Behaviour of the disease resistance gene *Asc* in protoplasts of *Lycopersicon esculentum* mill

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Action of *Asc*, a single dominant Mendelian gene controlling disease response at the whole plant level, was detected at the level of individual cells. Protoplasts, freshly isolated from resistant (*Asc/Asc*) and susceptible (*asc/asc*) tomato isolines, were differentially sensitive to AAL toxin as observed *in planta*. Protoplast mortality was toxin concentration dependent in both isolines and required the same 24–36 h time frame as necrosis in leaflet tissue, but *asc/asc* protoplasts were 10-fold less sensitive (0·15 μM) and *Asc/Asc* protoplasts were over 100-fold less sensitive (> 150 μM) than corresponding leaflet tissue. Light was required for necrosis in leaflets but not in protoplasts; *asc/asc* leaflets kept in darkness remained green for 48 h at 100-fold excess toxin concentration whereas protoplasts showed equivalent sensitivity in light or dark. Aging diminished toxin sensitivity in both leaflets and in protoplasts. Fully expanded *asc/asc* leaflets were less than half as sensitive to AAL toxin (~ 30% necrosis) as rapidly expanding leaflets (~ 87% necrosis) while protoplasts from equivalent leaflets were only 80% as sensitive to AAL toxin 2 days after isolation and insensitive after 9 days. These data indicated that the action of the *Asc* gene is expressed in protoplasts, the process of cell death is analogous in single cells and intact leaves, but that additional processes expressed in differentiated tissue can modify *Asc* response to AAL-toxin.

INTRODUCTION

Single dominant genes provide disease resistance at the whole plant level, but biochemical and molecular characterization of resistance gene function *in planta* are hampered by the complexity and asynchrony of cellular responses during disease development in differentiated tissue. Detection of the action of resistance genes at the level of individual cells could avoid problems associated with complex tissues and simplify the design of strategies to screen, isolate, characterize, and modify genes regulating plant disease. It has been argued that the molecular characterization of resistance genes will occur most readily in plants that are genetically well defined and which can be manipulated at the whole plant, cellular, and molecular levels [*1*, *10*, *12*]. The cultivated tomato has the requisite attributes for studies of plant response to disease at each of these levels [*10*, *16*], if resistance gene function occurs in isolated cells.

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Host-specific phytotoxins, acting as primary determinants of disease, are direct chemical probes for the activity of genes regulating disease in plants [*9*, *22*]. Maize protoplasts have been reported to express the cytoplasmic gene controlling resistance to *Helminthosporium maydis* Hmt toxin [*6*] but the specific action of a nuclear resistance gene has not been reported in protoplast or cell cultures. A host–pathogen system with the potential for detecting expression of a nuclear resistance gene in single cells is alternaria stem canker of tomato. The pathogen, *Alternaria alternata* f. sp. *lycopersici* [*8*, *17*], produces a host-specific toxin (AAL toxin) which elicits characteristic necrotic symptoms of the disease when infiltrated into tomato leaflets [*8*]. Genetic studies revealed that the reaction of tomato to both pathogen and toxin is controlled by the *Asc* gene [*4*] which has been mapped to chromosome 3 at position 93 [*20*]. Near-isogenic lines of tomato (F_9) were developed to test various hypotheses regarding the action of the *Asc* gene product [*4*]. AAL toxin has been purified both from liquid culture of *A. alternata* f. sp. *lycopersici* and from field grown tomato plants exhibiting symptoms of the alternaria stem canker disease [*5*, *18*] and the structure of the toxin has been reported [*2*, *3*].

The objective of this study was to determine if the action of the *Asc* gene could be detected in single tomato cells even though previous attempts with tomato cell cultures failed to detect the action of the *Asc* gene [*7*,*21*]. The approach taken here was to use the AAL toxin as a probe for the action of the *Asc* gene in genetically equivalent leaflet lamina and protoplasts derived from tomato lines near-isogenic for the *Asc* gene. The sensitivity of various tomato tissues to AAL toxin was assessed in the homozygous *Asc* isolines in relation to: (a) differential sensitivity to AAL toxin in freshly isolated protoplast cells, (b) the effect of cellular organization on relative sensitivity, response time, and light requirement in leaflets *v.* freshly isolated protoplasts, and (c) the effect of aging on sensitivity of leaflets and protoplasts of the *asc*/*asc* isoline to the toxin. Results presented here indicate the *Asc* gene is expressed functionally in protoplasts, but leaflet organization and developmental stage can affect the response of *Asc* to AAL toxin.

