Biphasic Superoxide Generation in Potato Tubers. A Self-Amplifying Response to Stress[1]

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Potato (*Solanum tuberosum*) cultivars differ quantitatively in their responses to mechanical stress including the ability to synthesize melanin pigments in tuber tissues. Investigations into the cellular events induced by mechanical stress on tuber tissues have shown that an early cellular response is a significant and rapid synthesis of superoxide radicals. This burst of radical production distinctively displays a reproducible biphasic pattern over time with peaks of generation at 2 and 5 h. A concomitant consequence of the generation of these free radicals is elevated levels of oxidatively modified tuber proteins. Both radical generation and protein modification vary between cultivars but both are directly proportional to the amount of melanin pigments produced. Cell-free extracts of mechanically stressed tissues, pectic fragments, and scission products generated from cell walls are able to induce superoxide generation in non-stressed tissues, indicating the participation of a biologically active factor that induces a further a phase of radical synthesis.

Mechanical stress is imposed on plant cells by a variety of physical stimuli and leads to a wide range of cellular responses. Early studies on mechanical stress used simple physical contact with bean stem tissues to examine responses in cell growth (thigmomorphogenesis), callose deposition, and ethylene sythesis (Jaffe, 1973; Jaffe et al., 1985; Jaffe and Forbes, 1993). Legendre et al. (1993), Yahraus et al. (1995), and Cazalé et al. (1998) more recently exposed soybean (*Glycine max*) and tobacco (*Nicotiana tabacum*) cells to mechanical stress by agitation of suspension cultures or to hypo-osmotic medium to increase cell turgor. Using these systems, they were able to demonstrate short-term generation of hydrogen peroxide by the cells. Yahraus et al. (1995) exerted direct pressure on cultured soybean cells on a microscope slide and were able to show hydrogen peroxide generation histochemically.

Such stress responses are often associated with transcriptional activation. Shirsat et al. (1996) and Elliott and Shirsat (1998) applied weights to tobacco leaf petioles to exert tensile stress on axial cells and demonstrated transcriptional regulation of expression of cell wall-strengthening proteins in the stressed cells. Braam and Davis (1990) described the *TCH* (touch) genes in Arabidopsis responsive to mechanical stimulation by touching the leaves, by bending the plants, or by simulated wind movement. Other than these examples, few studies have been undertaken to investigate the primary biochemical responses to and the perception of mechanical stress. The potato (*Solanum tuberosum*) tuber is a modified, underground stem adapted as a storage organ, which due to its size and weight is particularly susceptible to mechanical stresses. Physically damaged tuber tissues respond by producing melanin-based pigments, leading to a blue-black discoloration of subdermal tissues known agronomically as blackspot bruising. This is a serious agronomic problem manifested during harvesting, transport, and storage leading to significant levels of rejection of potato harvests (van es Rastovski, 1987; Kleinschmidt and Thornton, 1991; Potato Marketing Board, 1994). Melanin synthesis in tubers is catalyzed in part by the enzyme polyphenol oxidase (PPO; EC 1.14.18.1) in pathways closely similar to animal melanin pigment synthesis (Valverde et al., 1996). Cellular disruption following mechanical stress leads to decompartmentalization of the amyloplast-located PPO, which then mixes with monophenolic substrates such as Tyr and chlorogenic acid. A series of oxygen-dependent reactions produces initially red-brown 3,4-dihydroxy-Phe intermediates and ultimately leads to the formation of blue-black melanin pigments which can polymerize to water-insoluble complexes (Corsini et al., 1992; Stevens and Davelaar, 1996; Friedman, 1997). Recent evidence also points to the involvement of covalently cross-linked protein in these complexes (Stevens and Davelaar, 1996, 1997). The synthesis of melanin is thought to be a defense mechanism in which the polymerized, insoluble complexes form a resistant barrier, sealing tuber tissues against the entry and spread of pathogens. The predisposition of tubers to

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melanin synthesis depends on the growing and storage conditions and exhibits a wide range of genetic variation (O'Leary and Iritani, 1969; Hudson, 1975; Skrobacki et al., 1989; Dixon, 1992; Sieczka and Thornton, 1993; British Potato Council, 2000). Transgenic plants expressing anti-PPO gene constructs now form the basis of novel potato varieties highly resistant to bruising and confirm the catalytic involvement of PPO enzymes in this phenomenon (Bachem et al., 1994; Krohn et al., 1998; Coetzer et al., 2001). Valverde et al. (1996) recently implicated the use of oxygen-free radicals by PPO in the synthesis of animal melanin-based pigments.

The oxidative burst is a rapid production of highly active oxygen species (AOS), which include various oxygen-free radicals (superoxide, hydroxyl, and hydroperoxyl radicals) and hydrogen peroxide, and is a well-characterized response of plant cells to pathogen challenge (Bolwell and Wojtaszek, 1997; Lamb and Dixon, 1997; Wojtaszek, 1997). Generation of superoxide radicals is the first step in the production of AOS. Addition of a single electron to molecular oxygen generates the superoxide anion:

$$O_2 + e^{-1} > \cdot O_2^-$$

and is believed to be catalyzed by a plasma membrane-located enzyme complex, NADPH-dependent oxidase (EC 1.6.99.6; Doke, 1995). The possible involvement of an alternative superoxide-generating pathway involving a pH-activated cell wall peroxidase has also been suggested (Bolwell et al., 1995). Under normal cellular conditions, superoxide radicals are rapidly converted to hydrogen peroxide and oxygen by the enzyme superoxide dismutase (SOD). However, during an oxidative burst, a large excess of AOS are produced, which due to their high reactivity with proteins, lipids, and nucleic acids have been proposed as potent local inhibitors of pathogen spread (Tenhaken et al., 1995; Mehdy et al., 1996). AOS have also been implicated in signaling roles and in scission and cross-linking of cell wall components (Brisson et al., 1994; Low and Merida, 1996; Fry, 1998), and it is possible that they also influence and participate in a wide range of metabolic events.

