USING GLUCOSE OXIDASE TO CONTROL VERTICILLIUM WILT IN COTTON

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DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge, this thesis contains no material previously published, or the result of any work by another person, except where due reference is make in the text.

The research described in this thesis is my original work, with the following exceptions:

Celia Miller embedded, sectioned and mounted tissue for dark field microscopy (Chapter 4). Erika Merkel attempted the cotton transformations with pSEGON (Chapter 4). Merran Brown and Danny Llewellyn germinated and analysed glucose oxidase expression in the T_1 cotton seedlings (Chapter 4).

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ABSTRACT

The biocontrol fungus *Talaromyces flavus* is antagonistic to several fungal pathogens, including *Verticillium dahliae* (Kleb), the soil fungus responsible for Verticillium wilt in many plant species. The mechanism(s) involved in the antagonism are unclear, however *T. flavus* secretes the enzyme glucose oxidase which in the presence of glucose results in the production of hydrogen peroxide. This hydrogen peroxide has been shown to be toxic to *V. dahliae* at low concentrations (Kim *et al.*, 1990b). As Verticillium wilt in cotton is very difficult to control using conventional methods, there is interest in this interaction. This thesis examines the importance of glucose oxidase in the antagonism between *T. flavus* and *V. dahliae* and how glucose oxidase may be used to control Verticillium wilt in cotton.

Using the *A. niger* glucose oxidase gene as a probe, the *T. flavus* glucose oxidase gene was isolated and sequenced. The gene was subsequently transformed into *T. macrosporus* (a non-glucose oxidase-producing *Talaromyces* species) to create *T. macrosporus* variants which secreted high levels of glucose oxidase. In addition, the glucose oxidase gene was modified and gene replacement techniques used to create a glucose oxidase-deficient *T. flavus* mutant. This glucose oxidase-deficient mutant, the best glucose oxidase-producing *T. macrosporus* variant and both wild-type parents were examined *in vitro* and in pot trials for their ability to inhibit *V. dahliae*. *In vitro*, glucose oxidase was found to be predominantly responsible for the inhibition of *V. dahliae* growth. However in pot trials, *Talaromyces* isolates secreting glucose oxidase were found to be no more effective at controlling Verticillium wilt in cotton than non-glucose oxidase-secreting isolates. These results suggest that although hydrogen peroxide produced by glucose oxidase effectively inhibits *V. dahliae* growth *in vitro*, in the field, glucose oxidase does not play an important role in the biocontrol of Verticillium wilt by *T. flavus*.

In addition to being toxic to *V. dahliae* at low concentrations, hydrogen peroxide is also thought to have a role in natural plant defence mechanisms (Bradley *et al.*, 1992). Therefore transgenic plants producing glucose oxidase may have increased

resistance to Verticillium wilt either because the hydrogen peroxide generated by glucose oxidase is directly toxic to *V. dahliae* or because it stimulates existing plant defence mechanisms. To explore this possibility, the *T. flavus* glucose oxidase gene was modified for plant expression and used to transform tobacco as this plant is more amenable to molecular manipulation than cotton. Some toxic effects (stunted growth and poor seed formation) were associated with high levels of glucose oxidase expression, particularly in tissue culture, but a significant number of independent transgenic tobacco plants expressing glucose oxidase were obtained. As tobacco is not susceptible to Australian isolates of *V. dahliae*, infection trials were performed with *R. solani*, another fungal pathogen whose growth was found to be inhibited by low concentrations of hydrogen peroxide. The incidence and severity of *R. solani* infection in T_1 seedlings expressing glucose oxidase was found to be significantly lower than the infection levels in non-producing seedlings, suggesting that expression of glucose oxidase in plants results in increased resistance to *R. solani*. However to ensure pathogen infection in these trials it was necessary to include glucose in the growth medium. The extent to which increased *R. solani* resistance is dependent on this exogenous glucose is unknown.

Cotton plants expressing glucose oxidase were also generated and seed has been obtained from at least ten independent lines. Progeny homozygous for the glucose oxidase gene are still being generated and in the future will be analysed for increased resistance to Verticillium wilt.

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CHAPTER 1
GENERAL INTRODUCTION

1.1 OVERVIEW

Fungal diseases are a major problem in most agricultural production areas. Currently, the main methods of controlling fungal disease involve the use of chemical fungicides, the breeding and use of fungus-resistant cultivars and the adoption of agricultural practices which reduce the buildup and spread of infested soil and pathogen carrying plant materials. Although these methods have helped in managing many diseases, there are limitations to the current technologies available. For instance, the breeding of fungus-resistant cultivars is time consuming and resistance is usually only effective against subspecies of certain pathogens. In addition, some pathogens quickly evolve and develop resistance so a resistant variety may only have a short practical lifetime. Chemical solutions are expensive and over time they also become less effective due to resistance development in the pathogen. There is also growing concern because the production and use of such chemicals is damaging to the environment. Therefore, improved solutions to disease control are required.

Biological control is an alternative way to control fungal disease which has received considerable attention over the last five years because biocontrol organisms have the potential to provide protection against fungi without damage to the environment. However to date, little commercial success with such organisms has been achieved. The rapid developments in the field of genetic engineering over the past ten years has raised the possibility of transferring potentially useful genes from diverse species into biocontrol organisms to improve their biocontrol ability. Furthermore, these techniques could be used to transfer desirable genes into plants to increase their disease resistance. Such genetic engineering solutions would increase the diversity of biocontrol organisms and disease-resistant plants and also reduce the dependency of the farmer on agrochemicals. This study focuses on the possible use of a potentially useful antifungal enzyme, glucose oxidase from the biocontrol fungus *Talaromyces flavus*. Molecular techniques are used to investigate how this enzyme

may be used either in a biocontrol organism or in transgenic plants to control the fungal disease, Verticillium wilt in cotton.

1.2 VERTICILLIUM WILT

1.2.1 Taxonomy

Verticillium wilt is a vascular wilt disease affecting many different plant species. It is caused by several species in the genus *Verticillium* Nees ex Link 1824. This genus, which is classified within the Class Deuteromycetes, subclass Hyphomycetidae, contains over 30 species, all of which are characterized by verticillately (produced in whorls) branched conidiophores with phialides producing conidia terminally in mucilaginous 'heads'. Hyphal cells, phialides and conidia are uninucleate but hyphal tips may be multinucleate (reviewed by Domsch *et al.*, 1980a).

Five species of Verticillium are known to cause wilt disease in plants; these are *V. albo-atrum* Reinke and Berthhold 1879, *V. dahliae* Klebahn 1913, *V. nigrescens* Pethybridge 1919, *V. nubilum* Pethybridge 1919 and *V. tricorpus* Issac 1953. These species are distinguished from other members of the genus by their ability to infect plants and produce black resting structures. They are classified individually depending on the type and occurrence of the resting structures produced. *V. nubilum* and *V. nigrescens* form chlamydospores (thick walled spores usually arising from a terminal or lateral swelling of a conidiophore or hypha and separated from the mother cell by one or two septa) either singularly (*V. nubilum*) or in chains (*V. nigrescens*). *V. dahliae* produces discrete black microsclerotia (brown to black resting structures which arise by repeated budding and multilateral septation of a single or a few adjacent hyphae) but no chlamydospores. *V. tricorpus* forms microsclerotia and chlamydospores whereas *V. albo-atrum* produces dark resting mycelium but no microsclerotia or chlamydospores (Domsch *et al.*,1980a).

Verticillium wilt is most commonly caused by *V. albo-atrum* and *V. dahliae*. As there was much controversy regarding the relationship between these two species until the early 1970s, reports prior to this date often refer to both species as *V. albo-atrum*. However *V. dahliae* is more common, infects a wider range of hosts and is

distributed more widely around the world than *V. albo-atrum*. Under field conditions, Verticillium wilt in cotton is detrimental only when caused by *V. dahliae*. At low temperatures (20-24°C) *V. albo-atrum* can cause severe wilt of cotton in the glasshouse, however, in the field, mean temperatures are not normally this low until the crop is almost mature. Therefore, wilt caused by this species does little damage to the cotton crop (Bell, 1992a).

Isolates of *V. dahliae* have been classified into different vegetative incompatibility groups (VCGs) based on their ability to form heterokaryons with each other. Using nitrate non-utilizing mutants different investigators (Joaquim and Rowe, 1990 & 1991; Strausbaugh, *et al.*, 1992; Chen, 1994) have proposed the existence of four different VCGs. In general, there does not seem to be a relationship between the pathogenicity to a particular host species and a particular VCG, however on certain hosts there does appear to be some correlation with virulence. For instance, isolates from cotton that cause severe defoliation belong to VCG 1 whereas non-defoliating isolates belong to VCG 2 and 4 (Joaquim and Rowe, 1990; Bell, 1992b). Australian *V. dahliae* strains isolated from cotton have been found to belong to VCG 4, a group causing only mild to moderate symptoms, even in susceptible cotton cultivars (Bell, 1992b). More recently, restriction fragment length polymorphisms (RFLPs) and randomly amplified polymorphic DNAs (RAPDs) are being used to try to characterize *V. dahliae* isolates (Okoli *et al.*, 1993; Nazar *et al.*, 1991). To date, these techniques have shown differences at a molecular level between different *Verticillium* species (Typas, *et al.*, 1992; Robb *et al.*, 1993) but, in general, little information has been obtained that would allow separation of *V. dahliae* into different subgroups within the species.

1.2.2 The disease

V. dahliae does not grow saprophytically through the soil but dormant microsclerotia present either freely in the soil or in plant debris provide a reservoir of soil-borne propagules. Structurally, microsclerotia have two regions of cells. There is a peripheral region composed of either degenerate cells or cells with moderate cytoplasmic content and an inner region consisting of cells containing dense cytoplasm. Melanin coats all the cells but is more abundant over cytoplasm rich cells

(Gordee and Porter, 1961; Brown and Wyllie, 1970; Perry and Evert, 1984). The microsclerotia can survive for many years in the soil at depths down to 40cm, presumably because the melanin granules in their outer cell walls and surrounding matrix protect the propagule against desiccation, parasitism by micro-organisms and toxic chemicals in the soil (Bell and Wheeler, 1986). Microsclerotia germinate in response to root exudate and produce both hyphae and conidia which subsequently colonize root surfaces. This usually occurs 3-10mm back from the root tip where the level of root exudate is high (Gerik and Huisman, 1988).

Hyphae from germinated microsclerotia invade the root, usually entering either intercellularly or intracellularly through the root tip where the concentration of antifungal compounds such as tannins and phytoalexins is low. However, infection through the region of elongation, root hairs or lower hypocotyl region has also been reported (Garber and Houston, 1966; Fitzell *et al.*, 1980). Wounds are not required for infection but may increase the incidence and severity of the wilt (Bell, 1992a).

Two distinct types of infection occur. In 'immune' hosts infection is confined to the cortical region of the root. The host displays no symptoms but conidia and microsclerotia formation occurs, resulting in further soil infestation (Evans and Gleeson, 1973). In 'susceptible' hosts, the fungus successfully penetrates the plant's xylem vessels. In cotton, germination, penetration and movement of *V. dahliae* to the vascular tissue can occur within 3 days of inoculation (Garber, 1973). Once in the xylem vessels the fungus proliferates and produces conidia which travel upwards to the xylem end walls where they germinate, penetrate the next vessel segment and produce more conidia. Some hyphae penetrate pit membranes and form conidia in adjoining vessels. Repetition of these processes many times spreads the fungus completely throughout the xylem vessels of the plant (Bell, 1992a).

Once spread throughout the xylem, the pathogen severely disrupts the passage of water through the stem. This may be attributed to the physical presence of the pathogen's mycelium or to the resistance responses of the plant to the pathogen. Responses involve the formation of tyloses (growths across the xylem vessel originating from paravascular parenchyma cells) (Mace, 1978) and the production of

gums within the vessels (Garber, 1973, cited in Schnathorst, 1981). Both responses block infected xylem vessels in an attempt to confine the pathogen. Further xylem occlusion is caused by high molecular weight polysaccharides either produced by the pathogen or cleaved from the plant's cell walls by fungal hydrolytic enzymes. Together this results in a significant increase in the resistance of the stem to water flow and thus the characteristic wilting of leaves (Ayer and Racok, 1990).

Symptoms usually develop after most of the xylem vessels in a leaf become blocked. In cotton, more obvious symptoms include a yellowing of lower leaves with discolouration first occurring along the margins and between the major veins, vascular discolouration and stunting of plant growth. As the tissue of *Verticillium*-infected plants senesce and die, microsclerotia begin to form. This may take several weeks or months because microsclerotia are not produced in dying tissues until they are moistened by rain, irrigation water or turned under moist soil (Schnathorst, 1981). Tissue decay and cultivation usually results in microsclerotia being returned to the soil where the disease cycle begins again.

The severity and incidence of Verticillium wilt in cotton is affected by many factors including inoculum potential, temperature, pathogen virulence, cultivar tolerance, soil conditions (pH, moisture content and nutrient content) and the presence of other microorganisms in the soil (reviewed by Bell, 1992a). The two most important parameters are inoculum density and temperature. Infection trials carried out in California have shown clearly that incidence of disease in cotton increases progressively as inoculum density increases (De Vay and Pullman, 1984) and similar trends have been observed in Australia (Allen, 1994). Temperature is important as *V. dahliae* does not grow above 30°C so the disease does not occur when soil and air temperatures exceed this. Australian isolates (VCG 4) are particularly susceptible to temperature and Verticillium wilt rarely occurs when temperatures are in excess of 27°C. Wet conditions, either due to heavy rainfall or irrigation, usually decrease soil temperature and so increase the incidence of Verticillium wilt. However, prolonged flooding of soil often decreases wilt severity because the conditions favour other antagonists or result in accumulation of toxic metabolites in the soil.

Susceptibility of cotton plants to Verticillium wilt is also dependent on the age, physiological condition and genetic constitution of the plants. In general, younger plants are more susceptible to the disease than older plants and rapidly growing, succulent plants are less tolerant to the disease compared to those hardened by unfavourable growing conditions. Some varieties have increased tolerance to the disease, however none of these varieties stops infection of xylem tissue, they only slow the rate and incidence of the disease.

Soil conditions either directly or indirectly also influence wilt incidence. Verticillium wilt is most prevalent in neutral or slightly alkaline soils (pH 6-9) presumably because this is the optimal pH range for *V. dahliae* growth. *V. dahliae* growth, microsclerotial production and survival is inhibited in soil below pH 5.5, but this inhibition may be due to higher concentrations of manganese and aluminium ions which accumulate both in the soil and the leaves of plants as the pH of soil is lowered. Soils deficient in potassium or high in nitrogen can also significantly increase the incidence of Verticillium wilt.

As *V. dahliae* does not compete well with other micro-organisms in the soil, the presence of antagonists in the soil can decrease wilt severity. Other soil inhabitants such as the reniform and root-knot nematodes which wound roots and facilitate penetration by the pathogen have been reported to increase the incidence of Verticillium wilt in glasshouse trials. However, the importance of this interaction has yet to be proven in the field. (Bell, 1992a)

1.2.3 The economic importance of Verticillium wilt in cotton

In Australia, as in many of the other major cotton producing countries (including the USA and the former Soviet Union), Verticillium wilt is responsible for the largest losses to cotton crops of any disease. The disease significantly reduces lint yields and affects fibre quality as yarn spun from wilt infected plants is inferior in appearance and of a lower grade. Seeds from diseased plants are also lower in weight and vigour.

The disease was first observed on cotton in Australia in 1959 in the Namoi Valley. Today, Verticillium wilt is observed in most cotton growing areas of Australia. In

particular, the disease can be a problem in the cooler growing areas (New South Wales and Southern Queensland) where cooler, wetter conditions promote vigorous growth of *V. dahliae*.

1.2.4 Control of Verticillium wilt in cotton

Verticillium wilt in cotton is particularly difficult to control for several reasons. The microsclerotia produced are resistant to many soil fumigants and remain viable in the soil for long periods. The few chemicals which do effectively kill *V. dahliae* in the soil (for example methyl bromide, chloropicrin and metham-sodium) are very expensive and some such as methyl bromide are phytotoxic and can stunt cotton plants. Such broad-spectrum biocides can also create additional difficulties as pathogens can easily invade areas of low biological diversity (Marois *et al.*, 1983). Irrigated cotton monoculture further favours Verticillium wilt as it causes the inoculum density of the fungus in the soil to increase and the irrigation water quickly spreads the disease from field to field.

Cultivars of cotton with some tolerance to Verticillium wilt are now available in many countries. In Australia the recently released Sicala V-I and Sicala V-2 show good tolerance to the disease and are now used in areas where Verticillium wilt is a problem. The tolerance in these cultivars, like other overseas varieties is probably due to the speed of the defence response. Resistant plants analysed in the USA have been found to generate antifungal chemicals (phytoalexins) and physical barriers (gels and tyloses) more rapidly than susceptible cultivars (Bell *et al.*, 1986; Mace, 1989). In this way the plants are able to contain the pathogen and delay the onset and severity of the disease. However resistance in different cotton cultivars is multigenic (Devey and Roose, 1987; Wilhelm *et al.*, 1974; P. Reid, per communication) so it is difficult to introduce this very desirable trait into more agronomically desirable cultivars. To date, no single dominant resistance gene for Verticillium wilt has been identified in cotton although such a gene has been identified in some varieties of tomato and sunflower (Schaible *et al.*, 1951; Fick and Zimmer, 1974).

Although selection of an appropriate cotton cultivar is essential to minimize losses to Verticillium wilt, good cultural practices are also necessary to control the disease

(reviewed by Bell, 1992a). Such practices either help prevent the introduction, spread and build up of inoculum of the pathogen in the soil or reduce *V. dahliae* inoculum density in the soil. Methods include not using susceptible cotton cultivars in infected fields (a practice which causes the inoculum density of strains pathogenic to cotton to increase greatly in the field) and rotation of cotton with other plants such as grasses (eg. perennial ryegrass) or cereals (barley or wheat) which are poor hosts for the pathogen. However, crop rotation is limited as many other crops such as potato, tomato and sunflower are infected by *V. dahliae* and although cotton strains are often less pathogenic on other plant species (although there are exceptions) the crop may still act as a host and further propagate the pathogen.

Weed control can also be important. Evans (1971) isolated *V. dahliae* from twenty-six of fifty-four species of weeds growing in infested soils in the Namoi Valley of New South Wales, Australia. In particular, microsclerotia were plentiful in Noogoora burr (*Xanthium pungens*), Bathurst burr (*Xanthium spinosum*) and Saffron thistle (*Carthamus lanatus*). Proper control of these and other weeds is important to stop high disease incidence and spread of *V. dahliae*.

Similarly, crop residues can cause build-up of inoculum potential in the soil and can rapidly spread the disease by transporting the pathogen in irrigation systems to new fields. In Australia, the mean incidence of Verticillium wilt increased from 4.1% in the 1984/1985 season to 16.5% in the 1989/1990 season. This increase coincided with the increased adoption of minimum tillage practices and permanent bed systems which resulted in the greater retention of crop residues from season to season (Allen, 1994). Today methods which allow the rapid removal and decomposition of crop residues are favoured as these generally decrease the production of microsclerotia. This usually involves shredding stalks and completely ploughing them under as soon as possible after harvest or raking and burning of crop residues. Solarization is also used in many countries to help control Verticillium wilt. This involves placing a clear plastic sheet over previously irrigated soil for several weeks. Irradiation from the sun usually causes the soil temperature to rise to 40-50°C to depths of 25cm which over a period of 3-4 weeks kills *V. dahliae* propagules (Pullman *et al.*, 1981).

Another possible way of controlling Verticillium wilt is biological control of *V. dahliae* with other organisms. This method is becoming increasingly popular because it is an attractive alternative to the use of potentially harmful chemical sprays. Biopreparations containing the organism are usually either applied as a seed coat, or used with fertilizer prior to planting. Good biocontrol organisms establish well in field soil and compete with or parasitize the *V. dahliae* pathogen. The only biocontrol agent which has been used commercially to control Verticillium wilt in cotton is *Trichoderma viride*. Preparations of this fungus which parasitizes *V. dahliae* have been used on a limited scale in Russia for more than 20 years (Fedorinchik, 1964, cited in Bell, 1992a). Several other organisms including *Gliocladium roseum* and *Talaromyces flavus* have been found to be antagonistic to *V. dahliae* in pot trials and in the field (Keinath, *et al.*, 1991; Marois *et al.*, 1982) but as yet are not used routinely to control *V. dahliae*.

1.3 *TALAROMYCES FLAVUS*

1.3.1 Taxonomy

Talaromyces is a genus of the ascomycete fungi which is comprised of twenty species. Characteristic features of genus members are soft, white to yellow ascoma and asci which are formed in chains (reviewed by Domsch *et al.*, 1980b). All known species are homothallic with the exception of the recently described heterothallic *T. derxii* (Takada and Udagawa, 1988). *Talaromyces flavus* (Klöcker) Stolk and Samson 1972 (the perfect stage of *Penicillium vermiculatum* Dangeard 1907; also called *Penicillium dangeardii*) is the type species of the genus. It is distinguished from other members of the genus by the presence of vermiform (worm-shaped) ascogonia which have antheridia coiled around them. Ascospores are yellow and produced within 3 weeks, conidiophores usually form greyish-green sectors and produce conidia 2-3.5 × 2.0-2.5 µm which are round to ellipsoidal. The species is widely distributed around the world but it is more common in warmer regions such as tropical Australia, Egypt, equatorial West Africa and Central America. It is usually isolated from the soil or from organic substrates such as rotting fruit. The optimum temperature for fungal growth is 37°C and growth is inhibited at 5°C.

