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PRODUCTION, MOLECULAR EVALUATION AND DISEASE RESPONSE OF TRANSGENIC POTATO PLANTS (SOLANUM TUBEROSUM L.) EXPRESSING A FUNGAL GLUCOSE OXIDASE GENE

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By

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ABSTRACT

PRODUCTION, MOLECULAR EVALUATION AND DISEASE RESPONSE OF TRANSGENIC POTATO PLANTS (*SOLANUM TUBEROSUM* L.) EXPRESSING A FUNGAL GLUCOSE OXIDASE GENE

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Late blight, (*Phytophthora infestans* (Mont.) De Bary), is one of the most devastating diseases of potato worldwide and the development of cultivars resistant to this disease is an important objective for potato breeding programs. Current emphasis is on the utilization of horizontal resistance rather than vertical resistance conferred by specific R (resistance) genes. This research was undertaken to determine if enhanced resistance to late blight could be obtained by combining natural resistance and engineered resistance conferred by the fungal transgene glucose oxidase. Late blight susceptible cultivars 'Atlantic', 'Snowden' and 'Spunta' and partially resistant cultivars 'Zarevo' and 'Libertas' were transformed with the glucose oxidase gene under the control of the Gelvin Super Promoter. Copy number ranged from 1 to 8 and glucose oxidase enzyme activity ranged from 0 to 93.96 Units/mg plant tissue * 10^5. In agronomic trials, 44% of the transgenic lines tested were determined to be "off-type" compared to appropriate non-transgenic controls based on plant and/or tuber phenotypic characteristics. Because several off-type lines did not express the glucose oxidase protein, this phenomenon can not be attributed to the glucose oxidase transgene.

Lines expressing the glucose oxidase transgene were evaluated for reaction to *P. infestans* in growth chambers and inoculated field studies. Results from growth chamber trials suggested a small effect in 'Spunta'-derived transgenic lines but high coefficient of

variation (CV) values prevented the identification of any significant differences between the transgenic lines and their respective non-transgenic controls. Field studies revealed no significant effect of the glucose oxidase transgene in either late blight susceptible or partially late blight resistant cultivars.

Transgenic lines were also evaluated for response to *Erwinia. carotovora* ssp. *carotovora* (soft rot) and *Fusarium sambucinum* (dry rot). Following inoculation, disease progression was determined by measuring the diameter and depth of lesions over time for *Fusarium* dry rot and by measuring lesion diameters over time for *Erwinia* soft rot.

The glucose oxidase gene had a minimal effect on resistance of the cultivars to *Fusarium* dry rot and *Erwinia* soft rot although differences between lines were difficult to detect due to high error variation. Lines SGO-1 and SGO-2 were the only ones significantly different from the non-transgenic control for response to *Fusarium* dry rot and lines SGO-9, SGO-12, LGO-1 and LGO-3 were the only lines significantly different from the non-transgenic controls for response to *Erwinia* soft rot. There did not appear to be any correlation between resistance to *Fusarium* and resistance to *Erwinia* among the transgenic lines.

These results indicate that the glucose oxidase gene alone or in combination with natural host plant resistance will not produce completely late blight resistant cultivars nor will it produce high levels of resistance to *Fusarium* dry rot or *Erwinia* soft rot. However, potato cultivars with moderate levels of disease resistance may be useful in combination with reduced input chemical control programs. This potential benefit would need to be weighed against the time and cost required for the development of transgenic potato cultivars and the potential for off-type tubers and plants.

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Wow! After five years of hard work I've finally finished my Ph.D.! However, saying, "I've" finished is horribly inaccurate due to the many people who have contributed their time, knowledge and emotional support. I would like to thank my committee members Dr. Jim Kelly, Dr. Ken Sink and Dr. Ray Hammerschmidt for lending their expertise to the completion of this research. My major professor, Dr. Dave Douches, deserves a special thanks for his input on the design and execution of my research and for hours of conversation and debate on my research, various plant breeding topics and things totally unrelated to science. I would also like to thank the "potato crew", Dilson Bisognin, Joe Coombs, Chris Long, Anne Lund, Beth Leary and Kelly Zarka for all their help and for the fun and friendship we experienced together. Special thanks to Kelly for putting up with my mishaps in "her" lab. I also need to thank Dr. Willie Kirk and the late blight crew for their help both in the lab and in the field. Last but not least, thanks to my husband Rob for his support, encouragement, humor (he told everyone that I was getting my Ph.D. in light bright) and for not proposing until after I passed my comprehensive exams! Thanks again to you all for your invaluable part in this chapter of my life.

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Chapter I

GENERAL INTRODUCTION

The cultivated potato, *Solanum tuberosum,* has had an interesting history ranging from its role in the Irish potato famine of the 1840's, to its current importance in the fast-food industry. Though it was slow to be accepted as a food crop when introduced into Europe from South America, the potato has become one of the most important food crops in the world. Although it is enjoyed in the United States for its taste and versatility, in other parts of the world the potato is desired for its high nutrient value for humans and for livestock. Ranking fifth in area among crop plants grown for human consumption and fourth in yield, the potato is superior to many crop plants in protein production per unit area and time, energy production and the presence of essential amino acids (Ross, 1986).

Although the origin of the cultivated potato is not certain, archaeological evidence and the current distribution of primitive cultivated potatoes and wild species suggests that the potato was first domesticated in northern Bolivia near Lake Titicaca. It then spread rapidly throughout the high Andes of Bolivia and Peru where other staple crops such as maize did not thrive (Hawkes, 1992). In addition to the cultivated potato, there are many wild potato species that range in ploidy level from diploid (2n=2x=24) to hexaploid (2n=6x=72) with most species functioning as autopolyploids and only a few as allopolyploids. These species occupy a large geographical area ranging from the southwestern United States, through Mexico and Central America and throughout many South American countries. It is from these wild species that the cultivated potato was derived. Although the exact progenitor of cultivated potato is unknown, it is generally assumed to be a diploid species whose natural distribution area coincides with the

proposed region of domestication (high Andes from central Peru southwards to central Bolivia) (Hawkes, 1990). The wild species *S. leptophyes* is found in the appropriate area and has been proposed as similar or identical to the progenitor species for cultivated diploid potato (Hawkes, 1990) (Figure 1.1). Other species such as *S. canasense* have been proposed as the progenitor species for diploid cultivated potato. Based on chloroplast genome type (ct-genome), Hosaka (1986) suggest that *S. canasense* is one of the most probable ancestral species as it has the C type ct-genome which is ancestral to the S type ct-genome common in cultivated diploid species (Table 1.1; Figure 1.2). Although many of the cultivated potatoes and wild species are short-day adapted, selection from *S. tuberosum* ssp. *andigena* both in Europe and Chile produced the long-day adapted *S. tuberosum* ssp. *tuberosum* which is cultivated in many areas of the world. Although it is generally agreed that the cultivated potato arrived in Spain around 1570 and in England around 1590 (Hawkes, 1992) the nature and source of the early European potato has been disputed. Some botanists believed that the European potato must have been imported from Chile already adapted to long day lengths (*S. tuberosum* ssp. *tuberosum*) (Hawkes, 1992). However, several pieces of information refute this assertion leading both Salaman (1946) and Hawkes (1967) to suggest that the European potato was derived from Andean sources of the short day adapted *S. tuberosum* ssp. *andigena*. First of all, herbarium specimens and descriptions from the late 16th and early seventeenth centuries more closely resemble subspecies *andigena* than subspecies tuberosum (Hawkes, 1967). Secondly, the trip from Chile to Europe would have taken too long for potato tubers to survive thus it is more likely that the first shipments came from Peru or Colombia (Salaman 1946). In addition, the conversion of *andigena* to *tuberosum* has

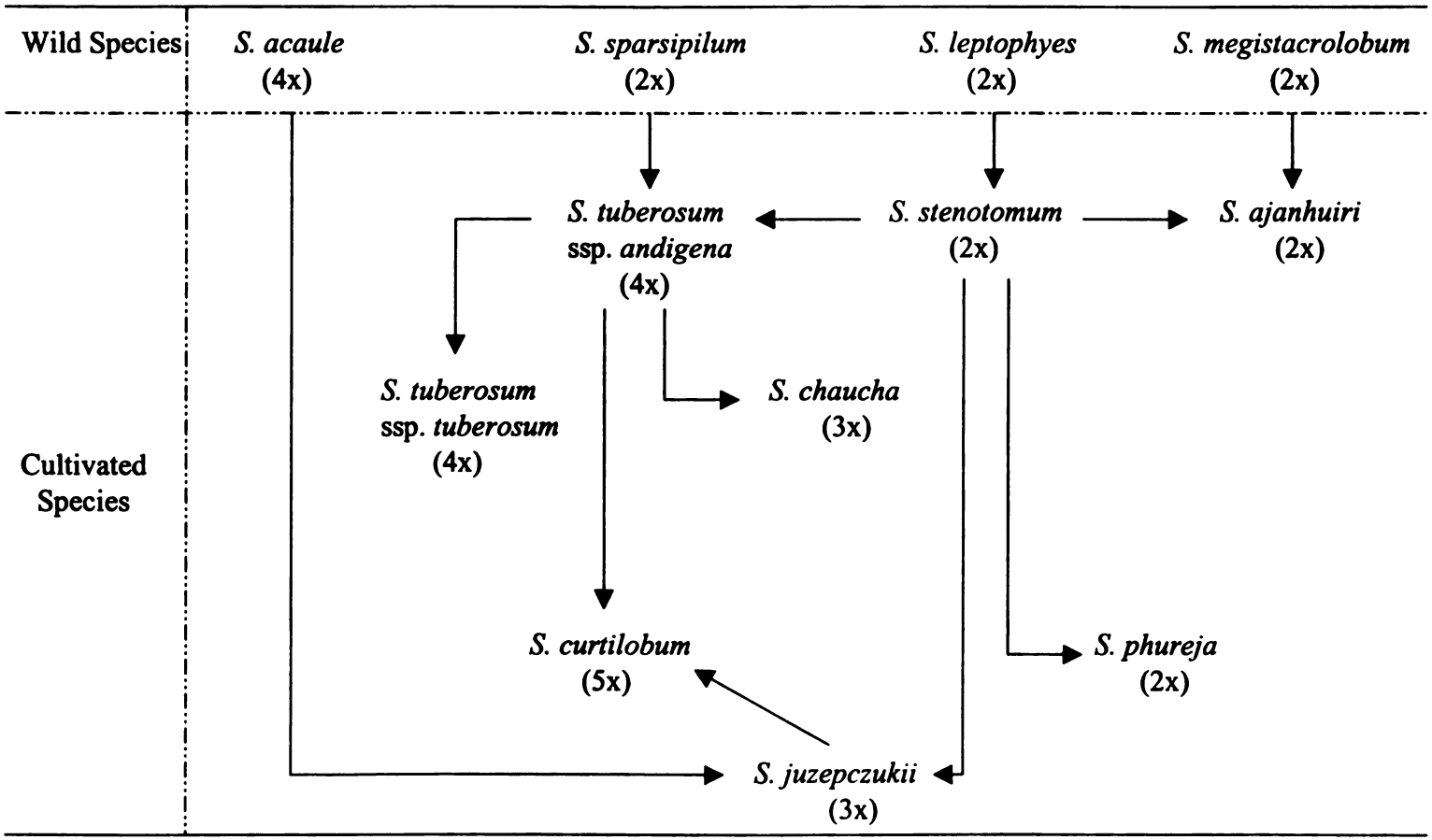

Figure 1.1. Evolutionary relationships of cultivated potatoes and their ploidy levels (adapted from Hawkes, 1990).

Table 1.1 Chloroplast DNA (ct-DNA) types and ploidy levels of wild and cultivated potato species.

Species	Chloroplast DNA type	Ploidy
S. *leptophyes*	W	2x
S. sparsipilum	W	2x
S. acaule	C	4x
S. canasense	C	2x
S. stenotomum	S	2x
S. tuberosum ssp. *andigena*	A	4x
S. tuberosum ssp. tuberosum	T	4x
S. chaucha	S	3x
S. curtilobum	S	5x
S. phureja	S	2x
S. juzepczukii	C	3x

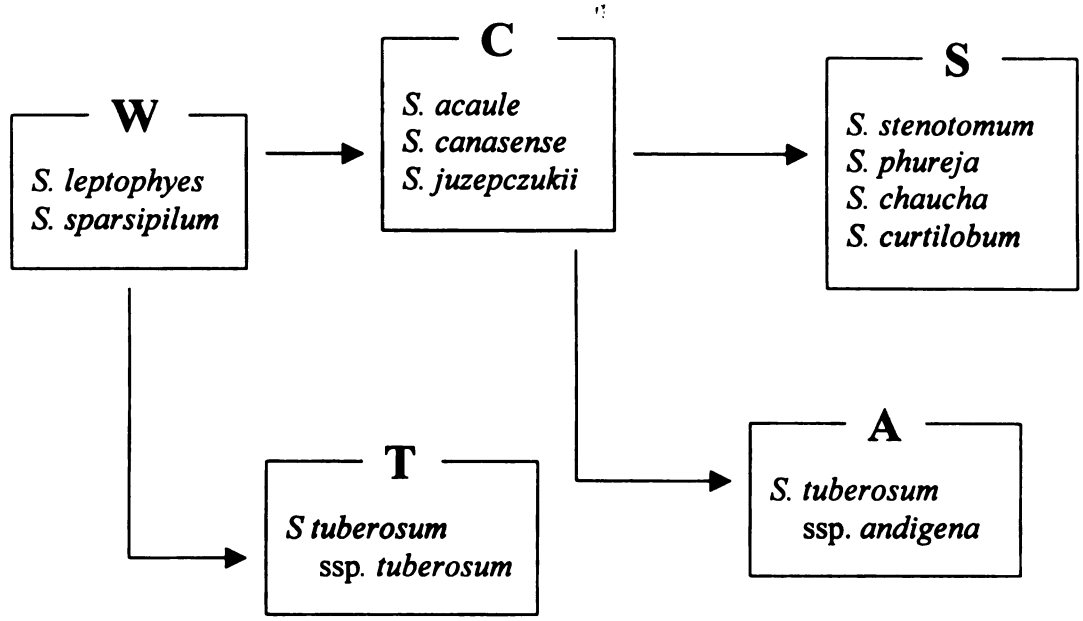

Figure 1.2. The relationship of five potato chloroplast DNA (ct-DNA) types based on restriction fragment length polymorphisms (adapted from Hosaka, 1986).

been accomplished by Simmonds (1966) in only a few generations of selection. Thus, it would be quite possible to develop *tuberosum* from imported *andigena* in the years between the first introduction to Europe (circa 1570) and the description of the European *tuberosum* by Linnaeus (circa 1750). Lastly, the early European potato was regarded as a botanical curiosity until the mid-eighteenth century due to poor yields and late crops indicating adverse photoperiodic response (Hawkes, 1992). Hosaka and Hanneman (1988) provided further evidence supporting *andigena* as the first European potato. These authors evaluated the ct-genome of hybrids from "Myatt's Ashleaf", which is one of the relics of the first European potato, and determined that these hybrids had the A type ct-genome found in ssp. *andigena* but not in ssp. *tuberosum*. Based on their ct-genome determinations, Hosaka and Hanneman (1988) proposed that the original European potato was ssp. *andigena* that was adapted to long day lengths. These potatoes prevailed in Europe until the mid 1800's when they were mostly destroyed by disease and were replaced by the American cultivars derived from "Rough Purple Chili" which was selected from Chilean ssp. *tuberosum* (Salaman, 1949). Therefore, current European and American potatoes carry the T type ct-genome found only in ssp. *tuberosum* (Hosaka and Hanneman, 1988).