MATERIALS AND METHODS

Plant material and growth conditions

Plants used for leaflet bioassays were grown from seeds of F_9 derived lines of *Lycopersicon esculentum* Mill, developed as isolines for the *Asc* gene [*4*]. The seed was sown in 15 cm pots containing UC-mix soil [*13*] and thinned to one plant per pot following emergence. Plants were grown under greenhouse conditions for 4 weeks, transferred to growth chambers (25 °C/21 °C day/night, 16 h photoperiod) at the 5-leaf stage, and used in experiments at the 7-leaf stage. Transfer to the growth chamber for the final period of development allowed time for the plants to adapt to environmental conditions equivalent to the bioassay. Plants were illuminated from 0600 h to 2100 h with a combination of cool white fluorescent and incandescent lamps giving an intensity of 112 Wm^{-2} irradiance at the leaf canopy during the photoperiod and under continuous light during the bioassay period.

Plants used as a source for protoplasts were grown from seed of *Asc* lines under aseptic conditions in MS10 medium [*15*] under a 15 h photoperiod at 112 Wm^{-2} under warm

white and grolux fluorescent lighting, 25 °C and 60% RH. After 3–4 weeks growth, leaflets were harvested and protoplasts isolated as described earlier [*1*, *12*]. Following isolation and washing, protoplasts were resuspended in 1/2 VKM plating medium [*12*] amended with 1 mg ml^{-1} NAA and 0·5 mg ml^{-1} BAP at a cell density of 100000 protoplasts ml^{-1}. Protoplasts receiving light treatments were isolated in light and incubated in 96-well plates under continuous light at an intensity of 112 Wm^{-2}. Protoplasts receiving dark treatments were isolated under a red safe-light, placed in 96-well plates, and wrapped immediately in 2 layers of aluminium foil before being incubated in the same growth chambers as the light treated protoplasts. AAL-toxin was produced, purified, and quantified as described previously [*5*].

Assessment of AAL toxin responses

Bioassay studies to determine toxin sensitivity of excised terminal leaflets from designated leaves were conducted at various concentrations of AAL toxin as described previously [*4*]. The effect of leaflet age on relative sensitivity to AAL toxin was evaluated on the youngest 5 cm long terminal leaflet lamina (leaf position 1) and the next two older terminal leaflets (leaf positions 2 and 3, respectively) taken from 7-leaf plants. In a 7-leaf plant the youngest terminal leaflet occurred on the 5th formed leaf (rapidly expanding). Leaf position 2, the intermediate age tested, occurred on the 4th formed leaf (still expanding). Leaf position 3, the oldest tested, occurred on the 3rd formed leaf (fully expanded). Excised leaflets were incubated in controlled environment chambers during the bioassays at 25 °C under continuous warm white and grolux fluorescent lighting with an intensity of 112 Wm^{-2} irradiance at the surface of the leaflets. Bioassay plates requiring total darkness were placed in plastic boxes, wrapped in aluminium foil and black velvet, and incubated in the same controlled environment chambers as the light exposed leaflets. Necrosis ratings were determined visually by assessing the percentage of the leaflet lamina area with necrotic lesions in comparison to a standard scale [*4*].

Cell viability was determined using the vital stain fluorescein diacetate [*19*] and measured by counting protoplasts exhibiting u.v. light induced fluorescence of the viable cells on a Nikon Diaphot-TMD inverted microscope. Each of three views per well of the 96 well microtitre plate were counted to determine the average number of live protoplasts per view for each treatment. Each view consisted of counting 100 cells. Final assessments, except where noted differently, were taken 5 days after toxin application.

Statistical analysis

Leaflet and protoplast bioassay treatments were arranged in a complete block factorial design containing 3 replicates for each treatment. The mean number of viable control protoplasts per view was 71 out of 100 cells counted where 27 views were recorded per treatment. Percentage cell mortality was calculated from the number of viable cells in a treatment divided by a number of viable control cells, subtracted from 1·0 and multiplied by 100%. Error bars represent the standard errors determined from 3 replications of each treatment where each experiment was performed at least 3 times. Estimates of percentage necrotic leaf area were converted to arc sine values prior to

statistical analyses. Calculations, analysis of variance, and LSD were accomplished using the SAS computer package [SAS Institute Inc. (1985) SAS User's Guide: Basics, Version 5 Edition. Cary, NC: SAS Institute Inc.].