Stolk and Sampson (1972) recognized two varieties in *T. flavus*: var. *flavus* and var. *macrosporus*. *T. flavus* var. *macrosporus*, unlike *T. flavus* var. *flavus* produces large (5-6.5 × 3.5-5.2 μm), heat resistant ascospores (Beuchat, 1988), however both varieties produce a metabolites known as mitorubins and both produce a conspicuous profile of blue, violet, yellow and orange metabolites on thin layer chromatography (TLC) plates. Frisvad (1990) examined *T. flavus* (var. *flavus* and var. *macrosporus*) secondary metabolite profiles using TLC and found all isolates of var. *macrosporus* unlike var. *flavus*, produced large amounts of duclauxin and other specific metabolites. Strains of var. *flavus* produced vermiculine, vermicelline, glucose oxidase and other unknown secondary metabolites not found in var. *macrosporus*. Based on these differences and the previously observed differences in ascospore size and heat resistance, they proposed that the two varieties be classed as two separate species. For the purposes of this thesis, these two varieties will be recognized as different species and referred to as *T. flavus* and *T. macrosporus* respectively.

1.3.2 *T. flavus* as a potential biocontrol agent

T. flavus and its anamorph *P. vermiculatum* have shown antagonistic activity against several plant pathogenic fungi including *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *V. dahliae* (Boosalis, 1956; Su and Leu, 1980; Marois *et al.*, 1982). The fungus is also a good soil competitor and has been shown to establish itself in the rhizosphere of cotton and potato grown in field soil and eggplant grown in potting mix (Marois *et al.*, 1984). It has been used as a biocontrol agent against the fungi mentioned above with varied success. Boosalis (1956) found the incidence of *Rhizoctonia*-incited damping off and seedling root rot was not affected when peas were grown in unsterilized soil infested with *P. vermiculatum*. However, McLaren *et al.* (1983) obtained 68-92% disease control of Sclerotinia wilt of sunflowers when *T. flavus* and sclerotia of *S. sclerotiorum* were buried together in the field. Experimentally, *T. flavus* has also been reported to suppress Verticillium wilt of eggplant (Marois *et al.*, 1982; Fravel and Roberts, 1991; Saito *et al.*, 1994), tomatoes (Dutta, 1981) and potatoes (Fravel *et al.*, 1986; Nagtzaam, 1994). However, results with potatoes have been inconsistent and some workers (Spink and Rowe, 1989)

have found *T. flavus* to have no significant effect on the incidence and severity of Verticillium wilt on this plant.

Mycoparasitism is thought to be involved in antagonism of *T. flavus* towards each of the fungi mentioned above as *T. flavus* has been shown to parasitize both the hyphae and sclerotia of *S. sclerotiorum* (McLaren *et al.*, 1986 & 1989), the hyphae of *R. solani* (Boosalis, 1956) and the microsclerotia of *V. dahliae* (Fahima *et al.*, 1992). *T. flavus* parasitizes all three fungi in a similar way. On hyphae, the fungus coils around the developing hyphal branches of the host and then the hyphae tips directly invade into the host's cell. Deterioration of the cytoplasm follows with the infected cell eventually collapsing, although the cell walls remain intact. Sclerotia and microsclerotia are also invaded directly without the formation of appressoria. The fungus proliferates inside the structure spreading intracellularly and intercellularly and simultaneously degrading the cell contents of the host. *T. flavus* is a 'passive' mycoparasite of *Sclerotinia* sclerotia (Adams and Ayers, 1979). Such parasites; unlike 'aggressive' mycoparasites, are unable to grow out from infected sclerotia into the soil and parasitize healthy sclerotia (Adams, 1990). The ability of *T. flavus* to aggressively parasitize *V. dahliae* microsclerotia has not been determined.

Toxic metabolites have also been implicated in biocontrol activity of *T. flavus* especially with respect to control of *V. dahliae.* Fravel (1987) found *T. flavus* secreted a metabolite into liquid medium which in the presence of glucose was toxic to *V. dahliae* microsclerotia and inhibited growth of *V. dahliae* mycelia. Purification of the toxic substance and HPLC analysis subsequently identified the active component as glucose oxidase which had been secreted from the fungal hyphae (Kim *et al.*, 1988). Talaron, an antifungal antibiotic produced by *T. flavus* when it was grown in culture media containing 8% glucose (Mizuno *et al.*, 1974) was also later confirmed to be glucose oxidase (Kim *et al.*, 1990b).

Glucose oxidase leads to the production of hydrogen peroxide (H_2O_2) as a byproduct of glucose oxidation.

$$\textbf{Glucose} + \textbf{H}_2\textbf{O} + \textbf{O}_2 \xrightarrow[\textit{Oxidase}]{\textit{Glucose}} \textbf{Gluconic Acid} + \textbf{H}_2\textbf{O}_2$$

When added to growth media, hydrogen peroxide inhibits microsclerotial germination and mycelial growth. The other reaction components, glucose oxidase (no glucose present), gluconic acid and glucose do not inhibit fungal growth (Kim *et al.*, 1988). Therefore it was concluded that it is the hydrogen peroxide produced by glucose oxidase which inhibits *V. dahliae* growth and microsclerotial germination. Interestingly, *V. dahliae* has been found to be approximately 10-fold more sensitive to hydrogen peroxide than some other fungi and bacteria tested. In addition, low concentrations of hydrogen peroxide have been found to be toxic to other fungal pathogens such as *R. solani*, *Sclerotinia minor* and *Pythium ultum* (Kim *et al.*, 1990b).

It is not clear whether glucose oxidase plays a major role in the control of Verticillium wilt in the field. Doubts have been raised as to whether enough glucose is present in the soil to allow *T. flavus* to produce inhibitory quantities of hydrogen peroxide. Glucose oxidase alone does not affect microsclerotia (Fravel *et al.*, 1987), suggesting intact microsclerotia do not supply enough glucose to allow production of toxic quantities of hydrogen peroxide. However, Fravel and Roberts (1991) showed that in pot trials, eggplant roots have the potential to supply glucose oxidase with glucose (reviewed in Chapter 3 introduction). Spatial distribution studies have also shown that *T. flavus* preferentially colonizes the surface of root tips of potatoes, tomatoes and eggplant (Tjamos and Fravel., 1994). This is the region of the root from which glucose is released so adequate glucose may be available to allow production of significant amounts of hydrogen peroxide. Fravel and Roberts (1991) also conducted biocontrol trials using different isolates of *T. flavus* which produced either large or small amounts of glucose oxidase. They found that the amount of glucose oxidase produced by *T. flavus* isolates correlated with ability of *T. flavus* to control Verticillium wilt in eggplants (also reviewed in Chapter 3 Introduction). However, some doubt still exists because non-isogenic isolates were used and other proteins or metabolites produced by these isolates (or different levels of these compounds) may have affected the results.

Experiments conducted by Madi *et al.* (1989) suggest that other inhibitory compounds are involved in the inhibition of *V. dahliae* by *T. flavus*. They found that addition of the peroxide scavenger, catalase, to the culture filtrate of *T. flavus* isolates resulted in a loss of only 50% of its toxic activity towards *V. dahliae*. Other potentially harmful compounds were found in the filtrate including the cell-wall degrading enzymes, β-1-3 glucanase, cellulase and chitinase. These enzymes are probably necessary for digestion of sclerotia and microsclerotia cell walls prior to invasion by *T. flavus* hyphae but to date no clear correlation has been found between levels of these enzymes and the ability of *T. flavus* to control Verticillium wilt (Madi *et al.*, 1992). *T. flavus* also produces the antibiotics vermiculine and vermicellin (Fuska *et al.*, 1972 & 1979; Frisvad *et al.*, 1990). These and other secondary metabolites produced by the fungus could also inhibit *V. dahliae* growth.

The antagonistic activity of *T. flavus* towards *V. dahliae* may therefore be due to a combined effect of mycoparasitism, lytic enzymes and toxic metabolites. Glucose oxidase may be directly involved, with the hydrogen peroxide generated by the enzyme directly inhibiting *V. dahliae* growth and/or predisposing *Verticillium* hyphae and sclerotia to infection before contact occurs.

1.4 GLUCOSE OXIDASE

Glucose oxidase (β-D-glucose: oxygen 1-oxido-reductase, EC 1.1.3.4) catalyses oxidation of β-D-glucose to hydrogen peroxide and D-glucono-δ-lactone. The D-glucono-δ-lactone subsequently hydrolyses rapidly to D-gluconic acid:

$$\text{Glucose} + O_2 \longrightarrow \text{Glucono-}\delta\text{-lactone} + H_2O_2$$

$$\text{Glucono-}\delta\text{-lactone} + H_2O \longrightarrow \text{Gluconic acid}$$

1.4.1 Occurrence and function

Glucose oxidase was first isolated from the mycelia of *Aspergillus niger* and *Penicillium glaucum* by Müller in 1928. Since then the enzyme has been reported in several fungi including various species of the genus *Penicillium* (Coulthard *et al.*,

1945; Keilen and Hartree, 1948; Nakamatsu *et al.*, 1975; Petruccioli, *et al.*, 1993), the ectomycorrhizal fungus *Tricholoma robustum* (Iwase, 1992), *Phanerochaete chrysosporium* (Kelley and Reddy, 1986), *Alternaria alternata* (Caridis *et al.*, 1991) and both *Talaromyces stipitatus* (Nakamatsu *et al.*, 1975) and *Talaromyces flavus* (Kim *et al.*, 1988). The enzyme is also widely distributed in nature, it has been discovered in cell extracts from the bacterium *Malleomyces pseudomallei* (Dowling and Levine, 1956) and the red alga *Iridophycus flaccidum*, (Bean and Hassid, 1956) in the juice sacs of citrus fruit, (Bean *et al.*, 1961) in honey and in the pharyngeal gland of the honeybee (Schepartz and Subers, 1964).

The biological function of glucose oxidase in most organisms is unknown. In honey, glucose oxidase-mediated hydrogen peroxide probably functions as an antibacterial factor (Schepartz and Subers, 1964). In *P. chrysosporium*, a fungus which causes rotting in many tree species, the hydrogen peroxide produced by glucose oxidase is required by a ligninase enzyme for the degradation of lignin (reviewed in Kelley and Reddy, 1986). As many of the other fungi which produce glucose oxidase such as *A. alternata*, *A. niger*, *T. flavus* and *T. robustum* are either leaf litter decomposers or mycorrhizal fungi which colonize plant roots, these fungi probably require the ability to break down lignin to gain nutrients. Therefore, in these fungi as in *P. chrysosporium*, hydrogen peroxide generated by glucose oxidase may be required by ligninase enzymes for the degradation of lignin.

1.4.2 Industrial use

Industrially, glucose oxidase has considerable importance. It is widely used in medical diagnosis, the food industry and in future may be useful in agriculture (see later). The enzyme is normally produced by controlled fermentation of *A. niger* or *P. amagasakiense*. Traditional methods involving random mutagenesis and subsequent selection of mutant strains with increased productivity have been employed to obtain over-producing glucose oxidase strains. However only relatively small increases in glucose oxidase activity have been obtained (Fiedurek *et al.*, 1992). De Baetselier *et al.* (1991) have recently developed a fermentation process to produce glucose oxidase from a recombinant *S. cerevisiae*. The recombinant enzyme is stable to higher temperatures and a wider pH range than the commercially produced *A. niger* enzyme.

It is also free of contaminating amylase, cellulase and catalase so it is particularly useful in some areas where enzyme impurities impair the use of the enzyme.

Glucose oxidase is used in medicine primarily to detect and quantitate the amount of glucose in body fluids (reviewed by Richter, 1983). The complete test is available commercially in the form of test strips impregnated with glucose oxidase, peroxidase and a dye. After initial oxidation of glucose by glucose oxidase, the peroxidase oxidises the hydrogen peroxide and dye resulting in water and oxidised dye which is often a green to brown colour. The method is very accurate, reliable and fast (the glucose response can be read after 30 seconds (Barker and Shirley, 1980)) and requires very little sample volume. However, the glucose oxidase used in this method must be free of catalase and other enzymes such as amylase and maltase. These enzymes must be removed or inactivated prior to use.

Glucose oxidase is widely used in the food industry, largely because the enzyme occurs naturally and is therefore considered safe for use in foods. It is mainly used as an antioxidant to help prevent changes in the colour and flavour of food products both during processing and in storage. For instance, it is used as a preservative in canned soft drinks, citrus drinks, salad dressings and dried foods such as instant coffee and milk powders. The second most important market for glucose oxidase in the food industry is the removal of glucose from egg whites and whole eggs. This prevents the browning and development of offensive flavours which can occur in the manufacture of dried eggs if the glucose is not removed.

As previously mentioned, glucose oxidase has been implicated in the antagonism of *V. dahliae* by *T. flavus* and low concentrations of the hydrogen peroxide generated by the enzyme have been shown to be toxic to several plant pathogenic fungi. Hydrogen peroxide has also been implicated in natural plant pathogen defence mechanisms (see later). With the aid of genetic engineering, glucose oxidase, may in future, be useful in agriculture to help improve plant disease resistance.

1.4.3 Enzyme properties

Glucose oxidase has been purified from four of the fungal genera known to produce it (*Aspergillus*, *Penicillium, Talaromyces* and *Phanerochaete*) and partially purified

from several other sources (*I. flaccidum*, citrus fruit, honeybees and honey). In general, the glucose oxidases from fungi are very similar whereas those from other sources are more diverse (with the exception of glucose oxidase from honey and the honeybee which are almost identical to each other (Schepartz and Subers, 1964)). For instance, the glucose oxidases from *I. flaccidum* and citrus fruit can oxidise several different sugars including glucose, galactose, xylose, maltose and cellobiose whereas *M. pseudomallei* glucose oxidase utilizes only glucose and galactose and glucose oxidase from honey and honeybees is highly specific for glucose. Also, the enzyme from citrus fruits is thought to be a flavoprotein whereas spectral studies of the honey, honeybee and red alga glucose oxidases indicates that these enzymes are not. Other disparate characteristics are seen in the glucose oxidases from honey and honeybees. These enzymes, unlike the others which have a pH optimum of 5.0 to 5.5, have a pH optimum of 6.1 and 6.7 (honey and honeybee enzyme respectively). They also require an extremely high substrate concentration (1.5M and 2M). In contrast, the *A. niger* glucose oxidase requires a substrate concentration of only 0.1M (Barker and Shirley, 1980).

The glucose oxidases which have been purified from the fungi *Aspergillus*, *Penicillium, Talaromyces* and *Phanerochaete* are all flavoproteins with each enzyme molecule containing two molecules of FAD. Most are dimeric and are thought to consist of two identical polypeptide chain subunits covalently linked by disulphide bonds. The exception is glucose oxidase from *Penicillium amagasakiense*. This enzyme consists of four equally sized polypeptide chains each of which has a molecular weight of 45 000. Two of these polypeptide chains are held together by a disulphide bond to form a dimer (81 000) and two dimeric units associate non-covalently to form a tetramer (164 000) (Yoshimura and Isemura, 1971).

The characteristics of each fungal glucose oxidase is shown in Table 1.1. The enzymes all have similar molecular weights (approximately 160 000) and all have pH optima of around 5.0. Glucose oxidase from *T. flavus* is the most pH tolerant enzyme. It is stable from pH 3.0 to 7.0, unlike *P. amagasakiense* and *A. niger* which are stable from pH 3.5 to 7.0 and pH 3.5 to 6.5 respectively. All enzymes tested are inhibited by heavy metals such as silver and mercury. The most distinct enzyme is

CHARACTERISTICS OF FUNGAL GLUCOSE OXIDASES				
	T. flavus	*P. amagasakiense*	*A. niger*	*P. chrysosporium*[g]
Molecular weight	164 000[f]	150 000[a] - 158 000[b]	150 000 - 186 000[c,d]	180 000
Km (glucose)	10.9mM[f]	11 - 15mM[d,h]	26 - 33mM[d,h,i]	38mM
Isoelectric point(s)	5 - 6 pH 4.40 - 4.55[f]	1 pH 4.35[a]	6 pH 3.9 -4.3[i]	*nd*
pH range	3.0 - 7.0[f]	3.5 - 7.0[n]	3.5 - 6.5[b]	*nd*
pH optimum	5.0[f]	4.0 - 6.5[b]	4.5 - 6.5[e]	4.6 - 5.0
Glycosylation	yes[f]	yes[h]	yes[c,d]	no
Substrate specificity	glucose[l]	glucose[k]	glucose[k,i]	glucose, sorbose, xylose, maltose
Inhibitors	*nd*	$HgCl_2$, $AgCl_2$[a]	Ag^+, Hg_2^+, Cu_2^+[m]	Ag^+, *o*-phthalate

Table 1.1

[a] Yoshimura and Isemura, 1971
[b] Nakamura and Fujiki, 1968
[c] Pazur et al., 1965
[d] Swoboda and Massey, 1965
[e] Richter, 1983
[f] Kim *et al.*, 1990a
[g] Kelley and Reddy, 1986
[h] Hayashi and Nakamura, 1976
[i] Hayashi and Nakamura, 1981
[k] Adams *et al.*, 1960
[l] Kim *et al.*, 1988
[m] Nakamura and Ogura, 1968
[n] Kusai *et al.*, 1960
nd not determined

that from the basdiomycete, *P. chrysosporium.* Unlike the others, it is not glycosylated and although glucose is its primary substrate it can also utilize a small amount of sorbose, xylose and maltose (Kelley and Reddy, 1986). The glucose oxidases from the ascomycetes are highly specific for β-D-glucose. The enzymes all have a relatively low affinity for glucose with the Km values for glucose ranging from 10.9 to 38mM. However, the glucose oxidases from *T. flavus* and *P. amagasakiense* tend to have a slightly higher affinity for glucose (Km 10.9 to 15mM) than those from *A. niger* and *P. chrysosporium* (Km 26 to 38mM respectively). Up to six isozymes have been observed for glucose oxidase from *T. flavus* and *A. niger.* In *A. niger*, the multiple bands have been shown to be due to differences in carbohydrate content and structure rather than differences in amino acid sequence (Hayashi and Nakamura, 1981). Such differences are also thought to account for the multiple bands seen for *T. flavus* glucose oxidase.

The amino acid composition of glucose oxidase has only been determined for *A. niger*, *P. amagasakiense* and *T. flavus* (Nakamura and Fujiki, 1968; Pazur *et al.*, 1965; Kim *et al.*, 1990a). With a few exceptions, the amino acid compositions are the same (± 10%). The most abundant amino acids are aspartic acid, glutamic acid, glycine, alanine and leucine with cysteine least abundant. There is more lysine and phenylalanine (approximately 30%) and less histidine and arginine (approximately 50% and 30% respectively) in *T. flavus* and *P. amagasakiense* compared with the *Aspergillus* enzyme.

Glucose oxidase is excreted into the surrounding medium by both *P. amagasakiense* and *T. flavus* (Barker and Shirley, 1980; Kim *et al.*, 1990a). The subcellular localization of glucose oxidase in *A. niger* has been a point of discussion for some time. Ultrastructural studies performed by Van Dijken and Veenhuis (1980) indicated that the enzyme was located in peroxisomes. However, Mishak *et al.* (1985) showed that under manganese-deficient growth conditions, *A. niger* glucose oxidase was predominantly found in the culture fluid and Witteveen *et al.* (1992) showed using immunocytochemical methods that the bulk of *A. niger* glucose oxidase is localized in the cell wall. Also, the *A. niger* glucose oxidase is strongly glycosylated, which has never been observed for any peroxisomal protein, and the

amino acid sequence derived from the glucose oxidase DNA sequence shows a typical secretion signal peptide (Frederick *et al.*, 1990). Therefore, *A. niger* glucose oxidase, like that of the other ascomycete fungi, is thought to have an extracellular location and the results of van Dijken and Veenhuis are thought to be incorrect and due to artifacts of the method used. The subcellular location of *P. chrysosporum* glucose oxidase has not been precisely determined but Forney *et al.* (1982) have shown that production of hydrogen peroxide is localized in microbodies in the periplasmic space of the fungal cells. It is likely therefore that glucose oxidase is also extracellular in this fungus but confined to microbodies in the periplasm.

Most work concerning the induction of glucose oxidase has been performed with *A. niger*. In this fungus, high levels of glucose, high dissolved oxygen levels and an appropriate pH (usually around 5.5) are required to efficiently induce glucose oxidase (Zetelaki and Vas, 1968; Zetelaki, 1970). Of these three parameters, glucose and oxygen levels are thought to be the most important (Witteveen *et al.*, 1990) and Northern-blot analysis has shown that both regulate glucose oxidase expression at the steady state RNA level (Witteveen *et al.*, 1993). The presence of manganese and low nitrogen levels have also been reported to influence induction (Müller, 1986). However, Mishak *et al.* (1985) showed that manganese is not required for glucose oxidase induction and Witteveen *et al.* (1990) concluded that while the level and nature of the nitrogen source may influence induction, a low level of nitrogen is not required for induction.

1.4.4 Gene structure

The gene for glucose oxidase has been cloned from *A. niger* by several groups (Kriechbaum *et al.*, 1989; Frederick *et al.*, 1990 and Whittington *et al.*, 1990). Southern analysis indicates only one copy of the gene is present in the *A. niger* genome. The coding sequence consists of 1815bp which encodes 605 amino acid residues. The mature protein contains 583 amino acids, the difference being due to 22 amino acids which comprise the secretion signal presequence. No introns were present in the coding region. Eight potential *N*-linked glycosylation sites are contained within the mature enzyme, five contain the sequence Asn-X-Thr and three the alternative Asn-X-Ser.