In addition to its poor initial performance the potato was not well accepted in Europe for several other reasons. First of all, the attributes of known plants with similar characteristics were applied to the potato. Thus, the undesirable flatulence and lust inciting qualities of sweet potato and the toxicity of black nightshade were associated with potatoes (Salaman, 1949). In addition, many doctors and herbalists attributed leprosy, consumption, rickets and many other diseases to the consumption of potatoes

(Salaman, 1949). Lastly, the potato was the first edible plant in Europe to be grown from tubers and have the edible portion grown on underground stems. Therefore, the potato was immediately under suspicion simply because it was an oddity (Salaman, 1949). The potato was mostly disfavored and was slow to be accepted in parts of England, France and Russia. However, in Ireland, the potato was more readily accepted for several reasons (Salaman, 1949). The Irish climate and soil were generally well suited to growing potatoes and the crop required limited time, energy and skill to produce. The potato was also easily incorporated into the traditional diet and preparation techniques. Perhaps more importantly, potatoes could be stored through the winter to feed both family and livestock after all the surplus of grain had been used to pay rent.

A large population dependent on the potato for sustenance, the economic and political environment of Ireland in the mid-1800's, and the arrival of the potato late blight pathogen, *Phytophthora infestans* mont. DeBary, lead to the Irish Potato famine in 1845 and 1846. An outbreak of late blight in England was reported in August of 1845 and by the end of September the disease had spread through Poland, Germany, Belgium, France and Ireland (Salaman, 1949). Although early varieties escaped infection most of the Irish potato crop was a late variety called 'Lumper' which either rotted in the field or later in storage. The disease reappeared early in the season of 1846, spread rapidly across Ireland and destroyed most of the crop (Salaman, 1949). With little aide from the ruling English government, the result in Ireland from 1845-1852 was the death of around one million people who relied on potatoes for food and the emigration of another million and a half people fleeing from poverty and starvation (Kinealy, 1996).

Late blight continues to be a devastating disease of potatoes and much research has been done to understand the causal pathogen (*P. infestans*) and to develop methods of control. Not a true fungus, *P. infestans* belongs to the Oomycete class and is classified as a "water mold" due to its adaptation for thriving where there is free water and cool temperatures. The life cycle of *P. infestans* can be divided into a highly efficient asexual life cycle that can be repeated in 5-7 days under ideal conditions (Kirk, 1996) and a sexual cycle (Figure 1.3). The asexual cycle is carried out via sporangia that can fruit quickly on leaf and stem tissues. Sporangia are non-motile and rely on wind and rain for dispersal but can undergo indirect germination to produce mobile zoospores (Lacy and Hammerschmidt, 1995). Tuber infection occurs when zoospores or sporangia swim or are washed into the soil and enter the tubers through lenticels or cracks or when healthy tubers physically contact diseased foliage at harvest. *P. infestans* is heterothallic with two known mating types, A^1 and A^2. If mycelia of different mating types interact, sexual propagation can occur through the formation of oospores. Sexual reproduction in this pathogen is of great importance for two reasons. First, oospores have been shown to survive freezing and thawing, long-term storage, and passage through the digestive system of snails (Umaerus and Umaerus, 1994). Thus, these spores may survive in soils separate from the host plant for long periods of time and may serve as an inoculum source for newly planted potatoes. Secondly, sexual reproduction implies genetic recombination and the possibility of new genotypes of the pathogen with enhanced virulence and greater resistance to chemical control. Prior to the 1980's, *P. infestans* populations in Europe and North America consisted solely of the US1 genotype and the A^1 mating type. However, the US8 genotype (A^2 mating type) was reported in Europe

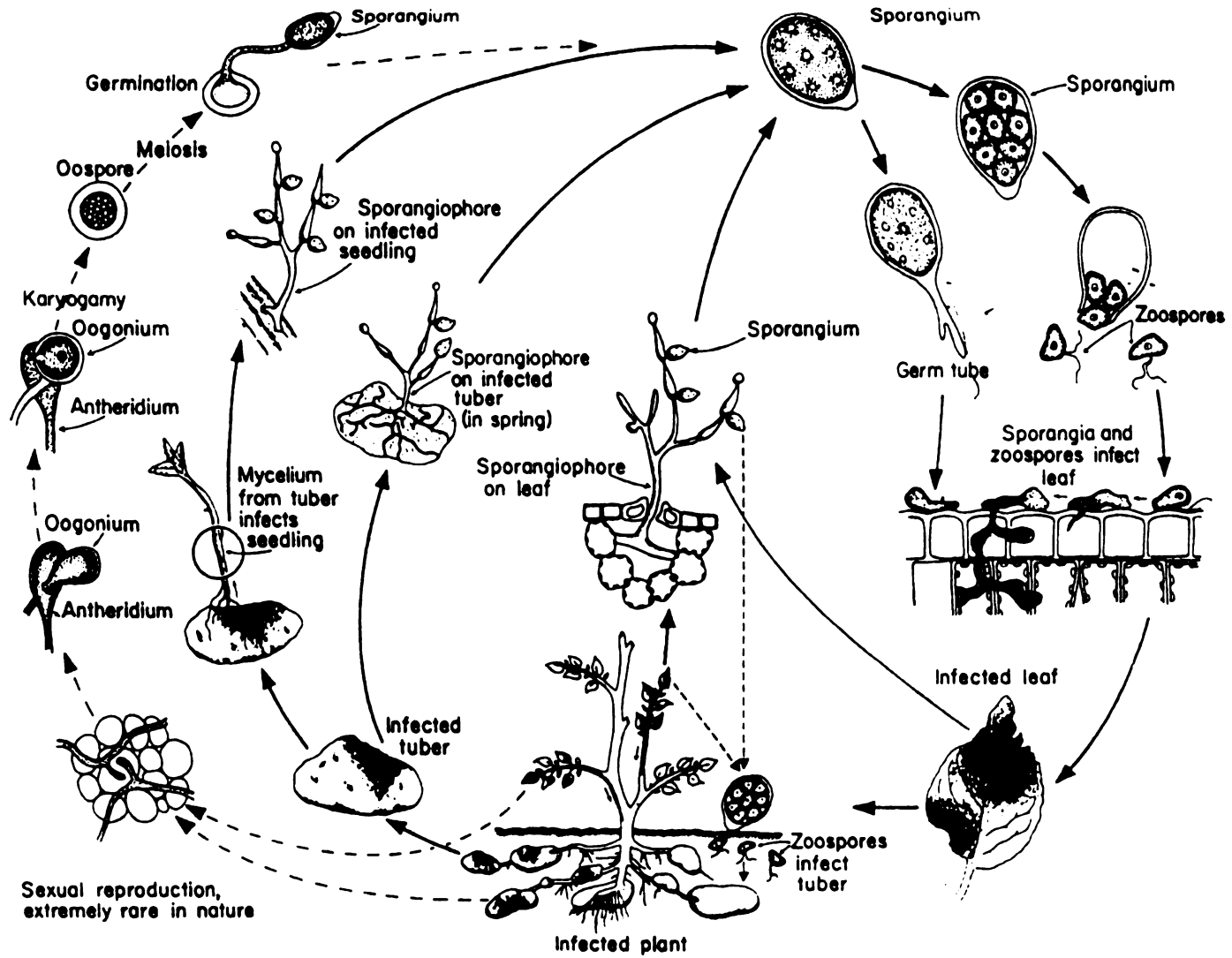

Figure 1.3. Disease cycle of late blight of potato and tomato caused by *Phytophthora infestans* (Agrios, 1988).

during the mid 1980's and in North America in 1992 making sexual reproduction a threat to late blight control.

Since the devastation of the Irish Potato Famine, research efforts have lead to disease management programs that integrate cultural practices and chemical controls. It is believed that asexual spores of *P. infestans* can only overwinter in living plant tissue. Thus, an important control measure is to eliminate cull piles and volunteer potatoes. Other cultural practices such as planting certified seed and regular scouting help to minimize the development and spread of late blight. Chemical control of late blight began during the 1880's with the development of the Bordeaux mixture and other copper-based compounds. Since then several classes of residual and systemic, fungicides have been commercialized and are being used to prevent late blight infection in potato crops. Although chemical control is an important component of integrated management of late blight, there is growing public concern over the use of chemicals. Special permission (Section 18 registration) is required in order to use certain fungicides in the United States. In addition, the US8 genotype, that is now present in North America, is resistant to the systemic fungicide, metalaxyl, which had been the predominant chemical used for late blight prevention and eradication. There is also concern that the heavy use of chemicals will lead to the development of chemical-resistant late blight genotypes.

Another component of late blight management in potatoes has been to breed resistant cultivars. The first efforts to develop late blight-resistant potato cultivars emphasized vertical, or specific resistance, conferred by major resistance genes (R genes). These R genes were initially found in wild *Solanum* species from Mexico where the *P. infestans* populations are mixtures of many races and provide ample opportunity

for the host plant to evolve various resistances to the pathogen. *S. demissum*, a Mexican hexaploid wild species, was initially the predominant source of late blight resistance used for breeding (Ross, 1986). A series of crosses between cultivated potato and *S. demissum* resulted in backcross hybrids that were completely resistant to late blight. Although *S. demissum*-based resistance was highly successful it was short-lived as *P. infestans* quickly evolved virulence to the deployed R genes. The rapid breakdown in this resistance coinciding with the development of effective chemical controls for late blight discouraged progress in late blight-resistance breeding for many decades.

Due to the presence of the US8 genotype of *P. infestans* in Europe and North America and the concern over the use of chemicals in agriculture, breeding for late blight resistance is a priority once again. However, the emphasis now is on exploiting horizontal resistance. Also referred to as general or field resistance, horizontal resistance is polygenic in nature and is assumed to be more stable than a monogenic system such as vertical resistance. The genetic basis for horizontal resistance to late blight has yet to be resolved. However, studies have shown that both general (GCA) and specific combining ability (SCA) contribute to the variability of horizontal resistance to late blight. SCA has been reported for foliar resistance (Killick and Malcolmson, 1973) and for tuber resistance (Stewart *et al.*, 1992; Bradshaw *et al.*, 1995) and several studies (Malcolmson and Killick, 1980; Wastie *et al.*, 1993; Bradshaw *et al.*, 1995) have reported GCA for foliar and tuber resistance. In addition, Tai and Hodgson (1975) showed GCA to be the largest source of variability for horizontal late blight resistance but suggested that there was also an important SCA component as well. The importance of maternal effects and the influence of R genes on horizontal late blight resistance is also unclear. Killick and

Malcolmson (1973) noted no maternal effects in crosses involving a late blight resistant parent. However, Lishak and Nazar (1977, cited in Umaerus *et al.*, 1983) demonstrated that the resistance of the progeny was better if the late blight resistant parent was female. Some authors have suggested that horizontal and vertical resistance are not genetically distinct mechanisms but that "minor" genes (quantitative trait loci or QTL) are alleles of "major" (R) genes (Nelson, 1978; Robertson, 1985). Recent studies have provided molecular evidence to support this theory. Leonards-Schippers *et al.*, (1994) found a QTL for late blight resistance that mapped to the same chromosome region as the dominant allele, R1, conferring race specific resistance to *P. infestans*. In addition, the clustered R genes R3, R6 and R7 on chromosome XI are also linked to a QTL for quantitative late blight resistance (Gebhardt, 1999).

One of the difficulties in breeding for horizontal resistance to late blight is that it is hard to maintain during crossing and backcrossing which is often necessary to recover commercially acceptable tuber types (Black, 1970). The mean resistance of the progenies is usually less than the parental mean. Another problem encountered in breeding for horizontal resistance is the positive correlation between late maturity and late blight resistance. Late maturity is not suitable for potato growing regions in North America but has been closely associated with late blight resistance from many sources. It is also more difficult to evaluate horizontal late blight resistance than it is to evaluate vertical resistance. Horizontal resistance can be influenced by environmental factors such as day length and light intensity (Schumann and Thurston, 1977) and can be masked by the presence of R genes, which are found in most cultivars and wild species.

Although most potato breeding programs emphasize the use of horizontal late blight resistance, it is important to note that single gene resistance sources are still being explored. Helgeson *et al.* (1998) developed late blight resistant breeding lines via the somatic fusion of *Solanum bulbocastanum* with *S. tuberosum* cultivars. Backcross progeny of these somatic hybrids have been tested for several years in the Toluca Valley of Mexico and have thus far demonstrated durable late blight resistance in the presence of numerous *P. infestans* races (Helgeson *et al.*, 1998; Helgeson, personal communication). Subsequent evaluation of the resistance has implicated a single gene coming from *S. bulbocastanum* that is currently being cloned to evaluate the sequence and identify any known functional sequence motifs (Helgeson, personal communication). In addition Douches *et al* (unpublished data) have developed a highly resistant breeding line derived from the Mexican cultivar 'Tollocan'. This cultivar has been identified as having durable resistance in the Toluca Valley of Mexico (personal communication, Niederhauser). However, the transmission of this resistance would indicate that it is monogenic or at most oligogenic (Douches, unpublished data). Similarly, the horizontal late blight resistance in the cultivar 'Sterling' has been mapped to a few major effect quantitative trait loci (QTL) (Collins et al., 1999). Thus, it may be unwise to completely abandon the use of what has been considered to be major -gene resistance to late blight.

Pyramiding resistance genes has been proposed as a means of stabilizing resistance against variable plant pathogens (Nelson, 1978). This breeding strategy involves incorporating several different resistance genes into one cultivar in order to expand the range of pathogen genotypes to which a cultivar is resistant and/or to increase the durability of resistance. Gene pyramiding has been highly successful for controlling

stem rust of wheat for 40 years in Canada and in the north-central United States (Line and Chen, 1995). This strategy has also been developed and utilized to breed for resistance to anthracnose (Young and Kelly, 1996; Young and Kelly, 1997), bean common mosaic virus (Haley *et al.*, 1994a; Haley *et al.*, 1994b) and rust (Miklas *et al.*, 1993; Haley *et al.*, 1993) in dry bean (*Phaseolus vulgaris*). For more complex resistance systems, an analogous strategy would be to combine resistance components from various sources.

There are several different components of horizontal resistance to fungal infection including resistance to infection, slower growth of the pathogen through tissues, an extended incubation period and a reduction in the number of spores produced (Hide and Lapwood, 1992). The predominant mechanism of resistance may vary from species to species. Ross (1986) noted that some Polish cultivars resisted infection by *P. infestans*, whereas others resisted spread and sporulation. Additionally, in a study of eight wild potato species, Colon *et al.*, (1995) reported that the species differed in the importance of various components of resistance. By crossing different sources of late blight resistance it may be possible to combine several resistance mechanisms and thus improve upon the level and durability of late blight resistance. The International Potato Center (CIP) in Peru has developed an R gene free population (population B) in which it has been possible to identify and combine sources of horizontal resistance (Landeo *et al.*, 1997). Many other sources of late blight resistance have been reported (Douches *et al.*, 1997a; Douches *et al.*, 1997b; Colon and Budding, 1988; Helgeson *et al.*, 1998; Bamberg *et al.*, 1994; Huaman, 1987) and several groups are utilizing this broad genetic base of materials to develop late blight resistant potato cultivars.

Although late blight is the most significant disease of potato worldwide, the potato is prone to more than one hundred diseases caused either by bacteria, fungi, viruses or mycoplasms (Hide and Lapwood, 1992). Fortunately, only a few of these diseases become serious problems in any one growing region. Some of the most important diseases of potato in North America other than late blight include: early blight (*Alternaria solani*), early die (*Verticillium dahliae*), dry rot (*Fusarium sp.*), soft rot (*Erwinia carotovora ssp.*), common scab (*Streptomyces scabies*), potato virus X and Y, and potato leaf-roll virus. In addition to these pathogens, the potato also falls prey to insect pests such as the Colorado potato beetle, which severely defoliates the plants, aphids, which spread viruses, and potato leaf-hoppers which damage foliage. Many of these pathogens and insect pests can currently be controlled with chemicals however the development of chemical resistant genotypes is possible if not probable. Therefore, potato breeders also focus on developing cultivars with resistance to multiple pathogens and/or insect pests. As with late blight, the sources for resistance to these diseases and pests are most often wild *Solanum* species.