RESULTS

Genotypic differential in AAL toxin sensitivity

Both leaflets and freshly isolated protoplasts from *Asc* isolines expressed genotype-specific differential sensitivity to AAL toxin. Leaflets from the sensitive *asc/asc* line expressed 7% interveinal necrosis when exposed to AAL toxin at 0·015 μM compared to *Asc/Asc* leaflets which required toxin amounts in excess of 1·5 μM to induce an equivalent amount of necrosis [Fig. 1(a)]. Freshly isolated *asc/asc* protoplasts showed significant mortality (14%) at 0·15 μM AAL toxin [Fig. 1(b)]. Although this

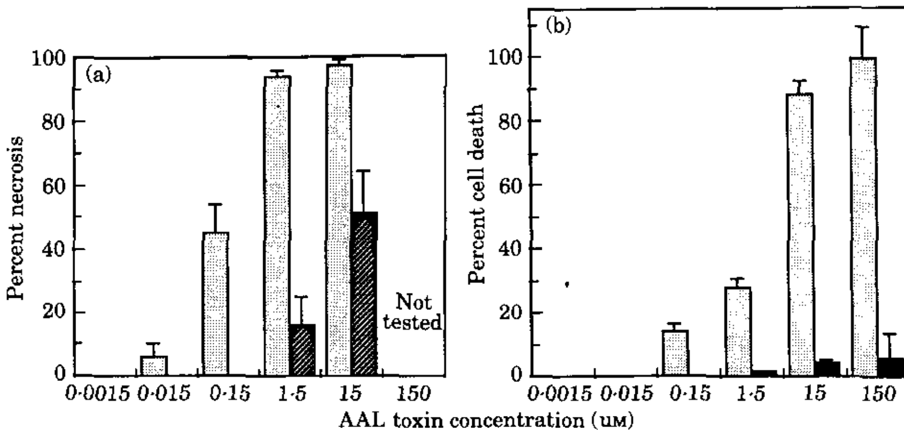

FIG. 1. Genotype-specific sensitivity of leaflets and protoplast to AAL toxin. (a) Sensitivity of leaflets to AAL toxin was tested in leaf bioassays consisting of excised leaflets put on filter paper saturated with 3 ml of toxin solution in Petri dishes. Petri dishes were subsequently sealed with Nescofilm and incubated in a growth chamber at 25 °C with continuous light. Results scored after 48 h were expressed as percentage leaf necrosis. Experiments were performed in triplicate with 3 leaflets per experiment. Error bars represent the S.E. of 3 experimental means. (b) Protoplasts of susceptible and resistant tomato lines, near isogenic for the *Asc* gene, were isolated as described previously [*12*], cultured in 125 ul 1/2 VKM [*12*] in 96 well plates, with a concentration of 1×10^5 protoplasts ml^{-1}. AAL toxin, dissolved in water was added in 5 ul aliquots per well at the concentrations indicated. Plates were incubated in a growth chamber at 25 °C. Viability of protoplasts was assessed after 5 days by FDA staining [*19*]. Viable cells were counted in 3 wells with 3 views per well with each view containing 100 cells. Data was normalized as described in Materials and Methods. Experiments were performed in triplicate. Error bars represent the S.E. of 3 experimental means. Error bars exceeding 100% were due to values less than 100% being averaged into the mean. Solid bars indicate the *asc/asc* genotype and dark striped bars indicate the *Asc/Asc* genotype.

concentration was 10 times higher than that required to elicit detectable leaflet necrosis in the sensitive genotype, the genotypic differential was clearly evident since toxin concentrations up to 1000-fold greater (150 μM) did not significantly decrease viability of *Asc/Asc* protoplasts.

Cellular organization and AAL toxin sensitivity

Comparison of protoplasts from the two genotypes indicated that protoplasts are less sensitive to AAL toxin than comparable cells in leaflets. Ten times more AAL toxin was required to initiate the onset of cell mortality in *asc/asc* protoplasts (0·15 μM) [Fig. 1(b)] than in leaflets (0·015 μM) [Fig. 1(a)]. AAL toxin at 1·5 μM caused 16% necrosis in *Asc/Asc* leaflets but negligible protoplast mortality. Ten-fold higher AAL toxin concentration increased *Asc/Asc* leaflet necrosis to 51% but protoplast mortality measured only 4% and reached a maximum level of 6% with 150 μM AAL toxin. The net result is that although protoplasts of both genotypes appear to be less sensitive to AAL toxin than corresponding interveinal lamina areas of leaflets from which they were isolated, the genotype-specific differential was expressed at both levels.