Several promoter elements are present in the 5' untranslated region. A TATAA sequence is present at -81 with respect to the initiation codon and the sequence between the TATAA and the ATG is highly pyrimidine rich (74%). Similar pyrimidine rich regions have been found in other fungal promoters and may be important in determining the transcription initiation site (Hamer and Timberlake, 1987; Punt *et al.*, 1990). No consensus poly-A-addition site (AATAAA) is present in the 3' untranslated region.

The gene has been introduced into *Aspergillus nidulans* and the yeasts *Saccharomyces cerevisiae* and *Hansenula polymorpha* (Frederick *et al.*, 1990; Whittington *et al.*, 1990; Hodgkins *et al.*, 1993). In all cases the gene was correctly expressed and secreted, however in both yeasts the glucose oxidase secreted was of a higher molecular weight than that produced by *A. niger*. This is presumably due to differential glycosylation of the enzyme in yeast. The *A. niger* glucose oxidase gene has also been reintroduced into *A. niger* where increased copy number increased glucose oxidase production (Whittington *et al.*, 1990).

1.5 USING GLUCOSE OXIDASE TO IMPROVE PLANT DISEASE RESISTANCE

1.5.1 A brief overview of the plant defence response

The way in which a plant responds to a particular pathogen depends upon the genetic characteristics of both the pathogen and the plant host. Two types of interactions are generally recognized; compatible (between a susceptible host and a virulent pathogen) and incompatible (between a resistant host and an avirulent pathogen). Incompatible interactions are often characterized by rapid plant cell death in the immediate vicinity of the pathogen (hypersensitive response). In a compatible interaction, attack by a virulent strain does not elicit rapid localized cell death.

In both types of interaction, tissue attacked by the pathogen responds with a battery of defence mechanisms (although the speed of the response may be significantly slower in a compatible interaction). One of the first defence responses is the rapid release of activated oxygen species such as hydrogen peroxide and superoxide in what is termed an 'oxidative burst'. Other defence responses include the deposition

of callose, β-1,3 glucan and lignin as well as the induction of genes coding for enzymes involved in the synthesis of complex antimicrobial compounds (phytoalexins) (Dixon and Lamb, 1990). These localised responses are often followed by the establishment of systemic acquired resistance (SAR) in which tissues distant from the original infection site show increased resistance to subsequent infection not only to the original pathogen but to many other pathogens as well (reviewed by Ryals *et al.*, 1994). Salicylic acid, another compound induced in the defence response has been associated with this type of resistance (Gaffney *et al.*, 1993; Vernooij *et al.*, 1994).

Signals for activation of these various defences are thought to be initiated in response to recognition of pathogen elicitors by plant receptors. The defence response may be specifically induced (determined by the avirulent genotype of the pathogen race and the resistant genotype of the host cultivar) or nonspecifically induced by a range of biotic and abiotic elicitors. Structurally characterized fungal elicitors include the β 1-6, 1-3 linked β-glucans from the cell walls of *Phytophthora megasperma* sp. *glycinera*, chitin, chitosan and the peptide product of the *avr9* gene of *Cladosporium fulvum* (Basse *et al.*, 1992; Van den Ackerveken *et al.*, 1992). Only the latter elicitor is race-cultivar specific.

1.5.2 A role for hydrogen peroxide in the plant defence response

Hydrogen peroxide performs a variety of roles in the plant (reviewed by Elstner, 1987) and recently the compound has been implicated in several aspects of the plant's defence response system. Initially hydrogen peroxide is produced with other activated oxygen species such as superoxide (O_2^-) in the oxidative burst. This 'burst' occurs within minutes when leaf tissues and cell cultures are exposed to pathogenic bacteria, fungi, fungal cell wall components and elicitors and may last from minutes to a few hours (Doke, 1983 & 1985; Epperlein, 1986; Keppler *et al.*, 1989). This response is therefore rapid and transient and occurs in both compatible and incompatible interactions.

Hydrogen peroxide produced by the oxidative burst is thought to have several roles. Small amounts of hydrogen peroxide are toxic to living organisms so it is probably

directly antimicrobial. Also the hydrogen peroxide produced results in a rapid cross-linking of the cell wall (Bradley *et al.*, 1992). Such cross-linking appears to make the cell wall more resistant to pathogen digestion and may serve to slow pathogen ingress prior to the deployment of transcription dependent defences (Brisson *et al.*, 1994).

Of particular interest is a second and larger oxidative burst observed by Levine and coworkers (1994) approximately three hours after inoculation of soybean cells with an avirulent pathogen strain. This oxidative burst was much larger and more prolonged than the initial burst and was observed only when inoculation was performed with an avirulent pathogen. Further experiments indicated that the hydrogen peroxide from this second burst may be the trigger for programmed cell death of challenged cells in an incompatible interaction. They also propose that hydrogen peroxide acts as a signal for induction of cellular protectant genes such as glutathione S-transferase (GST) in adjacent cells. This is suggested in experiments in which two soybean cell cultures were separated from each other by a pair of permeable membranes filled with culture medium. Inoculation of one set of cells with the an avirulent pathogen induced cell death only in the challenged cells, not in the second set of cells separated from the challenged cells by the membrane. However GST transcripts accumulated not only in the challenged cells but also in the second set of cells, implying transmission of a low molecular weight signal for gene activation. Northern analysis showed that the presence of catalase (which breaks down hydrogen peroxide to water and oxygen) in the culture medium between the two sets of cells completely blocked GST induction in the uninfected cells suggesting that hydrogen peroxide is the local diffusible signal in this situation.

The components responsible for the generation of hydrogen peroxide and the other oxygen species involved in the oxidative burst in plant cells have yet to be identified. However, it has been reported (Levine *et al.*, 1994) that plant cells contain proteins immunologically related to the p22 subunit of the mammalian NADPH oxidase involved in the phagocyte oxidative burst. Moreover, experiments in which elicited plant cells are treated with G protein and protein kinase inhibitors indicate that the signalling pathway may resemble that of the phagocyte oxidative burst. Thus the

mechanism for hydrogen peroxide production in the plant cells may be similar to that used during phagocyte activation in the immune system (Chasan, 1994).

A further role for hydrogen peroxide in cell signalling is suggested by the experiments of Apostol *et al.*, (1989). They found that addition of catalase, 24 hours after elicitor induction, blocks phytoalexin accumulation in soybean cells. Other groups (Devlin and Gustine, 1992; Davis *et al.*, 1993) have shown that the oxidative burst is not necessary for phytoalexin formation and Levine *et al.* (1994) found that hydrogen peroxide was not a primary signal for induction of phenylalanine ammonia-lyase and chalcone synthase, two enzymes involved in the synthesis of some phytoalexins. The response observed by Apostol and coworkers is therefore not associated with the oxidative burst.

Recent experiments by Chen *et al.* (1993) in which a salicylic-acid binding protein was identified as a catalase, have also implicated hydrogen peroxide in SAR. The enzyme has a dissociation constant for salicylic acid of 14μM which is within the concentration of salicylic acid found in plants following infection (2 to 15 μM). Crude extracts of salicylic acid were found to inhibit catalase activity by approximately 70% and hydrogen peroxide in tobacco leaves treated with salicylic acid was found to increase by 50-60%. They propose that increased levels of hydrogen peroxide resulting from inhibition of catalase by salicylic acid is responsible for some of the effects of salicylic acid in SAR. It is unlikely that salicylic acid is responsible for the oxidative burst, as the kinetics of the oxidative burst are much faster than those of the salicylic acid increase. However, salicylic acid does accumulate on a time scale that is consistent with a role in mediating the hydrogen peroxide increases that are responsible for defence gene activation (Chasan, 1994). As oxidative stress induces salicylic acid (Yalpani *et al.*, 1994), Jones (1994) has suggested that a loop may be set up in which salicylic acid accumulation is activated by oxidative stress, which leads to a rise in hydrogen peroxide which leads to a rise in salicylic acid and so on. This cycle could eventually deplete the cell of NADPH, resulting in its death and/or it could cause hydrogen peroxide to increase to levels sufficient for cell death.

As hydrogen peroxide appears to play an important role in the plant defence response system it is possible that overproducing hydrogen peroxide in plants may increase disease resistance. In particular, such transgenic plants may have increased resistance to Verticillium wilt as *V. dahliae* is especially sensitive to hydrogen peroxide (Kim *et al.*, 1990b).

1.5.3 Current strategies for engineering plants resistant to fungal pathogens

Several strategies have emerged over the past ten years to try and genetically engineer plants with increased resistance to fungal disease. These include (1) introducing a gene (or genes) into a plant that synthesizes a protein with activity against the pathogen, (2) prematurely activating expression of some endogenous gene(s) normally induced in response to pathogen infection or (3) introducing a gene to deactivate some pathogen-produced factor required for pathogenesis.

Many approaches to date have involved introducing a single gene into plants which codes for a protein which is inhibitory to the pathogen. In particular, antifungal compounds which interfere with or degrade the pathogen's cell wall and/or membrane have been investigated. Such proteins which have been shown to inhibit fungal growth *in vitro* include: chitinases, β 1-3 glucanases, thionins, permatins, lysozyme and lectins (reviewed by Ward *et al.*, 1994). Results from infection trials of transgenic plants expressing these proteins are still limited and concerned mainly with expression of chitinases and glucanases. In these experiments variable results have been obtained. Broglie *et al.*, (1991) obtained increased resistance to infection by the soil-borne fungus *Rhizoctonia solani*, when they constitutively expressed the bean (*Phaseolus vulgaris* L.) class I chitinase gene in both tobacco and oilseed rape (*Brassica napus* L.). Similarly, Howie *et al.*, (1994) obtained increased resistance to *R. solani* when they expressed the chitinase gene from *Serratia marcescens* in tobacco. However, Neuhaus *et al.*, (1991) found that constitutively expressing a tobacco class I chitinase in *Nicotiana sylvestris* did not alter the susceptibility of the transgenic plants to the fungal leaf pathogen *Cercospora nicotianae* suggesting that disease resistance may be confined to *R. solani.* Glucanases have also been expressed in plants but very little increase in disease resistance has been reported (Lamb *et al.*, 1992). However, glucanases and chitinases can act synergistically

against fungi *in vitro* (Mauch *et al.*, 1988) and a report by Logemann *et al.*, (1994) suggests that a similar effect occurs *in vivo*. They found that tomato plants expressing either a class I chitinase or a class I β 1-3 glucanase alone were not protected against *Fusarium oxysporum* whereas tomato plants simultaneously expressing both genes showed a substantial increase in resistance to the pathogen.

Other antifungal proteins which have been investigated include the ribosome-inactivating proteins (RIPs) which inhibit protein synthesis by depurinating a specific residue near the 3' end of the 28S ribosomal RNA. Most RIPs show some activity in a variety of systems but they exhibit highest activity towards ribosomes from distantly related species (Roberts and Selitrennikoff, 1986; Stirpe and Hughes, 1989). A purified barley RIP has been reported to inhibit the growth of fungi *in vitro* (Leah *et al.*, 1991) and expression of the barley cDNA in tobacco under the control of a wound-inducible promoter confers resistance to *R. solani* (Logemann *et al.*, 1992). Another antifungal protein which has been expressed in plants is pathogenesis-related protein 1 (PR-1a). PR-1a is the most strongly inducible of the genes that are activated during SAR however no function has yet been assigned to it and the gene shows no resemblance to any gene sequence with known biochemical function (Ward *et al.*, 1994). Tobacco plants expressing PR-1 have been found to be resistant to only the two oomycete pathogens *Peronospora tabacina* and *Phytophthora parasitica* (Alexander *et al.*, 1993).

Modification of a plant's metabolism is another way in which to improve disease resistance. An example of this was recently reported by Hain and coworkers (1993) who transferred the grape stilbene synthase gene into tobacco. This enzyme is required for synthesis of the phytoalexin resveratrol which is associated with resistance to the fungus *Botrytis cinerea*. The substrates for the enzyme (malonyl-CoA and *para*-coumaroyl CoA) are present in most plant species but the enzyme is not. Constitutively expressing this gene in tobacco was found to significantly reduce disease symptoms following infection with *B. cinerea*.

Increased disease resistance may also be obtained by altering the metabolism of the pathogen. Polygalacturonase inhibitor proteins (PGIPs) are thought to act against

fungal polygalacturonases (PGs) which are important in the metabolism of host pectins. Pear PGIP has been found to be an effective inhibitor of *B. cinerea* PGs and expression of this gene in tomatoes has been reported to increase resistance to this pathogen (Powell *et al.*, 1994).

Therefore, to date, a range of proteins have been introduced into plants in an attempt to increase disease resistance. Work in this thesis focuses on attempting to increase disease resistance in plants by creating transgenic plants which express glucose oxidase. If such expression increases hydrogen peroxide levels, increased disease resistance could result either because the hydrogen peroxide is directly toxic to the pathogen or, as outlined earlier, because other plant defence mechanisms are stimulated.

1.6 GENERAL AIMS OF THIS THESIS

There are two general aims of the work described in this thesis. One is to try and elucidate the importance of glucose oxidase in the antagonism between *T. flavus* and *V. dahliae*. The second is to determine if and how *T. flavus* glucose oxidase may be used to control Verticillium wilt in cotton. As a first step towards these goals the *T. flavus* glucose oxidase gene was cloned and characterised. A transformation system for both *T. macrosporus* (a non-glucose oxidase-producing *Talaromyces* species) and *T. flavus* was developed. The putative *T. flavus* glucose oxidase gene was introduced into *T. macrosporus* and glucose oxidase-producing transformants obtained. The glucose oxidase gene was also mutated and gene replacement techniques used to create a glucose oxidase-deficient *T. flavus* strain. To investigate the significance of glucose oxidase in the inhibition of *V. dahliae* by *T. flavus*, this glucose oxidase-deficient strain and a glucose oxidase-producing *T. macrosporus* transformant were each examined for their ability to inhibit *V. dahliae* growth *in vitro* and to control Verticillium wilt in cotton.

The above experiments indicated that although glucose oxidase effectively inhibited *V. dahliae* growth *in vitro*, glucose oxidase secreted by *T. flavus* in soil had no significant effect on the occurrence of Verticillium wilt in cotton. The possibility that expression of glucose oxidase in the cotton plant would increase the plant's

resistance to Verticillium wilt was therefore explored. The *T. flavus* glucose oxidase gene was modified and initially introduced into tobacco, a plant more amenable to transformation than cotton. Glucose oxidase expression levels in T_0 plants were determined and gene constructs resulting in successful glucose oxidase expression in tobacco transformed into cotton. To gain some indication of the effect of glucose oxidase expression on disease resistance, preliminary infection trials with T_1 tobacco seedlings were performed. Seedlings were assessed for their ability to resist infection by *Rhizoctonia solani*, another soilborne fungal pathogen whose growth was found to be inhibited by glucose oxidase.

CHAPTER 2
ISOLATION AND EXPRESSION OF THE GLUCOSE OXIDASE GENE FROM *TALAROMYCES FLAVUS*

2.1 INTRODUCTION

Glucose oxidase (β-D-glucose: oxygen 1-oxido-reductase, EC 1.1.3.4) catalyses the oxidation of glucose to gluconate and hydrogen peroxide in the presence of molecular oxygen. The enzyme is produced by several fungi but most notably by species of *Aspergillus* and *Penicillium*. It is of considerable importance in the food industry for the removal of residual glucose or oxygen from foods and beverages. It is also frequently used in medical diagnosis where it forms the basis of methods for the detection and quantitation of glucose in body fluids such as blood and urine (Richter, 1983). More recently the enzyme has been implicated in the biological control activity of the fungus, *Talaromyces flavus* (Kim *et al.*, 1988).

T. flavus (Klöcker) Stolk and Samson (anamorph: *Penicillium dangeardii* Pitt) is a potential biocontrol agent of several pathogenic fungi, including *Verticillium dahliae* (Kleb) (Marois *et al.*, 1982) the soil fungus responsible for Verticillium wilt in cotton and many other plant species. As Verticillium wilt is very difficult to control using conventional methods, there is considerable interest in the biology and biochemistry of the interaction between these two fungi. Although the mode of action of *T. flavus* against *V. dahliae* is not completely established, it is known that the fungus secretes glucose oxidase and that this enzyme, in the presence of glucose, inhibits the growth of *V. dahliae* microsclerotia and hyphae *in vitro*. Previous studies have shown that it is the hydrogen peroxide released on the catalytic oxidation of glucose which is highly toxic to *V. dahliae* (Kim *et al.*, 1988).

Kim *et al.* (1990a) purified and analysed the *T. flavus* glucose oxidase enzyme. Isoelectric focusing revealed six different isozymes in purified protein. However, the protein is probably the product of a single gene as the isozymes observed are thought to result from minor variations in the glycosylation pattern of one gene, rather than being the products of different genes. The gene for *A. niger* glucose oxidase had

already been cloned (Kriechbaum *et al.*, 1989; Frederick *et al.*, 1990; Whittington *et al.*, 1990). This gene, which codes for 605 amino acids, has a 22 amino acid secretion signal presequence and contains no introns. Southern blot analysis indicates it is a single copy gene.

In order to investigate the role of glucose oxidase in the antagonism between *T. flavus* and *V. dahliae* at a molecular level, the *T. flavus* glucose oxidase gene has been cloned. As the *A. niger* and *T. flavus* glucose oxidase proteins are biochemically very similar (Kim *et al.*, 1990a) it was assumed they would be similar at a nucleic acid level and the *A. niger* gene was used as a probe to isolate the *T. flavus* gene from a genomic library. Reported here is the successful isolation of the *T. flavus* glucose oxidase gene using heterologous hybridization. The complete sequence of the gene is described and its relationship to other oxidoreductases discussed. In addition, a transformation system for the *Talaromyces macrosporus* species is described and used to demonstrate the identity of the glucose oxidase clone. This latter species, formerly *T. flavus var macrosporus*, is very closely related to *T. flavus* and is an ideal system in which to express the putative glucose oxidase gene as it does not itself produce glucose oxidase (Frisvad *et al.*, 1990). Correct expression of the putative *T. flavus* glucose oxidase gene in this species is demonstrated, confirming that the gene isolated encodes a functional glucose oxidase.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strains and plasmids

E. coli strains DH5α and JM101 were used for subcloning and sequencing of fungal DNA. *E. coli* strain LE392 was used to generate the genomic library. Plasmid pAN7-1 was kindly provided by Barry Scott, Massey University, NZ. Plasmid DNA was purified as previously described (Murray *et al.*, 1992).

2.2.2 Fungal isolates and growth conditions

T. macrosporus isolates FRR2417, FRR2268 and FRR2386 were kindly supplied by CSIRO Food Research, Australia. *T. flavus* 32908 and *A. niger* 9029 were obtained

from the American Type Culture Collection (ATCC). All strains were maintained on potato dextrose (PD) agar (Difco). Cultures were grown at 26°C in either liquid PD broth, or on solid PD media. Single-spore purified isolates of the *T. macrosporus* transformants were stored as spore suspensions in 10% glycerol at -80°C.

2.2.2.1 Production of *T. flavus* and *T. macrosporus* conidia

Isolates were grown on a glucose, NaCl, corn steep liquor, molasses agar (Katan *et al.*, 1984) under continuous fluorescent light at 26°C for 7-10 days. Spore suspensions were prepared by washing the agar surface with sterile distilled water. Spore concentrations in the aqueous suspension were determined with a haemocytometer.

2.2.2.2 Production of *T. flavus* and *T. macrosporus* ascospores

Isolates were grown in the dark at 26°C on PD agar for 3 weeks to allow the formation of cleistothecia. These were collected in sterile distilled water and homogenized to release the ascospores. After heat activation (Katan, 1985) ascospores were counted with a haemocytometer and plated on PD agar.

2.2.3 Amplification of the *A. niger* glucose oxidase gene

Primers complementary to the *A. niger* glucose oxidase coding region at the 5' end (5'-GGATCCATGCAGACTCTCCTTGTGAGCTCG-3') and the opposite strand of the 3' end (5'-AAGGATCCTCACTGCATGGAAGCATAATCTTC-3') were made. Mycelia used for genomic DNA extraction was obtained by growing *A. niger* in PD broth at 26°C for approximately 30 hours. Mycelia was harvested by filtering fungal culture through 2 layers of autoclaved Whatman #2 filter paper, washed twice with sterile water and then freeze dried overnight. Genomic DNA was isolated from *A. niger* freeze dried fungal material using the method of Raeder and Broder (1985). PCR (Polymerase Chain Reaction) was carried out using 200ng of DNA, *Taq* DNA polymerase buffer (Promega); 1mM $MgCl_2$; 200μM each dNTP; 1μM each primer and 2.5 units of *Taq* DNA polymerase (Promega). The reaction consisted of 35 cycles where one cycle = 1min @ 94°C, 1min @ 55°C, 3min @ 72°C.

2.2.4 Construction of *T. flavus* genomic library

T. flavus mycelia and DNA was obtained as for *A. niger* except *T. flavus* was grown in PD broth culture for approximately 48 hours before harvesting and freeze drying. All recombinant DNA manipulations were done essentially according to Sambrook *et al.*, 1989. DNA was partially digested with *Sau*3A, size fractionated on a glycerol gradient and fragments of 18-22kb pooled. DNA was ligated into *Bam*H1 cut EMBL 3 arms (Promega) overnight, packaged and used to infect *E. coli* LE392 cells. A total of 50 000 plaques were obtained.