In addition to the sources of disease resistance previously mentioned, it may also be possible to utilize engineered resistance mechanisms. The potato is very amenable to tissue culture propagation, to infection by *Agrobacterium tumefaciens* and to regeneration. Numerous potato transformation and regeneration protocols have been published utilizing both leaf and stem sections (Yadav and Sticklen, 1995; DeBlock, 1988; Wenzler *et al.*, 1989; Hulme *et al*, 1992). Thus, transformation of potato with foreign genes has been relatively easy. In recent years, potatoes have been genetically modified to incorporate transgenes for resistance to Colorado potato beetle (Perlak *et al.*,

1993), potato virus Y (PVY) (Pehu *et al.*, 1995; Hefferon *et al.*, 1997) and potato leaf roll virus (PLRV) (Brown *et al.*, 1995; Kawchuck *et al.*, 1991) as well as transgenes affecting carbohydrate metabolism (Stark *et al.*, 1992) and disease resistance (Allefs *et al.*, 1996, Liu *et al.*, 1994). As our understanding of plant disease resistance mechanisms develops, more and more defense-related genes are being cloned and thus becoming available for insertion into plant genomes. Many of these transgenes show promising results in tobacco or tomato, which are members of the same family (*Solanaceae*) as the potato. By utilizing some of these promising genes alone or in combination with various sources of resistance from wild species and existing cultivars it may be possible to develop potato cultivars that are not only resistant to late blight but also resistant to pests and other diseases as well.

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Chapter II

PRODUCTION, MOLECULAR CHARACTERIZATION AND LATE BLIGHT REACTION OF TRANSGENIC POTATO PLANTS (*SOLANUM TUBEROSUM* L.) EXPRESSING A FUNGAL GLUCOSE OXIDASE GENE

ABSTRACT

Late blight, (*Phytophthora infestans* (Mont.) De Bary), is one of the most devastating diseases of potato worldwide and the development of resistant cultivars for late blight management in commercial production is important. Current emphasis is on the utilization of horizontal resistance rather than vertical resistance conferred by specific R genes. Our research was undertaken to determine if enhanced resistance to late blight could be obtained by combining natural resistance and engineered resistance conferred by the fungal transgene glucose oxidase from *Aspergillus niger*. This gene was selected due to its reported effect against multiple pathogens including *P. infestans, Erwinia carotovora, Alternaria solani,* and *Fusarium sambucinum*. Late blight susceptible cultivars 'Atlantic', 'Snowden' and 'Spunta' and partially resistant cultivars 'Zarevo' and 'Libertas' were transformed with the glucose oxidase gene under the control of the Gelvin "super promoter". Putative transgenic lines were verified and copy number determined via Southern blotting. Copy number ranged from 1 to 8. Northern and western analyses were used to determine which transgenic lines transcribed and/or translated the glucose oxidase transgene. Only those lines in which the glucose oxidase protein was detected via western blotting were included in disease evaluations. Glucose oxidase enzyme activity was estimated for each line that was positive in the western analyses and ranged

from 0 to 93.96 Units/mg plant tissue * 10^5. There was no apparent correlation between level of transgene mRNA and enzyme activity or between copy number and level of transgene mRNA.

In agronomic trials, 44% of the transgenic lines were phenotypically "off-type" compared to non-transgenic controls based on plant and/or tuber characteristics. Because several off-type lines did not express the glucose oxidase protein, this phenomenon cannot be attributed solely to the glucose oxidase transgene.

Lines expressing the glucose oxidase transgene were evaluated for reaction to *P. infestans* in growth chambers and inoculated field studies. Results from trials conducted in growth chambers suggest a small effect in 'Spunta'-derived and 'Libertas' derived transgenic lines but high coefficient of variation (CV) values prevented the identification of any significant differences between the transgenic lines and their respective non-transgenic controls. Field studies revealed no significant effect of the glucose oxidase transgene in either late blight susceptible or partially late blight resistant cultivars.

These results indicated that the glucose oxidase gene alone or in combination with natural host plant resistance will not produce completely late blight resistant cultivars. Potato cultivars with moderate levels of late blight resistance may be useful for reducing the amount of fungicide (active ingredient) used in late blight control programs. Therefore, transgenic lines that exhibit lower levels of late blight infection in growth chamber trials may be useful in combination with reduced rates of fungicide application. This potential benefit would need to be evaluated against the time and cost required for development of transgenic potato cultivars and the potential for off-type tubers and plants.

INTRODUCTION

Late blight, caused by the oomycete, *Phytophthora infestans* (Mont.) deBary, is the most serious disease of potato (*Solanum tuberosum* subsp. *Tuberosum* L.) in many areas of the world. Unchecked, this disease can rapidly devastate entire potato crops as witnessed by the Irish potato famine of the 1840's. Bourke (1964) noted that "In about ten weeks, potato murrain (as the disease was sometimes called) spread unchecked through Belgium and became widespread in the Netherlands, France, Britain and Ireland where almost half of the staple food crop was lost. In the following year, the blight reappeared first in Ireland and by August 1846 the entire Irish crop was destroyed."

Economic losses due to this disease occur by reduced photosynthetic capacity resulting in yield reduction and by tuber infection resulting in storage losses. Foliar infection first appears as water-soaked lesions that enlarge rapidly under moist, cool conditions and become brown and irregular in shape. White mycelial growth is often be observed on the lower leaf surfaces. Under continuously wet conditions, the disease develops rapidly resulting in the destruction of both leaves and stems. Tuber infection is indicated by irregular, dark patches on the tuber surface and by reddish-brown discoloration extending into the tuber tissue. During storage, infected tubers are often invaded by secondary pathogens leading to tuber decay.

Currently, the strategy for controlling potato late blight in the United States is a combination of cultural and management practices such as frequent field scouting and elimination of cull piles and the use of protectant fungicides. Until recently, the systemic fungicide metalaxyl was used to prevent the spread of late blight once the disease was identified in a field. However, in 1992 the metalaxyl resistant, US8 genotype of

P. infestans was identified in North America making this important chemical virtually obsolete. At present there are no commercially acceptable late blight resistant potato cultivars. However, many different sources of moderate to high levels of resistance have been identified in wild species and various cultivars (Douches *et al.*, 1997a; Douches *et al.*, 1997b; Helgeson *et al.*, 1998; Bamberg *et al.*, 1994). By combining these different sources of resistance, it may be possible to pyramid different mechanisms of resistance into one genotype and thereby develop stronger and more durable resistance to potato late blight. Different sources of resistance might also be combined through the use of genetic engineering. Combining a transgenic source of resistance with moderate levels of natural late blight resistance may provide another means to develop a potato cultivar with stronger more durable resistance.

One gene that has been used to increase disease resistance is the glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) gene isolated from the fungus, *Aspergillus niger.* Frederick *et al.* (1990) cloned and sequenced this gene and it was subsequently transformed into the potato cultivar 'Russet Burbank' (Wu *et al.*, 1995). Transgenic plants were shown to have increased resistance to bacterial soft rot caused by *Erwinia carotovora* ssp. *carotovora*, *Verticillium* wilt caused by *V. dahliae* and to late blight caused by *P. infestans.* This increased resistance has been attributed to the accumulation of hydrogen peroxide from the oxidation of β-D-glucose by glucose oxidase (glucose ---- glucono-δ-lactone + H_2O_2). Further support for the role of H_2O_2 in host plant resistance was demonstrated using catalase deficient tobacco plants. When induced by overexposure to light, these plants accumulated H_2O_2, acidic and basic pathogenesis related proteins (PR proteins) and salicylic acid as well as demonstrating

increased resistance to the bacterial pathogen *Pseudomonas syringae* pv. *syringae* (Chamnongpol *et al.*, 1998).

The chain of activity that occurs within a plant upon contact with a pathogen has been studied in depth. One of the first plant defense responses to both compatible and incompatible pathogens is a rapid oxidative burst resulting in the production of active oxygen species (AOS) including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^·$) (Mehdy, 1994; Baker and Orlandi, 1995; Low and Merida, 1996). In a compatible reaction, a second, prolonged oxidative burst occurs following hypersensitive cell death (Baker and Orlandi, 1995; Low and Merida, 1996). Two mechanisms have been proposed for the production of H_2O_2 during the oxidative burst: production via a plasma membrane bound NAD(P)H oxidase and production via cellular peroxidases. Both mechanisms have been demonstrated in different plant species (Bolwell, *et al.*, 1998).

The AOS, in particular H_2O_2, play several important roles in plant defense systems. Hydrogen peroxide has been shown to have direct antifungal activity at low concentrations (Peng and Kuc, 1992; Wu, *et al.*, 1995) and to alter cell wall components. Structural changes in the cell wall include lignification (Dean and Kuc, 1987; Hammerschmidt and Kuc, 1982) and cross-linking of abundant, hydroxyproline-rich cell wall proteins (Brisson *et al.*, 1994; Bradley *et al.*, 1992). Such alterations in cell wall structure could delay pathogen invasion and/or trap pathogens in cells that will undergo hypersensitive cell death. Although this would not provide complete resistance, it may give the plant time to mobilize transcription-dependent defense mechanisms. Hydrogen peroxide also contributes to hypersensitive cell death via lipid peroxidation and

generation of lipid free radicals (Keppler and Baker, 1989). Tenhaken *et al.* (1995) reported that endogenous H_2O_2 produced by the oxidative burst is sufficient to trigger cell death in an infected cell and serves as a diffusable signal for induction of cellular protectant genes in neighboring cells. Expression of protectant genes encoding glutathione S-transferase, glutathione peroxidase and ubiquitin limits the spread of cell death by detoxifying and or metabolizing many products of oxidative stress (Tenhaken *et al.*, 1995). In contrast, Baker *et al.*, (1997) demonstrated that it was possible to elicit the oxidative burst without causing hypersensitive cell death. It has also been suggested that in the cowpea-cowpea rust fungus pathosystem the oxidative burst does not trigger hypersensitive cell death (Heath, 1998). In catalase deficient plants low levels of H_2O_2 were sufficient to activate defense mechanisms such as PR proteins without causing cell death. However, the strength and speed of the induction was impaired compared to plants in which high levels of H_2O_2 were present and cell death occurred (Chamnongpol *et al.*, 1998).

Activation of phytoalexin synthesis has also been reported as a role of H_2O_2 in the plant disease response. Phytoalexins are low molecular weight antimicrobial compounds formed during a hypersensitive reaction to a compatible pathogen. Activation of phytoalexin synthesis by H_20_2 has been reported in suspension cells (Apostol *et al.*, 1989) and in potato tubers (Chai and Doke, 1987). However, recent evidence suggests that pathways independent of the oxidative burst also contribute to the regulation of phytoalexin accumulation (Mehdy, 1994).

Hydrogen peroxide has not only been implicated in the hypersensitive response but also in systemic acquired resistance (SAR) in plants. SAR is an induced defense

response triggered by infection with a necrosis-causing pathogen or by organic compounds such as salicylic acid (SA). Establishment of SAR has been correlated with the expression of nine gene families coding for proteins whose possible causal role in resistance has attracted considerable attention (Ward *et al.*, 1991). These genes, called pathogenesis-related (PR) genes, are induced upon infection with a compatible pathogen, but are also induced by SA via a secondary messenger. Chen *et al.*, (1993) suggested that H_2O_2 is the secondary messenger by which PR genes are activated. These authors found that salicylic acid (SA) bound a protein (SABP) which was capable of catalyzing H_2O_2 to form O_2 and H_2O. When bound to SA, the catalytic activity of SABP would be disabled, H_2O_2 would accumulate, and PR genes would be activated. Conrath *et al.* (1995) provided support for this theory by demonstrating that a synthetic inducer of SAR also inhibited catalase activity. However, Bi *et al.*, (1995) found that H_2O_2 and the catalase inhibitor 3-AT were weak activators of PR1-a in wild type tobacco and were not able to activate PR1-a in transgenic tobacco that lacked the ability to accumulate SA. Neuenschwander *et al.* (1995) reported similar results suggesting that H_2O_2 does not function downstream of SA in SAR signaling but that SA inhibition of catalase may be important for the hypersensitive response. Alvarez et al., (1998) provided further evidence for the role of H_2O_2 in SAR. These authors reported that the primary oxidative burst leads to secondary oxidative bursts in small collections of cells distant from the infection site resulting in low frequency systemic micro-hypersensitive responses (micro-HR). Blocking the primary oxidative burst inhibited the formation of micro-HRs and the induction of glutathione-S-transferase (GST) (an indicator of SAR) in uninfected leaves. These authors speculated that micro-oxidative bursts mediate cell death, defense

induction and signal propagation in parallel with the primary oxidative burst and that both the primary and secondary oxidative bursts are necessary for SAR.

Other molecules such as nitric oxide (NO) and ethylene have also been implicated in oxidative cell death and may function in a complex cycle designed to perpetuate and control H_2O_2 during the plant disease response. As with H_2O_2, NO may have several important roles. One function of NO is to inhibit cytochrome c oxidase activity in mitochondria which may increase electron flow towards oxygen and thus stimulate superoxide and H_2O_2 formation (VanCamp *et al.*, 1998). Nitric oxide was also found to enhance H_2O_2-mediated cell death and the removal of NO by either NO scavengers or biosynthetic inhibitors blocked the HR as well as the induction of phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) in pathogen challenged plants (Delledone *et al.*, 1998). GST and PR1 genes were also induced by NO but the induction was dependent on both H_2O_2 and SA (Levine *et al*, 1994). Thus, NO appears to function in two pathways, one leading to phenylpropanoid (PAL) and flavanoid (CHS) biosynthesis and one leading to antioxidant (GST) and PR gene expression (VanCamp *et al.*, 1998). In addition, Noritake *et al,* (1996) reported that NO was capable of inducing synthesis of the phytoalexin rishitin in potato tissues. Ethylene has also been implicated in oxidative cell death. Lund *et al.*, (1998) reported that ethylene promotes lesion formation around infection sites but doesn't seem to be present in leaves distant from the infection site.

Although the exact function of H_2O_2 in the plant disease response process is not fully elucidated, there appears to be complex interactions between H_2O_2, SA, NO and ethylene which serve to carefully control cell death and pathogen defense pathways

involving hazardous molecules such as NO and H_2O_2. VanCamp *et al.*, (1998) suggested a model for oxidative cell death (OCD) in which H_2O_2, SA, and cell death are linked in a self-amplifying process which is reiterated in distant leaves (Figure 2.1). In this model, H_2O_2 alone or in combination with SA activates cell death, which leads to enhanced production and accumulation of AOS. This cycle would perpetuate H_2O_2 and lead to expression of various defense related genes. SA and ethylene may enhance H_2O_2-mediated cell death as indicated by dotted lines. The model depicts NO as functioning in the OCD cycle (SA/ H_2O_2 dependent pathway) and separate from the OCD cycle leading to PAL and CHS expression (SA/ H_2O_2 independent pathway).

Regardless of the precise function of H_2O_2 in plant disease resistance, manipulation of H_2O_2 levels in potatoes via the incorporation of the glucose oxidase gene has been shown to provide some resistance to late blight and other diseases (Wu *et al.*, 1995; Wu *et al.*, 1997). To date only one cultivar (Russet Burbank) has been transformed with the glucose oxidase gene and these transformants had only a reduced rate of infection when challenged with *P. infestans* and other pathogens (Wu *et al.*, 1995; Wu *et al.*, 1997). Developing potato cultivars with less susceptibility to late blight may be useful for reducing the amount of fungicide necessary for control. However, the ultimate goal is to develop completely resistant cultivars. One way this may be accomplished is by pyramiding engineered resistance mechanisms with natural host plant resistance. Therefore, we attempted to build stronger resistance to late blight by combining the glucose oxidase gene with horizontal resistance to late blight found in existing potato cultivars.