We have used a novel tetrazolium dye-based assay to directly measure superoxide radical production in tissues from mechanically stressed potato tubers. The results show that levels of superoxide synthesis vary between potato cultivars correlating closely with differing susceptibilities to blackspot bruising. This synthesis is biphasic with two peaks of superoxide accumulation over a 6-h period. We have also examined changes in the level of protein modification in mechanically stressed tubers and show a similar correlation with susceptibilities to blackspot bruising. A definitive role for superoxide radicals over other associated AOS has been shown through inhibitor and AOS-scavenging studies. Also induction of superoxide generation is demonstrated in a non-stressed tuber tissues by exposure to extracts prepared from mechanically stressed tubers and by hydrolysates from cell wall materials. We discuss the possible correlations between superoxide production, protein modification, and the synthesis of melanin bruise pigments and also the merits of a self-elicited oxidative burst, which amplifies the localized response signal.

RESULTS

Different Genetic Lines of Potatoes Exhibit a Variable, Quantitative Response to Mechanical Stress

The level of susceptibility of tuber tissues to mechanical stress is conveniently assessed by quantitation of melanin pigment synthesis in cortical tissues after a standardized impact (blackspot bruise index). In this study, potato cultivars were selected on the basis of their genetic predisposition to respond to mechanical stress through pigment synthesis (Dixon, 1992; Sieczka and Thornton, 1993; British Potato Council, 2000). The selected cultivars exhibited a wide range of susceptibilities to mechanical stress and our estimates (Table I) of the bruise indices of these cultivars were in accord with the published values in which potato cv Russet Burbank was most susceptible, whereas potato cv Maris Piper was least susceptible.

Level of Superoxide Generation Is Dependent upon the Magnitude of the Impact Energy

Tissue explants excised from impact sites on tubers were shown to respond to mechanical stress by generating superoxide radicals. When the energy of impact on tuber tissues from a mechanically sensitive line (potato cv Russet Burbank) was varied, the resulting maximum level of superoxide production also varied (Fig. 1). An impact energy as low as 0.1 J was sufficient to initiate detectable levels of radical generation, and this increased linearly with increasing energies up to 0.7 J. Above 0.7 J, impact energy

Table I. *Assessment of responses of different potato lines to mechanical stress*

Thirty tubers of each cultivar were exposed to a standard impact energy (0.7 J) and incubated at 28°C for 48 h. Tubers were quartered at the point of impact, and the extent of pigment synthesis was assessed. Indices were calculated according to the intensity of pigmentation and the volume of affected tissue. The results were expressed on a scale of 1 to 10.

Cultivar	Bruise Index
Russet Burbank	9.2
Saturna	7.9
Cara	5.5
King Edward	4.0
Maris Piper	3.1

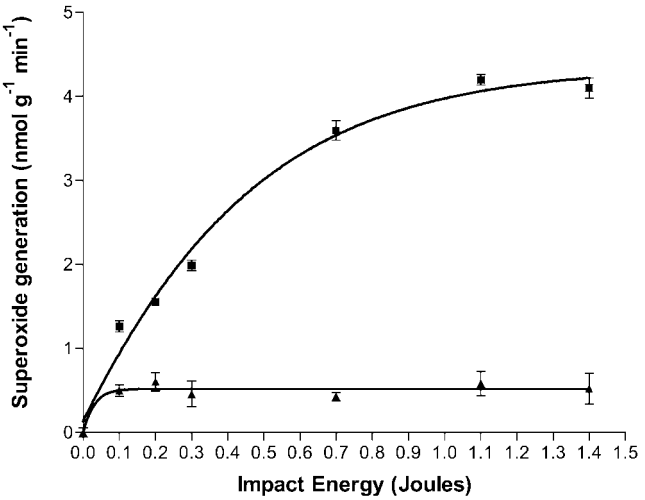

Figure 1. Superoxide generation by tuber cells exposed to increasing mechanical stress. Tubers of two different potato lines with differing mechanical susceptibilities (susceptible potato cv Russet Burbank [■] and resistant potato cv Cara [▲]) were exposed to impact energies ranging between 0.1 and 1.4 J, and the resulting generation of superoxide above background levels was measured as described in "Materials and Methods." The data plotted are the results of duplicate estimates (two samples from the same tuber) from two independent experiments. Error bars are SD; where not visible, error bars are hidden by symbols.

did not result in a further linear increase but rather reached a plateau at maximal superoxide production. In contrast, a more mechanically resistant line (potato cv Cara) was comparatively insensitive to the energy of impact. All energies tested induced only a small, constant superoxide production above background level, amounting to about 10% of the maximal response of the susceptible line. We selected 0.7 J as the standard energy of impact for all subsequent experiments.

Superoxide Radical Generation Is Directly Correlated to the Degree of Susceptibility to Mechanical Stress

Tissue explants excised from impact sites on tubers were shown to respond to mechanical stress by generating superoxide radicals (Fig. 2, A–E). The magnitude of superoxide generation was found to be highly variable between different genetic lines. Tissues from those varieties highly susceptible to mechanical stress (Fig. 2, A and B) generated much higher levels of superoxide compared with the more resistant varieties (Fig. 2, C–E). The kinetics of radical synthesis were nonlinear. Radical generation above background levels was first detectable in the most susceptible lines within about 1 h postimpact. It is intriguing that in all the varieties tested, superoxide production occurred in a distinctive and reproducible two-phase pattern. In the first phase, superoxide generation reached a peak 1 to 2 h after impact. This was followed by a second phase with maximal generation 4 to 5 h after impact. The response of potato cv Saturna (Fig. 2B) was typical of the biphasic responses by susceptible lines, and invariably the second peak was of greater intensity than the first. Both peaks were reduced in magnitude with increasing varietal resistance to mechanical stress, whereas in the most resistant varieties, the first peak was barely discernable above background, control levels. No significant increase in superoxide generation was seen in control tissues sampled distal to the point of impact, suggesting that the oxidative burst was specifically a localized response to mechanical stress.