2.2.5 Library screening and DNA sequencing

Plaques were transferred onto nitrocellulose filters and hybridized with the 1.8kb *A. niger* glucose oxidase gene generated by PCR. The probe was purified from a 0.6% seaplaque gel using the method of Thuring *et al.*, (1975) and labelled with ^{32}P-dCTP by random primer extension using an Amersham Multiprime DNA labelling kit. Unincorporated nucleotides were separated from the incorporated nucleotides through Sephadex G-50 spin column. Filters were prehybridized for 6 hours at 30°C in 50% formamide, 10% dextran sulphate, 5× SSC, 5× Denhardts solution, 0.1% SDS, 50mM $NaPO_4$ (pH 6.5) and 0.5mg/ml of denatured salmon sperm DNA. Hybridization was performed overnight at 30°C in essentially the same mixture except with only 20mM $NaPO_4$ (pH 6.5) and 2-5 × 10^6 counts per minute of probe added per 10ml of hybridization solution. After hybridization filters were washed twice with 2×SSC, 0.1%SDS at room temperature, air dried and autoradiographed for 1-2 days at -80°C. Potential positives were replated and screened again with the same probe. Positive clones were purified and analysed using Southern blotting. Hybridising *Eco*R1 and *Bam*H1 fragments were subcloned into pUC119 and mapped by restriction digest analysis. Fragments to be sequenced were subcloned into either pUC118, pUC119, or pBluescript SK$^-$ and sequenced by the dideoxynucleotide chain termination method using universal dye primers and *Taq* polymerase. Sequencing reactions were run on an ABI sequencing machine.

2.2.6 Transformation and cotransformation of *T. macrosporus*

2.2.6.1 Preparation of protoplasts

This method is based on that of Murray *et al.* (1992). Fungal cultures were grown in PD broth at 26°C for 40 hours, and then the mycelium harvested by filtering through a sterile sintered glass funnel. The mycelium was washed twice with sterile distilled H_2O and approximately 2.5g of mycelium (wet weight) transferred into 10ml of filter sterile osmotic medium containing 13mg/ml of Novozym 234 (Novo Industry). The mixture was incubated at 26°C on a slow shaker (70 rpm) for 1½ hours. The mixture was then transferred to centrifuge tubes, overlaid with ST buffer (Yelton *et al.*, 1984) and spun at 750 × *g* for 5 minutes. The protoplasts which band at the interface of the two solutions were removed, mixed with 3ml of STC buffer (Yelton *et al.*, 1984) and centrifuged at 750 × *g* for 10 minutes. Protoplasts were washed twice more in STC buffer before being resuspended in STC buffer to a final concentration of 1×10^8 per ml.

2.2.6.2 Transformation and cotransformation

Transformation and cotransformation of *Talaromyces* isolates was carried out as described in Murray *et al.* (1992) except plates were inverted and incubated in the dark at 26°C for 7-10 days.

2.2.7 Glucose oxidase assays

2.2.7.1 Indicator plates

Fungal transformants were tested for acid production on agar plates using a petri dish method based on that developed by Witteveen *et al.* (1990). In a petri dish a two layer system was used. The bottom layer consisted of PD medium containing 25g/L glucose and 15g/L agar. The top layer contained per litre: 0.2g KH_2PO_4, 0.15g $MgSO_4.7H_2O$, 0.3g $(NH_4)_2HPO_4$, 25g glucose, 9g $CaCO_3$ and 15g agar. Inoculated plates were incubated for 3-4 days and acid produced during growth dissolved the $CaCO_3$. Production of hydrogen peroxide by fungal cultures was determined using a modification of an indicator plate developed by Fiedurek *et al.* (1986). The agar plate consisted of two layers: the lower one consisted of PD media containing 25g/L glucose and 15g/L agar. The top layer contained per litre: 80g glucose, 10g starch, 1.7g potassium iodide and 0.2g of sodium deoxycholate and was adjusted to pH 5.8.

Inoculated plates were incubated overnight at 26°C and violet-blue zones were formed when iodine that was released from the KI by hydrogen peroxide combined with starch.

2.2.7.2 Fungal spores

Single spore isolates originating from conidiospores or ascospores were tested for glucose oxidase activity by submerging small pieces of hyphal material from each colony into a stain solution containing the reagents used in the top layer of the KI indicator plates described above. Hyphae were left for 12-16 hours at room temperature, the presence of glucose oxidase was indicated by the stain solution turning a violet-blue colour.

2.2.7.3 Enzyme assays

I *Freeze dried fungal tissue*

Tissue was ground by hand in 0.1M Na_2PO_4 buffer (pH 6.0) with a glass grinder and then centrifuged at 4°C (13000 rpm) for 10 minutes. The supernatant was removed and assayed for glucose oxidase activity using a modification of the method used by Frederick *et al.* (1990). Supernatant (100-200µl) was added to 700-800 µl of 0.1M Na_2PO_4 (pH 6.5) containing 2mg/ml glucose, 30µg/ml horse radish peroxidase (Sigma), and 0.6mg/ml *o*-dianisidine (Sigma). After 30 minutes incubation at 30°C the enzymatic reaction was stopped by adding 100µl of 4M H_2SO_4 (total volume 1ml). The absorbance at 405nm was measured using a Labsystems Multiskan Plus. Values were converted to glucose oxidase units/ml by comparison with a standard curve of 0-0.7 units/ml prepared with commercially available *A. niger* glucose oxidase (Sigma). One unit of activity is defined as that amount which will oxidise 1.0 µmole of β-D glucose to D-gluconic acid and H_2O_2 per minute at pH 5.1 and 25°C

II *Culture filtrate*

One hundred ml of PD broth containing 5% glucose was inoculated with four circular agar disks 4mm in diameter (cut with a sterile cork borer) from 6 day old cultures of *T. flavus*. Replica flasks of the liquid cultures were maintained at 26°C on a rotary shaker at 100 rpm for 26 hours and then samples were filtered through a 0.45µm

filter to remove fungal hyphae and spores. The resulting cell free culture filtrates were assayed for glucose oxidase as described above.

2.2.8 Southern analysis

Genomic DNA from different fungi (3µg) was digested with various restriction enzymes and electrophoresed on a 0.7% agarose gel. Denatured DNA was transferred to Hybond-N nitrocellulose filters overnight by the method of Southern (1975). Probe DNA was labelled as previously described and hybridizations were carried out at 42°C overnight in the dextran sulphate buffer previously detailed. Filters were washed twice in 2×SSC, 0.1% SDS, then twice in 0.1×SSC, 0.1% SDS at room temperature, air dried and autoradiographed for 1-2 days at -80°C.

2.3 RESULTS

2.3.1 Generation of the *A. niger* glucose oxidase gene by PCR

A heterologous probe was generated by PCR amplification of *A. niger* DNA. Specific primers were designed to the 5' and 3' ends of the coding region of the published *A. niger* glucose oxidase gene sequence (Frederick *et al.*, 1990). PCR was carried out as described in Materials and Methods and the expected 1.8kb fragment amplified. Southern blot and restriction enzyme analysis confirmed the fragment to be the *A. niger* glucose oxidase gene (results not shown).

2.3.2 Cloning and characterisation of the *T. flavus* glucose oxidase gene.

The PCR fragment isolated above was used to screen an EMBL 3 genomic library of *T. flavus* 32908 as described in Materials and Methods. A genomic library was used because the *A. niger* glucose oxidase gene contained no introns and it was assumed that the *T. flavus* gene would be similar. Once isolated, it was intended to express the glucose oxidase gene in several different organisms, but the lack of introns in the gene made the availability of a cDNA clone unnecessary.

A total of 8000 plaques were screened at reduced stringency and four hybridizing plaques detected. Secondary screening confirmed that two of the four were real hybridizations, of which one hybridized more strongly than the other. DNA from each was isolated and both were found to contain inserts of approximately 20kb.

DNA from the strongest hybridizing clone was digested with several restriction enzymes, blotted and then probed with the *A. niger* gene. Only a single band hybridized in DNA digested with either *Eco*R1 (7.7kb) or *Bam*H1 (2.2kb) (results not shown) so these fragments were thought to contain the *T. flavus* glucose oxidase gene. DNA from the weaker hybridizing band was also analysed. It was found to contain only the 2.2kb *Bam*H1 fragment. Both the *Eco*R1 and *Bam*H1 fragments were cloned into pUC119 and mapped further. The 7.7kb *Eco*R1 fragment was found to contain the *Bam*HI 2.2kb fragment plus 2.6kb further upstream and 2.9kb further downstream (Figure 2.1). The gene was assumed to be centred around the 2.2kb *Bam*H1 fragment as this region hybridized strongly to the *A. niger* probe. Overlapping fragments from this region were subcloned into M13 and sequenced. The strategy followed to determine the nucleotide sequence of the glucose oxidase gene of *T. flavus* and its flanking regions is shown in Figure 2.2.

A total of 3032bp of DNA was sequenced and this sequence is shown in Figure 2.3. Analysis of the sequence revealed a 1815bp open reading frame (ORF) from base 1032 to base 2847. This ORF is exactly the same size as the *A. niger* glucose oxidase coding region and is 65% similar to it at a nucleotide level. The 605 amino acid protein which it encodes is 64% identical (77% similar) to the *A. niger* glucose oxidase protein (Figure 2.4). Seven consensus sites for potential N-linked glycosylation were identified in the *T. flavus* protein sequence, while in the *A. niger* glucose oxidase protein eight potential glycosylation sites were identified. Four of these potential glycosylation sites are in identical positions in the *T. flavus* and *A. niger* glucose oxidase gene sequences (Figure 2.4). Another potential site (*T. flavus*, aa 375-377) is within 2bp of a potential site in *A. niger* (aa 377-379). As in *A. niger*, the *T. flavus* glucose oxidase protein has a putative secretion signal sequence at the amino terminus of the protein. A hydropathy plot of this region shows it to be predominantly hydrophobic (Figure 2.5) and it extends for 22 amino acids according to the rules of Von Heijne (1985). The cleavage site (Leu-X-Ala) is not unusual and frequently precedes signal peptidase cleavage sites. Cleavage of this putative 22 amino-acid signal peptide from the precursor is predicted to yield a mature protein of 583 amino acids which has an estimated molecular weight of 63.5kDa and an isoelectic point of 4.88. This compares well with the molecular weight of 71kDa and

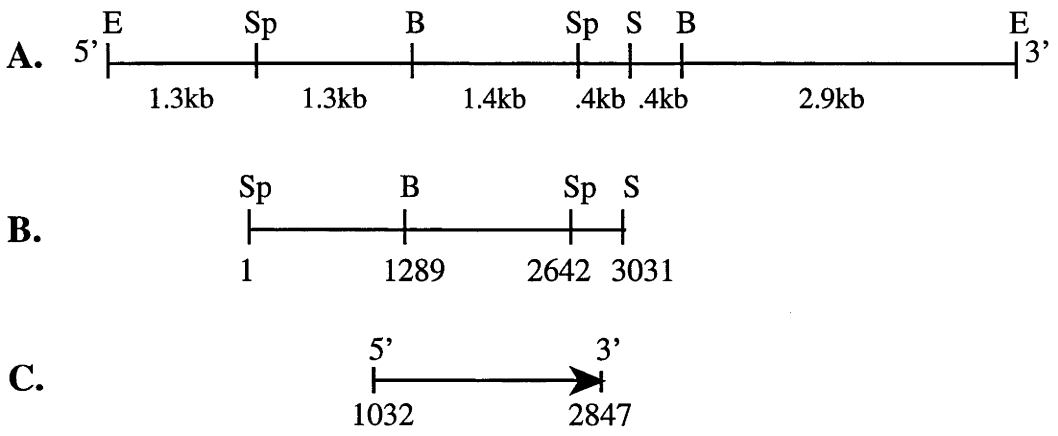

Figure 2.1 **A.** Simplified restriction map of 7.7kb *Eco*RI fragment containing the glucose oxidase gene. Shown are the restriction sites for *Eco*RI (E), *Sph*I (Sp), *Bam*HI (B), and *Sac*I (S). **B.** Region of the 7.7kb fragment sequenced. Numbers correspond to base pairs sequenced starting at the 5' *Sph*I restriction site. **C.** Localization of glucose oxidase open reading frame in the region sequenced .

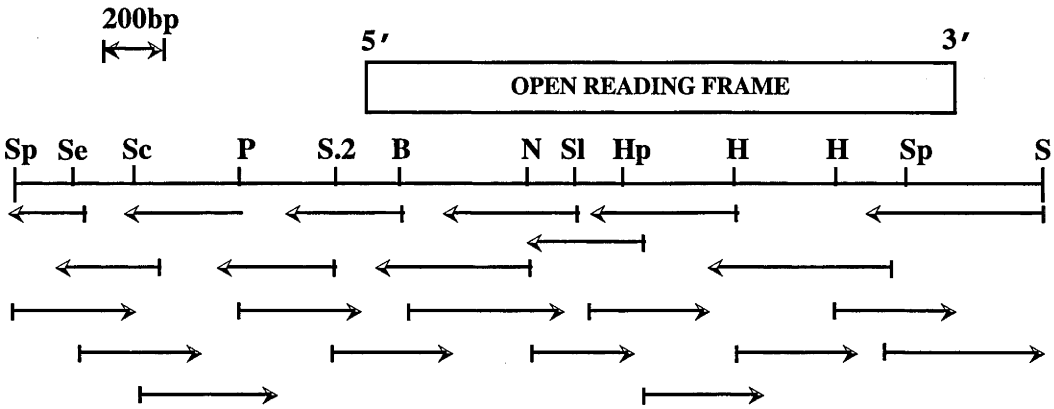

Figure 2.2 Sequencing strategy. Arrows indicate the positions, lengths and directions of sequencing from restriction enzyme cloning sites. The position of the open reading frame is also shown. Abbreviations: **B**, *BamH*I; **H**, *Hin*dIII; **Hp**, *Hpa*I; **N**, *Nco*I; **P**, *Pst*I; **S**, *Sac*I; **S.2**, *Sac*II; **Sc**, *Sca*I; **Se**, *Spe*I; **Sl**, *Sal*I; **Sp**, *Sph*I.