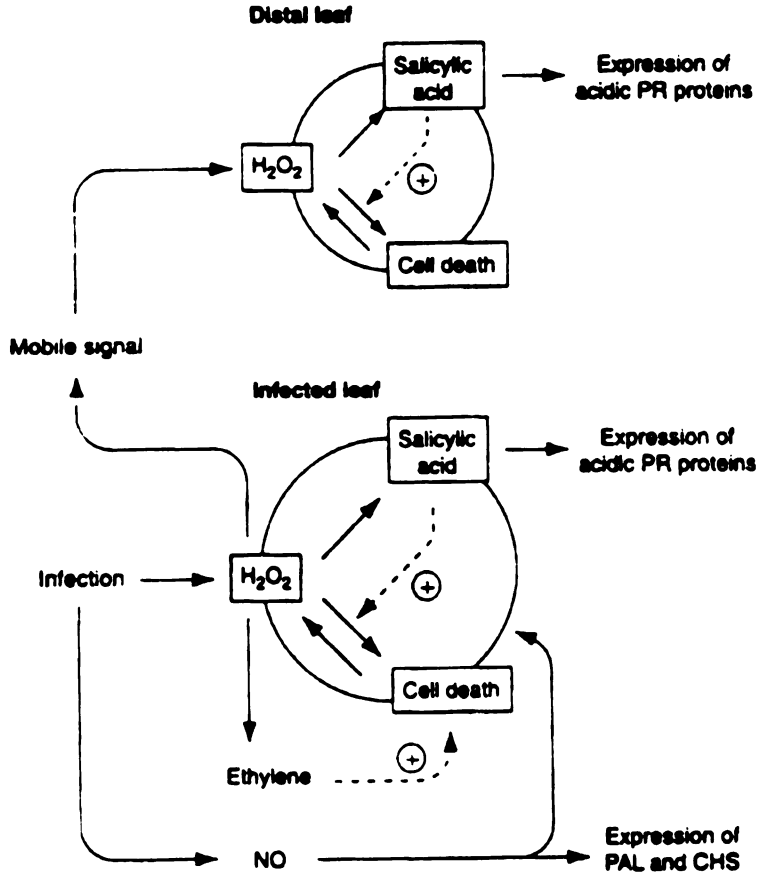

Figure 2.1. Hypothetical model showing the role of H_2O_2 and NO in the induction of gene expression and cell death during plant-pathogen interactions. H_2O_2, salicylic acid, and cell death are linked in a self-amplifying process, termed the oxidative cell death (OCD) cycle. The OCD cycle is reiterated in distal leaves, leading to SAR. Cell death in infected leaves is initiated through several routes, including, besides the OCD cycle, ethylene- and NO-dependent pathways. Broken arrows marked by a plus indicate putative agonistic effects of salicylic acid and ethylene on H_2O_2 mediated cell death (VanCamp et al., 1998).

MATERIALS AND METHODS

Cloning of the glucose oxidase gene

The glucose oxidase gene was cloned from *Aspergillus niger* using the polymerase chain reaction (PCR) and primers designed according to Frederick *et al.* (1990) to which *Sma*I and *Xba*I restriction sites had been added. The resulting DNA fragment was sequenced for verification, compared to the published sequence (Frederick *et al.*, 1990) and incorporated into the pE1102 vector. The resulting plasmid, pSPUD11 (Fig. 2.2), had the glucose oxidase gene under the control of the Gelvin "super promoter" (Ni *et al.*, 1995). This promoter is a combination of a trimer octopine synthase upstream activating sequence $(ocs)_3$ joined to a mannopine synthase (mas) activator and promoter. PSPUD11 also contained the *Nos* terminator sequence, right and left borders for plant transformation purposes and the *NptII* gene (kanamycin resistance) as a selectable marker. The pSPUD11 plasmid was transformed into *Agrobacterium tumefaciens* strain LBA4404 (Ooms *et al.*, 1982) via tri-parental mating.

Transformation of potato cultivars

The cultivars 'Spunta', 'Snowden', 'Atlantic', 'Zarevo' and 'Libertas' were micropropagated in GA-7 Magenta vessels each containing 25 ml of modified MS basal medium (Douches *et al.*, 1998). Of these cultivars, 'Spunta', 'Snowden' and 'Atlantic' are susceptible to *P. infestans*, whereas 'Zarevo' and 'Libertas' have shown moderate levels of resistance in greenhouse and/or field experiments (Douches unpublished data). Transformations of potato cultivars were done according to Li *et al.*, (1999) using the

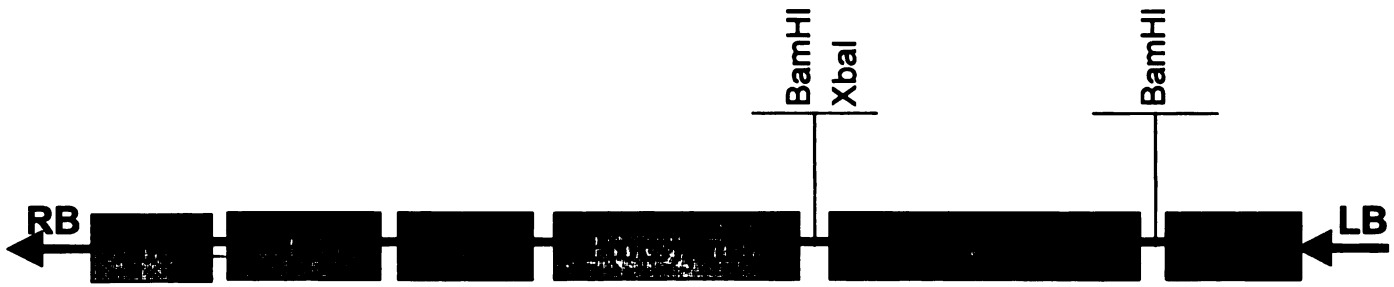

Figure 2.2 Diagram of the pSPUD11 vector containing the glucose oxidase gene from *Aspergillus niger* under the control of the Gelvin "super promoter" [$P(ocs)_3$ mas]. Restriction sites used for Southern blotting are shown.

A. tumefaciens culture harboring the pSPUD11 vector. Regenerating shoots (> 5 mm) were excised and transferred individually into 25 x 100 mm glass tubes each containing 20 ml of MS medium (Douches *et al.*, 1998) supplemented with 200 $mg \cdot L^{-1}$ Timentin (SmithKline Beecham, Philadelphia, Pa.) and 50 $mg \cdot L^{-1}$ kanamycin. Rooted plantlets were designated as SGO-# (derived from 'Spunta'), AGO-# (derived from 'Atlantic'), SNDGO-# (derived from 'Snowden'), ZGO-# (derived from 'Zarevo') and LGO-# (derived from 'Libertas'). Named plantlets were subcultured and subsequently transplanted into 10 cm pots containing Bacto potting medium (Michigan Peat Co. Houston, Texas) and grown in the greenhouse under fluorescent lights (16 h photoperiod) at 23 to 27°C. Leaf tissue from these plants was used for the molecular expression assays.

Molecular characterization of transgenic potato lines

Southern Analysis

DNA was extracted from each putative, transgenic line (Saghai-Maroof *et al.*, 1984) and used for Southern blotting to verify incorporation of the glucose oxidase transgene and to determine copy number. Genomic DNA (20 µg) was digested with *BamH*I to excise the glucose oxidase gene for hybridization with a glucose oxidase DNA probe. Digestion with *Xba*I was used to determine copy number. DNA fragments were electrophoretically separated through a 1% agarose gel and transferred onto a nylon membrane (Hybond N, Amersham, U.K.). Prehybridization was conducted for 2 hr. at 42°C followed by an overnight hybridization (Douches *et al.*, 1998). The hybridization solution contained a probe that was random primed labeled using DIG-11-dUTP

according to the manufacturer's instructions (Roche Molecular Biochemicals, Germany). Following hybridization, the membrane was washed twice in 2x SSC, 0.1% SDS for 15 min. at room temperature and then twice in 0.5x SSC, 0.1% SDS for 15 minutes at 65°C. Chemiluminescent detection was according to Li *et al.*, 1999. Each line was scored for the presence or absence of the glucose oxidase gene and the number of inserts.

Northern analysis

Greenhouse-grown plants were also used as the source for RNA. Young, healthy leaf tissue (1 mg) was collected from each transgenic potato line and frozen immediately in liquid nitrogen. Frozen tissue was stored at –80°C. Total RNA was isolated using Qiagen RNeasy Plant Total RNA Kit (Qiagen, Chatsworth, CA) and quantified by spectrophotometer. Northern analysis was conducted according to Li *et al.* (1999) to verify the presence of the glucose oxidase transcript. To prepare a DIG-11-dUTP labeled RNA probe, the first half of the glucose oxidase (~1.5 kb) was transferred to the Bluescript®$SK^{+/-}$ plasmid (Stratagene, LaJolla, CA). The resulting vector was transformed into *Eschericia coli* strain DH5α and the glucose oxidase RNA probe was synthesized by *in vitro* RNA transcription using an RNA labeling kit (Roche Molecular Biochemicals, Germany). The entire gene sequence could not be used in this vector/host combination because the glucose oxidase gene was expressed and toxic to the bacteria. After detection of the glucose oxidase transcript via northern analysis, each blot was then hybridized with a DIG-11-dUTP labeled 18S ribosomal probe to determine if the amount of total RNA in each lane was approximately equal. For this probe, a prehybridization/hybridization temperature of 42°C was used but all other conditions

were as described by Li *et al.* (1999). Relative levels of glucose oxidase transcript were determined on each individual blot.

Western Analysis

Total protein was extracted from fresh leaf tissue (0.2 g) by grinding in 800 ml of extraction buffer (50 mM Tris HCl pH 8.0, 1 mM EDTA, 10 mM diethyldithiocarbamic acid, 0.05% Tween 20). A Bradford protein assay (BioRad, Hercules, CA) was conducted on the soluble leaf extracts to quantify the amount of protein in each sample. Based on the estimated amount of protein in each sample, equal amounts (200 mg) of total protein were loaded on a 10% SDS-PAGE gel in addition to 50 ng purified glucose oxidase enzyme (Sigma, St. Louis MO). Identical samples were loaded onto a second gel and both gels were subjected to electrophoresis at 45 v and run overnight. One of the gels was stained with Coomassie Brilliant Blue R-250 solution to visually compare loading. The other gel was transferred, via western blotting, to a membrane and probed with a 1:40,000 dilution of glucose oxidase rabbit polyclonal antibody (Polysciences, Inc., Warrington, PA) and an alkaline phosphatase conjugated antirabbit IgG (Li *et al.*, 1999). Antibody binding was detected with CSPD (disodium 3-(4-methoxyspiro{1,2-dioxetance-3,2'(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenylphosphate) according to the manufacturer's instructions (Roche Molecular Biochemicals, Germany) and the blot was exposed to X-ray film (Hyperfilm, Amersham).

Glucose oxidase enzyme activity assay

Transgenic plants that produced the glucose oxidase protein (based on western analysis) were evaluated for the amount of glucose oxidase enzyme activity using a method modified from Gallo (1981) by Wu *et al.* (1995). Enzyme activity in this assay is indicated by the production of a rose colored pigment. Proteins were extracted from 200 mg fresh leaf tissue by grinding in a potassium phosphate buffer (25 mM, pH 7.0, 5 mM EDTA) at 4°C. A dilution series was made from each sample by mixing 10, 20, 30 or 40 µl of sample with 990, 980, 970 or 960 µl reagent mixture respectively (70 mM KH_2PO_4 buffer, pH 5.8, 0.57 mM 4-aminoantipyrine, 0.35 mL/L Triton X-100, 10 mM crystalline phenol, 23 units/mL horseradish peroxidase and 175 mM glucose) for a total reaction volume of 1.0 mL. Known concentrations of purified glucose oxidase were also mixed with the reagent mixture in order to create a standard curve. Reaction mixtures were incubated at 22°C for at least 10 minutes but not longer than 90 minutes as the color begins to fade. Following the incubation period, the absorbance of each sample was measured at 510 nm. Using the standard curve, the amount of enzyme (mg enzyme/mg of fresh tissue) was determined for each dilution of a sample. One mg of the glucose oxidase enzyme that was used to create the standard curve has 15 to 25 Units (U) of activity/ mg enzyme without added oxygen (Sigma-Aldrich Co.). Therefore, we used the average value, 20 U/mg enzyme, to convert mg enzyme/mg fresh tissue into units of enzyme activity. Because the resulting numbers were small, all values were multiplied by 10^5 in order to simplify analysis. The value of the non-transgenic control was then subtracted from each of the transgenic lines. Using dilutions as replications, ANOVA

was done using SAS proc glm (SAS Institute) and lines were compared to non-transgenic controls with both Dunnett's T test and Tukey's Studentized Range Test (SAS Institute).

Late blight screening of transgenic lines

Foliar resistance

Foliar resistance to late blight was tested both in greenhouse mist chambers and in isolation plots at the MSU Muck Soils Research Farm (Bath, MI). Transgenic potato plants were subcultured in 20 mL of MS medium (Douches *et al*, 1998) and then planted in the greenhouse 10 cm pots containing Bacto potting medium (Michigan Peat Co., Houston, TX). Prior to flowering, plants were placed on trays in the mist chamber in a randomized complete block design (RCBD) with four replications and were inoculated with zoospore suspension cultures of *P. infestans* (US8) as outlined by Douches *et al.* (1997). Plants were rated over time for percent area infected. Percentage data were normalized by transformation using the arcsine function. Analysis of variance (ANOVA) was conducted using SAS proc glm and transgenic lines were compared to the non-transgenic control cultivar from which they were derived using both Dunnett's T Test and Tukey's Studentized Range Test (SAS Institute). Some plantlets did not survive the transfer from tissue culture to the greenhouse and therefore not all lines tested were included in all four replications. Therefore, least-squares means (LSmeans) were used for comparisons.

Transplants from tissue culture were also used for field studies with the exception of SGO plants in the 1999 field-season which were grown from tubers produced at the Montcalm Research Farm the previous year (1998). Plants were grown in a RCBD with

four replications and five plants per plot with two feet between plots to facilitate evaluation. Forty-two days (1998) and 55 days (1999) after planting, plants were inoculated with zoospore suspension cultures of *P. infestans* via a sprinkler irrigation system. Moisture was maintained on the foliage via frequent misting irrigation. Plots were evaluated over time for percent area infected. These data were used to calculate area under the disease progress curve (AUDPC) (Shaner and Finney, 1977). The AUDPC was converted to relative area under the disease progress curve (RAUDPC) by dividing the calculated AUDPC by the maximum area under the disease progress curve. Statistical analyses and comparisons were conducted as noted above for greenhouse data.

Tuber resistance

Tuber late blight response was evaluated in each of the transgenic lines. Ten field-grown tubers of each line were removed from cold storage (3 months at 5.6°C) and brought to room temperature for 24 hrs. The tubers were surface sterilized by immersion in a 10% Clorox solution for 10 minutes and then rinsed 3X with distilled water. In 1998, tubers were injected with homogenized mycelial cultures after surface sterilization and then incubated at 12°C and 95% relative humidity for 48 days. Tubers were visually rated using a 1-9 scale (Niemira *et al.*, 1999) and then tuber slices (apical, middle and terminal slices) were scanned and digitally analyzed for reflective intensity (Niemira *et al.*, 1999). In 1999, tubers were prepared for inoculation as in 1998. However, instead of injecting the inoculum, ten tubers of each line were scuffed with a wire brush to simulate harvest damage and were submerged in a zoospore suspension culture (1×10^5 sporangia/ml) for 3 minutes (Platt and Tai, 1998). All tubers were incubated as in 1998

and evaluated using the 1-9 scale. Numerical data were analyzed using SAS proc glm and transgenic lines were compared to their respective non-transgenic controls using both Dunnett's T Test and Tukey's Studentized Range Tests (SAS Institute). Ordinal data from visual ratings were analyzed by ordinal analysis (Karcher and Schabenberger, 2000; Karcher, 2000). Photographic images in this dissertation are presented in color.