Cellular organization and Asc response time

Onset of AAL toxin induced leaflet necrosis and protoplast mortality occurred within the same time frame in the respective isolines. Leaflets of the *asc/asc* isoline began to show visible necrosis 24–36 h after AAL toxin treatment [*4*, *14*] which reached a concentration dependent maximum level at approximately 48 h. A concentration of 37·5 μM induced 17% mortality within 1 day after exposure to AAL toxin and 24% after 2 days ($P > F = 0{\cdot}07$) (Fig. 2). However, at lower toxin concentrations an extra 1–2 day incubation period was required for significant mortality to occur. The mean number of viable control protoplasts per view (100 cells counted) in these experiments was 81 (where 81 viable cells = 0% mortality).

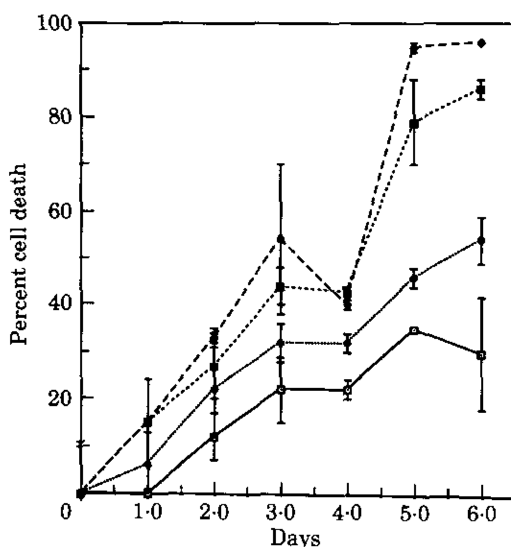

FIG. 2. Temporal response of protoplasts to AAL toxin. The amount of time required for *asc/asc* sensitivity to AAL toxin in protoplasts was determined by incubating protoplasts with 0 (—▣—), 1·5 (······◆······), 7·5 (----■---) and 37·5 μM AAL toxin (- -◇- -) and analysed as described in the legend to Fig. 1.

Cellular organization and the effect of light on cell death

Light was required for expression of toxin-induced necrosis in *asc/asc* leaflets but not in protoplasts [Fig. 3]. Toxin treated leaflets kept in total darkness for 48 h did not express necrosis at toxin concentrations up to 1·5 μM [Fig. 3(a)]. Equivalent tissue incubated in continuous light expressed 29% necrosis at 0·015 μM and 100% necrosis at 1·5 μM AAL toxin. A minimum of 12 h light was required for significant necrosis (49%) to occur at 1·5 μM AAL toxin [Table I]. Conversely, *asc/asc* protoplasts did not

TABLE I

Light exposure required to induce AAL toxin associated necrosis asc/asc *leaflets*

	Light exposure time (h)											
	0	*1*	*2*	*3*	*4*	*5*	*6*	*12*	*18*	*24*	*36*	*48*
% Necrosis*	0a	14a	23a	31a	17a	24a	27a	49b	61b	84c	71c	78c

*Statistical analysis performed on arcsine values of % necrosis. $n = 20$. Leaflets treated with 1·5 μM AAL toxin were exposed to increasing hours of mixed incandescent/cool white fluorescent light at 112 Wm^{-2} and then placed in total darkness for the duration of the 48 h AAL toxin treatment. The % necrosis induced by AAL toxin was assessed after 48 h. Means with the same letters are not significantly different at $P > F \leqslant 0{\cdot}05$.

require light to express sensitivity to AAL toxin [Fig. 3(b)]. Protoplasts exhibited 30% mortality at 1·5 μM toxin in either light or dark treatments after 5 days. Similar effects on protoplast mortality in light or dark were obtained at higher AAL toxin concentrations [Fig. 3(b)]. The mean number of viable control protoplasts was 65 per view for the light treatment and 63 for the dark treatment in these experiments.