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1032 CCACAAGTCCTAGAGAAGACACACAGTCTCGAGCCCAAAGTAAGAATGGATATTGTGACT
-976 TCCTAAAGGCCTCACCGGGCAGTGAGGTATTTGATGTTTACCAAACGCTAGTATGGGTAG
-912 CATAATCGGTGATACCTAGGTATATCATATGTTCATCCACAGGGCTGGGTTTGTGAAGAA
-852 ACTGTAGCACTAGTGCTGCTTAGTTGCATATGGAGTTTCTATCTGCACTATTCCGTTGGA
-792 GGAAGGAAGAAAAGGGCAAGAGAGATACTGTCAAATGAATGTACTCGGGGGTCACTGAAT
-732 ACGTGAAAGCGTACTTAGGTGATCTATTGCGAGAATAGTTCAATGATATCGATGTCCTCT
-672 CGGCGCTCCACTCTCTCTATTCGTATCTGATTCTGATCTGCTCTTCATTCACAACTTTAT
-612 GTATCTGTCATGCCAGTTTTACGAGTACTGGGAAAGTTGGCGCTCAGAGCTGGGATTCTT
-552 GGGTTTCATTGACGCTCAACCTAGAGTTTGAATGATATCGCTTTATCTTTAGATAATCTT
-492 CAACGTAACAATGTGCTTGAGCTTCTAGCGCCAAGATGCGTAGACTTTCGTAAATGGTAG
-432 TTCAAGCTAATAATTCAGGAAAATATTGCAGAGGATTATCGCCACACATGCCGATGGAGC
-372 ATACAGACTCCTCTTGATACGATGCTTTGACCACTCACATCCTCCAGCCTTCCATCCAGG
-312 TCCCTAGGTTCAGCCGTGCTTCCAGCACTTACTGATCAAACCCCTGTAGCACGGCTAGTA
-252 TCTCATATCTTTCCGTCTGCAGCATGAGTCGCTCATGTCTGCACGAGTCCATTTTCAGAA
-192 AGTGGGATAATCTAACCTGGTGGCGAGGCCAAGATACGACATAAAGGAAATGTTTGCTTC
-132 TTGCAAGTCTATAAATTGAGCGACATCTACCGCTGTTCAGACAAGTTCTTCAGCACAACA
 -72 ATCAGGTAATTTCACCACTCTCCTTGCAATCCCGTTTATCTTCTCCATCTCCTTGACCTT
                 M  V  S  V  F  L  S  T  L  L  L  A  A  A  T  V
 -12 GCCGGATCGAAATGGTGTCTGTATTTCTCAGCACTCTTCTTTTAGCCGCGGCTACGGTC
      Q  A  Y  L  P  A  Q  Q  I  D  V  Q  S  S  L  L  S  D  P  S
  47 CAAGCCTACCTGCCTGCCCAACAGATTGATGTCCAGTCTAGTCTTCTCAGTGACCCTAGC
      K  V  A  G  K  T  Y  D  Y  I  I  A  G  G  G  L  T  G  L  T
 107 AAGGTCGCCGGAAAGACCTATGATTACATTATTGCTGGTGGTGGTTTGACTGGCCTTACT
      V  A  A  K  L  T  E  N  P  K  I  K  V  L  V  I  E  K  G  F
 167 GTTGCCGCCAAACTGACAGAAAACCCCAAGATCAAAGTCCTGGTTATTGAAAAGGGCTTC
      Y  E  S  N  D  G  A  I  I  E  D  P  N  A  Y  G  Q  I  F  G
 227 TATGAGTCCAACGATGGAGCCATCATCGAGGATCCAAATGCTTACGGACAAATCTTCGGC
      T  T  V  D  Q  N  Y  L  T  V  P  L  I  N  N* R* T* N  N  I
 287 ACCACTGTTGACCAGAACTACCTCACCGTTCCCCTGATCAACAACCGCACGAACAATATC
      K  A  G  K  G  L  G  G  S  T  L  I  N  G  D  S  W  T  R  P
 347 AAGGCCGGCAAGGGTCTTGGAGGATCAACCTTGATAAACGGTGACTCTTGGACTCGCCCG
      D  K  V  Q  I  D  S  W  E  K  V  F  G  M  E  G  W  N  W  D
 407 GACAAAGTCCAGATTGATTCTTGGGAGAAGGTCTTTGGCATGGAAGGTTGGAATTGGGAC
      S  M  F  E  Y  M  K  K  A  E  A  A  R  A  P  T  A  A  Q  L
 467 AGTATGTTTGAGTACATGAAGAAGGCCGAGGCTGCACGTGCCCCTACTGCTGCTCAACTT
      A  A  G  H  Y  F  N* A* T* C  H  G  T  N* G* T* V  Q  S  G
 527 GCTGCCGGTCACTACTTCAATGCTACCTGCCATGGAACTAACGGTACTGTTCAATCCGGA
      A  R  D  N  G  Q  P  W  S  P  I  M  K  A  L  M  N  T  V  S
 587 GCCCGTGACAACGGTCAACCTTGGTCTCCTATTATGAAGGCCCTTATGAACACCGTCTCG
      A  L  G  V  P  V  Q  Q  D  F  L  C  G  H  P  R  G  V  S  M
 647 GCCCTTGGTGTCCCCGTACAGCAAGACTTTCTCTGCGGTCATCCTCGAGGTGTCTCTATG
      I  M  N  N  V  D  E  N  Q  V  R  V  D  A  A  R  A  W  L  L
 707 ATCATGAACAATGTCGACGAAAACCAAGTTCGTGTTGATGCTGCCCGTGCATGGCTGCTT
      P  S  Y  Q  R  P  N  L  E  I  L  T  G  Q  M  V  G  K  V  L
 767 CCCAGCTACCAGCGCCCCAACTTGGAGATCCTTACTGGTCAGATGGTTGGAAAGGTTCTG
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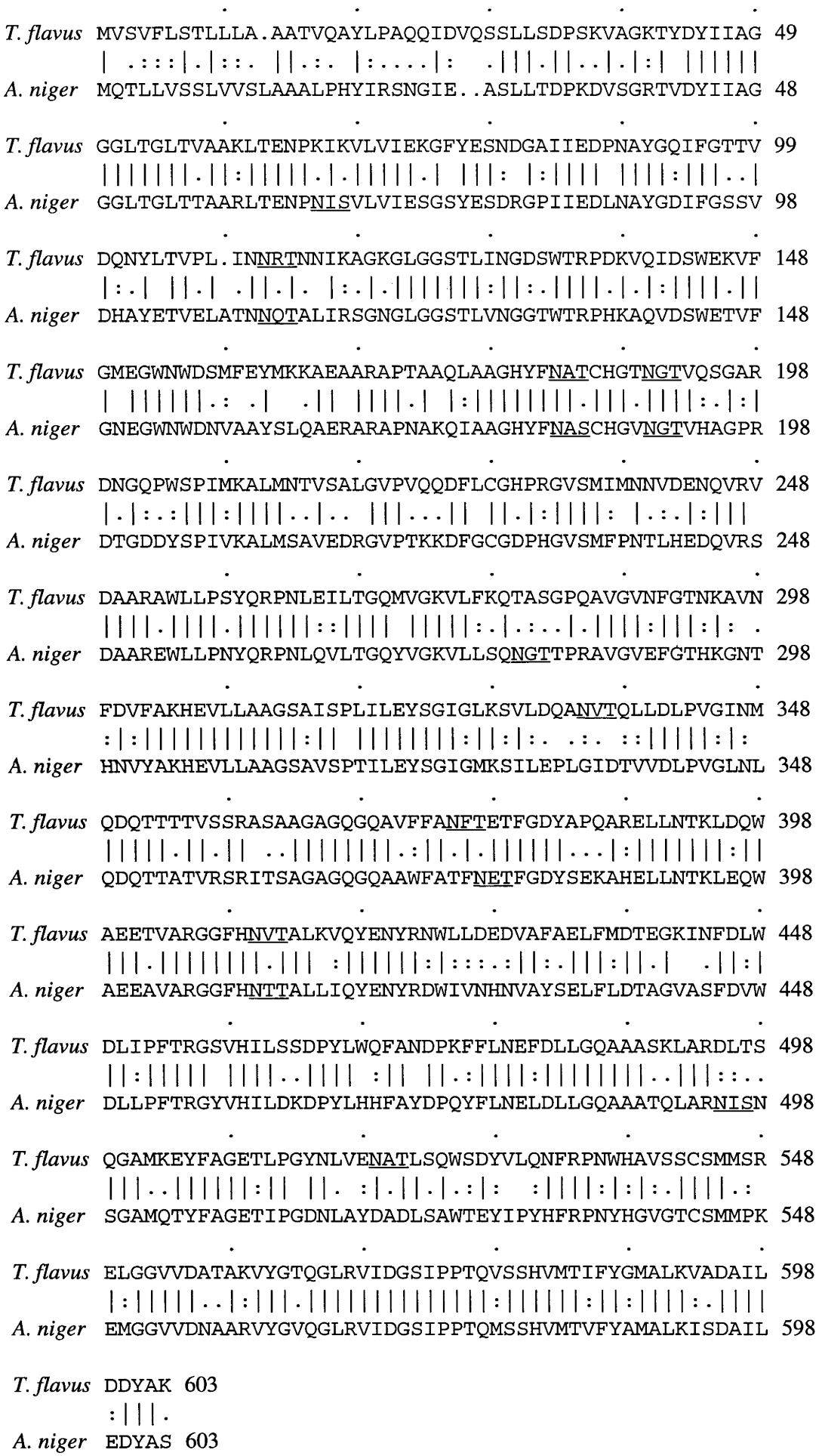
     F  K  Q  T  A  S  G  P  Q  A  V  G  V  N  F  G  T  N  K  A
827  TTTAAACAGACCGCATCCGGTCCCCAGGCTGTTGGTGTGAACTTCGGTACTAATAAGGCC
     V  N  F  D  V  F  A  K  H  E  V  L  L  A  A  G  S  A  I  S
887  GTTAACTTTGACGTCTTTGCTAAGCATGAGGTCCTTTTGGCTGCCGGCTCAGCTATCTCT
     P  L  I  L  E  Y  S  G  I  G  L  K  S  V  L  D  Q  A  N* V*
947  CCGCTGATCTTGGAATATTCTGGCATAGGCTTGAAGTCTGTTCTTGATCAGGCCAATGTC
     T* Q  L  L  D  L  P  V  G  I  N  M  Q  D  Q  T  T  T  T  V
1007 ACTCAGCTTCTTGATCTTCCTGTTGGTATCAATATGCAAGACCAGACCACAACCACTGTC
     S  S  R  A  S  A  A  G  A  G  Q  G  Q  A  V  F  F  A  N* F*
1067 AGTTCCCGTGCTAGTGCCGCTGGTGCTGGTCAGGGTCAGGCCGTCTTCTTCGCCAATTTC
     T* E  T  F  G  D  Y  A  P  Q  A  R  E  L  L  N  T  K  L  D
1127 ACTGAAACCTTCGGTGACTACGCCCCCCAGGCCAGAGAGTTACTCAACACCAAGCTTGAC
     Q  W  A  E  E  T  V  A  R  G  G  F  H  N* V* T* A  L  K  V
1187 CAATGGGCTGAGGAGACCGTTGCGCGAGGTGGTTTCCATAATGTAACTGCTCTCAAAGTT
     Q  Y  E  N  Y  R  N  W  L  L  D  E  D  V  A  F  A  E  L  F
1247 CAATATGAAAACTATCGTAACTGGCTCCTTGACGAAGACGTTGCCTTCGCCGAGCTTTTC
     M  D  T  E  G  K  I  N  F  D  L  W  D  L  I  P  F  T  R  G
1307 ATGGATACCGAGGGCAAGATCAACTTCGACTTATGGGATCTCATCCCTTTCACTCGTGGT
     S  V  H  I  L  S  S  D  P  Y  L  W  Q  F  A  N  D  P  K  F
1367 TCCGTCCATATCCTCAGTAGCGACCCTTACCTATGGCAATTCGCCAACGACCCCAAATTC
     F  L  N  E  F  D  L  L  G  Q  A  A  A  S  K  L  A  R  D  L
1427 TTCCTGAACGAGTTTGACCTCCTTGGTCAAGCCGCTGCTTCCAAGCTTGCTCGTGATCTT
     T  S  Q  G  A  M  K  E  Y  F  A  G  E  T  L  P  G  Y  N  L
1487 ACCAGCCAAGGTGCTATGAAGGAGTACTTCGCCGGAGAGACTCTTCCAGGATACAACTTG
     V  E  N* A* T* L  S  Q  W  S  D  Y  V  L  Q  N  F  R  P  N
1547 GTCGAGAATGCTACTCTTTCCCAGTGGTCGGATTATGTCTTACAGAACTTCCGTCCCAAC
     W  H  A  V  S  S  C  S  M  M  S  R  E  L  G  G  V  V  D  A
1607 TGGCATGCTGTCAGCAGCTGCTCTATGATGTCTAGAGAGCTTGGTGGTGTCGTTGATGCT
     T  A  K  V  Y  G  T  Q  G  L  R  V  I  D  G  S  I  P  P  T
1667 ACTGCCAAGGTGTACGGTACGCAGGGCCTACGTGTCATTGATGGCTCTATTCCTCCGACT
     Q  V  S  S  H  V  M  T  I  F  Y  G  M  A  L  K  V  A  D  A
1727 CAGGTGTCTTCTCATGTCATGACCATTTTCTACGGAATGGCTTTGAAAGTTGCTGATGCG
     I  L  D  D  Y  A  K  S  A  *
1787 ATTCTGGACGACTATGCCAAAAGTGCCTAGAGGTGTCATGAATCGCGGTTCGTCAGCGAA
1847 TTTGCTAGGGTTTAGATCACCGATTTTTTCTCCTCGCTCATACATTGTTAGATTCTCGCA
1907 CATATAGATCGATTTAAATTGCTTATAGACAACGTGAAATTTACTACTTATTCATCGAAC
1967 TTACATTCTTCAAAATATTCAAGAGAGCTC
```

Figure 2.3 The nucleotide and deduced amino acid sequence of the *T. flavus* glucose oxidase gene. Translation of the entire sequence is shown beginning at the ATG start site, (nucleotide #1). The putative signal sequence is underlined. Possible TATAA and CAAT boxes are in bold and double underlined. Potential glycosylation sites are marked with an asterisk.

Figure 2.4 Alignment of the *T. flavus* and *A. niger* glucose oxidase amino acid sequences. *A. niger* sequence from Frederick *et al.*, 1990. The sequences were aligned for maximum homology using the GCG bestfit program. Solid lines indicate amino acid matches, the symbol : indicates a conservative replacement and the symbol . a less conservative replacement. Potential glycosylation sites are underlined.


T. flavus MVSVFLSTLLLA.AATVQAYLPAQQIDVQSSLLSDPSKVAGKTYDYIIAG 49
A. niger MQTLLVSSLVVSLAAALPHYIRSNGIE..ASLLTDPKDVSGRTVDYIIAG 48
T. flavus GGLTGLTVAAKLTENPKIKVLVIEKGFYESNDGAIIEDPNAYGQIFGTTV 99
A. niger GGLTGLTTAARLTENPNISVLVIESGSYESDRGPIIEDLNAYGDIFGSSV 98
T. flavus DQNYLTVPL.INNRTNNIKAGKGLGGSTLINGDSWTRPDKVQIDSWEKVF 148
A. niger DHAYETVELATNNQTALIRSGNGLGGSTLVNGGTWTRPHKAQVDSWETVF 148
T. flavus GMEGWNWDSMFEYMKKAEAARAPTAAQLAAGHYFNATCHGTNGTVQSGAR 198
A. niger GNEGWNWDNVAAYSLQAERARAPNAKQIAAGHYFNASCHGVNGTVHAGPR 198
T. flavus DNGQPWSPIMKALMNTVSALGVPVQQDFLCGHPRGVSMIMNNVDENQVRV 248
A. niger DTGDDYSPIVKALMSAVEDRGVPTKKDFGCGDPHGVSMFPNTLHEDQVRS 248
T. flavus DAARAWLLPSYQRPNLEILTGQMVGKVLFKQTASGPQAVGVNFGTNKAVN 298
A. niger DAAREWLLPNYQRPNLQVLTGQYVGKVLLSQNGTTPRAVGVEFGTHKGNT 298
T. flavus FDVFAKHEVLLAAGSAISPLILEYSGIGLKSVLDQANVTQLLDLPVGINM 348
A. niger HNVYAKHEVLLAAGSAVSPTILEYSGIGMKSILEPLGIDTVVDLPVGLNL 348
T. flavus QDQTTTTVSSRASAAGAGQGQAVFFANFTETFGDYAPQARELLNTKLDQW 398
A. niger QDQTTATVRSRITSAGAGQGQAAWFATFNETFGDYSEKAHELLNTKLEQW 398
T. flavus AEETVARGGFHNVTALKVQYENYRNWLLDEDVAFAELFMDTEGKINFDLW 448
A. niger AEEAVARGGFHNTTALLIQYENYRDWIVNHNVAYSELFLDTAGVASFDVW 448
T. flavus DLIPFTRGSVHILSSDPYLWQFANDPKFFLNEFDLLGQAAASKLARDLTS 498
A. niger DLLPFTRGYVHILDKDPYLHHFAYDPQYFLNELDLLGQAAATQLARNISN 498
T. flavus QGAMKEYFAGETLPGYNLVENATLSQWSDYVLQNFRPNWHAVSSCSMMSR 548
A. niger SGAMQTYFAGETIPGDNLAYDADLSAWTEYIPYHFRPNYHGVGTCSMMPK 548
T. flavus ELGGVVDATAKVYGTQGLRVIDGSIPPTQVSSHVMTIFYGMALKVADAIL 598
A. niger EMGGVVDNAARVYGVQGLRVIDGSIPPTQMSSHVMTVFYAMALKISDAIL 598
T. flavus DDYAK 603
A. niger EDYAS 603

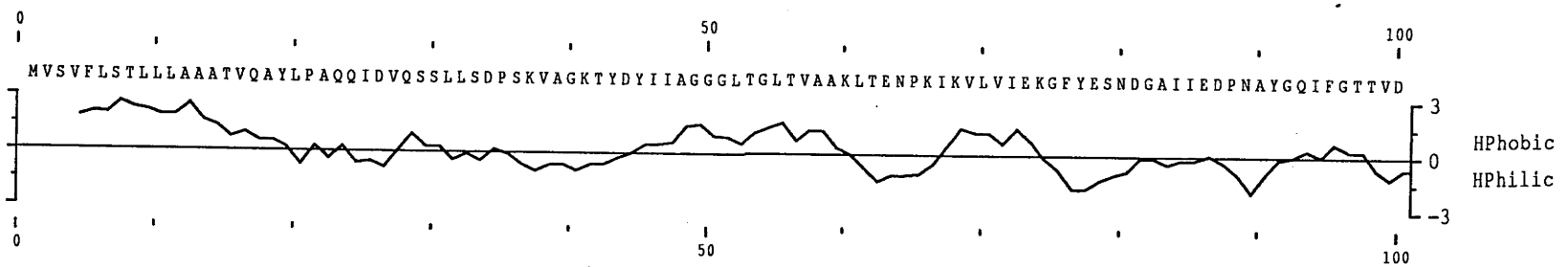


Figure 2.5 Average hydrophobicity profile of *T. flavus* glucose oxidase. The GCG program based on that of Kyte and Doolittle (1982) was used. The numbers refer to amino acid residues. Areas above the median line are hydrophobic and areas below are hydrophilic.

isoelectric point of 4.45 determined for denatured *T. flavus* glucose oxidase by Kim *et al.* (1990a). In addition, the amino acid composition of glucose oxidase derived from the DNA sequence is in close agreement with that measured by Kim *et al.* (Table 2.1).

Analysis of the 5' non-coding region of the *T. flavus* glucose oxidase sequence reveals the presence of several potential promoter elements. A TATAAA sequence is located at -122 (wrt the ATG initiation codon) and several CAAT boxes are present at -43, -73 and -483 (Figure 2.3). However, it is unlikely any of these elements are functional as CAAT boxes usually fall at least 50bp upstream of TATAAA boxes (Nussinov, 1986). In addition, two of the CAAT boxes are within a very pyrimidine rich region (80%) which spans from -12 to -63 bases upstream of the coding region. A similar pyrimidine rich region is found -12 to -80 bases upstream of the initiating ATG in the *A niger* glucose oxidase gene. The *A. niger* 5' region also contains a TATAA sequence (at -122) but no CAAT boxes are present.

No consensus polyadenylation sequence (AATAAA) is found in the 3' untranslated region of the *T. flavus* glucose oxidase gene. The AAACAA sequence identified by Frederick *et al.* (1990) as being polyadenylated in *A. niger* glucose oxidase (at +151 with respect to the stop codon) is also not present. However, 162 bases downstream of the stop codon in *T. flavus* glucose oxidase there is an AAATA sequence. This sequence may function as the polyadenylation sequence in *T. flavus* glucose oxidase. In addition, the 3' region contains a 120bp AT rich region (70%) which may also be involved in transcription termination.

The *A. niger* glucose oxidase gene is induced only in the presence of glucose and high oxygen concentrations (Witteveen *et al.*, 1993). Although the regulation of *T. flavus* glucose oxidase gene expression has not been analysed it is possible this gene is regulated in a similar manner. To determine if any consensus sequences specific to the regulation of the glucose oxidase genes existed, the 5' and 3' untranslated regions of the *A. niger* and the *T. flavus* glucose oxidase genes were compared. No significant total or localized homology between these regions was found.

	Mole percent of amino acids in *T. flavus* glucose oxidase	
Amino acid	**From gene sequence[a]**	**From purified enzyme[b]**
Asp	6.0	11.9
Asn	6.0	-
Thr	6.5	6.6
Ser	6.4	7.2
Glu	4.3	9.9
Gln	5.5	-
Pro	4.0	4.6
Gly	8.8	9.2
Ala	10.1	10.6
Cys	0.52	0.2
Val	7.5	6.8
Met	2.5	2.4
Ile	4.4	3.8
Leu	8.9	10.4
Tyr	3.3	3.4
Phe	4.4	4.5
His	1.4	1.4
Lys	4.6	4.5
Arg	2.9	2.9
Trp	2.1	*nd*

Table 2.1 Amino acid composition of *T. flavus* glucose oxidase based on **a**, the *T. flavus* glucose oxidase gene sequence (this study) and **b**, amino acid analysis of the purified enzyme (Kim *et al.*, 1990a). Abbreviations: *nd*, not determined.

2.3.3 Homology of *T. flavus* glucose oxidase with other protein sequences

The primary structure of *T. flavus* glucose oxidase was compared with the protein sequences compiled in the Swiss-Prot protein sequence data base (Pearson and Lipman, 1988). Overall the protein is most homologous (64%) with *A. niger* glucose oxidase (Figure 2.4) however it also shows significant homology in several other regions to five other flavin proteins. These are *Candida boidinii* alcohol oxidase, (AOX(C); Sakai and Tani, 1992), *Hansenula polymorpha* alcohol oxidase (AOX(H); Ledeboer *et al.*, 1985), *Pseudomonas oleovorans* alcohol dehydrogenase (AlkJ; Van Beilen *et al.*, 1992), *E. coli* choline dehydrogenase (BetA; Lamark *et al.*, 1991) and *Drosophila melanogaster* glucose dehydrogenase (GLD; Krasney *et al.*, 1990).

These proteins all belong to a group of enzymes known as GMC oxidoreductases (Cavener, 1992). The proteins share a number of regions of sequence similarity, one of which corresponds to the βαβ segment of the FAD ADP-binding domain. This domain which is located at or near the N-terminus consists of 11 amino acid positions at which specific amino acids occur. Sequences that contain the expected amino acids at only nine or ten of the positions can still generate ADP-binding folds (Wierenga *et al.*, 1986). *T. flavus* glucose oxidase contains nine of the consensus amino acids at the correct positions (Figure 2.6a). It deviates from the consensus in having an aspartic acid residue at the first position and a tyrosine residue at the second position. The first of these changes is not unusual as 38% of the proteins found in the Swiss-Prot database with a recognizable ADP-binding fold (Release 28), have an aspartate at position one. However, other than BetA and AlkJ, only two proteins (glutathione reductases) were found to have a tyrosine residue at position two.

The GMC oxidoreductases have sequence similarity in four other regions, however the function of these domains is not yet known. One region is located in the N-terminal section, about 50 residues after the ADP-binding domain. It contains six conserved residues within a stretch of 22 amino acids. Two conserved regions are located in the central section of the proteins which contain seven and five conserved amino acids, respectively. The fourth area of conservation is near the C-terminus where there are six out of 22 residues conserved. All of these amino acids are

Figure 2.6 Regions of conserved amino acid sequence in: *T. flavus* glucose oxidase, GOD(T); *A. niger* glucose oxidase, GOD(A); *H. polymorpha* alcohol oxidase, AOX(H); *C. boidinii* S2 alcohol oxidase, AOX(C); *E. coli* choline dehydrogenase, BetA; *P. oleovorans* alcohol dehydrogenase, AlkJ and *D. melanogaster* glucose dehydrogenase, GLD. Numbers indicate the position of amino acids with respect to the initiating methionine.

A. Putative ADP-binding fold in GOD(T), GOD(A), AOX(H), AOX(C), BetA, AlkJ, and GLD. The top rows show the ADP-binding consensus sequence that can fold in a βαβ structure with ADP-binding properties (Wierenga *et al.*, 1986). Symbols indicate which amino acids should occur at the given positions: (●) K, R, H, S, T, Q, N; (■) A, I, L, V, M, C, G; (▲) D, E. Letters in bold are in agreement with the consensus sequence.

B. Conserved regions of amino acid sequence from *T. flavus* glucose oxidase and other related GMC oxidoreductases. Conserved residues are shown in bold.

A.

		--- β ---- ------- α ----- ----- β ----
Consensus sequence		●■-■- G-G--G---■ --■ ■-■-▲
	aa position	
GOD(T)	41-75	KTYDYI**I**A **GGGL**T**GL**TV**A** AK**L**T..ENPK IK**V**L**V**I**E**KG
GOD(A)	40-74	RTVDYI**I**A **GGGL**T**GL**TT**A** AR**L**T..ENPN IS**V**L**V**I**E**SG
AOX(H)	5-41	DEFD**I**I**V**V **GGGS**T**G**CCI**A** GR**L**ANLDDQN LT**V**A**L**I**E**GG
AOX(C)	5-41	EEFD**V**I**V**C **GGGS**T**G**CVI**A** GR**L**ANVDE.N LK**V**L**L**I**E**NG
BetA	1-35	MQFDYI**I**I **GAGS**A**G**NVL**A** TR**L**T..EDPN TS**V**L**LLE**AG
AlkJ	1-35	MYDYI**I**V **GAGS**A**G**CVL**A** NR**L**S..ADPS KR**V**C**LLE**AG
GLD	63-97	YEYDFI**V**I **GGGS**A**G**SVV**A** SR**L**S..EVPQ WK**V**L**L**I**E**AG

B.

	aa position	
GOD(T)	117-151	KAGKG **LGG**STLI**N**GD SWT**R**PDKVQI DS**W**EKVF
GOD(A)	117-151	RSGNG **LGG**STLV**N**GG TWT**R**PHKAQV DS**W**ETVF
AOX(H)	86-117	PCANI **LGG**GSSI**N**FL MYT**R**ASASDY DD**W**ESEG
AOX(C)	85-117	PQANI **LGG**GSSI**N**FM MYT**R**ASASDY DD**W**ESEG
BetA	80-114	GRGKG **LGG**SSLI**N**GM CYI**R**GNALDL DN**W**AQEP
AlkJ	77-110	PRGKT **LGG**SSSI**N**AM VYI**R**GHEDDY HA**W**EQAA
GLD	142-175	PRGKV **LGG**TSVL**N**GM MYV**R**GNREDY DD**W**A.AD
GOD(T)	308-334	LLAA**G**SA IS**P**LI**L**EY**SG** I**G**LKSVLDQA
GOD(A)	308-334	LLAA**G**SA VS**P**TI**L**EY**SG** I**G**MKSILEPL
AOX(H)	266-292	VISC**G**TI SS**P**LV**L**QR**SG** I**G**AAHHLRSV
AOX(C)	265-291	VVSC**G**TV SS**P**MV**L**QR**SG** I**G**EPSKLRAA
BetA	255-281	LLCA**G**AI AS**P**QI**L**QR**SG** V**G**NAELLAEF
AlkJ	250-276	LLSL**G**AV GT**P**HL**L**ML**SG** V**G**AAAELKEH
GLD	312-338	VLSA**G**AV NS**P**HI**L**LL**SG** V**G**PKDELQQV
GOD(T)	338-362	QLLD**LP. VG**I**N**MQDQTT TTVSSRASA
GOD(A)	338-362	TVVD**LP. VG**L**N**LQDQTT ATVRSRITS
AOX(H)	296-321	PIVD**LP**G **VGEN**FQDHYC FFTPYYVKP
AOX(C)	295-320	PIVE**LP**G **VGRN**FQDHFC YFVPYRIKQ
BetA	285-306	LVHE**LP**G **VGEN**LQDHL..EMYLQYE C
AlkJ	280-301	LVHD**LPE VGKN**LQDH...DITLMCA A
GLD	342-367	TVHN**LP**G **VGKN**LHNHVT YFTNFFIDD.
GOD(T)	550-582	LGG **V**VDATAKVY**G** TQGLR**V**I**DGS** IPPTQVSSHV
GOD(A)	550-582	MGG **V**VDNAARVY**G** VQGLR**V**I**DGS** IPPTQMSSHV
AOX(H)	586-618	KGG **V**LDARLNVY**G** VQNLK**VADLS** VCPDNVGCNT