RESULTS

Transformation and Molecular Analysis

Transformation of 'Spunta' and 'Atlantic' was successful resulting in 12 transgenic lines from 'Spunta' and 14 transgenic lines from 'Atlantic' (Table 2.1 and Table 2.2). However, transformation of the cultivars 'Libertas' (7 transgenic lines) (Table 2.3), 'Zarevo' (1 transgenic line) and 'Snowden' (2 transgenic lines) was more difficult resulting in fewer transformed shoots. Regeneration of transgenic shoots from 'Spunta' and 'Atlantic' occurred within 3 to 5 weeks following co-cultivation and there were multiple shoots per explant. Very few shoots were recovered from the other transformed cultivars and regeneration occurred 8 to 12 weeks following co-cultivation (data not shown). Both 'Snowden' and 'Zarevo' explants quickly developed a dry, brown border along the cut edge of the leaf and were very sensitive to any mechanical damage during the transformation process. In addition, 'Zarevo' explants would begin to brown soon after transfer to selection media and would often die before regeneration occurred

The copy number of the transgenic lines recovered in this study ranged from 1 to as many as 7 copies in AGO-8 (Table 2.1; Table 2.2; Table 2.3; Figure 2.3). Gene silencing often results from having multiple copies of the same gene (Napoli et al., 1990; Van der Krol et al., 1990) and therefore transgenic lines with single gene insertions are preferred. However, in this study there is no correlation, positive or negative, between copy number and gene silencing. For example, AGO-2 has only 1 copy of the glucose oxidase transgene but northern analysis (Figure 2.4) revealed a lack of transgene transcription whereas, AGO-8 contains 7 transgene copies and produces both the

Table 2.1. Molecular analyses, field and greenhouse late blight experiments and tuber phenotype of 'Spunta' - derived (SGO) lines containing the glucose oxidase gene

SGO Line	Copy Number	Northern (leaf)[x]	Northern (tuber)	Western	Glucose Oxidase Enzyme Activity[y]	Greenhouse Late Blight Trials[z]			Late Blight Field Trials (RAUDPC)			Tuber Phenotype
						First	Third	Combined	1998	1999	Combined	
Spunta	----	-	-	-	0.00	68.2	75.7	84.9	.294	.221	.252	Typical
1	1	+++	-	+	19.65*	6.7*	64.6	41.0*	.290	.213	.251	Typical
2	1	+++	-	+	4.42	6.0*	65.7	41.2*	----	----	----	Knobby,
3	2	+++	-	+	18.56*	----	73.7	----	.350	.244	.297*	Knobby,
4	3	++	-	-	----	----	----	----	----	----	----	Knobby,
9	3	+	-	+	0.00	27.0	47.5	42.5*	.355	.236	.296*	Typical
10	1		-	-	----	----	----	----	----	----	----	Typical
12	1	+++	-	+	30.39*	8.1*	62.9	40.8*	.287	.221	.254	Typical
14	1	++	-	+	6.43	33.8	27.0	26.9*	.281	.231	.256	Typical
19	3	++	-	-	----	----	----	----	----	----	----	Typical
20	2	-	-	-	----	----	----	----	----	----	----	Typical
23	3	+	-	-	----	----	----	----	----	----	----	Typical
26	5	+++	-	-	----	----	----	----	----	----	----	Typical
CV						47%	36%	40%	11%	11%	11%	

*Significantly different from 'Spunta' based on Dunnet's T test ($P < 0.05$)

[x] (+) = lowest relative band intensity, (+++) = highest relative band intensity

[y]Units of activity in 1 mg of leaf tissue multiplied by 10^5

[z]LSmeans for % infection at the last evaluation

Table 2.2. Molecular analyses, field and greenhouse late blight experiments and tuber phenotype of 'Atlantic' - derived (AGO) lines containing the glucose oxidase gene.

AGO Line	Copy Number	Northern[x] (leaf tissue)	Northern (tuber tissue)	Western	Glucose Oxidase Enzyme Activity[y]	Greenhouse Late Blight Trial[z]	Greenhouse Late Blight Trial[z]	1999 Field Trial (RAUDPC)	Tuber Phenotype
Atlantic	0	-	-	-	0.00	30.2	74.7	.262	Typical
2	1	-	-	-	----	----	----	----	Knobby, elongated
4	3	++	-	+	44.23*	24.3	----	.247	Typical
5	1	+++	-	+	49.47*	97.4	78.7	.215	Typical
6	5	+++	-	+	21.75*	85.4	79.7	.287	Elongated
7	1	+++	-	+	22.74*	46.1	84.6	.269	Typical
8	7	+++	-	+	4.92	30.1	70.5	.252	Elongated
9	1	+++	-	-	----	----	----	----	Elongated
10	1	+++	-	-	----	----	----	----	Elongated
11	2	+++	-	+	22.26*	32.2	62.7	.258	Elongated
12	4	+++	-	+	13.74*	41.0	73.1	.270	Typical
16	3	++	-	-	----	----	----	----	Elongated
17	1	+++	-	+	17.04*	----	81.2	----	----
18	2	+++	-	+	93.96*	----	66.6	----	----
21	3	+++	-	-	----	----	----	----	Elongated
CV						42%	33%	14%	

*Significantly different from 'Atlantic' based on Dunnet's T test or LSmeans comparison ($P < 0.05$)

[x](**) = lowest relative band intensity, (***) = highest relative band intensity

[y]Units of activity in 1 mg of leaf tissue multiplied by 10^5

Table 2.3. Molecular analyses, field and greenhouse late blight experiments and tuber phenotype of 'Libertas' - derived (LGO) lines containing the glucose oxidase gene.

LGO Line	Copy Number	Northern[x] (leaf tissue)	Northern (tuber tissue)	Western Results	Glucose Oxidase Enzyme Activity[y]	Greenhouse Late Blight Trial[z]	Greenhouse Late Blight Trial[z]	1999 Field Trial (RAUDPC)	Tuber Phenotype
Libertas	0	-	-	-	0.00	16.5	73.0	.327	Typical
1	1	+++	+	+	53.27*	11.1	41.4	.446	Knobby, elongated
3	3	+	+	+	12.66*	25.3	95.2	.363	Typical
4	2	++++	-	+	42.64*	----	97.1	.343	Knobby, elongated
10	6	+++	-	+	6.05	41.9	77.0	.386	Typical
13	1	-	----	-	----	----	----	----	Typical
15	1	+++	-	-	----	----	70.2	.355	Typical
16	7	+++	-	+	2.12	66.7*	93.1	.341	Knobby, elongated
CV						42%	39%	13%	

*Significantly different from 'Libertas' based on Dunnett's T test or LSmeans comparisons ($P < 0.05$)

[x] (+) = lowest relative band intensity, (++++) = highest relative band intensity

[y] Units of activity in 1 mg of leaf tissue multiplied by 10^5

[z] LSmeans for % infection at last evaluation

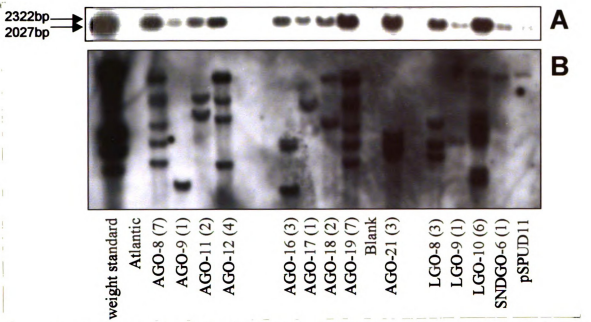

Figure 2.3. Southern blot of *Bam*HI digested DNA verifying insertion of the glucose oxidase gene (A) and *Xba*I digested DNA to determine transgene copy number (B).

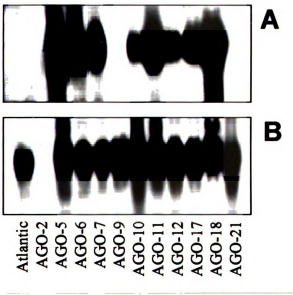

Figure 2.4. Northern blot of transgenic potato lines containing the glucose oxidase gene hybridized with the glucose oxidase probe (A) and a potato 18S ribosomal probe (B).

transgene transcript and the glucose oxidase protein (Table 2.2; Figure 2.5). There are other lines (SGO-1, LGO-1, AGO-5) with only one copy which produce relatively high levels of the transgene transcript as well as the glucose oxidase protein. In addition, relative level of transgene mRNA did not always reflect the amount of enzyme activity detected. Lines with relatively high transgene mRNA had both high (LGO-1, LGO-4) and low (LGO-10, SGO-2) glucose oxidase enzyme activity (Table 2.1; Table 2.3).

In this study there is evidence suggesting both transcriptional and post-transcriptional gene silencing. Lines in which the transgene is present but the transgene transcript is absent suggest transcriptional gene silencing whereas lines in which both the transgene and its transcript are present but the glucose oxidase protein is lacking suggest post transcriptional gene silencing (Table 2.1; Table 2.2; Table 2.3).

Of all the transgenic lines recovered, there were 9 AGO lines, 5 LGO lines, 6 SGO lines and one SNDGO line that produced the glucose oxidase protein (Table 2.1; Table 2.2; Table 2.3). The only ZGO line recovered did not express the glucose oxidase protein. Further analyses were limited to AGO, LGO and SGO lines in which western analysis revealed the presence of glucose oxidase protein (approximately 80 kDa).

Because elevated levels of H_2O_2 can have detrimental effects on plant cells, plant growth habit as well as plant and tuber morphology were closely monitored for each of the transgenic lines (Table 2.1; Table 2.2; Table 2.3; Figure 2.6). Lines SGO-3 and SGO-9 exhibited delayed shoot emergence in the trial at the Montcalm Research Farm. Although tubers of SGO-3 were noted to be small with numerous knobs, tubers of SGO-9 were similar in shape and size to untransformed 'Spunta'. Line SGO-4 did not express the glucose oxidase protein, even so, it was stunted in growth and produced small,

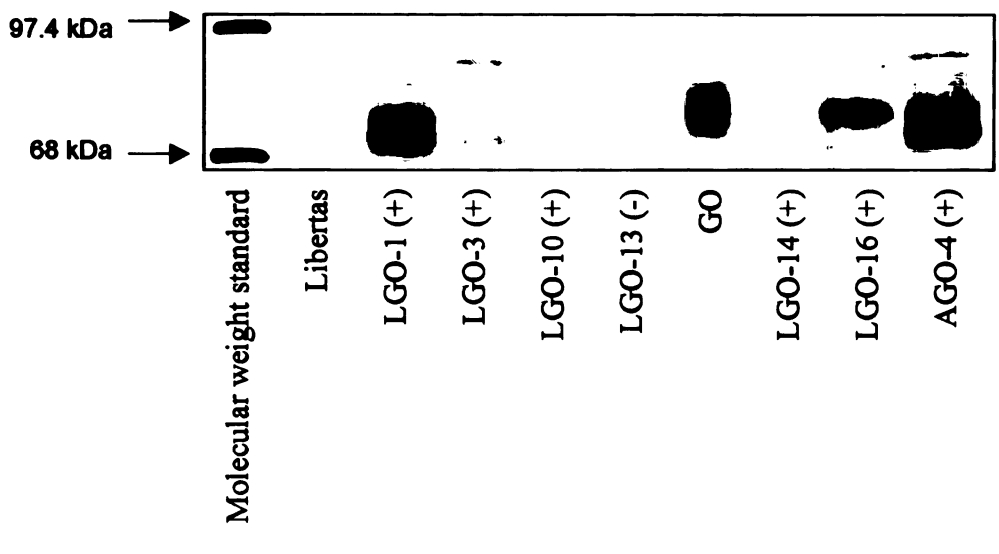

Figure 2.5. Western blot showing leaf protein hybridized to glucose oxidase polyclonal antibody.

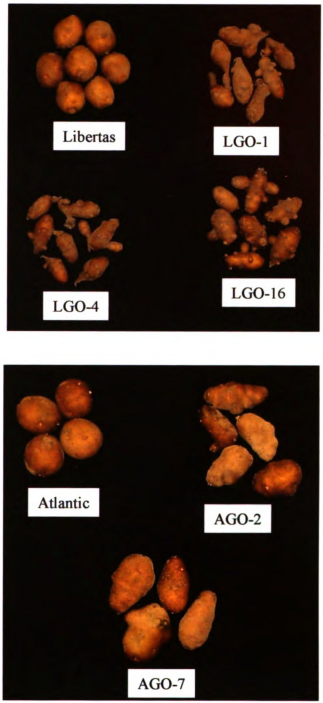

Figure 2.6. Photograph showing the off-type tuber morphology of several transgenic lines containing the glucose oxidase gene.

knobby tubers. None of the AGO lines differed from the untransformed 'Atlantic' in growth habit or plant morphology but many had slightly elongated tubers. The exception was AGO-2 which was off-type with very knobby and elongated tubers despite the lack of glucose oxidase protein. Among the LGO lines, LGO-1, 3, 4 and 16 were stunted in growth compared to the untransformed 'Libertas' whereas plants of LGO-10 were the same as the control. Because 'Libertas' is a late maturing variety and tissue culture transplants were used, the tubers from the LGO lines and the 'Libertas' control were small. However, tubers of LGO-1, 4 and 16 were also knobby and elongated. Glucose oxidase enzyme activity in lines LGO-1 and 4 was high compared to other transgenic plants but LGO-16 and LGO-3 had much lower enzyme activity (Table 2.3).

Foliar Late Blight Screening

The 'Spunta' derived SGO lines were tested in the greenhouse in three consecutive trials (Table 2.1). In the first trial lines were a significant source of variation only at 14 days post inoculation ($P < 0.0075$) and lines SGO-1, 2, 9 and 12 were less infected than the 'Spunta' control based on LSmeans comparisons of percent infection. Lines were not a significant source of variation at any evaluation point in the second or third trials. Using data combined over trials one and three, lines were a significant source of variation at 14 DPI ($p<0.0215$) and lines SGO-1, 2, 9, 12 and 14 were significantly less infected than the 'Spunta' control based on Dunnett's T tests (Table 2.1). Trial two could not be combined with either trial one or trial three due to non-homogenous variation.

The 'Atlantic' derived AGO lines were tested twice in the greenhouse for response to *P. infestans* (Table 2.2). In the first trial, lines were not significantly different at 7 DPI but were significant different at 10 and 14 DPI ($P < 0.0183$ and $P < 0.046$ respectively). Line AGO-6 had a higher infection level than the 'Atlantic' control ($P <$ .0022) at 10 DPI and lines AGO-5 and 6 more infected than the control ($P < 0.0224$ and $P < 0.0166$ respectively) at 14 DPI based on LS means comparisons. In the second trial, lines were not significant at any point following inoculation. In the first greenhouse trial of LGO lines, lines were a significant source of variation at 7 ($P < 0.0112$), 10 ($P <$ 0.0001) and 14 ($P < 0.0389$) DPI. Transgenic line LGO-16 had more infection than the 'Libertas' control at 7 DPI ($P < 0.006$), LGO-3, 10 and 16 were more infected than the control at 10 DPI and LGO-16 was the only line more infected than the control at 14 DPI. In the second trial, lines were not a significant source of variation at any point after inoculation (Table 2.3).

Field evaluations of response to *P. infestans* were done two years for SGO lines and one year for all other transgenic lines. In 1998, lines were a significant source of variation ($P < 0.0117$). However, based on both Tukey's Studentized Range Test and Dunnett's T test none of the SGO lines were significantly different ($P < 0.05$) from the 'Spunta' control. Transgenic lines SGO-1, 12 and 14, which were significantly less infected than the control in one of the greenhouse trials, did have a lower RAUDPC value than the control, but the difference was not significant (Table 2.1). In 1999, lines were not significantly different for the SGO ANOVA, however, when data were combined over years, lines were significant. In the combined analysis, lines SGO-3 and

SGO-9 were significantly more infected than the 'Spunta' control (Table 2.4). This is in contrast to the greenhouse trial in which SGO-9 ranked lower than 'Spunta' for percent area infected. Lines were not significantly different in the AGO field trial ($P < 0.2694$) and the LGO field trial ($P < 0.0568$) (Table 2.2 and Table 2.3).

Tuber Late Blight Evaluation

Tuber late blight evaluations were done in both 1998 and 1999 for SGO transgenic lines and in 1999 for AGO and LGO transgenic lines (Table 2.4). In 1998 lines were a significant source of variation ($P < 0.007$) although none of the SGO transgenic lines were different from the 'Spunta' control based on ordinal analysis of visual ratings. Lines were significant in the digital analysis of tuber flesh for the apical ($P < 0.0096$), middle ($P < 0.0089$) and terminal slices ($P < 0.0147$) as well as tuber average ($P < 0.0086$). However, none of the transgenic lines were significantly different from the 'Spunta' control. In the 1999 ordinal analysis, lines were not a significant source of variation for either the SGO lines or the LGO lines but were significant for AGO lines ($P < 0.05$). Lines AGO-6 and 8 were significantly less infected than the 'Atlantic' control. AGO-6 was among the most susceptible lines in both greenhouse and field evaluations of foliar response to late blight whereas AGO-8 was among the best lines in all evaluations. However, based on limited infection of control tubers ('Spunta', 'Atlantic' and 'Libertas') in 1999, the dipping method of inoculation did not provide adequate infection for determination of tuber late blight resistance.

