. brassicicola* genomic DNA was extracted by using a microbial DNA isolation kit (Mo Bio Laboratories Inc., Solana Beach, Calif.). Restriction digests were performed by digesting 3- μ g aliquots of genomic DNA with six individual restriction enzymes (*Bam*HI, *Hind*III, *Pst*I, *Eco*RI, *Eco*RV, and *Xba*I). Restriction digests were separated on a 1% agarose gel and blotted onto nylon membranes as for virtual Northern blots. DNA probe for Alt b 1 was labeled as described above for the virtual Northern probe. The concentration of the probe in hybridization solution was 50 ng/ml, and hybridization was carried out at 50°C. Membranes were washed in a final solution of $0.1 \times$ SSC and 0.1% sodium dodecyl sulfate at 68°C.

SSH identified a cDNA clone highly up-regulated in the tester cDNA population. Sequence analysis of this cDNA clone using BLASTN and BLASTX (3) revealed high homology (BLASTN, 1e-176; BLASTX, 2e-57) with the major *Alternaria* allergen Alt a 1. Subsequently, we named this gene Alt b 1. Figure 1 shows the cDNA sequence of Alt b 1 as well as the amino acid sequence homology between Alt a 1 (GenBank accession number P79085) and Alt b 1. The cDNA sequence is 815 bp long, including a 205-bp 5' untranslated region and a poly(A) tail. The initiation codon (ATG) starts at position 206, and the termination codon (TAA) ends at position 680. The 205-bp 5' untranslated region that exists in the Alt b 1 cDNA sequence also appears in the Alt a 1 sequence (data not shown). The cDNA sequence of Alt b 1 was 92% similar to that of Alt a 1, and the gene product can thus be considered an isoallergen (10). The amino acid sequence of Alt b 1 was 90% similar to the previously identified Alt a 1. The 18-amino-acid cleaved signal peptide located at the N terminus that directs Alt a 1 for export from the cell also appears to be present in Alt b 1.

Southern analysis of Alt b 1 (Fig. 2A) revealed the apparent presence of one copy of Alt b 1 in the *A. brassicicola* genome. The enzymes *Bam*HI and *Eco*RI gave two large fragments, while the enzyme *Hind*III gave one fragment of about 1,500 bp that hybridized with the Alt b 1 cDNA probe. The two large fragments produced by *Bam*HI and *Eco*RI can be explained by the presence of restriction sites for these two enzymes in the middle of the Alt b 1 cDNA clone used as a probe in Southern analysis. Virtual Northern analysis using Alt b 1 cDNA as a probe revealed drastic up-regulation on the leaf surface of Alt b 1 precursor compared with ungerminated spores and spores germinated only in water based on relative mRNA transcript abundance (Fig. 2B).

RT-PCR experiments (Fig. 2C) demonstrated that Alt b 1 is highly expressed in *A. brassicicola* cultures growing in various liquid media (glucose-yeast extract broth, minimal medium, and potato dextrose broth). RT-PCR experiments also demonstrated that Alt b 1 is expressed by spores germinating on the leaf surface of a more resistant *Arabidopsis* ecotype, Columbia.

To our knowledge, this is the first study to identify an Alt a 1 precursor homolog in an *Alternaria* species other than *A. alternata*. The presence of Alt b 1 in *A. brassicicola* may be another source of *Alternaria* allergen, particularly since *A. brassicicola* infects wild cruciferous species and domesticated spe-