When the maximal levels of superoxide generation were quantitatively compared (Fig. 3) to the bruise indices in the different genetic lines, a high degree of correlation was observed (Pearson correlation coefficient $r^2 = 0.9386$), indicating that the two events are closely related and potentially are causally linked.

Levels of Oxidatively Modified Tuber Proteins Are Also Correlated with Susceptibility to Mechanical Stress

One of the many consequences of elevated generation of radicals is oxidative damage to proteins resulting in modifications to amino acid side groups, notably the introduction of carbonyl groups into Lys residues. Quantification of the levels of secondary carbonyl groups in impacted tuber proteins showed a trend highly similar to the results for radical generation with mechanically susceptible varieties showing higher levels of oxidatively modified proteins. When carbonyl levels for the different potato lines were plotted against their bruise index values (Fig. 3), a tightly correlated linear relationship was observed (Pearson correlation coefficient $r^2 = 0.9448$). This substantiates the results for the direct measurement of superoxide generation, showing that carbonyl modification is also directly proportional to mechanical susceptibility of the genetic line. It further confirms the elevated generation of superoxide radicals. These data constitute the highest correlated set of factors to blackspot bruise so far described. It is likely that other radical-mediated modifications will follow similar linear relationships.

Superoxide Radicals and Not Hydrogen Peroxide Are Causally Linked to Pigment Synthesis

Previous studies of the responses of plant cells to mechanical stress have demonstrated the production of hydrogen peroxide as a key AOS. In this study, the development of melanin pigmentation in impacted tuber tissues was investigated using AOS-scavenging enzymes and an inhibitor of radical synthesis.

In the presence of the NADPH oxidase inhibitor, diphenylene iodonium chloride (DPI), synthesis of pigment was inhibited by more than 70% of the control level (Fig. 4). This supports the suggestion that initial superoxide generation and pigment syn-

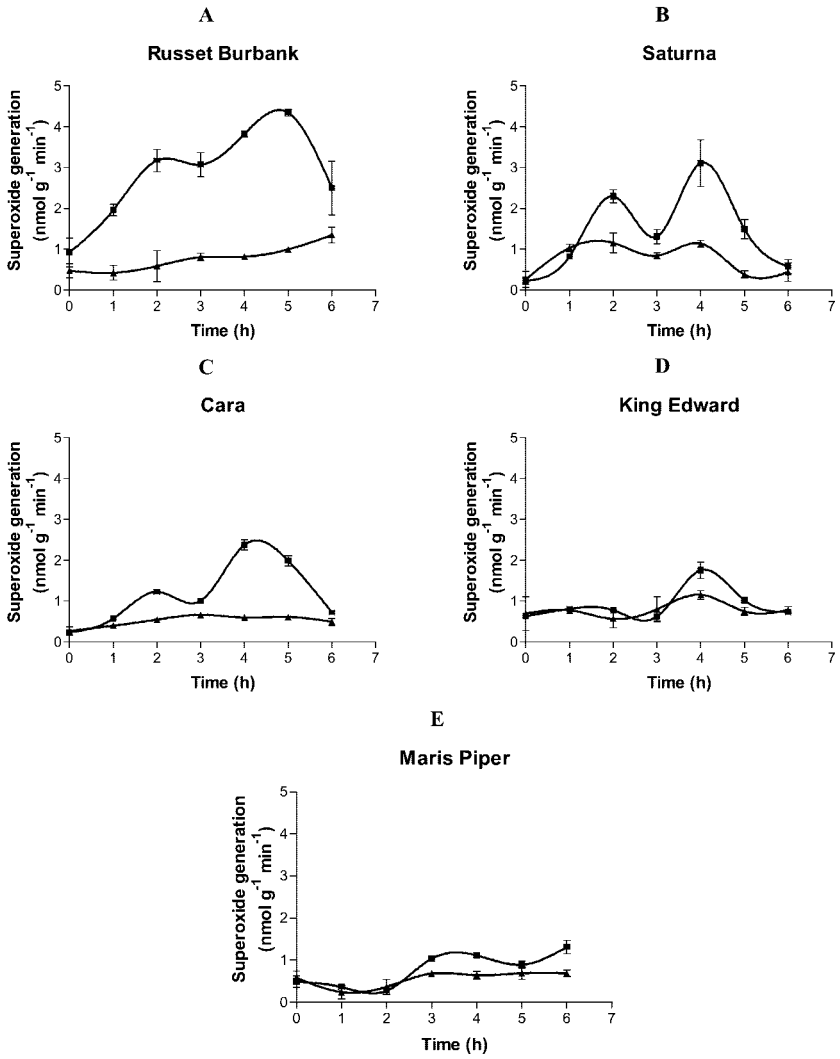

Figure 2. Time course of superoxide radical generation from impacted tuber tissues excised from potato cultivars displaying variation in mechanical susceptibility. Superoxide assays were carried out using the formazan dye 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) as indicated in "Materials and Methods." A through E represent results from (in order of increasing resistance) cvs Russet Burbank, Saturna, Cara, King Edward, and Maris Piper, respectively. Assays were performed on tissues excised from tubers 0 to 6 h after impact either from the impact site (■) or at a distal site (▲) on the same tuber. All assays were carried out in duplicate (two samples from the same tuber) and are the results from four independent experiments. Error bars are SD; where not visible, error bars are hidden by symbols.

thesis are linked. To investigate which AOS were involved, impacted tuber sections were exposed to SOD and catalase (Fig. 4). SOD, which actively removes superoxide radicals, had a strongly inhibitory effect upon pigment synthesis, reducing the values to 40% of the control. In contrast, catalase, which eliminates hydrogen peroxide, had little effect over the control pigment levels. This suggests that superoxide and not hydrogen peroxide is directly responsible for the effects upon bruise development. The results of the SOD experiments further indicated that superoxide production was necessary for pigment synthesis rather than merely a by-product thereof.