AOX(C)	585-617	EGG **V**LDPRLNVH**G** VKGLK**VADLS** VCPDNVGCNT
BetA	484-516	EMS **V**VDGEGRVH**G** LEGLR**VVDAS** IMPQIITGNL
AlkJ	480-512	PAS **V**VDPCLKIR**G** LANIR**VVDAS** IMPHLVAGNT
GLD	552-584	PMA **V**VNHELRVH**G** IRGLR**VMDTS** IMPKVSSGNT

conserved in both *T. flavus* and *A. niger* glucose oxidase at the expected positions (Figure 2.6b), further substantiating that these glucose oxidases fall into this class of enzymes.

2.3.4 Expression of the glucose oxidase gene in *T. macrosporus*

2.3.4.1 Transformation of *T. macrosporus*

Of the three different isolates of *T. macrosporus* obtained, FRR2268 and FRR2386 had been isolated overseas from decaying fruit and FRR2417 had been isolated from Australian soil. If transformants could be successfully generated, it was intended to test the highest expressing glucose oxidase producers in the soil for their ability to control Verticillium wilt in cotton (see Chapter 3). Isolate FRR2417 was already in Australia, so a transformation procedure was developed for this isolate in order to minimise potential quarantine restrictions on the use of transformants. Preliminary experiments showed regenerating protoplasts of this isolate to be sensitive to moderate concentrations of the antibiotic hygromycin (200µg/ml) (results not shown). Transformations were done with the vector pAN7-1 (Punt *et al.*, 1987) which carries the gene for hygromycin resistance under the control of *A. nidulans* 5' and 3' sequences (Figure 2.7). Before transformation, the vector was linearized with *Hin*dIII as linear DNA is thought to be more recombinogenic than circular DNA in fungi (Orr-Weaver *et al.*, 1981).

Hygromycin resistant colonies were observed 1-2 weeks after transformation. Approximately 20 transformants/µg of DNA were obtained and most of these colonies continued to grow when transferred to fresh selective medium, suggesting that few were 'abortive transformants' (Tilburn *et al.*, 1983). This corresponds to a transformation frequency of about 0.001% of the original number of protoplasts or 0.01 - 0.02% if one takes into account that only 5-10% of the protoplasts regenerate after incubation in PEG. Southern blotting and probing of transformant DNA with pAN7-1 confirmed the presence of the hygromycin gene in the fungal genome (results not shown).

A plasmid containing the 7.7kb *Eco*R1 fragment believed to contain the *T. flavus* glucose oxidase gene, pGO, was digested with *Eco*RI and transformed into isolate

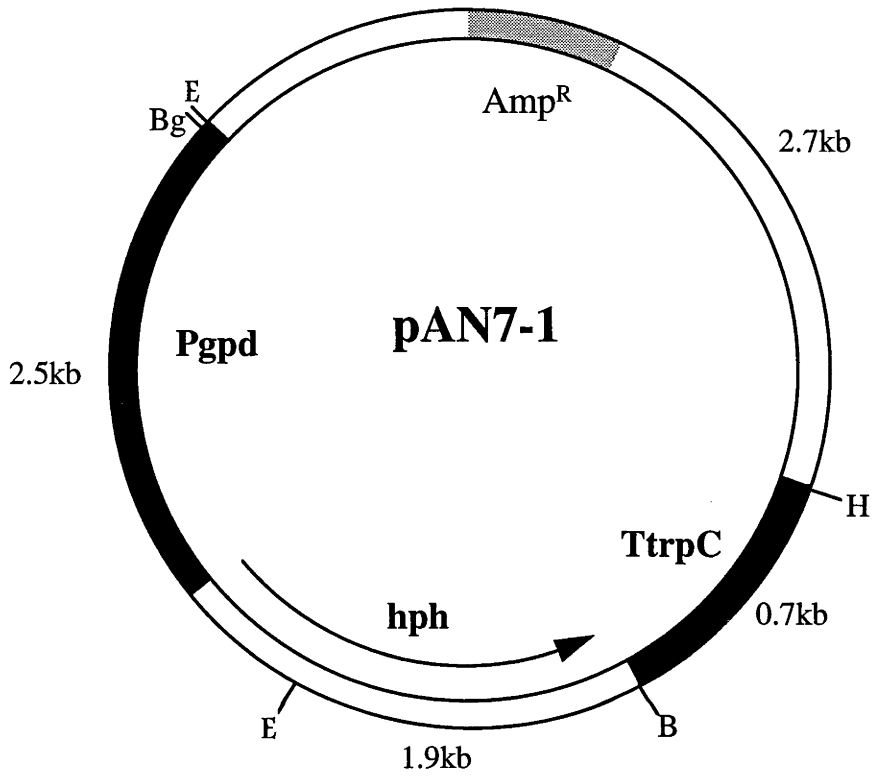


Figure 2.7 Restriction enzyme map of pAN7-1 redrawn from published map (Punt *et al.*, 1987). Restriction sites for *Eco*RI (E), *Bgl*II (Bg), *Bam*HI (B) and *Hin*dIII (H) are shown, as are the genes for ampicillin resistance ($Amp^{R)}$ and hygromycin resistance (hph). The *hph* gene is fused to the *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter fragment (Pgpd) and an *A. nidulans* trpC terminator (TtrpC). The size of various fragments is shown in kb.

FRR2417 by cotransformation with pAN7-1. Transformants were initially selected on hygromycin and then colonies were screened for glucose oxidase activity on two indicator plates. The transformation frequency obtained (0.01%) was similar to the frequency obtained when *T. macrosporus* was transformed with pAN7-1 alone. Of 29 hygromycin resistant transformants analysed, 18 (62%) were also glucose oxidase positive. Figure 2.8 shows a transformed and untransformed isolate on a calcium carbonate indicator media.

The stability of the primary transformants was evaluated by subculturing each transformant six times on PD media and then retesting each for glucose oxidase activity as described in Materials and Methods. Only one of the eighteen transformants was found to no longer produce glucose oxidase. This transformant was also no longer hygromycin resistant.

As PEG is known to stimulate protoplast fusion and hygromycin resistance is dominant, some of the transformants may have arisen as heterokaryons. To remove any heterokaryon transformants, single-spore purified cultures were isolated from ten of the primary transformants as described in Materials and Methods. To determine if the glucose oxidase gene was stably maintained through mitosis in these isolates, forty conidiospores derived from each of these spore purified cultures were tested for glucose oxidase activity as described in Materials and Methods. All the conidial colonies tested produced glucose oxidase. Ascospores resulting from selfing of each of the transformants were also analysed for glucose oxidase activity. All ascospore colonies tested (30 per transformant) produced glucose oxidase.

2.3.4.2 Analysis of glucose oxidase activity in cotransformants

To quantitate the amount of glucose oxidase produced by individual transformants, seven transformants were grown in broth containing 5% glucose, the fungal mycelia harvested and freeze dried. Cell free extracts for each were prepared as described in Materials and Methods and the glucose oxidase activity in each determined spectrophotometrically by assaying for the production of hydrogen peroxide (Frederick *et al.*, 1990). In order to determine the amount of glucose oxidase secreted by individual transformants, broth from the growth solution of each

Figure 2.8 A transformed and untransformed isolate of *T. macrosporus* FRR2417 on a calcium carbonate indicator plate. Clearing of agar is caused by acid dissolving the calcium carbonate and is correlated with glucose oxidase activity. The positive control is *T. flavus* 32908 from which the glucose oxidase gene was isolated.

transformant was collected 26 hours after inoculation, filter sterilized and assayed for glucose oxidase activity using the method described above. Experiments were performed in duplicate and repeated twice.

In vitro activities ranging from 91 to 790×10^{-3} units/mg were obtained for the freeze dried tissue and from 4 to 91×10^{-3} units/ml were obtained for the broth of the seven transformants analysed (units of activity as defined in the Materials and Methods). Approximately $4\text{-}5 \times 10^{-3}$ units of glucose oxidase activity were detected in extracts from the regenerated wild-type strain and GO5, a Hyg^{+}/GO^{-} transformant (Table 2.2). As no glucose oxidase activity was detected in the assay solution alone this activity is a measurement of the background level of hydrogen peroxide production in the fungal extract. No background activity was detected in the broth cultures.

The best transformant (GO10) secreted approximately 2.5 times as much glucose oxidase as the *T. flavus* 32908 isolate from which the gene was isolated. In general the level of activity between the two experiments correlated well, with the best glucose oxidase producers secreting the most glucose oxidase. The one exception is transformant GO13 in which the level of glucose oxidase secreted is low compared to the amount of enzyme produced. In this case the transformation event may have affected the fungal secretion pathway.

2.3.4.3 Molecular analysis of cotransformants

In order to examine the seven transformants at a molecular level, DNA from each was digested with *Xba*I. This enzyme cuts once within the *Eco*RI 7.7kb fragment resulting in 3.7kb and 4.0kb fragments. Southern blots of these digests were then probed with a 0.65kb ^{32}P-labelled *BamHI/Hpa*I fragment isolated from the *T. flavus* glucose oxidase gene. This fragment which is located within the coding region, hybridizes only to the 4.0kb *XbaI/Eco*RI fragment (Figure 2.9). Therefore, with the exception of tandem repeats, each hybridizing band should represent a single integration event and each band should be at least 4.0kb in size.

As shown in Figure 2.10 the *T. flavus* transformants analysed all have different hybridization patterns and as predicted all bands are greater than 4.0kb. Isolates GO4 and GO13 both show only one intense hybridization signal (20kb and 7kb

Fungal Isolate	Enzyme activity in freeze dried tissue[a] (Units/mg × 10^{-3})	Enzyme activity in broth[b] (Units/ml × 10^{-3})
Control	0.2 ± 0.3	0.1 ± 0.1
FRR 2417	4.9 ± 0.7	0.1 ± 0.1
GO3	91.5 ± 5.4	9.9 ± 2.7
GO4	308.7 ± 30.0	46.5 ± 3.6
GO5	3.9 ± 0.6	0.2 ± 0.1
GO8	791.4 ± 54.5	77.9 ± 4.2
GO10	861.7 ± 64.8	90.8 ± 5.6
GO13	102.7 ± 8.7	3.4 ± 1.7
GO21	450.2 ± 25.2	73.0 ± 3.6
GO27	498.3 ± 42.1	67.1 ± 2.5
***T. flavus* 32908**	356.6 ± 23.2	48.2 ± 3.1

Table 2.2 Glucose oxidase activity in *T. macrosporus* transformants. One unit of glucose oxidase activity is defined as the amount which will oxidise 1.0 μmole of β-D glucose to D-gluconic acid and H_2O_2 per minute at pH 5.1 and 25°C. Values are the means ± the standard errors. **a:** Glucose oxidase activity per mg of freeze dried tissue. **b:** Glucose oxidase activity in broth 26 hours after inoculation with equal amounts of fungus. The control assay contained all the assay components except fungal extract[a] or inoculated broth culture[b].

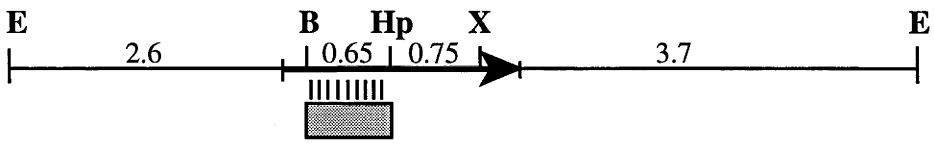


Figure 2.9 Hybridization strategy used to screen *T. macrosporus* transformants. The arrow indicates the size and direction of the glucose oxidase open reading frame. The grey box represents the ^{32}P-labelled *Hpa*I/*BamH*I fragment used to probe *T. macrosporus* transformant DNA digested with *Xba*I. Abbreviations: **E**, *Eco*RI; **B**, *BamH*I; **Hp**, *Hpa*I and **X**, *Xba*I. The size of various fragments is shown in kb.

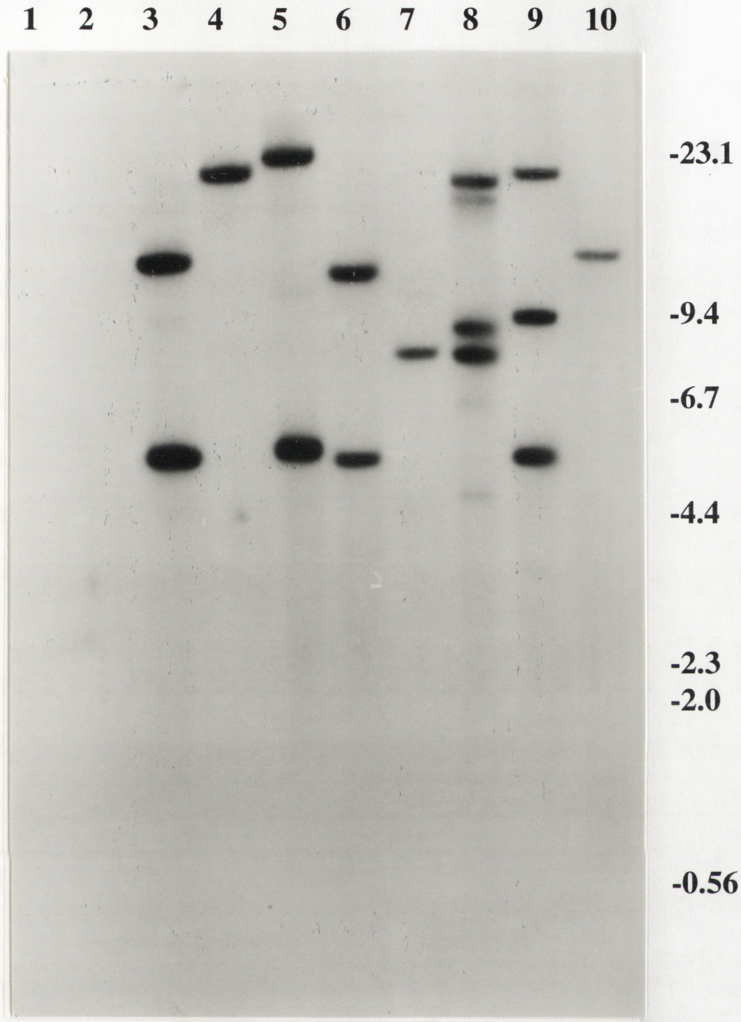


Figure 2.10 Autoradiograph of a Southern blot of *Xba*I digests of genomic DNA from *T. flavus* 32908, *T. macrosporus* FRR2417 and *T. macrosporus* transformants hybridized with a ^{32}P-labelled *BamHI*/*Hpa*I fragment from the *T. flavus* glucose oxidase gene. Lane 1, *T. macrosporus* FRR2417; lane 2, GO5; lane 3, GO3; lane 4, GO4; lane 5, GO8; lane 6, GO10; lane 7, GO13; lane 8, GO21; lane 9, GO27; lane 10, *T. flavus* 32908. The position of lambda *Hin*dIII DNA size standards are indicated in kb.

respectively) indicating that the glucose oxidase gene has integrated at a single site in these transformants. As expected *T. flavus* 32908 has only one band (approximately 11kb), indicating that only one copy of the gene is present in the genome. The lower intensity of hybridization seen is due to the loading of less DNA and is not a true representation of the intensity that would be expected for a single copy gene. The other transformants have two or three strong bands each, suggesting that in each case, the gene has integrated into two or three different places in the genome. It is possible that two of these transformants (GO21 and GO27) have tandem repeats of the *Eco*RI 7.7kb fragment integrated at a single site. Such an arrangement (head to head, tail to tail or tail to head) would create a 7.7kb and 8.0kb fragment respectively as well as one or two fragments of unknown length. Both GO21 and GO27 have bands of around 7-8kb however the intensity of the signals does not seem strong enough to suggest a long tandem repeat is involved in either case. No band is seen in FRR2417 DNA. As *T. macrosporus* and *T. flavus* are considered to be very closely related, this suggests the glucose oxidase gene has recently either inserted into the *T. flavus* genome or been deleted from the *T. macrosporus* genome.

A number of faint bands are seen in all lanes except for those containing untransformed wild-type DNA, GO5 (Hyg^+/Gox^-) DNA and *T. flavus* 32908 DNA. Most of these fragments are too small to be attributed to partial digestion and as none are seen in *T. flavus* 32908 DNA it is assumed they are not the result of the probe hybridising to the 3.7kb region of the *Eco*RI fragment. Similarly, an absence of bands in GO5 indicates the probe is not hybridizing weakly to pAN7-1. It is possible that the *Eco*RI 7.7kb fragment recombined with pAN7-1 or the pUC119 DNA before integrating into the genome. The use of two transforming plasmids does seem to increase the recombination process during chromosome integration (Nowak and Kück, 1994), and in this experiment three pieces of linear DNA were introduced. The faint bands observed could result if recombined DNA containing small regions of the glucose oxidase gene was present in the genome.

2.4 DISCUSSION

The *A. niger* glucose oxidase gene has been used to isolate the glucose oxidase gene from *T. flavus*. Confirmation that the gene is glucose oxidase has been established in two ways. First, the sequence contains a single ORF that predicts a protein sequence which has substantial homology with the *A. niger* protein sequence. Second, the gene has been successfully expressed in *T. macrosporus*, a fungus which does not naturally produce glucose oxidase.

Including 5' and 3' untranslated regions, a total region of 3032bp of DNA has been sequenced. Located within the 5' untranslated region there are several potential promoter elements. A TATAAA sequence is present 122bp upstream of the ATG. This motif, thought to be involved in binding of the TFIII component of the general transcription machinery is found in many fungal genes 50-150bp from the translation initiation site (Unkles, 1992). The TATAAA sequence identified is therefore placed in an appropriate position to be functional. Downstream of the TATAAA element are two CAAT sequences. However, if the TATAAA sequence is functional it seems unlikely that these sequences would have any significant role given that TATAAA elements are normally positioned closer to the initiation codon than CAAT elements (Nussinov, 1986). Another CAAT sequence is located 483bp upstream of the initiation codon. As CAAT elements have been found more than 300bp distant to transcriptional start points in filamentous fungi (Vanhanen *et al.*, 1991; Iturriaga *et al.*, 1990), it is possible that this region is significant. Also present in the promoter region, approximately 60bp downstream of the TATAAA sequence, are several CT boxes. These pyrimidine rich tracts have been found in many fungal promoters and may be important in determining the site of transcription initiation (Hamer and Timberlake, 1987; Punt *et al.*, 1990). However, to verify the significance of any of these elements in the transcription process, a functional analysis of the region is required.

No AATAAA polyadenylation signal was found in the 3' untranslated region; however a possible AAATA polyadenylation sequence is present 162bp downstream of the stop codon. In addition, the 3' region contains a large AT-rich region of 120bp (70% AT) which could be involved in termination of transcription.

The initiating ATG is located 1032bp downstream from the proximal end of the region sequenced. The sequence around this ATG is similar to the fungal consensus structure proposed by Ballance (1991). In particular there is a C at position -4 and an A at positions -1 and -2 (with respect to the initiation codon). The ORF it initiates codes for a protein of 605 amino acids, the first 22 amino acids of which codes for a secretion signal sequence. The protein has 7 potential sites for N-linked glycosylation, all of which contain the sequence Asn-X-Thr. The absence of any Asn-X-Ser sites may be an indication that Asn-X-Thr is the preferred glycosylation sequence in this fungus as it is in *S. cerevisiae* (Moehle *et al.*, 1987). Excluding the contribution of the putative signal peptide and carbohydrate structure, the predicted protein is estimated to have a molecular weight of 63.5kDa and an isoelectric point of 4.88. These values are similar to those determined by Kim *et al.* (1990a). Using SDS PAGE and isoelectric focusing they found that *T. flavus* glucose oxidase had a molecular weight of 71kDa and isoelectric point of 4.45. This therefore suggests that approximately 10% of the molecular mass of *T. flavus* glucose oxidase is due to carbohydrate. A similar proportion of carbohydrate is found in *A. niger* glucose oxidase (Frederick *et al.*, 1990).

When compared with other protein sequences, the *T. flavus* protein is most similar to *A. niger* glucose oxidase. The homology is spread over the entire protein with the exception of the secretion signal sequence which is only 25% identical to the signal sequence of *A. niger* glucose oxidase. However, the two signal sequences are 60% similar so although the amino acid sequence has not been strictly conserved, amino acids with similar properties are often present in identical positions in the two signal peptides. Such changes are probably not detrimental to the function of the signal peptide as signal peptides require amino acids with similar properties in specific regions of the presequence rather than specific amino acids. This flexibility within the signal peptide sequence may have resulted in the signal peptide sequence from the glucose oxidase gene diverging more quickly than the rest of the coding sequence. Interestingly, an area at the C-terminus (around aa 540, with respect to the initiating methionine) identified by Frederick *et al.* (1990) as potentially being related to the active site domain of disulfide oxidoreductases is not highly conserved. Only the cysteine residue conserved in most oxygen dependent flavin oxidases (glucose