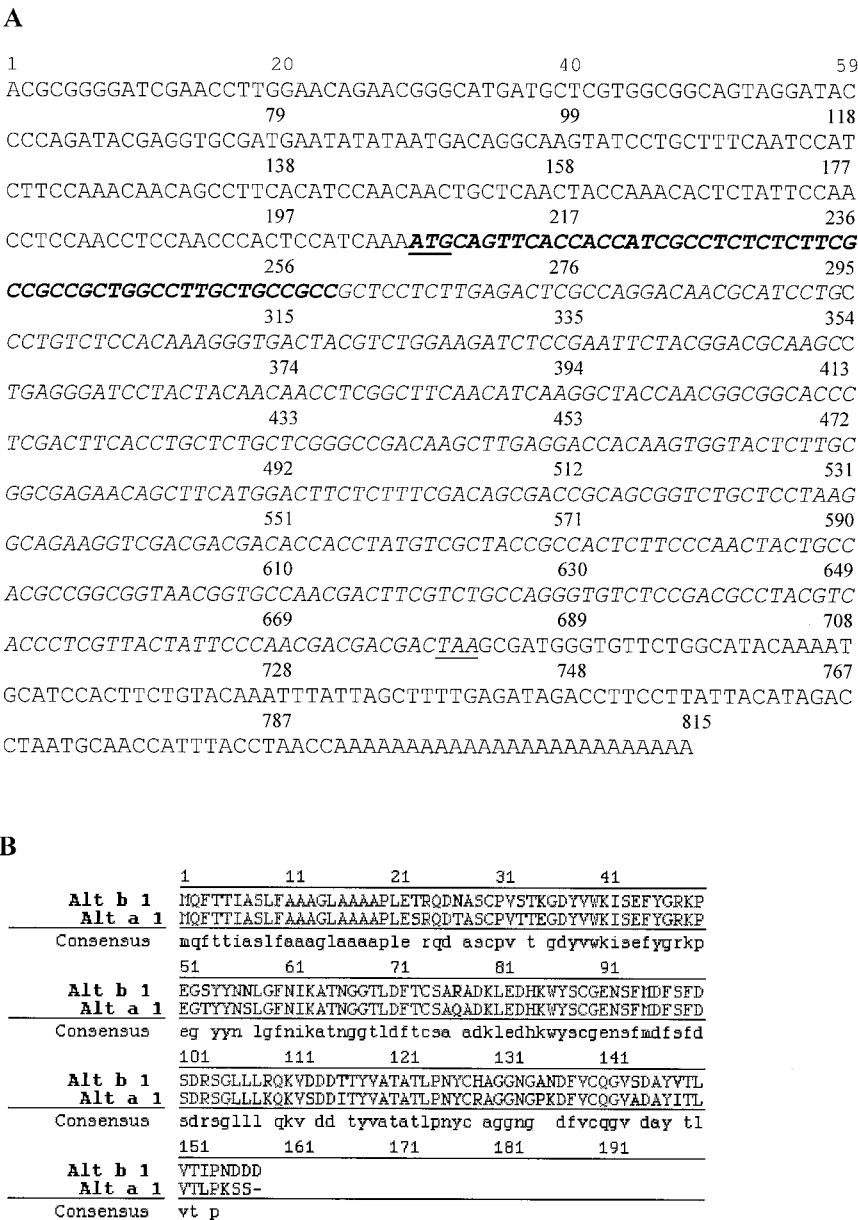


FIG. 1. (A) Nucleotide cDNA sequence of Alt b 1. Initiation (ATG) and stop (TAA) codons are underlined, and the sequence encoding the 18-amino-acid signal peptide is in bold. The open reading frame from position 206 to 680 is italicized. (B) Amino acid sequence homology between Alt a 1 precursor and Alt b 1 precursor open reading frames. Residues that are identical are shaded.

cies grown for food. Of potential interest to both allergen researchers and plant pathologists, Alt b 1 appears to be drastically up-regulated during the initial stages of infection on *Arabidopsis*. This may indicate that Alt b 1 has a role in fungal pathogenesis, though further studies are required to elucidate its requirement in infection. At present, it is not known whether allergenic proteins play a role in plant pathogenesis, and it is difficult to speculate on this concept due to the diverse structure and biological functions of known fungal allergens. Recently, it was reported that germinating spores of *Alternaria* release greater amounts of allergen than nongerminating spores (11). Our results, based on virtual Northern analysis, seem to confirm these results to a degree. If one assumes that

transcript abundance corresponds to allergen release, then clearly more allergen is released from *A. brassicicola* spores germinating in nutrient-rich environments than nongerminating spores. Interestingly, however, in our study, spores that were germinated in water alone did not have high levels of Alt b 1 transcript. We speculate that this is due to the nutrient-deficient environment of the sterile distilled water used to germinate the spores (though we did not test the water for the presence of sugars). Based on these results, simple spore germination does not seem to increase Alt b 1 transcription. Also, it is important to note that the study by Mitakakis et al. (11) used spores that were harvested from vegetable juice medium, which contains plant products. Our results along with those of