Cell-Free Extracts from Mechanically Stressed Tuber Tissue Elicit Superoxide Generation in Nonimpacted Tissues

The biphasic response to impact was indicative of two distinct events and possibly a reflection of the involvement of two different receptors. To investigate the nature of the initiation and biphasic generation of superoxide, a series of experiments were carried out to address the possibility of self-elicitation. Cell-free extracts were prepared from impacted tubers of susceptible (potato cv Russet Burbank) and resistant (potato cv Cara) tubers. These extracts were then tested for the ability to elicit superoxide generation in nonimpacted tuber explants. When extracts from an impacted susceptible cultivar (potato cv Russet Burbank) were exposed to nonimpacted tuber tissues, a substantial single burst of superoxide generation was observed with both susceptible (potato cv Russet Burbank) and resistant (potato cv Cara) varieties (Fig. 5A). In both cases, radical generation rose to a maximum level 1 to 3 h after exposure to the extract and then fell back to the background level. When an extract from an impacted resistant cultivar (potato cv Cara) was conversely exposed to the same two cultivars, little or no superoxide generation was observed (Fig. 5B). This suggested that a factor was uniquely produced in the susceptible cultivar during the response to mechanical stress and was subsequently able to induce a

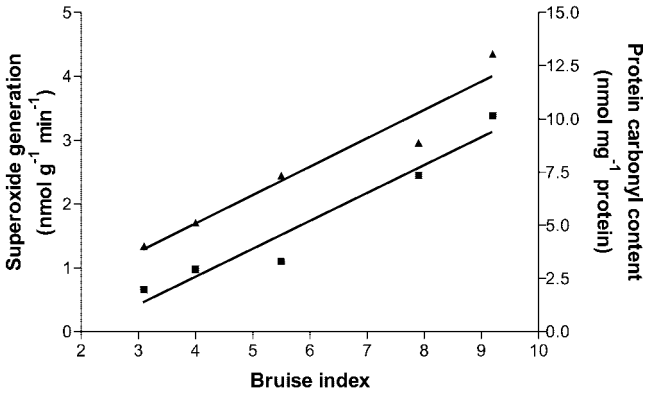

Figure 3. Relationship between levels of superoxide radical generation (▲), tuber protein carbonyl content (■), and tuber susceptibility to mechanical stress (bruise indices). The Pearson r^2 values of 0.9386 (radical generation) and 0.9448 (protein carbonyl content) indicate strong correlations with response to mechanical stress. The potato cultivars used to establish these correlations were Russet Burbank, Saturna, Cara, King Edward, and Maris Piper. Data plotted are the means of duplicates (two samples from the same tuber) from four independent experiments. Error bars are SD; where not visible, error bars are hidden by symbols.

superoxide burst even in a resistant cultivar, and that the same factor was not present in a resistant cultivar.

The Biphasic Oxidative Burst Is a Self-Induced Response Mediated by a Pectic Fragment Product of Radical Scission of a Cell Wall Polysaccharide

To investigate the nature of the factor produced in the susceptible varieties, nonimpacted tissues were exposed to various cell-free extracts or cell wall-derived fragments and then tested for initiation of superoxide synthesis. The results in Figure 6 were subjected to statistical analysis (ANOVA one-way analysis), and all data classified as significantly different had $P < 0.01$.

Control, nonimpacted tissues showed only low levels of superoxide generation (Fig. 6, B–C). Impacted susceptible (potato cv Russet Burbank) tissues showed elevated generation of superoxide, whereas impacted resistant (potato cv Cara) tissues showed only a small increase as observed previously. The two varieties both showed significantly elevated superoxide generation when nonimpacted tissues were exposed to pectinase or to a free radical-generating reaction (Fig. 6, B–D). The elevated superoxide generation in tuber tissues observed in potato cv Russet Burbank after impact could also be initiated in nonimpacted tissue by exposure to intact cells, purified cell walls, or pectin, treated with pectinase or purified polygalacturonidase enzyme (Fig. 6, B and E–G). The action of these enzymes clearly released a fragment from the pectic acid component of the cell walls, which then activated a receptor eliciting a burst of superoxide radical generation. In a similar fashion, exposure of the cell wall materials to radicals produced by an artificial generating system also produced a fragment that initiated a burst of radical synthesis. It is reasonable to assume that in this case, radical scission of cell wall polysaccharides including the pectic acid component, produced the same or a similar active pectic fragment that initiated the oxidative burst.

One of the objectives of these assays was to elucidate differences in response between potato varieties displaying different mechanical properties. It is clear that potato cv Russet Burbank tissue produces a large oxidative burst after impact, whereas potato cv Cara displays only a small increase over background levels (Figs. 5 and 6, B and C). However, it was unclear what the basis of this difference was. Interestingly, when potato cv Cara nonimpacted cells were exposed to pectinase- or radical-generated fragment preparations from potato cv Cara, a similar large oxidative burst was observed (Fig. 6C).

The results shown in Figure 5A proved that fragments generated from potato cv Russet Burbank cells were able to initiate a large oxidative burst in potato cv Cara cells. To prove that the same fragments were generated in resistant varieties, "cross-activation" experiments were set up in which superoxide generation was assayed from nonimpacted tissues of potato cvs Cara and Russet Burbank exposed to pectinase- or radical-generated fragments from potato cv Cara tuber cell walls. The results (Fig. 6D) showed that all potato cv Cara fragments were capable of eliciting a large oxidative burst in cells of both potato cvs Russet Burbank and Cara.