Developmental regulation of AAL toxin sensitivity

As *asc/asc* leaflets and protoplasts aged, sensitivity to AAL toxin decreased. The youngest leaflet (position 1) expressed the greatest amount of necrosis (86%) when exposed to 0·15 μM AAL toxin compared with the intermediate leaflet (position 2) (62% necrosis) and the oldest leaflet tested (28% necrosis) [Fig. 4(a)]. At 0·15 μM toxin concentration, there was an average of 33% difference in necrosis between oldest and intermediate leaflets, 24% difference in necrosis between intermediate and youngest leaflets and 57% difference in necrosis between oldest and youngest leaflets. The sensitivity differences were significant ($P > F = 0{\cdot}05$) between the expanding leaflets (intermediate and youngest) and the fully expanded leaflets (oldest).

In a fashion similar to leaflets, *asc/asc* protoplasts became less sensitive to AAL toxin as they aged following isolation. When protoplasts were treated with 37·5 μM toxin 0–24 h after isolation, 100% cell mortality was observed after 5 days [Fig. 4(b)]. When the toxin was added 2 days after isolation, mortality was reduced to 79%. There was a gradual decline in mortality at 3 days (57%), 4 days (30%) and 5 days (20%) after isolation. Nine days post-isolation, protoplasts were no longer sensitive.

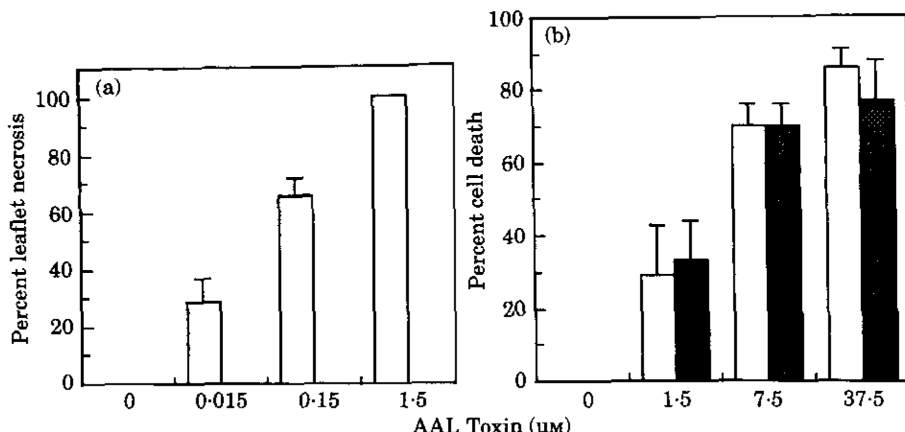

FIG. 3. Light regulation of toxin sensitivity. (a) Leaflet bioassays were carried out either in light (open bars) or in dark (dark, striped bars) as described in the legend to Fig. 1. (b) Protoplasts of the susceptible F_9 *asc/asc* genotype were incubated in light (open bars) or in dark (dark, striped bars) directly after toxin addition. Light conditions were as described in the legend to Fig. 1. For dark incubation, plates were wrapped in 2 layers of aluminium foil. Results were scored after 5 days. Viability of protoplasts was determined as described in the legend to Fig. 1.

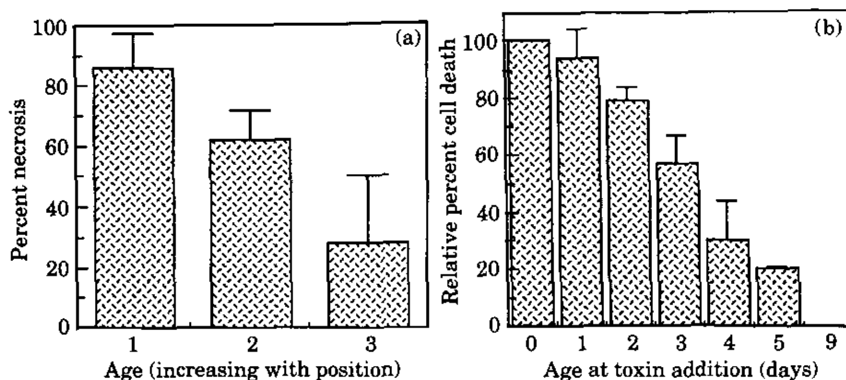

FIG. 4. Developmental regulation of sensitivity. (a) The effect of leaflet age on sensitivity to AAL toxin was determined by testing the first 5 cm terminal leaflet lamina (leaf position 1), the next older leaflet (position 2) and the next older leaflet (position 3) of the susceptible F_9 asc/asc genotype in leaflet bioassays at 0·15 μM AAL toxin. Plants were in the 7-leaf stage. Leaflet bioassays were performed as described in the legend to Fig. 1. (b) The effect of age on AAL toxin sensitivity of protoplasts of the susceptible F_9 *asc/asc* genotype was determined by adding 37·5 μM toxin 0, 1, 2, 3, 4, 5 and 9 days after protoplast isolation. Results were scored 5 days after toxin addition. Viability of protoplasts was assessed as described in legend to Fig. 1.