oxidase and alcohol oxidase) is present and correctly positioned in *T. flavus* glucose oxidase (Figure 2.11). This suggests the region identified by Frederick may not in fact be related to the disulfide active site. The *T. flavus* protein does however contain several regions that are similar to several areas in the GMC oxidoreductase proteins. One of these regions corresponds to the FAD ADP-binding domain, but the function of the other regions has yet to be determined.

In order to demonstrate the function of the isolated gene it was necessary to express the gene in a different fungal species. For this reason a transformation system for *T. macrosporus* was developed. The transformation frequency obtained (20 transformants/µg DNA) is low compared to other fungal species such as *Trichoderma spp* (200-800 transformants/µg DNA) (Herrera-Estrella *et al.*, 1990) and *Penicillium paxilli* (100-200 transformants µg/DNA) (Itoh *et al.*, 1994). It is however comparable to frequencies obtained for *Botrytis cinerea* (Hamada *et al.*, 1994) and *Phanerochaete chrysosporium* (Gessner and Raeder, 1994). In particular, it is very similar to the transformation frequency obtained for *Talaromyces* sp CL240 by Jain *et al.* (1992). They obtained a transformation frequency of 10 transformants/µg of DNA and the same protoplast regeneration frequency (5-10%), using a transformation procedure very similar to the one described here. They use phleomycin resistance as a dominant selectable marker but the resistance gene was under the control of the same *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter used here.

It is probable that the transformation frequency obtained for *T. macrosporus* can be improved given that many of the variables involved in the procedure were not optimized. Different factors, such as the age of the culture used, the choice of osmotic stabiliser and the time protoplasts are incubated in polyethylene glycol have all been found to influence transformation frequency (Kelly *et al.*, 1994). Optimization of these conditions could prove beneficial. Improvement in transformation efficiency has also been obtained when homologous expression signals are fused to the selection gene. In *Trichoderma reesei*, Mach *et al.*(1994) obtained a 15-20 fold increase in transformation frequency when they replaced the *A. nidulans* 5' and 3' sequences of pAN7-1 with expression signals from *T. reesei.* A

	aa position	
GOD(T)	536-548	NWHAVSS**C**SM---MS-R
GOD(A)	536-548	NYHGV**GTC**SM---M**P**-K
AOX(H)	566-578	TWHCL**GTC**SM---A**P**-R
AOX(C)	565-577	TWHCL**GT**NSM---A**P**-R
AOX(P)	565-577	TWHCL**GTC**SI---G**P**-R
GRDE	35-51	AKELG**GTC**VN**VGC**V**P**KK
MRED	129-145	RGTIG**GTC**VN**VGC**V**P**SK
LPDH	58-74	RGKLG**GTC**LN**VGC**I**P**SK

Figure 2.11 Comparison of the active site region of disulphide oxidoreductases with the sequence around amino acid (aa) 540 of glucose oxidase (with respect to the initiating methionine). Conserved residues are shown in bold. The enzymes are as in Figure 2.6 with the addition of AOX(P), alcohol oxidase from *Pichia pastoris* (Koutz *et al.*, 1989); GRDE, glutathione reductase from *E. coli* (Greer and Perham, 1986); MRED, mercuric reductase from *Pseudomonas aeruginosa* (Brown *et al.*, 1983) and LPDH, lipoamide dehydrogenase from *S. cerevisiae* (Ross *et al.*, 1988).

similar approach in *T. macrosporus* may also increase efficiency. Another option could be to use biolistic transformation. This method has been compared to the protoplast-mediated procedure for *Trichoderma harianum* and *Gliocladium virens* (Lorito *et al.*, 1993). In both cases the biolistic method was found to produce higher levels of transformation. However, for other fungi such as *Botrytis cinerea*, biolistic transformation is less efficient (Hilber *et al.*, 1994; Hamada *et al.*, 1994).

Very few abortive transformants were seen in this transformation system. Such transformants, which are often smaller than stable transformants, are thought to occur when the transforming DNA does not integrate stably into the genome and is only transiently expressed. Loss of this DNA results in the loss of the selectable marker and so is lethal when the transformant is grown on selection media. Given that the frequency of these transformants seems to vary from fungus to fungus it is not known why only one or two abortive transformants were seen here. Jain *et al.* (1992) also observed very few of these transformants when transforming *Talaromyces* sp CL240 so the low frequency observed may simply be a characteristic of this particular genus and the vectors used.

Transformation of the putative *T. flavus* glucose oxidase gene into *T. macrosporus* with pAN7-1 resulted in 62% of hygromycin resistant transformants producing glucose oxidase. This not only confirmed the identity of the gene but produced a number of potential biocontrol organisms as well (see Chapter 3). Also, as 2.4kb of promoter was transferred with the glucose oxidase gene into the transformants it is likely that any signal sequences required for the induction of glucose oxidase are present. Induction experiments with for example, glucose, could be performed to determine if the signal sequences required for induction are present or absent.

Good levels of enzyme activity were detected in the cotransformants with the best transformant secreting approximately two and a half times as much glucose oxidase as *T. flavus* 32908. As the two fungal species are very closely related, obtaining such good expression levels of a foreign protein is not surprising. The fact that even higher levels of the enzyme were not seen may be a reflection of the toxicity of the gene product. However, only a few transformants were analysed and examination of

more may reveal higher producers. The glucose oxidase gene was stably maintained in all but one of the eighteen cotransformants analysed. Single-spore isolates derived from ten of the seventeen glucose oxidase-producing transformants also stably maintained the glucose oxidase gene through mitosis and meiosis. This high degree of stability is not unusual and is consistent with the high degree of stability reported for transformants of other filamentous fungi (Ballance, 1991; Nara *et al.*, 1993; Hamada *et al.*, 1994).

Southern analysis confirmed that the transformed DNA had integrated into the genome of *T. macrosporus* with both the number and sites of integration varying between transformants. Several of the transformants had DNA integrated into single sites in the genome whereas in others the DNA had integrated into two or three locations. Two of the transformants examined may have integrated DNA present as tandem repeats however the intensity of the hybridization signal suggests the repeat is quite short in both transformants. As different hybridization patterns were obtained for individual transformants, it appears that the transforming DNA does not integrate preferentially into sites in the *T. flavus* genome. There is no correlation between the level of glucose oxidase expression and the number of integration sites. This has been observed for other fungal systems (Salch and Beremand, 1993) and probably reflects position effects created by integration of DNA at different sites within the chromosome. A number of faint bands seen in all the glucose oxidase-producing transformants may be the result of the DNA recombining with other introduced DNA before it integrates into the genome.

Southern analysis also showed that no glucose oxidase sequences were present in the wild-type *T. macrosporus* genome. As these two species are very closely related this is surprising but it does support the view of Frisvad *et al.* (1990) that *T. macrosporus* and *T. flavus* are two distinct species. Presumably, the glucose oxidase gene is either a recent addition to *T. flavus* or deletion from *T. macrosporus*.

CHAPTER 3
THE ROLE OF GLUCOSE OXIDASE IN THE BIOCONTROL OF VERTICILLIUM WILT OF COTTON BY *T. FLAVUS*

3.1 INTRODUCTION

Although *T. flavus* has been used experimentally as a biocontrol agent to help suppress Verticillium wilt in eggplants, tomatoes, potatoes, and artichokes, (Marois *et al.*, 1982; Dutta, 1981; Nagtzaam, 1994; Tjamos and Paplomatas, 1988) the mode of action(s) used by *T. flavus* to control *V. dahliae* is not fully understood. As previously mentioned in Chapter 2, *T. flavus* secretes an enzyme, glucose oxidase, that has been implicated in the suppression of *V. dahliae*. In the presence of glucose, glucose oxidase generates hydrogen peroxide that inhibits the growth of *V. dahliae* mycelia and microsclerotia *in vitro* (Fravel *et al.*, 1987; Kim *et al.*, 1988). The finding that *V. dahliae* is particularly sensitive to small amounts of hydrogen peroxide (Kim *et al.*, 1990b) supports a role for glucose oxidase in the suppression of Verticillium wilt.

Most inhibition experiments with glucose oxidase have been performed *in vitro* where there has been an abundant supply of glucose. The scarcity of glucose in the soil environment has raised doubts as to whether enough hydrogen peroxide would be produced *in vivo* for glucose oxidase to be an important factor in the control of *V. dahliae* in the field. Recently, Fravel and Roberts (1991) showed that in pot trials, purified glucose oxidase from a biocontrol strain of *T. flavus* significantly reduced the growth rate of *V. dahliae* microsclerotia in the presence, but not in the absence, of eggplant roots. This demonstrates that eggplant roots have the potential to supply glucose oxidase with glucose. However, adding extra glucose with the enzyme was found to kill microsclerotia, suggesting that the availability of glucose limits production of hydrogen peroxide *in vivo*. They also isolated a single ascospore variant of a *T. flavus* biocontrol strain which produced only 2% of the parent level of glucose oxidase. This isolate failed to control Verticillium wilt in eggplants whereas the parent significantly reduced the incidence of wilt. However, using SDS PAGE gels it was found that the variant produced two low molecular weight proteins at

much higher levels than the wild-type parent. So although these findings suggest glucose oxidase has an important role in the antagonism between *T. flavus* and *V. dahliae in vivo*, it is also possible the change in abundance of these low molecular weight proteins or other antimicrobial compounds may have affected the biocontrol ability of the *T. flavus* variant.

Other experiments have shown that glucose oxidase is not the only compound inhibitory to *V. dahliae* secreted by *T. flavus*. Madi *et al.* (1989) found that the addition of the peroxide scavenger catalase to the culture filtrate of various *T. flavus* isolates resulted in a loss of only 50% of its toxic activity towards *V. dahliae*. Also present in the culture filtrate were a number of lytic enzymes including cellulase, β 1-3 glucanase and chitinase. Such enzymes have been implicated in the antagonistic activity of other biocontrol agents such as *Trichoderma harzianum* and *Pythium nunn* where they are thought to be important in breaking down the pathogen's cell walls (Elad *et al.*, 1982; Elad *et al.*, 1985; Ridout *et al.*, 1986; Sivan and Chet, 1989).

Parasitism of *V. dahliae* microsclerotia by *T. flavus* hyphae has also been reported (Fahima and Henis, 1992). Transmission electron micrographs of microsclerotia incubated with *T. flavus* on agar show *T. flavus* produces small penetration pegs which lyse the microsclerotia cell wall at the site of contact with the hyphal tip. Inside the cell these penetration pegs give rise to hyphae which degrade the host's cell contents and use it as a nutrient source to produce more penetration pegs. These penetration pegs invade neighbouring host cells by penetrating their walls at specific sites, or by using natural openings between cells rather than lysing the cell. The lytic enzymes identified by Madi *et al.* are probably responsible for digesting the cell wall of the microsclerotia prior to penetration by *T. flavus* hyphae. In this situation, glucose oxidase may be important in weakening sclerotia and predisposing them to infection (Kim *et al.*, 1988).

The importance of glucose oxidase in the antagonism of *V. dahliae* by *T. flavus* is therefore not clear. The recent advances in the manipulation of filamentous fungi by molecular techniques have provided new tools with which to analyse fungal

organisms. In particular, the technique of targeted gene disruption in which homologous recombination permits the replacement of specific genomic sequences with sequences that have been altered *in vitro*, is useful to analyse the role of specific gene products. The cloning of the *T. flavus* glucose oxidase gene and the development of a transformation system for *T. macrosporus* (Chapter 2) allows this technique to be used to construct a *T. flavus* mutant in which only the glucose oxidase gene is inactivated. As *T. flavus* is haploid for most of its life cycle and the glucose oxidase gene appears to be present as only a single copy in the genome, the effect of this mutation can be analysed directly.

Described in this chapter is the creation of such a glucose oxidase-deficient *T. flavus* mutant. This glucose oxidase-deficient mutant, the best glucose oxidase-producing *T. macrosporus* transformant produced in Chapter 2 and both wild-type parents are examined *in vitro* and *in vivo* for their ability to inhibit *V. dahliae*. In this way, the importance of glucose oxidase in the inhibition of Verticillium wilt by *T. flavus* is examined. In addition, the catalase activity in *T. flavus*, *T. macrosporus* and *V. dahliae* are examined. As catalase very efficiently reduces hydrogen peroxide to oxygen and water, the level of activity in the different fungi would be expected to be an important factor in determining the tolerance of fungi to hydrogen peroxide. As *V. dahliae* is particularly sensitive to hydrogen peroxide, it would be expected to have less catalase activity than the *Talaromyces* isolates which would presumably have higher levels of the enzyme to protect themselves from oxidative damage by glucose oxidase generated hydrogen peroxide.

3.2 MATERIALS AND METHODS

3.2.1 *V. dahliae* strains

V. dahliae isolates (#2 and #13) previously isolated from diseased cotton were kindly supplied by Dr S. Allen, Agricultural Research Station, Narrabri, Australia. Isolates were maintained on Czapek-Dox agar (Difco) and grown at 21°C in the dark. Both isolates produced abundant microsclerotia after growing for 8-12 weeks in culture.

3.2.2 Construction of pHGO-1

A 4.4kb *Sac*I fragment from pGO containing the glucose oxidase ORF and approximately 2.4kb and 150bp of adjacent 5' and 3' DNA respectively, was cloned into plasmid pJKKm (Kirschman and Cramer, 1988) to create pGOK. The hygromycin resistance cassette from pAN7-1 (Punt *et al.*, 1987) was excised by digesting the plasmid with *Bgl*II and *Hin*dIII and both ends of the fragment were blunted by treatment with the Klenow fragment of DNA polymerase I. This fragment was then inserted into the *Hpa*I restriction site located in the middle of the glucose oxidase coding region in pGO-2 and restriction digests performed to determine the orientation of the antibiotic resistance cassette with respect to the glucose oxidase gene.

3.2.3 Glucose oxidase assays

Glucose oxidase enzyme assays, indicator plates and determination of glucose oxidase activity in conidiospores and ascospores were carried out as described in Chapter 2.

3.2.4 Southern analysis

As described in Chapter 2.

3.2.5 *In vitro* inhibition assays