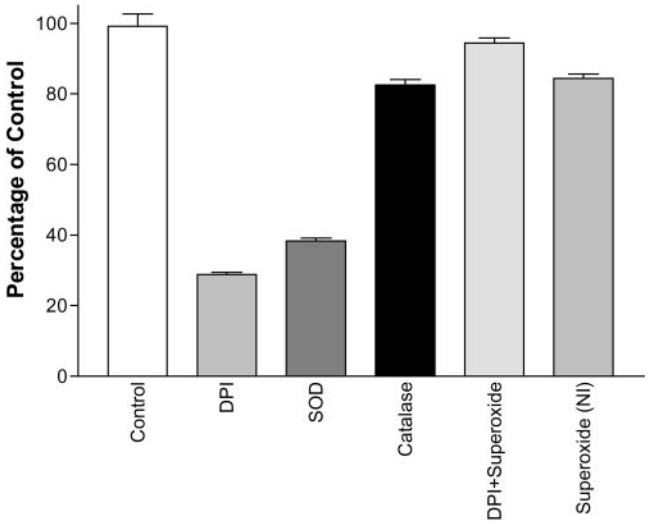

Figure 4. Effects of inhibitors of oxidative reactions on the generation of oxidatively modified proteins in mechanically impacted tubers of potato cv Russet Burbank. Inhibitors used were DPI, SOD, or catalase. Confirmation of alterations to AOS generation were made by analyzing secondary carbonyl accumulation in each of the three treatments (data not shown). The results were expressed as percentage of mean control value, and data plotted are duplicates (two samples from the same tuber) from four independent experiments. Error bars are SD; where not visible, error bars are hidden by symbols.

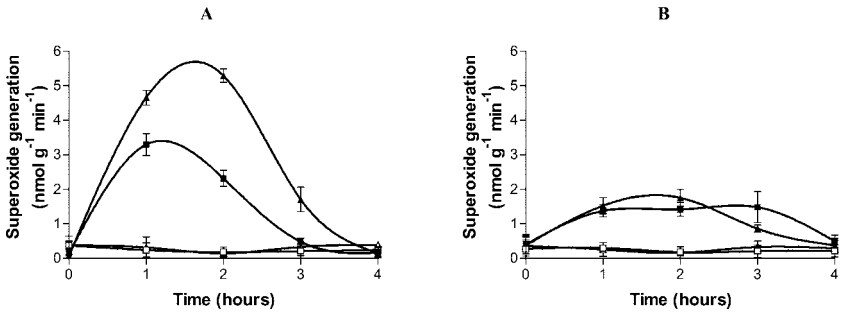

Figure 5. Elicitation of superoxide generation by cell-free extracts from impacted tuber tissues. A, Exposure of a nonimpacted bruise-resistant cultivar (potato cv Cara; ▲) and a nonimpacted bruise-susceptible cultivar (potato cv Russet Burbank; ■) to cell-free extracts of the impacted bruise-susceptible cultivar showing the generation of a single burst of superoxide generation after 1 to 2 h. Controls using extracts from nonimpacted tissue are potato cvs Cara (△) and Russet Burbank (□). B, Conversely, where the two cultivars (potato cvs Russet Burbank [▲] and Cara [■]) are exposed to a crude extract prepared from a bruise-resistant cultivar (potato cv Cara), no comparable superoxide generation resulted. Controls using nonimpacted tissue extracts are potato cvs Russet Burbank (△) and Cara (□). Data are the mean values for duplicates (two samples from the same tuber) from four independent experiments. Error bars are SD; where not visible, error bars are hidden by symbols.

DISCUSSION

We describe here the novel detection of superoxide radicals generated in potato tuber cells in response to mechanical stress and particularly differences in response between mechanically resistant and susceptible varieties. Detection is based on immersion of tissue explants from test tubers directly into a tetrazolium dye solution that sequesters the radicals immediately when they are synthesized. As a result, the assay provides a measure of generation potential rather than levels of accumulation. Due to the high reactivity of the free radicals and the low diffusion rate in the tuber apoplast, we believe that the observed results represent the responses of a few cell layers near the surface of the tissue cube. Assays in which the explants were bisected showed that radical production approximates more to the surface area rather than the volume of tissue.

We have used this assay to follow in detail the time course and factors affecting the level of radical generation in potato tuber cells after impact. Cells in tuber tissues exposed to mechanical stress respond with a rapid production of superoxide radicals. Susceptible potato varieties such as potato cv Russet Burbank display a large burst of radical synthesis, whereas more resistant varieties such as potato cv Cara show relatively low levels of radical production. Depending on the variety used, the response is proportional to the level of stress (impact energy) imparted up to a maximum level (Fig. 1). This indicates that the perception of the stress and the signal transduction to the superoxide synthesis complex are quantitatively linked, but there is an upper limit defined by other factors such as cell wall strength.

The level of oxidative burst is genetically highly variable because different potato varieties show diverse responses and quantities of superoxide generated (Fig. 2). We have followed superoxide generation over several hours after exposure to a standard impact. The phenomenon was most evident in potato cvs Russet Burbank and Saturna, cultivars that also exhibit the highest susceptibility to mechanical stress, i.e. display the highest bruise indices (Table I; Fig. 2). A novel feature of the superoxide synthesis was the biphasic pattern of generation with peak levels detectable at 1 to 2 and 4 to 5 h postimpact. This pattern is reminiscent of the response reported by Baker and Orlandi (1995) in which cultured plant cells treated with a pathogen-derived elicitor displayed two peaks of hydrogen peroxide generation over a period of 6 h. Phase 1 was suggested to be a nonspecific biological response to stress, whereas phase 2 was determined to be a specific interaction between the pathogen *hrp* complex and the plant receptors leading to hypersensitive cell death. In the present tuber system, we see an equivalent initial peak that probably corresponds to a response initiated by the shock wave passing through the cells causing a perturbation of the cell organization and ion balance or activation of a membrane-associated "stretch receptor' as proposed by Cazalé et al. (1999). The second peak is potentially of much greater interest. The observation that a second burst arises in mechanically stressed tissues in the absence of pathogens or pathogen-derived elicitors suggests activation via a cell receptor that further induces or amplifies the cells responses to the mechanical stimuli. Other studies have demonstrated AOS production in response to mechanical stress. Yahraus et al. (1995) exerted mechanical stress in soybean cell suspension cultures by hypo-osmotic media or by exerting physical pressure on cells under a microscope slide and were able to demonstrate peroxide production histochemically after a few minutes. Legendre et al. (1993) and Cazalé et al. (1998, 1999) used physical mixing of cell suspensions to impose mechanical stress and showed an oxidative burst of peroxide synthesis. However, these studies did not follow the longer time course described in the