Table 2.4. Tuber late blight evaluations for 1998 and 1999.

1998 tuber late blight evaluations					1999 tuber late blight evaluations	
Line	Mean visual rating	Apical	Middle	Terminal	Line	Mean visual rating
SGO-14	1.7	163.1	154.8	162.8	SGO-1	1.0
SGO-9	3.5	146.4	142.3	149.2	SGO-12	1.4
SGO-2	2.8	139.0	131.6	135.0	SGO-3	1.6
SGO-3	2.7	127.1	123.9	137.8	SGO-14	1.7
Spunta	3.5	118.6	124.7	137.0	SGO-2	2.2
SGO-1	5.1	114.6	111.3	110.3	Spunta	2.2
SGO-12	5.0	111.3	115.9	131.3	SGO-1	2.7
CV	-----	38.0	36.6	34.1		
					LGO-4	1.0
					LGO-10	1.2
					Libertas	1.4
					LGO-1	1.4
					LGO-15	1.4
					LGO-3	1.5
					LGO-16	1.5
					AGO-7	1.3
					Atlantic	1.3
					AGO-12	1.4
					AG0-11	1.5
					AG0-5	1.8
					AGO-6	2.2*
					AGO-8	2.2*

*Significantly different from control based on Dunnett's T test ($p<0.05$)

Enzyme activity assay

Lines differed in the ANOVA for enzyme activity among AGO, LGO and SGO transgenics ($P < 0\ 0001$). Based on Dunnet's T tests, all of the AGO lines except AGO-8 had higher glucose oxidase enzyme activity than the 'Atlantic' control (Table 2.2). Among LGO and SGO lines, only LGO-1, 3 and 4 and SGO-1, 3 and 12 had higher enzyme activity than their respective non-transgenic controls (Table 2.1 and Table 2.3).

DISCUSSION

The development of late blight resistant cultivars is an important objective in potato breeding programs. However, since the late blight pathogen readily overcomes R-gene based resistance, whether as single genes or as a pyramid of R genes (Ross, 1986), it is important to identify more complex mechanisms of resistance. Therefore, this study was undertaken to evaluate the effect of a fungal glucose oxidase gene in a late blight susceptible potato cultivar and in a cultivar possessing quantitative genetic resistance to blight.

The production of transgenic potato lines containing the glucose oxidase gene was readily achieved for the susceptible cultivars 'Atlantic' and 'Spunta'. However, lines with moderate resistance to late blight, 'Libertas' and 'Zarevo, were recalcitrant to transformation resulting in few transgenic lines. In addition, the transformed lines were recovered only after a lengthy time in *in vitro* culture. The difference between the cultivars in recovery of transgenic lines may be due to genetic variation for regeneration and transformation efficiency. Several authors have noted such a difference and have developed protocols for efficient regeneration of many cultivars (DeBlock, 1988; Hulme *et al.*, 1992; ; Wenzler *et al.*, 1989; Yadav and Sticklen, 1995). Another possible explanation may be an increased sensitivity to kanamycin. Breeding line MSG274-3 from the Michigan State University Potato Breeding Program, responded similarly to 'Zarevo' upon transformation and no regenerates had been recovered from this line using kanamycin selection (50 mg/L). However, transgenic regenerates from MSG274-3 were obtained if the explants were cultured on a reduced concentration of kanamycin (25

mg/L) (Douches unpublished data). A more formal study is needed to support this observation.

Although several of the plants recovered from the transformation process had the glucose oxidase transgene, many of them either did not transcribe the inserted gene, as indicated by Northern analysis, or did not translate the gene, as indicated by Western analysis. This phenomenon, known as gene silencing, results in the lack of expression of a transgene and/or any genes with similar homology (endogenous or exogenous). Gene silencing occurs either by prevention of transcription, transcriptional gene silencing (TGS) or by alteration and/or destruction of the gene transcript, post-transcriptional gene silencing (PTGS). TGS has been attributed to interactions between homologous promoters present at different genomic locations (trans silencing), interactions between different parts of a complex transgene locus, chromosome position into which a transgene inserts and the sequences surrounding the insertion site (Taylor, 1997). PTGS often occurs when transgene transcripts are abundant and it is generally thought to be triggered at the level of mRNA processing, localization, and/or degradation (Taylor, 1997). Several models have been proposed to explain this phenomenon. The "threshold model" suggests that when a specific transcript reaches a threshold level it and any homologous transcripts are targeted and degraded by the cell (Dougherty and Parks, 1995). Another model suggests that methylated transgenes produce aberrant RNA molecules that are selectively degraded along with related transcripts (Baulcombe and English, 1996). A third model suggests that base pairing of transcripts triggers the targeted degradation of homologous transcripts (Cameron and Jennings, 1991). Although the method of gene

silencing in our study has not been determined, it appears that both TGS and PTGS have occurred.

Although many of the glucose oxidase-containing lines exhibited altered plant growth habit and/or tuber phenotype, these changes cannot be attributed solely to the effects of H_20_2 because some of these lines did not produce the glucose oxidase protein and would therefore not have elevated levels of H_2O_2. For example, tubers of the AGO-2 lines were highly off-type, but neither the glucose oxidase transcript nor the protein was detected in this line (Table 2.1). Because morphological changes in either the plant or the tuber are not clearly associated with transgene expression, it is possible that these physical changes are due to the position of the transgene insertion and/or somaclonal variation that occurred during the transformation process. Somaclonal variation is defined as an increase of genetic variability in higher plants which takes place during *in vitro* culture. Several factors can influence the probability that somaclonal variation will occur including the method of vegetative propagation, type of tissue used, media components, and genotype of the plant (Pierik, 1987). In potato, certain genotypes have a greater tendency for somaclonal variation. Shepard *et al.* (1980) demonstrated that the cultivar 'Russet Burbank' was very unstable when plants originated from protoplasts. Similar results were found for the cultivar 'Bintje' (Ramula *et al.*, 1984). Regardless of the basis for the altered plant and/or tuber morphology, it is evident that an unusually large percentage of the transgenic plants recovered in this study (44%) were considered off-type. Transformation of the same cultivars used herein with other genes resulted in very few off-type plants despite using identical transformation protocols and transformation vectors (Douches, unpublished data). Therefore, if the glucose oxidase

gene is to be used in the development of potato cultivars, it may be necessary to produce and screen a larger population of plants than is required with other transgenes.

In previous research, the glucose oxidase gene was shown to reduce the growth of late blight lesions by 49% to 56% compared to controls in detached leaf assays (Wu *et al.*, 1995). In our study, greenhouse trials may indicate a reduction in late blight infection among SGO and LGO lines but large experimental error (CV's from 20.1 to 71.5%) limited our ablity to identify of differences between the control and transgenic lines. However, field trials indicate little if any effect of the glucose oxidase gene in any of the transgenic lines upon infection by the late blight pathogen *P. infestans*. It is not unusual for a potato line to have resistance in a greenhouse evaluation and exhibit less resistance in the field. A good example is the cultivar 'Zarevo' which is highly resistant in greenhouse studies, but is only moderately resistant in the field (Douches *et al.*, 2000). In the case of 'Zarevo' a possible explanation for the difference between field and greenhouse late blight evaluations is the behavior of *P. infestans* zoospores. Niemira *et al.*, (1998) demonstrated that on 'Zarevo', zoospores target stomata for penetration, whereas, on susceptible cultivars the zoospores penetrate directly through the leaf surface. Therefore, in 'Zarevo' the onset of infection may be delayed resulting in delayed growth and sporulation of the pathogen. In a short (one to two-week) greenhouse evaluation this may be detected as a high level of resistance. However, in a field trial, which is longer in duration, the amount of disease in 'Zarevo' will eventually reach levels at which the cultivar will be categorized as only moderately resistant.

There are several possible explanations for the disparity between the current research and that done by Wu *et al.*, (1995). Wu *et al,* (1995) measured the spread of

individual lesions in detached leaf assays and the area infected on four center leaves in whole plant analyses allowing them to limit error due to subjective measurements and identify small differences. In our study whole plants were evaluated for area infected in both the field and in the greenhouse. The subjectivity in this type of evaluation can lead to high experimental error and limit the ability to identify small differences. In an attempt to limit the subjectivity of these measurements all analyses were conducted by two or three member teams. Another difference between the two studies is the severity of environmental conditions in which whole plant analyses were conducted. Wu *et al.*, (1995) maintained high humidity in the experimental chamber for only the first 48 hours whereas we maintained high humidity for the entire experiment. Because high humidity favors late blight development our experimental conditions must be considered more severe and may have prohibited the measurement of small differences in disease severity. Another source of difference may be in the biotype of *P. infestans* used. In this study, several highly pathogenic isolates of the US-8 biotype of *P. infestans* were used. These isolates had been recovered from infected potato tissue discovered in various Michigan farm locations and would therefore be similar to what potatoes in the field would encounter. Wu *et al.*, (1995) do not report the source or biotype of *P. infestans* used in their research. Lastly, both studies used different glucose oxidase gene constructs for transformation. Our construct used the Gelvin super promoter and utilized the kanamycin resistance gene, *NptII*, as the selectable marker. The construct used by Wu *et al.*, (1995) used the constitutive figwort mosaic virus 35S promoter and a glyphosate resistance gene as the selectable marker. The DNA sequence of the glucose oxidase gene in both constructs should be the same as both were isolated from *Aspergillus niger* using

primers designed according to the published gene sequence (Frederick *et al.*, 1990). However, direct comparisons are not possible as the sequence of the construct used by Wu *et al* (1995) was not published.

Based on this study, the glucose oxidase gene, even in combination with moderate host plant resistance, will not provide complete protection against *P. infestans* but may slightly reduce susceptibility. Although the ultimate goal is to produce potato cultivars with complete resistance to late blight, cultivars with reduced susceptibility can lessen the frequency and quantity (active ingredient) of fungicide applied (Kirk *et al.*, 1999). Therefore, the glucose oxidase gene may be useful for slightly increasing the late blight resistance of otherwise desirable potato cultivars.

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Chapter III

RESPONSE OF TRANSGENIC POTATOES (*SOLANUM TUBEROSUM* L.) CONTAINING THE FUNGAL GLUCOSE OXIDASE GENE TO *FUSARIUM SAMBUCINUM* AND *ERWINIA CAROTOVORA* SSP. *CAROTOVORA*

ABSTRACT

Production of high quality, disease free potatoes is challenging due to the large number of pathogens that can attack the growing plant and/or the stored tubers. Two pathogens that are particularly troublesome during storage are *Fusarium sambucinum*, a fungus that causes dry rot of potato tubers, and *Erwinia carotovora* ssp. *carotovora*, a bacterium that causes soft rot of potato tubers. Currently, there are no commercial potato cultivars that are highly resistant to either disease and control measures are predominantly cultural. Transgenic potatoes containing a fungal glucose oxidase gene have been shown to have increased resistance to *E. carotovora* and several other pathogens Therefore, three potato cultivars ('Atlantic', 'Libertas' and 'Spunta') were transformed with the glucose oxidase gene and evaluated for their response to both *E. carotovora* ssp. *carotovora* and *F. sambucinum*.

Greenhouse and field grown tubers of transgenic lines were used to assess response to inoculation with both pathogens. Whole tubers were wounded and then inoculated with agar plugs of growing *F. sambucinum* cultures or tuber slices were inoculated with a suspension solution of *E. carotovora* ssp. *carotovora* (10^8 CFU·ml^{-1}). Disease progression was determined by measuring the diameter and depth of the lesion for *Fusarium* dry rot (14 to 28 days post inoculation based on tuber size) and by measuring lesion diameter 48 hours post inoculation for *Erwinia* soft rot.

The glucose oxidase gene had a no effect on the resistance of the cultivars to *Fusarium* dry rot but had a small effect against *Erwinia* soft rot. However, differences between lines were difficult to detect due to high error variations in many of the evaluations. Lines SGO-1 and 2 were the only lines significantly different from the non-transgenic control for response to *Fusarium* dry rot and lines SGO-9, 12, 1 and LGO-3 were the only lines significantly different from the non-transgenic controls for response to *Erwinia* soft rot. There does not appear to be any correlation between resistance to *Fusarium* and resistance to *Erwinia* among the transgenic lines. In addition, many of the lines had abnormal tuber phenotypes and/or abnormal plant morphology.

The glucose oxidase gene may be useful to develop potato cultivars with increased resistance to *Erwinia* soft rot. Future research will include evaluation of 1) the resistance of the transgenic lines to *Erwinia* soft rot under commercial storage conditions, 2) the resistance of the transgenic lines to *Erwinia* infection of the plant during the growing season, and 3) the possibility of producing lines with increased resistance and normal morphology.

INTRODUCTION

The potato is one of the most important crops worldwide ranking fourth in yield with worldwide production of at 194,833,731 metric tons of which the United States produces 21,725,182 metric tons (National Potato Council, 1999) many of which are held in storage until marketed. Producing high quality, disease-free potatoes is expensive and time consuming due to the large number of pathogens and insect pests to which the potato is susceptible. Hide and Lapwood (1992) estimated that there are over 100 pathogens that may infect the potato. Although many of these pathogens attack the growing crop, there are several important diseases that destroy stored potato tubers. Two of the most important of these are dry rot caused by the fungus *Fusarium* sp. and soft rot caused by the bacteria *Erwinia carotovora.*

Potato dry rot is often difficult to detect as tubers rot from the inside producing discolored cavities (Figure 3.1). However, infected tubers may exhibit wrinkled, sunken patches on the surface and fungal mycelia may be visible on the tuber surface as the disease progresses (Rich, 1983). The causal agent of this disease may be one of several *Fusarium* species including, but not limited to, *F. sambucinum* and *F. solani,* (Rich, 1983). This pathogen relies on wounding during harvest or seed cutting to infect. Therefore, careful handling of tubers during harvest and storage can reduce the occurrence of dry rot in storage. Other cultural practices that can limit the incidence of dry rot are harvesting at warmer temperatures (above 45°F) to avoid bruising, bruise/wound healing prior to storage, and long-term storage at low temperatures as *Fusarium* can develop at 40°F and above (Lacy and Hammerschmidt, 1993). The fungicide thiabendazole has been widely used by growers since the 1970's to control

A

B

Figure 3.1. Photographs of tubers inoculated with *Fusarium sambucinum* showing internal (A) and external (B) symptoms of infection.

potato dry rot however, several *Fusarium* isolates have demonstrated resistance to this chemical (Hanson *et al*, 1996). In their study, Desjardin *et al.*, (1993) found that all *Fusarium* isolates collected between 1963 and 1986 were sensitive to thiabendazole, whereas the majority of isolates collected in 1990 and 1991 were resistant. Hanson *et al.*, (1996) found that 42% of the isolates collected in the northeastern United States were resistant to thiabendazole. Currently, there are no commercial cultivars that are resistant to *Fusarium* dry rot thus, it is necessary to find alternative control measures for *Fusarium* dry rot and to develop resistant potato cultivars.

Testing for response to *Fusarium* dry rot is done either by wounding whole tubers and inoculating them with the pathogen (Vaughn and Spencer, 1994), or by directly inoculating freshly cut tuber surfaces with the fungus (Hanson *et al.*, 1996). Disease response is evaluated based on the presence and size of the lesion at the site of inoculation.

Unlike dry rot, potato soft rot is readily detectable due to the offensive odor produced through secondary infection by other bacteria such as *Pseudomonas* sp. *E. carotovora*, the causal agent of this disease penetrates the tuber via natural openings and wounds and destroys the tuber tissue through the activity of pectolytic enzymes (Bartz and Eckert, 1987). Bacterial soft rot is favored by warm temperatures (25° to 37°C), high moisture levels in the soil or during storage, wounding (Cromerty and Easton, 1973) and anaerobic conditions (DeBoer and Kelman, 1978). A number of chemicals, including chlorine-based chemicals, antibiotics and organic and synthetic bacteriocides have been tested for their ability to control soft rot in potato and other vegetables (Bartz and Eckert, 1987). However, public concern about the usage of toxic chemical on food has

encouraged the use of less controversial control measures to manage storage diseases (Wilson and Wisniewski, 1992). Therefore, control of soft rot in potatoes is predominantly cultural including precautions such as disinfecting storage bins and harvest equipment, avoiding excessive irrigation, careful handling during harvest and storage to avoid bruising and wounding, storage in cool dry conditions and air circulation to prevent depletion of oxygen (Rich, 1983). Currently, there are no commercial cultivars that are resistant to *Erwinia* soft rot.