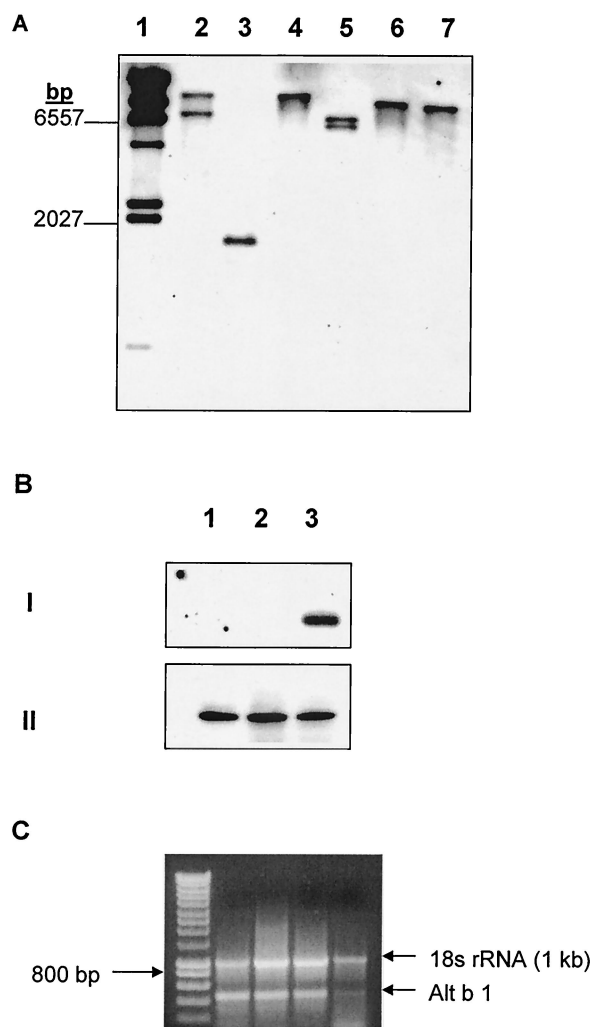


FIG. 2. (A) Southern analysis of Alt b 1. Lane 1, DIG ladder; lanes 2 to 7, *A. brassicicola* genomic DNA digested with *Bam*HI, *Hind*III, *Pst*I, *Eco*RI, *Eco*RV, and *Xba*I, respectively. Alt b 1 appears to be a single-copy gene. (B) Virtual Northern analysis of Alt b 1. Lane 1, cDNA derived from ungerminated spores; lane 2, cDNA derived from spores germinated in water only for 24 h; lane 3, cDNA derived from spores germinated for 24 h on *Arabidopsis* ecotype Landsberg leaf surfaces. I, Alt b 1; II, 18S rRNA control to demonstrate equal loading of lanes. (C) RT-PCR analysis of Alt b 1 expression. Lane 1, 1-kb ladder; lane 2, *A. brassicicola* grown in glucose-yeast extract broth for 6 days; lane 3, *A. brassicicola* grown in minimal medium for 6 days; lane 4, *A. brassicicola* grown in potato dextrose broth for 6 days; lane 5, *A. brassicicola* spores grown on *Arabidopsis* leaf surfaces for 24 h. The upper band corresponds to 18S rRNA, which allows comparison of Alt b 1 expression across samples. The bottom band corresponds to the Alt b 1 transcript.

Mitakakis et al. (11) suggest that a signal(s) present on the plant leaf surface may trigger an increase in Alt b 1 gene expression. RT-PCR experiments demonstrate that this signal is also present in nutrient-rich media, and thus, a nutrient-rich environment (such as the nasal passage) conducive to fungal growth may be the only requirement for Alt b 1 production. Identification of the specific environmental conditions that trigger Alt b 1 production and its release should help elucidate mechanisms that regulate its production and provide a clue to its potential biological function.

In this study we report the identification of an Alt a 1 isoallergen from the phytopathogenic fungus *A. brassicicola*. The degree of sequence homology between Alt a 1 and Alt b 1 (92% at the cDNA level) indicates that Alt b 1 may be considered an isoallergen (10; www.allergen.org). Further, Alt b 1 appears to have the 18-amino-acid signal peptide described for Alt a 1 that is required for secretion (6). Though fungi have long been known to be sources of allergens, recent studies have demonstrated their scope and impact on human health (5, 14). Successful control of allergies caused by fungal allergens will require knowledge of the potential sources of allergens, as well as an understanding of the biological functions of allergenic proteins. Future studies on Alt b 1 precursor will include examination of its role in the pathogenesis process on crucifer species by gene disruption as well as defining its allergenic properties. Identification of a biological function for Alt b 1 and its homolog Alt a 1 will certainly provide new ideas for potential control of this important fungal allergen and may help answer the question of how patients are sensitized to *Alternaria* allergens.