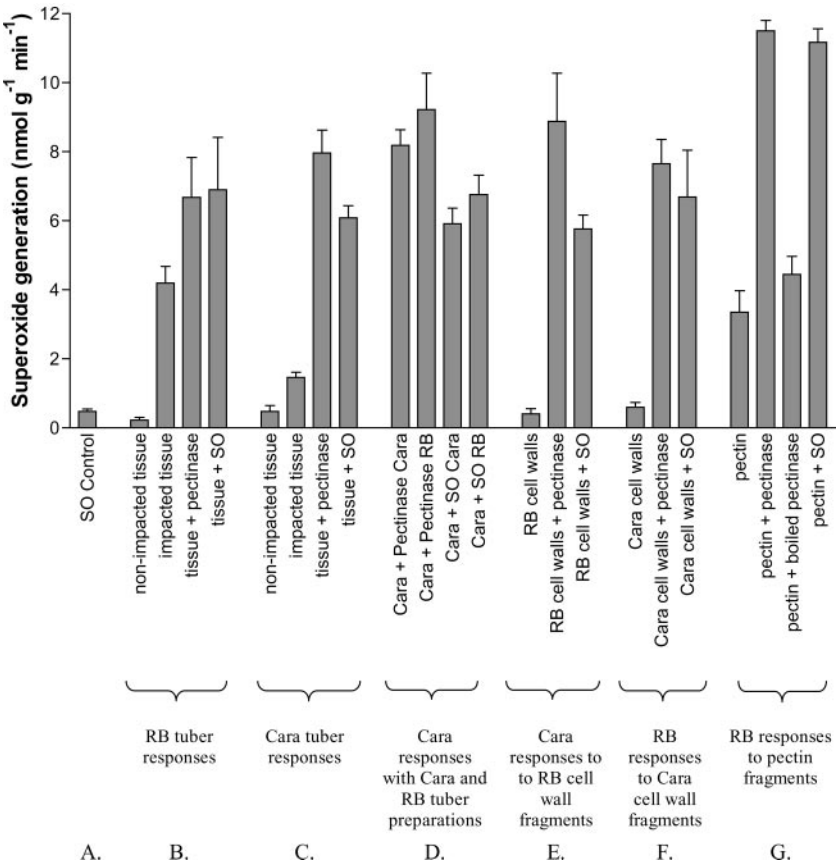

Figure 6. Superoxide generation by tuber cells in response to exposure to fragments generated by enzymic digestion or radical scission. The figure shows the extent of superoxide generation by tuber cells treated in various ways. Data are the mean values for duplicates (two samples from the same tuber) from four independent experiments. Error bars are SD; where not visible, error bars are hidden by symbols. A, SO control: nonimpacted tissue treated with exhausted superoxide generation solution; B, potato cv Russet Burbank tissue responses; C, potato cv Cara tissue responses; D, cross responses of potato cv Cara tuber preparations on potato cv Cara or potato cv Russet Burbank tissues; E, responses of potato cv Russet Burbank tissues exposed to potato cv Russet Burbank cell wall fragments; F, responses of potato cv Cara tissues exposed to potato cv Cara cell wall fragments; G, responses of potato cv Russet Burbank tissues exposed to pectin fragments. SO, Superoxide radical-generating system; RB/*Cara*, material derived from potato cvs Russet Burbank or Cara nonimpacted/impacted tissue − superoxide generation from nonimpacted tubers or tubers exposed to a standard impact; + pectinase, tissue samples or materials exposed to pectinase enzyme; + SO, tissue samples or materials exposed to superoxide radical-generating system; RB cell walls/*Cara* cell walls, cell walls purified from potato cvs Russet Burbank or Cara tubers; pectin, citrus peel pectin; cross-responses, responses of nonimpacted tissues of potato cvs Russet Burbank and Cara; *Cara*+Pectinase → *Cara*, nonimpacted potato cv Cara tissue exposed to an extract of pectinase-treated potato cv Cara tissue; *Cara*+Pectinase → RB, nonimpacted potato cv Russet Burbank tissue; exposed to an extract of pectinase-treated potato cv Cara tissue; *Cara*+SO → *Cara*, nonimpacted potato cv Cara tissue exposed to an extract of radical-treated potato cv Cara tissue; *Cara*+SO → RB, nonimpacted potato cv Russet Burbank tissue exposed to an extract of radical-treated potato cv Cara tissue.

present study and only measured hydrogen peroxide levels rather than superoxide radicals.

The observation that those varieties exhibiting a large oxidative burst were also the ones showing high mechanical susceptibility, as judged by high levels of melanin synthesis, posed the question of the relationship between these two processes. The correlation data presented in Figure 3 indicates that there is a direct and tight quantitative relationship between the degree of mechanical susceptibility of a variety and the level of superoxide generated by its cells on impact.