Variations of two inoculation procedures have been used to evaluate potato tuber resistance to soft rot: point titration and slice inoculation. Point titration involves the injection of the pathogen directly into intact tubers at a specified depth (Lojkowska and Kelman, 1994) whereas, in the slice method bacterial suspensions on filter paper disks are used to inoculate tuber slices (Lapwood, *et al.*, 1984). The relative resistance of the potato tuber is measured as the diameter of the macerated tissue for both inoculation procedures. Several authors have compared the results of both inoculation methods. Lojkowska and Kelman (1994) questioned the reliability of the tuber slice method due to the high level of variability that is often observed. However, Koppel (1993) suggested that the two methods measured different aspects of resistance. Point titration measured the resistance to progressive rot under anaerobic conditions, whereas, the slice method measured the reaction of the tuber to bacterial challenge at a wound site and the speed that wound healing occurred. Haynes *et al.* (1997) concluded that the slice test was valid provided a sufficient sample size was used and the appropriate parameters were measured.

In addition to inoculation method the *Erwinia* pathogen used for resistance screening is important. *Erwinia carotovora* ssp. *carotovora* (Ecc) and *Erwinia carotovora* ssp. *atroseptica* (Eca) are the predominant soft rot species in cooler potato growing areas (DeBoer and Kelman, 1978) whereas, *Erwinia chrysanthenmi* (Ech) prevails in the warmer growing areas (Hidalgo and Echandi, 1983). Several authors (Lapwood *et al.*, 1984; Austin *et al*, 1988; Wolters and Collins, 1994) have reported similar rankings of potato cultivars when inoculating with either Ecc, Eca or Ech. Therefore, the choice of *Erwinia* species used can be decided based upon the growing area for which variety development is targeted.

Several alternative treatments have been suggested to control both *Fusarium* dry rot and *Erwinia* soft rot in potatoes. Shashirekha and Narashimham, (1990) found that potatoes dipped in solutions containing trace elements such as iron, copper and zinc prior to storage significantly reduced the incidence of both dry and soft rots. Vaughn and Spencer (1994) tested three sprout inhibitors (salicylaldehyde, cinnamaldehyde and thymol) and found that they inhibited dry rot development if applied prior to penetration of the fungus into the tuber tissue. Biological control of both diseases was effective under experimental conditions (Wilson and Wisniewski, 1992; Bartz and Eckert, 1987), and ultraviolet irradiation eliminated dry rot and delayed soft rot in potatoes stored at 37°C without adversely affecting tuber quality (Ranganna *et al*, 1997). However, to date none of these control measures have found application at the commercial level.

As mentioned previously, no commercial potato cultivars have resistance to *Fusarium* dry rot and/or *Erwinia* soft rot. Various wild potato species have been reported as having some level of resistance to these diseases (Carputo *et al*, 1996; Bamberg, *et al.*,

1994) and some somatic hybrids have been reported to possess resistance to *Erwinia* soft rot (Helgeson *et al.*, 1993). In addition, Wu *et al.*, (1995) reported nearly complete resistance to *E. carotovora* ssp. *carotovora* in transgenic 'Russet Burbank' tubers containing a fungal glucose oxidase gene. As the name suggests, this gene encodes a protein that oxidizes glucose and produces hydrogen peroxide (H_2O_2) as a byproduct of the reaction. Hydrogen peroxide has been reported to function at several points in the plant disease response including: direct antimicrobial effect (Wu *et al.*, 1995), structural changes in the plant cell wall (Dean and Kuc, 1987; Brisson *et al.*, 1994), production of toxic, lipid free radicals (Keppler and Baker, 1989), activation of phytoalexin synthesis (Apostol *et al*, 1989; Chai and Doke, 1987) and induction of systemic acquired resistance (Chen *et al.*, 1993; Conrath *et al.*, 1995). The glucose oxidase transgene has been reported to be effective against a broad range of pathogens (*Phytophthora infestans, Alternaria solani, Verticillium dahliae, and E. carotovora*) (Wu *et al.*, 1995; Wu *et al.*,1997). Therefore, transgenic glucose oxidase-containing potato plants, which were developed to enhance foliar late blight (*P. infestans*) resistance (Felcher *et al.*, 2000), were also tested for their response to *Fusarium* dry rot and *Erwinia* soft rot.

MATERIALS AND METHODS

Cloning of the glucose oxidase gene

The glucose oxidase gene was cloned from *Aspergillus niger* using the polymerase chain reaction (PCR) and primers designed according to Frederick *et al.* (1990) to which SmaI and XbaI restriction sites had been added. The resulting DNA fragment was sequenced, compared to the published glucose oxidase sequence (Frederick *et al.*, 1990) and was incorporated into the pE1102 vector. The resulting plasmid, named pSPUD11 (Figure 3.2), had the glucose oxidase gene under the control of the Gelvin "super promoter" (Ni *et al.*, 1995). This promoter is a combination of a trimer octopine synthase upstream activating sequence $(ocs)_3$ joined to a mannopine synthase (mas) activator and promoter. The pSPUD11 vector also contained the *Nos* terminator sequence (Nos T), right and left borders for plant transformation purposes and the *NptII* gene (kanamycin resistance) to be used as a selectable marker. The pSPUD11 plasmid was transformed into *Agrobacterium tumefaciens* strain LBA4404 (Ooms *et al.*, 1982) via tri-parental mating.

Transformation of potato cultivars

The cultivars 'Spunta', 'Atlantic', and 'Libertas' were micropropagated in GA-7 Magenta vessels each containing 25 ml of modified MS basal medium (Douches *et al.*, 1998). Transformations of potato cultivars were done according to Li *et al.*, (1999) using the *A. tumefaciens* culture harboring the pSPUD11 vector. Regenerating shoots (> 5 mm) were excised and transferred individually into 25 x 100 mm glass tubes each containing 20 mL of MS medium (Douches *et al.*, 1998) supplemented with 200 $mg \cdot L^{-1}$ Timentin

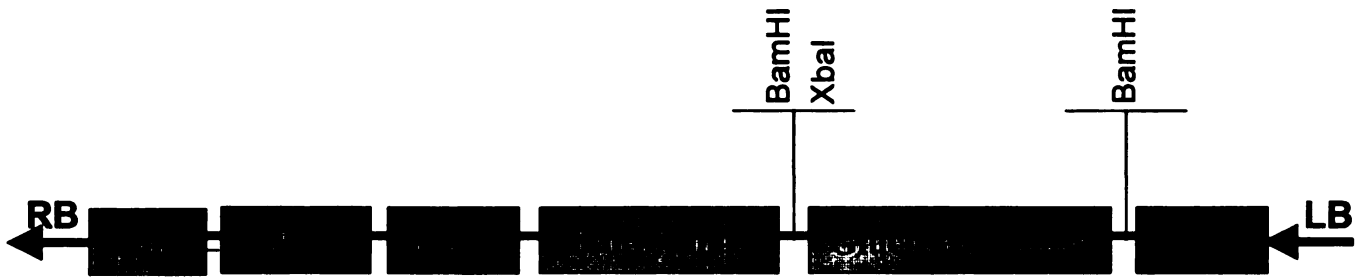

Figure 3.2 Diagram of the pSPUD11 vector containing the glucose oxidase gene from *Aspergillus niger* under the control of the Gelvin "super promoter" [$P(ocs)_3$ mas]. Restriction sites used for Southern blotting are shown.

(SmithKline Beecham, .Philadelphia, Pa.) and 50 $mg \cdot L^{-1}$ kanamycin. Rooted plantlets were designated as SGO-# (derived from 'Spunta'), AGO-# (derived from 'Atlantic'), and LGO-# (derived from 'Libertas'). Named plantlets were subcultured and subsequently transplanted into 10 cm pots containing Bacto potting medium (Michigan Peat Co. Houston, Texas) and grown in the greenhouse under fluorescent lights (16 h photoperiod) at 23 to 27°C. Leaf tissue from these plants was used for the molecular expression assays. All lines were characterized for transgene copy number, transgene transcription and translation, glucose oxidase enzyme activity and plant morphology (Felcher et al., 2000).

Fusarium dry rot screening of transgenic lines

Transgenic lines were tested for response to *Fusarium sambucinum* (*Fusarium* dry rot) using both greenhouse tubers (1998) and field grown tubers (1999). *F. sambucinum* cultures (isolate RN1), kindly provided by Dr. Hammerschmidt, were started by subculturing agar plugs of older cultures onto freshly prepared potato dextrose agar (PDA) medium. New cultures were incubated at 23°C in the dark under ambient conditions for four days prior to use. Small sections of the tuber surface were peeled back with a razor blade and the exposed tuber tissue was inoculated with an agar plug from the growing edge of the *F. sambucinum* mycelial culture. Tubers were labeled, placed in trays and incubated in the dark at 25-26°C for 14 days in 1998 due to small tuber size, and 28 days in 1999 with the exception of 'Libertas' and LGO lines (14 days) which were small in size. Following incubation, tubers were sliced perpendicularly through the inoculation site and lesion depth and diameter were measured (mm). Data

were analyzed using SAS proc glm and transgenic lines were compared to the respective non-transgenic controls using least squares means (LSmeans) comparisons for unbalanced data (1998) and Dunnett's T Test and Tukey's Studentized Range Test for balanced data in 1999 (SAS Institute, Inc).

Erwinia soft rot screening of transgenic lines

Cultures of *Erwinia carotovora* ssp. *carotovora* (Ecc) were provided by Dr. Hammerschmidt and were maintained as glycerol stocks in a -80°C freezer. Prior to use, a sample was taken from the stock, streaked onto solid PDA medium and incubated overnight at 25-26°C. A single colony was selected from the overnight culture and used to inoculate liquid LB media. The liquid culture was incubated overnight on a shaker (100 rpm) and then diluted to (10^8 CFU·ml^{-1}) based on spectrophotometer readings.

Ten field-grown tubers of each transgenic line were tested for *Erwinia* soft rot resistance. Tubers were removed from cold storage, washed and warmed to room temperature for 24 h prior to inoculation. A slice was taken from the middle of each tuber, placed in a Petri dish, and inoculated with a filter paper disk soaked in the Ecc suspension culture. In 1998, Petri dishes (15 x 100 mm) containing inoculated tuber slices were closed and placed in sealed plastic sleeves to maintain a near-anaerobic environment and maintain moisture levels. Lesion diameter (mm) was measured at 24 and 48 hrs. and lesion depth (mm) was measured at 48 hrs by cutting the tuber slice perpendicular to the previously cut surface. Due to variable data in 1998, the evaluation was modified in 1999. Tuber slices were placed in Petri dishes (25 x 100 mm) in which a thin layer of solid water agar had been poured. The dishes were stored and the tuber

slices evaluated as in the previous year. Data were analyzed in the same way as the *Fusarium* dry rot data.

RESULTS

Fusarium dry rot

In the ANOVA of the 1998 *Fusarium* dry rot evaluation of transgenic SGO lines, lines were significant ($P < 0.01$) accounting for approximately 58% of the variation (R^2 = .581) in lesion diameter . LSmeans ranged from 12.78 mm (SGO-2) to 18.17 mm (SGO-9) with only SGO-1 and 2 different from the 'Spunta' non-transgenic control (Table 3.1). For lesion depth, lines were also a significant source of variation in 1998 ($P < 0.01$) accounting for approximately 39% of the variation ($R^2 = 0.385$). Transgenic line SGO-3 was the only line different from the non-transgenic 'Spunta' control (Table 3.1). In the 1999 trial, lines were not significantly different for lesion diameter ($P < 0.56$) or lesion depth ($P < 0.22$). Lesion diameter ranged from 31.4 mm (SGO-9) to 24.9 mm (SGO-2) and lesion depth ranged from 3.45 mm ('Spunta) to 10.1 mm (SGO-9) (Table 3.1).

Ranking of lines varied between years and between lesion measurements within years although the most resistant and most susceptible lines tended to be the same across years and measurements (Table 3.1). For example, SGO-2 was the most resistant line, SGO-14 was the third most resistant line and SGO-9 was the most susceptible line in both 1998 and 1999 based on lesion diameter.

All other transgenic lines were only evaluated for response to *F. sambucinum* in 1999 due to the availability of tubers. Based on the ANOVA, lines were not significantly different for either the AGO lines or the LGO lines when lesion diameter or lesion depth was analyzed (Table 3.2 and Table 3.3). Lesion diameter ranged from 18.25 mm (AGO-12) to 23.9 mm (AGO-8) for 'Atlantic'-derived lines and from 15.8 mm (LGO-4) to 20.1 mm (Libertas) for 'Libertas'-derived lines. Lesion depth ranged from 3.7 mm

Table 3.1. Molecular analyses, *Fusarium* dry rot evaluations and tuber phenotype of 'Spunta' - derived (SGO) lines containing the glucose oxidase gene.

SGO Line	Copy Number	Northern (leaf)[x]	Northern (tuber)	Western	Glucose Oxidase Enzyme Activity[y]	1998 Lesion Measurements (mm)[y]		1999 Lesion Measurements (mm)[z]		Tuber Phenotype
						Diameter	Depth	Diameter	Depth	
Spunta	----	-	-	-	0.00	17.4	6.5	26.5	3.5	Typical
1	1	+++	-	+	19.65*	14.4*	6.5	27.9	6.6	Typical
2	1	+++	-	+	4.42	12.8*	6.2	24.9	5.1	Knobby,
3	2	+++	-	+	18.56*	18.2	4.6*	29.4	7.2	Knobby,
4	3	++	-	-	----	----	----	----	----	Knobby,
9	3	+	-	+	0.00	18.2	6.3	31.4	10.1	Typical
10	1		-	-	----	----	----	----	----	Typical
12	1	+++	-	+	30.39*	----	----	29.8	5.5	Typical
14	1	++	-	+	6.43	17.7	6.0	27.8	4.7	Typical
19	3	++	-	-	----	----	----	----		Typical
20	2	-	-	-	----	----	----	----		Typical
23	3	+	-	-	----	----	----	----		Typical
26	5	+++	-	-	----	----	----	----		Typical

*Significantly different from 'Spunta' ($P < 0.05$)

[x] (+) = loweset relative band intensity, (+++) = highest relative band intensity

[y] data reported as Lsmeans, compared to 'Spunta' using LSmeans comparisons

[z] data reported as means, compared to 'Spunta' using Dunnet's T test

Table 3.2. Molecular analyses, *Fusarium* dry rot experiments and tuber phenotype of 'Atlantic' - derived (AGO) lines containing the glucose oxidase gene.

AGO Line	Copy Number	Northern[x] (leaf tissue)	Northern (tuber tissue)	Western	Glucose Oxidase Enzyme Activity[y]	Lesion Diameter (mm)[z]	Lesion Depth (mm)[z]	Tuber Phenotype
Atlantic	0	-	-	-	0.00	18.8	4.3	Typical
2	1	-	-	-	----	----	----	Knobby, elongated
4	3	++	-	+	44.23*	18.6	4.8	Typical
5	1	+++	-	+	49.47*	22.1	4.9	Typical
6	5	+++	-	+	21.75*	18.5	3.7	Elongated
7	1	+++	-	+	22.74*	21.8	6.1	Typical
8	7	+++	-	+	4.92	23.9	4.7	Elongated
9	1	+++	-	-	----	----	----	Elongated
10	1	+++	-	-	----	----	----	Elongated
11	2	+++	-	+	22.26*	20.7	5.0	Elongated
12	4	+++	-	+	13.74*	18.3	4.5	Typical
16	3	++	-	-	----	----	----	Elongated
17	1	+++	-	+	17.04*	----	----	----
18	2	+++	-	+	93.96*	----	----	----
21	3	+++	-	-	----	----	----	Elongated

*Significantly different from 'Atlantic' ($P < 0.05$) based on Dunnet's T test

[x](++) = lowest relative band intensity, (+++) = highest relative band intensity

[y]Units of activity in 1 mg of leaf tissue multiplied by 10^5

[z]None of the lines were significantly different from 'Atlantic'

Table 3.3. Molecular analyses, *Fusarium* dry rot experiments and tuber phenotype of 'Libertas' - derived (LGO) lines containing the glucose oxidase gene.