DISCUSSION

The action of the *Asc* gene, conditioning reaction to AAL toxin and resistance to *A. alternata* f. sp. *lycopersici*, is expressed in protoplasts as well as in the differentiated leaf tissue of tomato. Resistant leaf tissue and protoplasts from the *Asc/Asc* genotype each required 100–1000 times more AAL toxin than corresponding material from the sensitive *asc/asc* line before significant cell death was induced. Cell death began within

24–36 h after toxin exposure in both *asc/asc* leaflets and protoplasts although *asc/asc* protoplasts were 10-fold less sensitive than corresponding leaflet tissue. Similar timing required for AAL toxin induced cell death for protoplasts and leaf tissue, along with the conserved genetic differences, suggests that the same programmed sequence of events leading to cell death is expressed in both tissues.

The reason for the apparent lower sensitivity of protoplasts to AAL toxin is unclear. Leaflets contain specialized transport systems which could both concentrate the toxin in areas adjacent to susceptible interveinal cells or facilitate intracellular toxin uptake. In either case, the effective concentration of AAL toxin in the leaf lamina relative to that experienced by individual protoplasts could be higher. When radiolabelled AAL toxin becomes available, relative uptake kinetics and specific activity of toxin in the respective tissues can be determined.

Sensitivity of *asc/asc* protoplasts to AAL toxin reported here differs from previous studies using cultured tomato cells [*7*, *21*] wherein higher toxin concentrations were required for cell death and the genetic differential expected of the *Asc* gene was not detected. Clearly, aging of protoplasts resulted in decreased sensitivity of the organized cells to AAL toxin in the present study. Suspension cells from AAL toxin sensitive leaflets of interspecific crosses of *L. esculentum* × *L. peruvianum* [*7*] required up to 200-fold higher toxin concentration to inhibit cell growth than the protoplasts reported herein. In addition, no genetic evidence was reported to confirm that alleles of the *Asc* gene were present in the interspecific hybrid plants used as a source of the callus cultures. Consequently, analyses of AAL toxin effects in such cell culture systems may not relate to the action of the *Asc* gene or normal physiological effects of toxin action. Our results suggest that mechanisms occurring within 2 days after protoplast isolation reduce sensitivity to AAL toxin. Microscopic observations suggested that onset of cell wall formation (indicated visually by calcofluor white staining under u.v. light) correlated with the reduction in AAL toxin sensitivity 2 days after protoplast isolation (data not shown). No intracellular mechanism is evident to explain this result though it may be simply a matter of reduced uptake by altered transport functions. Regardless, the current results indicate that freshly isolated *asc/asc* protoplasts can be used for physiological, genetic, and molecular studies of the *Asc* gene.

The requirement for light in toxin-induced cell death in leaflets presents an interesting dichotomy with the lack of a similar requirement for protoplasts. There is no clear explanation for the differences between the respective tissues although the protoplasts could be compensated for a light requirement by ingredients in the culture media. Alternatively, the effect of light on AAL toxin sensitivity may not be expressed at the cellular level, or the effect is lost during protoplast isolation. These results do suggest that light may not be involved directly with the primary action of the *Asc* gene product but is a secondary effector of cell death analogous to the final browning sequence associated with the hypersensitive response of tobacco leaves wherein browning only occurred when a lesion is exposed to light [*11*].

In summary, the regulation of AAL toxin sensitivity and resistance due to the alternate alleles at the *Asc* locus is expressed in protoplasts as was observed previously in leaflets. Hence, protoplasts can be used to study the physiological and molecular changes in tomato cells which lead to AAL toxin induced cell death in contrast to cell cultures where the genotypic differential is not observed. Use of protoplasts also

bypasses potentially complicating tissue-level regulatory mechanisms in leaflets such as the light requirement for cell death to occur in toxin treated leaf tissue.

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