Experiments designed to remove superoxide radicals or to inhibit superoxide generation also demonstrated that this radical was directly required for generation of melanin in response to mechanical stress (Fig. 4). Use of nitroblue tetrazolium as a histochemical stain for superoxide radicals indicated that the oxidative burst was spatially restricted to the impact zone and coincident with the region of melanin pigment synthesis (data not presented). Melanin and related complexes are known to be free radical scavengers, and melanin radicals have been demonstrated in animal pigments under conditions that

elevate free radicals (Qu et al., 2000), so it seems plausible that radical production and melanin synthesis may be causally related. Furthermore, work by Valverde et al. (1996) in mouse melanoma cells has provided direct evidence that PPO, the key enzyme involved in melanin pigment synthesis, may preferentially use superoxide radicals as a cosubstrate rather than any other form of molecular oxygen. In this way, a high level of superoxide radicals generated in a susceptible variety would provide an excess of a preferred cosubstrate for PPO.

One of the consequences of radical production is the oxidative modification of proteins in which carbonyl groups are introduced into protein side groups through modification of Lys, Pro, and Arg residues (Stadtman, 1993). Such protein modifications are well documented in animal systems, but the present report is the first demonstration, to our knowledge, of the effect occurring as an outcome of mechanical stress in plants. Because oxidative modification of tuber proteins arises through interaction with the oxygen-free radicals, it is not unexpected that levels of protein modification produced a similar direct relationship with the bruise indices (Fig. 4). This result also provides independent corroboration of the levels of radical production.

A large oxidative burst was inducible in both susceptible and resistant varieties by exposure of nonimpacted tuber cells to cell-free extracts from an impacted susceptible variety (Fig. 5). In contrast, an extract from an impacted resistant variety was unable to induce superoxide generation in nonimpacted cells of either a resistant or a susceptible variety. These results implicate a factor produced endogenously in impacted susceptible tuber cells but that is absent from impacted resistant cells. Because the factor can elicit a large oxidative burst in both susceptible and resistant varieties, this indicates that both are equally receptive to its presence and respond to the same extent. However, the resistant variety is incapable of producing or does not produce the factor on impact. Exposure to the factor initiated a single burst of superoxide generation of comparable magnitude to the second peak observed in the biphasic response to impact. This suggests that the second phase arises by the production of the fragment in vivo.

We extended these experiments further to try to elucidate the nature of this factor (Fig. 6, A–G). Pectinase and superoxide radicals produce fragments from both varieties that can elicit a superoxide burst. It is significant that fragments from the resistant variety, potato cv Cara, were able to induce a large oxidative burst in potato cv Cara tissues. This confirms that potato cv Cara can, but does not, produce the fragment in response to impact and precludes the inability to produce the fragment as suggested earlier (Fig. 5). Evidence from cross-activation experiments indicated that the fragments generated in susceptible and resistant varieties are most probably the same because potato cv Russet Burbank-generated fragments elicit a response in potato cv Cara and vice versa. The factor is clearly associated with cell walls. Treatment of purified cell walls by pectinase or superoxide action produced fragments causing elevated superoxide synthesis in nonimpacted tissues of both varieties.

The conclusion from the results shown in Figure 6G suggest that the most likely identity of the factor is a pectin-derived fragment. As suggested by other workers, active pectic fragments can be generated in the absence of enzymatic action by free radical oxidative scission. Fry (1998) suggested that scission of plant cell wall polysaccharides by hydroxyl radicals could yield biologically active fragments. These may act as ligands in the activation of a plasma membrane receptor, which initiates a signaling cascade leading to a large oxidative burst. Our results support this contention and furthermore provide an explanation for the observed biphasic superoxide synthesis. Because superoxide radicals rapidly dismutate to other AOS including hydroxyl radicals, the model proposed here could provide an in vivo example of the process of radical scission described by Fry and others (Fry, 1998: Fry et al., 2001; Miller and Fry, 2001).

CONCLUSIONS

The biphasic superoxide generation described here provides evidence of two possible receptor functions: first, a mechano-receptor that detects the transient shock-wave due to mechanical impact and induces an initial burst of superoxide synthesis into the apoplast of the tuber tissue. As a result of radical oxidative scission of cell wall pectin, biologically active pectic fragments are produced. Second, an elicitor-type receptor detects these active fragments and is activated leading to initiation of a signaling cascade which causes the second, larger burst of superoxide synthesis. The second phase of superoxide synthesis represents a significant amplification of the original response and suggests that in other stress situations a similar amplification may operate. Whether the elicitor-type receptor proposed here is unique or is the same as pathogen-type receptors remains to be elucidated.

The genetic variation observed between mechanically susceptible and resistant potato varieties appears to be largely based on the ability or inability of tuber cells to initiate the first synthesis of superoxide. It is clear from the present work that there are no compositional differences between the cell wall polysaccharides that preclude the generation of the active fragments. Nor is there significant variation in the level of receptor that initiates superoxide generation.

The amplification of the initial superoxide burst in susceptible potato varieties leads not only to the elevated synthesis of melanin pigmentation but also to

the manifestation of other deleterious effects including protein modification, membrane disruption, and cell death (Croy et al., 1998; Partington et al., 1999; Laerke et al., 2000). The genetic basis of the variation in initial response remains to be elucidated.

MATERIALS AND METHODS

Plant Materials

Five potato (*Solanum tuberosum*) varieties exhibiting different degrees of susceptibility to mechanical damage were studied: cv Cara (5.5), cv King Edward (4.0), cv Maris Piper (3.1), cv Russet Burbank (9.2), and cv Saturna (7.9). Figures in brackets refer to the bruise indices for these varieties. Tubers were specifically grown and harvested manually to avoid any mechanical stress. Harvested tubers were stored in the dark at 10°C to inhibit greening and sprouting.