Nucleotide sequence accession number. The sequence of the Alt b 1 gene has been deposited in GenBank under accession number AF499002.

REFERENCES

- Aden, E., B. Weber, J. Bossert, R. Wahl, M. Teppke, E. Frank, R. Wahl, H. Fiebig, and O. Cromwell. 1999. Standardization of *Alternaria alternata*: extraction and quantification of Alt a 1 by using an mAb-based binding assay. *J. Allergy Clin. Immunol.* **104**:128–135.
- Al-Doory, Y., and J. F. Domson. 1984. Mould allergy. Lea & Febiger, Philadelphia, Pa.
- Altschul, S. F., T. L. Madden, A. A. Schaffer, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389–3402.
- Barnes, C. S., F. Pacheco, J. Landuyt, D. Rosenthal, F. Hu, and J. Portnoy. 1996. Production of a recombinant protein from *Alternaria* containing the reported N-terminal of the Alt a 1 allergen. *Adv. Exp. Med. Biol.* **409**:197–203.
- Black, P. N., A. A. Udy, and S. M. Brodie. 2000. Sensitivity to fungal allergens is a risk factor for life-threatening asthma. *Allergy* **55**:501.
- DeVoue, M. W., A. M. Thacker, I. H. A. Curran, L. Zhang, G. Muradia, H. Rode, and H. M. Vijay. 1996. Isolation and expression of a cDNA encoding an *Alternaria alternata* Alt 1 a subunit. *Int. Arch. Allergy Immunol.* **111**:385–395.
- Diatychenko, L., Y. F. C. Lau, A. Campbell, F. Moqadam, B. Huang, S. Lukyanov, K. Lukyanov, N. Gurskaya, E. D. Sverdlov, and P. D. Siebert. 1996. Suppression subtractive hybridization: a method for generating differentially regulated or tissue specific cDNA probes and libraries. *Proc. Natl. Acad. Sci. USA* **93**:6025–6030.
- Endege, W. O., K. E. Steinmann, L. A. Boardman, S. N. Thibodeau, and R. Schelgel. 1999. Representative cDNA libraries and their utility in gene expression profiling. *BioTechniques* **26**:542–550.
- Halonen, M., D. A. Stern, A. L. Wright, L. M. Taussig, and F. D. Martinez. 1997. *Alternaria* as a major allergen for asthma in children raised in a desert environment. *Am. J. Respir. Crit. Care Med.* **155**:1356–1361.
- Hoffman, D., H. Lowenstein, D. G. Marsh, T. A. E. Platts-Mills, and W. Thomas. 1994. Allergen nomenclature. *Bull. W. H. O.* **72**:796–806.
- Mitakakis, T. Z., C. Barnes, and E. R. Tovey. 2001. Spore germination increases allergen release from *Alternaria*. *J. Allergy Clin. Immunol.* **107**:388–390.
- Otani, H., A. Kohnobe, M. Narita, H. Shiomi, M. Kodama, and K. Kohmoto. 2001. A new type of host-selective toxin, a protein from *Alternaria brassicicola*, p.68–76. In N. T. Keen, S. Mayama, J. E. Leach, and S. Tsuyumu (ed.), *Delivery and perception of pathogen signals in plants*. APS Press, St. Paul, Minn.
- Rotem, J. 1994. The genus *Alternaria*: biology, epidemiology, and pathogenicity. APS Press, St. Paul, Minn.
- Shelton, B. G., K. H. Kirkland, D. Flanders, and G. K. Morris. 2002. Profiles of airborne fungi in buildings and outdoor environments in the United States. *Appl. Environ. Microbiol.* **68**:1743–1753.
- Sporik, R. B., L. K. Arruda, J. Woodfolk, M. D. Chapman, and T. A. E. Platts-Mills. 1993. Environmental exposure to *Aspergillus fumigatus* allergen (*Asp f 1*). *Clin. Exp. Allergy* **23**:326–331.
- Zhang, L., I. H. A. Curran, G. Muradia, M. W. DeVoue, H. Rode, and J. M. Vijay. 1995. N terminus of a major allergen, Alt 1 a, of *Alternaria alternata* defined to be an epitope. *Int. Arch. Allergy Immunol.* **108**:254–259.