LGO Line	Copy Number	Northern[x] (leaf tissue)	Northern (tuber tissue)	Western Results	Glucose Oxidase Enzyme Activity[y]	Lesion Diameter (mm)[z]	Lesion Depth (mm)[z]	Tuber Phenotype
Libertas	0	-	-	-	0.00	20.1	8.4	Typical
1	1	+++	+	+	53.27*	17.6	7.5	Knobby, elongated
3	3	+	+	+	12.66*	18.8	6.9	Typical
4	2	++++	-	+	42.64*	15.8	6.8	Knobby, elongated
10	6	+++	-	+	6.05	18.3	7.3	Typical
13	1	-	----	-	----	----	----	Typical
15	1	+++	-	-	----	----	----	Typical
16	7	+++	-	+	2.12	18.9	9.7	Knobby, elongated

*Significantly different from 'Libertas' ($P < 0.05$) based on Dunnet's T test

[x] (+) = lowest relative band intensity, (++++) = highest relative band intensity

[y]Units of activity in 1 mg of leaf tissue multiplied by 10^5

[z]None of the lines were significantly different from 'Libertas'

(AGO-6) to 6.0 mm (AGO-7) for 'Atlantic'-derived lines and from 6.8 mm (LGO-4) to 10.1 mm (LGO-14) for 'Libertas'-derived lines. Ranking of lines based on lesion diameter differed from that based on lesion depth for both AGO and LGO lines but the rankings for LGO lines were more similar with LGO-4 ranked as the most resistant line for both measurements.

Erwinia soft rot

In 1998 two *Erwinia* soft rot inoculation experiments were conducted using tubers of SGO lines. The first experiment indicated that all transgenic SGO lines had smaller lesion diameters than the 'Spunta' control whereas, in the second experiment, lines did not differ . In 1999, a third experiment was conducted with results more similar to the first experiment in 1998. The level of infection, based on lesion diameter, was lower in the second experiment (16.7 mm) than in the first (23.5 mm) or third (29.10 mm) experiments. Based on this .information only the first (1998) and third (1999) experiments were considered in the analysis. In both experiments, several SGO transgenic lines had significantly smaller lesion diameters than the 'Spunta' control (Table 3.4). Analysis of data combined across experiments (F_{max} = 1.57) indicated that SGO-9 and 12 had smaller lesion diameters than the 'Spunta' control (Table 3.4).

Due to low tuber availability, LGO lines and AGO lines were tested only following the 1999 field season. Transgenic lines LGO-1, 3, and 16 had significantly smaller lesion diameters than the 'Libertas' control and lines AGO-4 and 5 had significantly smaller lesion diameters than the 'Atlantic' control (Table 3.5; Table 3.6; Figure 3.3 and 3.4).

Table 3.4. Molecular analyses, *Erwinia* soft rot evaluations and tuber phenotype of 'Spunta' - derived (SGO) lines containing the glucose oxidase gene.

SGO Line	Copy Number	Northern (leaf)[x]	Northern (tuber)	Western	Glucose Oxidase Enzyme Activity[y]	1998 Lesion Diameter (mm)	1999 Lesion Diameter (mm)	Lesion Diameter Years Combined (mm)	Tuber Phenotype
Spunta	----	-	-	-	0.00	23.5	29.1	26.3	Typical
1	1	+++	-	+	19.65*	16.0*	37.0	26.5	Typical
2	1	+++	-	+	4.42	----	22.0	----	Knobby
3	2	+++	-	+	18.56*	15.2*	28.7	22.0	Knobby
4	3	++	-	-	----	----	----	----	Knobby
9	3	+	-	+	0.00	13.3*	24.8	19.1*	Typical
10	1		-	-	----	----	----	----	Typical
12	1	+++	-	+	30.39*	12.1*	25.6	18.9*	Typical
14	1	++	-	+	6.43	12.8*	37.6	25.7	Typical
19	3	++	-	-	----	----	----	----	Typical
20	2	-	-	-	----	----	----	----	Typical
23	3	+	-	-	----	----	----	----	Typical
26	5	+++	-	-	----	----	----	----	Typical

*Significantly different from 'Spunta' ($P < 0.05$)

[x] (+) = loweset relative band intensity, (+++) = highest relative band intensity

[y] Units of activity in 1 mg of leaf tissue multiplied by 10^5

Table 3.5. Molecular analyses, *Erwinia* soft rot experiments and tuber phenotype of 'Atlantic' - derived (AGO) lines containing the glucose oxidase gene.

AGO Line	Copy Number	Northern[x] (leaf tissue)	Northern (tuber tissue)	Western	Glucose Oxidase Enzyme Activity[y]	Lesion Diameter (mm)	Tuber Phenotype
Atlantic	0	-	-	-	0.00	35.1	Typical
2	1	-	-	-	----	----	Knobby, elongated
4	3	++	-	+	44.23*	23.8*	Typical
5	1	+++	-	+	49.47*	25.3*	Typical
6	5	+++	-	+	21.75*	27.5	Elongated
7	1	+++	-	+	22.74*	31.4	Typical
8	7	+++	-	+	4.92	27.3	Elongated
9	1	+++	-	-	----	----	Elongated
10	1	+++	-	-	----	----	Elongated
11	2	+++	-	+	22.26*	31.5	Elongated
12	4	+++	-	+	13.74*	34.1	Typical
16	3	++	-	-	----	----	Elongated
17	1	+++	-	+	17.04*	----	----
18	2	+++	-	+	93.96*	----	----
21	3	+++	-	-	----	----	Elongated

[x](++) = lowest relative band intensity, (+++) = highest relative band intensity

[y]Units of activity in 1 mg of leaf tissue multiplied by 10^5

*Significantly different from 'Atalantic' ($P < 0.05$) based on Dunnet's T test

Table 3.6. Molecular analyses, *Erwinia* soft rot experiments and tuber phenotype of 'Libertas' - derived (LGO) lines containing the glucose oxidase gene.

LGO Line	Copy Number	Northern[x] (leaf tissue)	Northern (tuber tissue)	Western Results	Glucose Oxidase Enzyme Activity[y]	Lesion Diameter (mm)	Tuber Phenotype
Libertas	0	-	-	-	0.00	21.6	Typical
1	1	+++	+	+	53.27*	11.7*	Knobby, elongated
3	3	+	+	+	12.66*	14.0*	Typical
4	2	++++	-	+	42.64*	17.1	Knobby, elongated
10	6	+++	-	+	6.05	16.7	Typical
13	1	-	----	-	----	----	Typical
15	1	+++	-	-	----	----	Typical
16	7	+++	-	+	2.12	11.1*	Knobby, elongated

[x] (+) = lowest relative band intensity, (++++) = highest relative band intensity
[y]Units of activity in 1 mg of leaf tissue multiplied by 10^5
*Significantly different from 'Libertas' ($P < 0.05$) based on Dunnet's T test

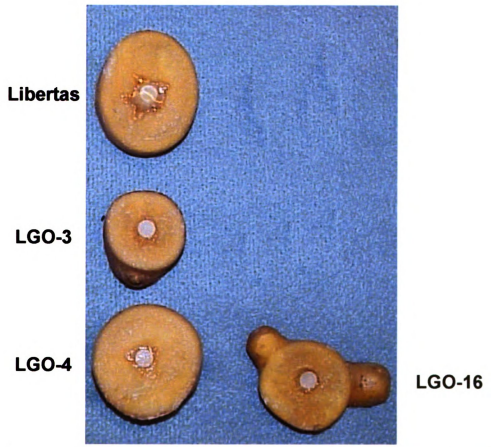

Figure 3.3. Photograph of 'Libertas' and LGO lines inoculated with *Erwinia carotovora* ssp. *carotovora*. All LGO lines shown had lesion diameters significantly less than the 'Libertas' control ($p<0.05$).

Figure 3.4. Photograph of 'Atlantic' and AGO-4 tubers inoculated with *Erwinia carotovora* ssp. *carotovora*. Lesion diameter of AGO-4 was significantly less than that of the non-transgenic 'Atlantic' control ($P < 0.05$).

DISCUSSION

For all of the 1999 *Fusarium* dry rot evaluations the coefficients of variation (CV) for the ANOVA's were quite high, ranging from 26.5 % for LGO lesion diameter to 93.7 % for SGO lesion depth. Therefore, identifying small differences between lines was difficult. In contrast, CV's for the 1998 trials were lower (ranging from 12.2 for SGO lesion diameter to 16.1 for SGO lesion depth) and differences were detected between lines for both measurements. However, only three AGO transgenic lines had smaller mean lesion diameters and only two had mean lesion depths smaller than the non-transgenic 'Atlantic' control. Therefore, even if the CV's were lower it is not likely that any of the lines would be significantly less infected than the control. In addition, glucose oxidase mRNA was not detected in northern analysis of RNA samples from tuber tissue of any AGO line. Thus, we would expect neither an increase in H_2O_2 levels in tubers nor an increased resistance to pathogens in tubers detached from the plant. In addition, previous research (Douches and Hammerschmidt, unpublished data) indicated that 'Atlantic' is highly susceptible to *Fusarium* dry rot and *Erwinia* soft rot due to slow suberization. Therefore, in 'Atlantic', even if endogenous H_2O_2 levels were increased it might not be sufficient to deter the pathogen and result in detectable differences in lesion size. Cultivars that are more tolerant of *Fusarium* might benefit more from the glucose oxidase gene. All of the LGO lines had smaller mean lesion diameters than the 'Libertas' control and four of the LGO lines had smaller mean lesion depths than the control. Thus for the LGO lines, lower CV's might have permitted us to identify small differences between lines. Northern analysis of LGO tuber tissues indicated the presence of glucose

oxidase mRNA in both LGO-1 and 3, two lines which had both small lesion diameter and lesion depth.

Similar to the dry rot evaluations, the CV's for the analyses of the soft rot trials were quite high ranging from 21.0%, for the first SGO trial in 1998, to 32.3% for the LGO trial in 1999. However, despite the relatively high CV's in these trials, differences existed among the SGO lines and the LGO lines. Two of the LGO lines with the smallest lesion diameters in the soft rot trials (LGO-1 and 3) also had some of the smallest dry rot lesion diameters. These two LGO lines were the only ones that had the glucose oxidase mRNA in the tubers. Taken together, these data suggest that the reduction in lesion size in LGO-1 and 3 is not a false positive result and is due to the presence of the glucose oxidase transgene. In contrast, glucose oxidase mRNA was not detected in any SGO line although differences were observed between lines for soft rot lesion diameter. It is possible that the glucose oxidase mRNA represents a small portion of total tuber RNA in most of the transgenic lines that it is undetectable in the amount of total RNA used for Northern analysis. Alternately, glucose oxidase mRNA may be transient, whereas the protein persists and remains active. Among AGO and SGO lines there did not appear to be any relationship between resistance to *Fusarium* dry rot and resistance to *Erwinia* soft rot. Wu *et al.* (1995), also found that glucose oxidase could inhibit *E. carotovora*, however, their results indicated nearly complete inhibition in aerobic conditions. The difference in these results could be due to differences in the amount of H_2O_2 produced in the tubers and this difference could be due to the different gene promoters used in each experiment, the lines used, the *E. carotovora* isolate used or any combination of these factors. Wu *et al.* (1995) utilized the figwort mosaic virus 35S promoter and transformed

'Russet Burbank' whereas, we transformed three different potato cultivars with the glucose oxidase gene under the control of the Gelvin "super promoter" (Ni *et al.*, 1995). Based on the methods used to clone the glucose oxidase, our gene sequences should be identical to that of Wu *et al.*, (1995) but differences are possible. Such differences would only be detected through direct comparisons which were not possible as Wu *et al.*, (1995) did not publish their gene sequence.

Based on these data, expression of the glucose oxidase gene seems to have very little effect on the response of potato lines to infection by *F. sambucinum* but may have a small effect on response to infection by *E. carotovora*. Wu *et al.*, (1995) found that the concentration of H_2O_2 required to inhibit bacterial growth was less than the concentration required to inhibit fungal growth. At 50 μM H_2O_2 bacterial growth was inhibited by about 80%, whereas fungal growth was only inhibited by about 60%. Inhibition of both bacteria and fungi was nearly 100% at 100 μM H_2O_2. Therefore, any increase in H_2O_2 levels in the transgenic glucose oxidase plants developed for this study may not be sufficient to be directly fungicidal, but may still have detrimental effects on bacteria such as *E. carotovora*.

Many of the transgenic lines containing the glucose oxidase gene exhibited tuber characteristics that were off-type compared to non-transgenic controls (Figure 3.5). Of the 33 transgenic plants evaluated, 16 (48%) were classified as off-type based on tuber appearance and some of these were also off-type for foliar characteristics (data not shown). When transgenic lines were compared to their non-transgenic controls based on shape and size, lines that had smaller dry rot and/or soft rot lesions than the controls (SGO-2, LGO-1 and -4) were often misshapen and severely off-type. All sectors of the

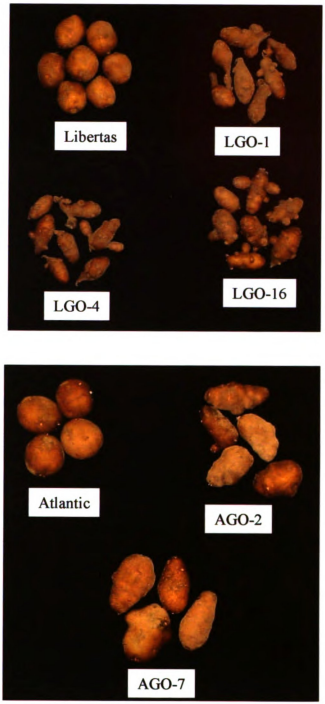

Figure 3.5. Photograph showing the off-type tuber morphology of several transgenic lines containing the glucose oxidase gene.

potato market have specific requirements for tuber shape and any new cultivar must conform if it is to be successful. Therefore, it would be necessary to develop and test many more transgenic lines in order to identify ones with an effect against soft and/or dry rot and an acceptable tuber appearance.

Based on these results some concerns arise about the use of the glucose oxidase gene to increase the resistance of stored potato tubers to *F. sambucinum* or *E. carotovora* ssp. *carotovora*. The effect of the glucose oxidase gene against both diseases appeared to be minimal under the experimental conditions that we employed. Therefore, the time, expense and effort required to develop transgenic lines with this gene may not prove worthwhile. However, small improvements gained under laboratory conditions may translate into more significant differences under commercial storage conditions. For example, the cultivar 'Snowden' is slightly less susceptible to both dry and soft rot in laboratory experiments but when combined with proper cultural practices, the use of 'Snowden' can significantly reduce the occurrence of these diseases in storage (Hammerschmidt, personal communication). Thus, it would be useful to test our transgenic lines in a commercial storage to determine the future role of the glucose oxidase gene in a breeding program. Secondly, because glucose oxidase produces H_2O_2, which is involved in several plant physiological processes, there is a stronger likelihood that it will adversely affect the physiology and/or morphology of transgenic plants than a gene that has a very limited and specific function.

If both of these concerns can be addressed then using the glucose oxidase gene in a potato breeding program could be beneficial. In addition, *Erwinia* can cause wilting of potato plants in the field and transgenic plants expressing the glucose oxidase gene in the

leaf tissue may be less susceptible to infection by these pathogens. Therefore, future research should include evaluation of 1) the resistance of the transgenic lines to *Erwinia* soft rot under commercial storage conditions, 2) the resistance of the transgenic lines to *Erwinia* infection of the plant during the growing season, and 3) the possibility of producing lines with increased resistance and normal morphology.

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