Tuber Mechanical Stress

Tubers were incubated for 48 h at 4°C in the dark and then impacted at the stolon end using a falling weight of 240 g and a 300-mm drop height imparting a standard energy of 0.7 J (Croy et al., 1998). Impacted and control tubers were then incubated at 26°C to promote maximal synthesis of bruise pigments. For bruise index calculations, tubers were incubated for 48 h and then cut in quarters centered at the impact site. The volume of affected tissue was measured, and the intensity of pigmentation was estimated on a scale of 0 (no discoloration) to 3 (deep blue-black coloration). Thirty tubers for each of the five cultivars were used, and the mean bruise index was calculated based on bruise extent and intensity. Values were expressed as percentages of a theoretical maximum bruise volume and intensity.

Assay of Superoxide Radical Generation in Tuber Tissues

Tuber tissues exposed to the standard mechanical stress were incubated in the dark at 26°C for various times and then assayed for superoxide generation. Five-millimeter cubes (125 mm^3 = 35 mg) of tuber tissue were excised from the center of impact sites and from distal control sites on the same tubers, washed thoroughly with distilled water, blotted dry, and then incubated at 20°C for 20 min in 200 μL of 0.12 mM XTT in 50 mM phosphate buffer, pH 8.2 (Able et al., 1998). The tissue cube was removed, and the assay solution was centrifuged (13,000*g* × 5 min). The A_{450} of the supernatant was measured and expressed as micromoles of superoxide generated per minute using the molar extinction coefficient for the XTT formazan product of 23,600 M^{-1} cm^{-1} (Sutherland and Learmonth, 1997; M. Sutherland, personal communication). Superoxide estimations were carried out in duplicate, and all assays were replicated. Verification of superoxide detection was confirmed by adding SOD at the start of the assay (Able et al., 1998).

Estimation of Protein Modification in Tuber Tissues

The carbonyl content of oxidatively modified tuber proteins was quantified by the spectrophotometric assay method of Levine et al. (1994). Tubers were exposed to standard mechanical stress and incubated for 48 h. Proteins were extracted from 150 mg of control and impacted tissue in 3 mL of 50 mM phosphate buffer, pH 7.4. The carbonyl groups on extracted proteins were reacted with 2,4-dinitrophenylhydrazine, and the resulting hydrazone derivatives were estimated from the peak absorbance at 355 to 390 nm using a molar extinction coefficient of 22,000 M^{-1} cm^{-1}. Protein contents of duplicate sample extracts were estimated from the A_{280}. Values were expressed as nanomoles of carbonyl per milligram of tuber protein.

Exposure of Tubers to Inhibitors and Free Radical Scavengers

Tubers were mechanically impacted followed immediately by bisection of the entire tuber through the point of impact. Each one-half was then extensively washed three times in distilled water before being gently blotted dry. One-half was placed in 5 mL of 50 mM phosphate buffer (pH 7.2) and acted as the uninhibited control; the other one-half was placed in 5 mL of test solution (10 μM DPI or 1 μg mL^{-1} catalase or 0.5 μg mL^{-1} SOD in 50 mM phosphate buffer, pH 7.2) in a petri dish. Each tuber half was then incubated at 27°C in the dark for the required time.

Response of Tuber Cells to Extracts from Mechanically Stressed Tissues and Cleavage Products of Cell Wall Materials

Five-millimeter cubes (125 mm^3) of tuber tissue were exposed to various tissue extracts and assayed for superoxide generation as described previously. Extracts were prepared from a 5-mm tuber cube excised from the center of impact sites, 2 and 4 h postimpact, and from nonimpacted tubers. Cubes were washed thoroughly with distilled water, homogenized in 500 μL of 50 mM phosphate buffer (pH 7.2) and centrifuged (13,000*g*) for 5 min. Two hundred microliters of each supernatant was added to a fresh, washed, 5-mm test cube from a nonimpacted tuber and incubated at 20°C for 2 h. The tuber tissue was then removed, washed, and immediately assayed for superoxide generation as described earlier.

Tissue cubes were also tested for superoxide generation after exposure to exogenously generated fragments from cell walls or pectin extract. Fifty microliters of 1% (w/v) aqueous suspension of purified tuber cell walls (see below) or 50 μL of 5% (w/v) citrus peel pectin in 200 μL of 50 mM sodium phosphate buffer, pH 7.8, was treated with 5 units of polygalacturonase, 5 μL of pectinase (Pectinex 3× L, Novozyme/Sigma-Aldrich, Poole, UK), or a superoxide generation solution. Superoxide generation was initiated by the addition of 50 μL of 2.8 μM phenazine methosulphate to 25 μL of 196 μM NADH and 0.2 μM EDTA in 50 mM sodium phosphate buffer, pH 7.8; superoxide generation lasted around 20 min. Mixtures were incubated for 30 min at room temperature before centrifugation (15,000*g*, 10 min, 16°C) and retention of the supernatants.

Cell walls were prepared from tubers of the potato cvs Cara and Russet Burbank. One hundred grams of tuber cortex (stolon end) was grated and extensively homogenized in 100 mL of 2 mM dithiothreitol, using a mortar and pestle. The homogenate was filtered through muslin and the cell walls, retained in the muslin, recovered, and rehomogenized until most of the starch grains were removed. The cell walls were then purified from residual starch by centrifugation onto a cushion of 100% (w/v) Percoll (Pharmacia AB, Uppsala) at 2,000*g*, 5 min, and 10°C. The starch grains passed through the Percoll cushion, and the cell walls collected at the interface. These were judged pure by fluorescence microscopy using calcofluor stain and by the absence of starch grains.

Statistical Analyses

Statistical analyses were performed on all replicated data sets using Prism software (v3.02, Graphpad Software, San Diego). Error bars on Figures 1 through 3 and 5 are of SD. All data used for graphs were from independent experiments replicated either two or four times as indicated. One-way ANOVA analysis (Newman-Keuls test) was used for pair wise comparisons of all data sets for Figures 4 and 6. Pearson *r* values for correlations were calculated for data in Figure 3.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

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