For production of culture filtrate, five circular agar disks, 4mm in diameter (cut with a sterile cork borer) from 6 day old cultures of *T. flavus* were transferred to 100ml of PD broth containing 5% glucose. Replica flasks of the liquid cultures were maintained at 26°C on a rotary shaker at 100 rpm for 48 hours. If necessary, the broth was then adjusted to pH 5.4 with 1M KOH before being filtered through a 0.45μm filter to remove fungal hyphae and spores. Conidiospores of *V. dahliae* were generated by growing *V. dahliae* in 100ml of PD broth on slow shakers (80 rpm) at 21°C in the light for 2-3 weeks. Spores were harvested by filtering the culture through 2 layers of sterile Whatman #2 paper and the concentration of spores determined microscopically using a haemocytometer. Filtrate was assayed for ability to inhibit *V. dahliae* growth using the method of Broekaert *et al.* (1990) but with the following modifications. Filtrate or filtrate diluted with PD broth was dispensed into

wells of a sterile microtitre plate (total volume 170µl) and 1×10^5 *V. dahliae* conidiospores (in a volume of 30µl) were added to each well. Spores were allowed to sediment for 20 minutes, after which the absorbance of the wells was measured at 595nm with a Multiskan plus microplate reader. The plate was incubated at 21°C in the dark on a slow shaker (80 rpm) and absorbance readings taken after appropriate incubation periods. Given absorbance values were calculated by subtracting the values of the first measurement from those of the second and following measurements.

3.2.6 Calculation of IC_{50} values

Culture filtrates were prepared as described above except glucose oxidase activity in the filtrate was determined as previously described in Chapter 2. A range of glucose oxidase concentrations were prepared by diluting the filtrate with different amounts of PD broth and the growth of *V. dahliae* in the different filtrates was determined as described above. Percent growth inhibition was calculated by comparing *V. dahliae* growth in culture filtrate to *V. dahliae* growth in unaltered PD broth.

3.2.7 Biocontrol trials

Cotton seedlings (*cv.* Coker 315) were surface sterilized by placing seeds in 70% ethanol for 30 seconds and then transferring to a 30% bleach solution containing 2 drops/100ml of Tween 20 for 20 minutes. After washing 6 times with sterile distilled water, seeds were transferred to pots containing germination media (MS (Murashige and Skoog, 1962) solidified with 2.0g/L Gelrite and 0.94g/L $MgCL_2$) and placed in low light conditions at 26°C. Six days later seedlings were removed from pots and their roots washed in sterile water before being infected with *V. dahliae*. A *V. dahliae* microsclerotial suspension was prepared by blending water and agar containing microsclerotia in a Waring blender for 30 seconds. The suspension was filtered through a sterile 2mm nylon mesh to remove large particles of agar or fungal material and the number of infective *V. dahliae* propagules/ml of solution determined using a Weber scientific counting chamber (0.2mm depth). Roots of the cotton seedlings were dipped in the *V. dahliae* microsclerotial suspension (1×10^8 propagules/ml) or water (control) before being planted in soil (previously steam stearilized). Five ml of biocontrol agent (2×10^6 propagules/ml - prepared as for the

microsclerotial suspension except 7 day-old *T. flavus* or *T. macrosporus* cultures were used) or water (control) was placed around the roots of each seedling prior to the roots being covered with soil (9 seedlings equally spaced in a 22cm square container). Eighteen hours after planting, 1ml of *V. dahliae* microsclerotial suspension (1×10^7 propagules/ml) and 1ml of biocontrol suspension (1×10^6 propagules/ml) was poured around the hypocotyl of each seedling. Plants were grown in an 'Environ Air' growth cabinet under the following conditions: 24°C, 16 hours light and 16°C, 8 hours dark for 9 weeks.

3.2.8 Catalase assays

Mycelia from 7 day old fungal cultures grown on agar were removed from plates with a spatula and emulsified in a mortar and pestle with 50mM KH_2PO_4 (pH 7.2). After centrifugation at 13000 rpm for 15 minutes the supernatant was removed and filtered through a 0.45µm filter. Total protein in each extract was determined using the method of Bradford (1976). Catalase activity was determined using a modification of the method of Ueda *et al.* (1990). In a quartz curvette 600µl of 40mM H_2O_2 and 50mM KH_2PO_4 (pH 7.2) and fungal extract to a total volume of 1.6ml was mixed and let stand for 30 seconds at room temperature. The decrease in absorbance at 240 nm was then followed spectrophotometrically and the rate of hydrogen peroxide decomposition calculated in units/µg total protein by comparing values to a standard curve prepared with commercially available catalase isolated from bovine liver (Sigma). One unit is defined as that amount which will decompose 1.0 µmole of H_2O_2 per minute at pH 7 and 25°C.

3.3 RESULTS

3.3.1 Targeted disruption of the *T. flavus* glucose oxidase gene

In order to specifically disrupt the *T. flavus* glucose oxidase gene, the plasmid pHGO-1 was constructed. This plasmid contains the complete glucose oxidase coding region plus approximately 2.2kb of adjacent 5' region and 200bp of 3' region (4.2kb total homologous DNA). Inserted into the *Hpa*I site in the middle of the glucose oxidase ORF is the cassette coding for hygromycin resistance from pAN7-1 (Figure 3.1). Disruption of the glucose oxidase gene in this way should inactivate the

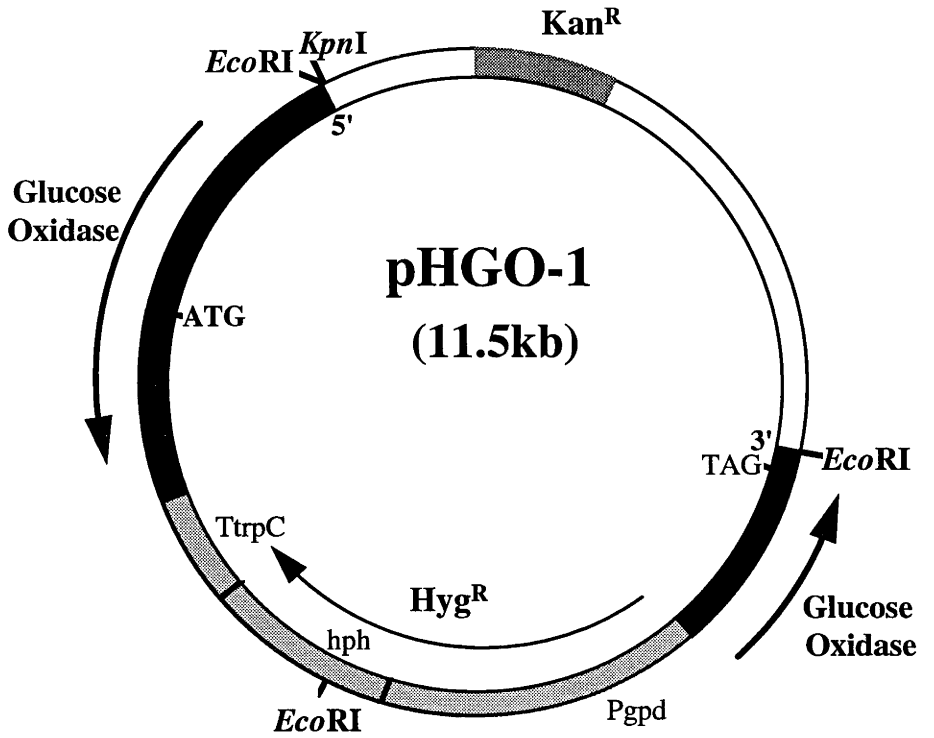


Figure 3.1 Schematic diagram of plasmid pHGO-1. Black regions represent the glucose oxidase gene and adjacent 5' and 3' regions. The ATG and TAG indicate the start and finish of glucose oxidase open reading frame. The grey region inserted into the open reading frame of the glucose oxidase gene represents the cassette coding for hygromycin resistance (Hyg^R) from pAN7-1 (Punt *et al.*, 1987). This consists of the hygromycin resistance gene (hph) fused to the *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter fragment (Pgpd) and a *A. nidulans* trpC terminator (TtrpC). Also shown is the gene coding for kanamycin resistance (Kan^R). Arrows indicate the orientation of the genes.

gene, in addition the hygromycin gene acts as the selectable marker in the transformation of *T. flavus*. As gene replacement has been shown to occur more frequently with linearized DNA (Fotheringham and Holloman, 1989; Hoskins *et al.*, 1990) plasmid pHGO-1 was linearized with *Kpn*I before being transformed into *T. flavus* 32908 using the procedure developed for *T. macrosporus*. Transformants were selected on hygromycin and then screened for loss of glucose oxidase activity on two indicator plates. As a control, *T. flavus* 32908 was also separately transformed with linearized pAN7-1.

Only four transformants were obtained from the transformation with pAN7-1 and only one transformant, GOH-1, arose from transformation with pHGO-1. Fortuitously, this single transformant failed to secrete glucose oxidase on either indicator plate. Enzyme assays performed on a crude protein extract from GOH-1 confirmed that no glucose oxidase was being produced by the transformant (results not shown). The mutant appeared to grow at the same rate as wild-type *T. flavus* 32908 and sporulated extensively producing both conidia and cleistothecia. The stability of the mutant phenotype was confirmed by subculturing GOH-1 six times on PD media without selection and retesting hyphae for glucose oxidase activity. Also fifty single conidiospore isolates and thirty single ascospores resulting from selfing of GOH-1 were analysed for glucose oxidase activity. Neither the hyphae nor any of the single spore derivatives tested produced glucose oxidase, confirming that the mutant phenotype was stable through mitosis and meiosis.

To obtain more detailed information on the integration of vector DNA into the genome of GOH-1, a Southern analysis was performed. Genomic DNA isolated from *T. flavus* 32908 and GOH-1 were both separately digested with *Eco*RI and *Hpa*I. Southern blots were then probed with ^{32}P-labelled pHGO-1. If homologous recombination and gene replacement had occurred, GOH-1 DNA digested with *Hpa*I should have a hybridization pattern different to that of *T. flavus* 32908, as the *Hpa*I site in the glucose oxidase gene was destroyed when the hygromycin resistance cassette was cloned into it. Also, as no *Hpa*I sites are present in pHGO-1, each hybridization band should correspond to a single integration site in the *T. flavus* genome. Similarly, as an *Eco*RI site is present in the hygromycin resistance gene a

different hybridization pattern should be observed in wild-type and GOH-1 DNA digested with *Eco*RI. As the 7.7kb *Eco*RI fragment has been mapped (Figure 2.1) it can be predicted that two hybridization bands of 4.7kb and 7.0kb would be seen if gene disruption occurred by homologous recombination as outlined in Figure 3.2.

As can be seen in Figure 3.3, GOH-1 and *T. flavus* 32908 DNA digested with *Hpa*I and *Eco*RI do indeed have different hybridization patterns. As expected when digested with *Hpa*I, two bands (approximately 4.4kb and 20kb) are seen in wild-type *T. flavus* 32908 DNA. The larger band is probably fainter than the 4.4kb band because it contains a smaller region of homology to the probe. Only a single 23kb band in seen in GOH-1 DNA digested with *Hpa*I. Although it is possible this large band results from vector DNA integrating into several sites in the genome, the intensity of the band suggests it represents a single integration event. When digested with *Eco*RI the expected 7.7kb fragment is seen in wild-type *T. flavus* 32908 DNA and two bands of the sizes predicted are seen in GOH-1 DNA. The large, very faint bands seen in both lanes result from partial digestion of the DNA. The absence of any other significant bands in GOH-1 confirms that only a single copy of the vector DNA has integrated into the glucose oxidase gene.

3.3.2 *In vitro* inhibition studies on *V. dahliae* using wild-type and genetically modified isolates of *T. flavus* and *T. macrosporus*

Four different fungal isolates (*T. flavus* 32908, GOH-1, *T. macrosporus* FRR2417 and GO10) were examined for their ability to inhibit *V. dahliae* growth *in vitro*. Filter sterilized culture filtrate derived from each isolate was inoculated with *V. dahliae* spores and fungal growth was monitored spectrophotometrically. Duplicates were performed for each experiment and the whole experiment repeated three times. The profile of *V. dahliae* growth in all culture filtrates can be seen in Figure 3.4.

To further quantify the data, the growth rate of *V. dahliae* was calculated using the linear period of growth that occurred 24 to 48 hours after the start of incubation. This data is shown in Table 3.1. No difference in *V. dahliae* growth rate is observed between *T. macrosporus* FRR2417 filtrate and uninoculated broth. Growth in filtrate from the *T. macrosporus* glucose oxidase-producing transformant, GO10, is much

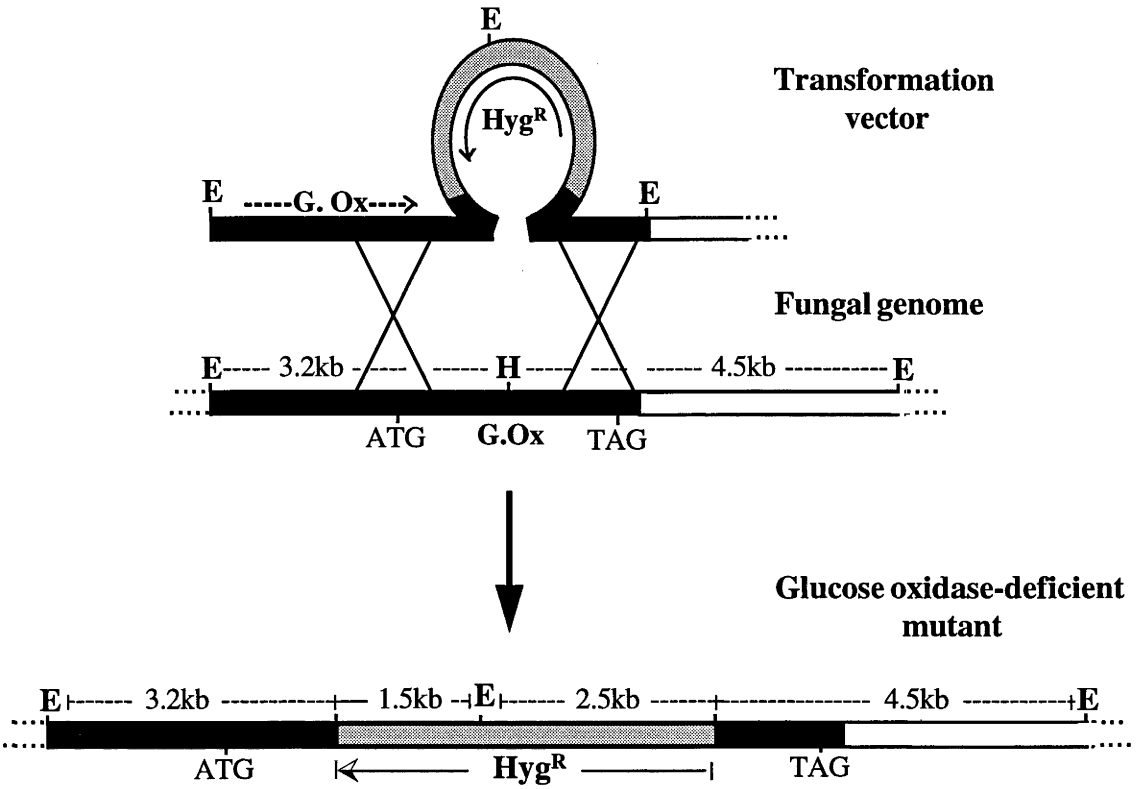


Figure 3.2 Diagrammatic representation of the creation of the *T. flavus* glucose oxidase-deficient mutant. The glucose oxidase gene is replaced by the disrupted glucose oxidase gene from the transformation vector by twofold homologous recombination. Black regions represent regions of homology between the plasmid and the fungal genome. The ATG and TAG indicate the start and finish of the glucose oxidase open reading frame. The grey region represents the cassette coding for hygromycin resistance (Hyg^R). Arrows indicate the orientation of genes. Abbreviations are as follows, E, *Eco*RI; H, *Hpa*I; G.Ox, glucose oxidase.

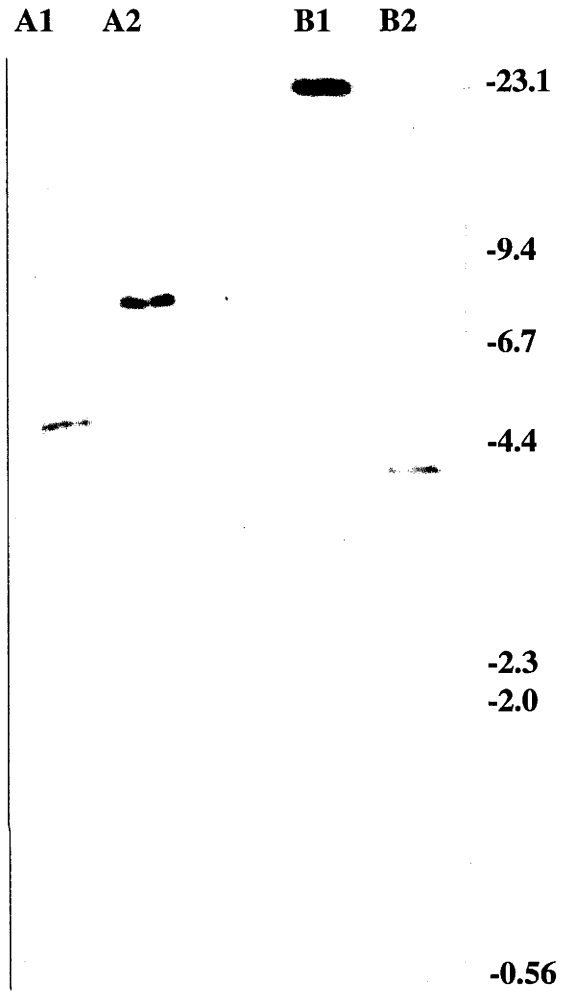


Figure 3.3 Southern hybridization analysis of glucose oxidase-deficient transformant GOH-1. DNA was hybridized with ^{32}P-labelled pHGO-1. **A1**: GOH-1 genomic DNA digested with *Eco*RI; **A2**: *T. flavus* 32908 genomic DNA digested with *Eco*RI; **B1**: GOH-1 genomic DNA digested with *Hpa*I; **B2**: *T. flavus* 32908 genomic DNA digested with *Hpa*I. Sizes of molecular weight markers are shown in kb.

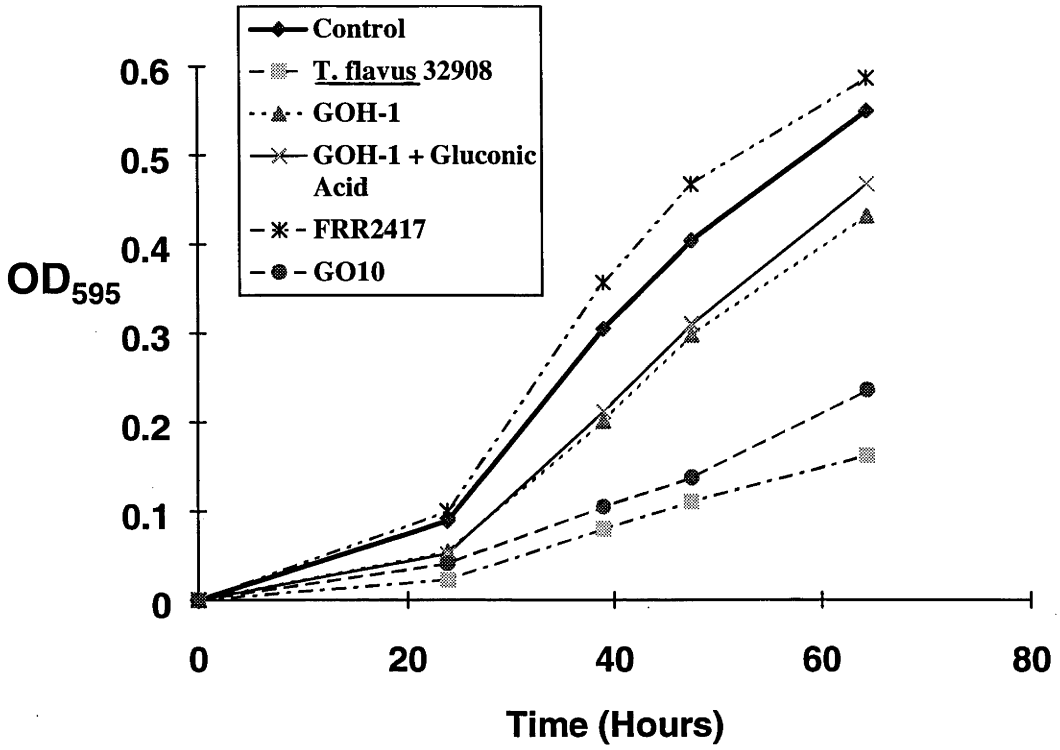


Figure 3.4 *In vitro* inhibition of *V. dahliae* with culture filtrate from different *Talaromyces* isolates. Growth over 65 hours in a microtitre plate was monitored by measurements of absorbance at 595nm. Inoculum density was 1×10^5 spores/well. Maximum standard error was ± 7% of the values obtained. For the sake of clarity standard error bars on the graph are omitted.

Culture Filtrate	Rate of *V. dahliae* growth OD Units/day
Uninoculated broth	0.315
***T flavus* 32908**	0.088
GOH-1	0.244
GOH-1 + gluconic acid	0.258
***T. macrosporus* FRR 2417**	0.313
GO10	0.096

Table 3.1 Growth rate of *V. dahliae* in culture filtrate derived from different *Talaromyces* isolates. Rates were calculated from data used to construct Figure 3.4 using the the linear period of growth which occurred 24 to 48 hours after the start of incubation.

slower, approximately 35% of the rate seen in the wild-type filtrate. Wild-type *T. flavus* 32908 filtrate also substantially inhibits the rate of *V. dahliae* growth. Growth in filtrate from the glucose oxidase-deficient mutant, GOH-1 is approximately 2.8 times faster that seen in *T. flavus* 32908 filtrate, however it is still only 78% of the rate observed in uninoculated broth. The presence of gluconic acid in the broth of GOH-1 during its growth does not affect the rate of *V. dahliae* growth in its filtrate. However, it was found that diluting the GOH-1 filtrate by 50% with water increased the *V. dahliae* growth rate by 8 to 11% to approximately 86 to 89% of that seen in uninoculated broth. Autoclaving the GOH-1 filtrate had no effect on *V. dahliae* growth rate (results not shown).

To compare the inhibitory ability of the filtrate from *T. flavus* 32908 and *T. macrosporus* GO10, the concentration of glucose oxidase in the filtrate required for 50% inhibition of *V. dahliae* (IC_{50}) was calculated (Figure 3.5). The amount of glucose oxidase in the culture filtrate was determined by comparison with standard concentrations of *A. niger* glucose oxidase. Units of activity are as defined in Chapter 2 Materials and Methods.

After 28 hours the IC_{50} for *T. flavus* 32908 was 0.04 units/ml (equivalent to 2.2µg of *A. niger* glucose oxidase) and for *T. macrosporus* GO10 was 0.11 units/ml (5.9µg/ml of *A. niger* glucose oxidase). The extent of growth inhibition tended to decrease as the incubation time increased; after 42 hours the IC_{50} values for *T. flavus* 32908 and *T. macrosporus* GO10 were 0.06 units/ml and 0.15 units/ml respectively. This increase in IC_{50} value over time has also been found by Cammue *et al.* (1992) when assaying the activity of antifungal proteins and is probably due to loss of enzyme activity over time. In both cases however, approximately 2.5 times more glucose oxidase activity was required in *T. macrosporus* GO10 filtrate to inhibit *V. dahliae* growth by 50%.

3.3.3 Biocontrol efficacy of wild-type and genetically modified *T. flavus* and *T. macrosporus* isolates

Cotton plants grown in soil containing *V. dahliae* and one each of the test organisms *T. flavus* 32908, *T. flavus* GOH-1, *T. macrosporus* FRR2417 or *T. macrosporus*

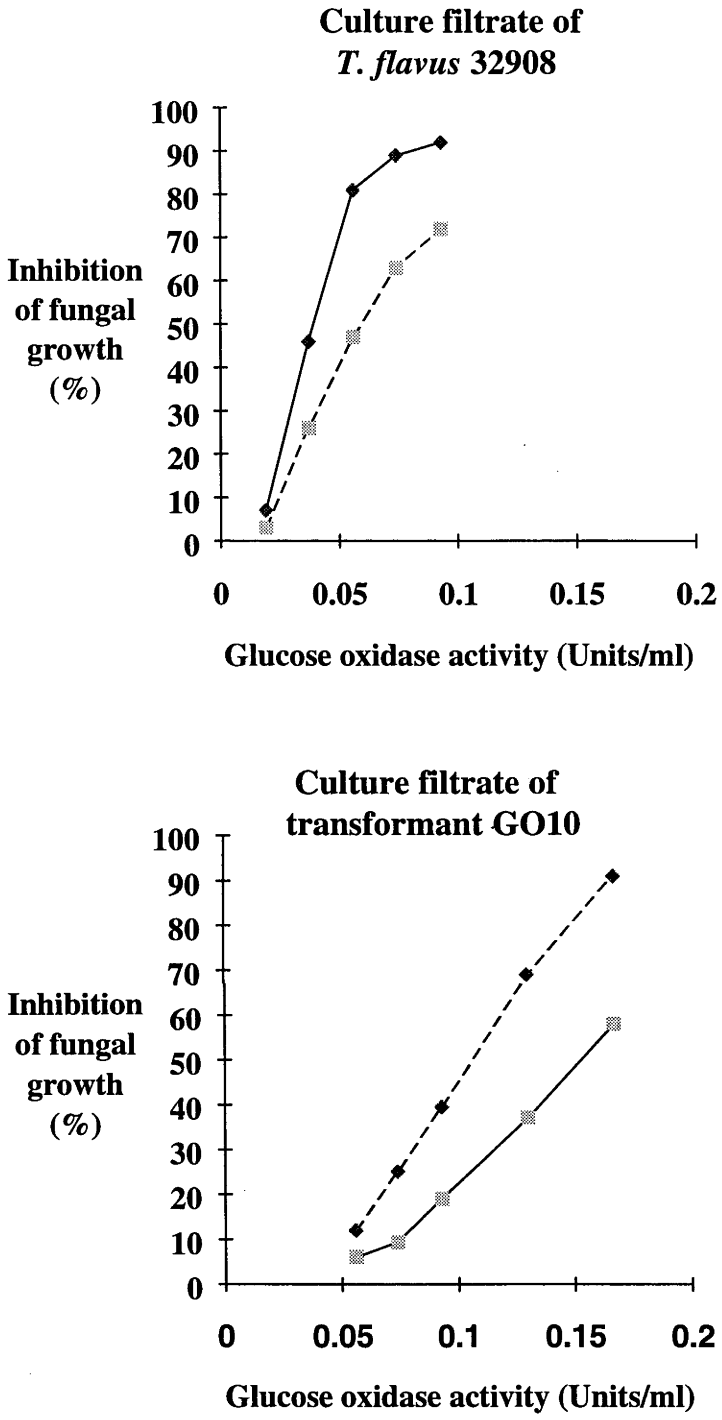


Figure 3.5 Time-dependent growth inhibition curves. Growth inhibition of *V. dahliae* was measured in different amounts of *T. flavus* and *T. macrosporus* culture filtrates. The amount of glucose oxidase in the culture filtrate was determined by comparison to standard concentrations of *A. niger* glucose oxidase. The percent growth inhibition was recorded after 28 hours (------) and 42 hours (———). One unit of activity is defined as that amount which will oxidise 1.0 μmole of β-D glucose to D-gluconic acid and H_2O_2 per minute at pH 5.1 and 25°C.

GO10, were assessed for incidence of Verticillium wilt over a period of nine weeks. As the aim of the experiment was to determine what effect glucose oxidase has on the biocontrol ability of *T. flavus* in untreated soil, glucose was not incorporated into the soil prior to its infestation with fungi. Typical symptoms of the disease, leaf wilting and chlorosis (Figure 3.6) were first observed 4-5 weeks after planting. Plants were scored for the presence or absence of the disease based upon observation of these symptoms at 5, 7 and 9 weeks after planting (Figure 3.7). In previous experiments *V. dahliae* had been recovered from the stem of similarly symptomatic plants confirming that *V. dahliae* was the cause of infection in these plants. Experiments were repeated three times.

On average, 45% of plants infected with *V. dahliae* alone displayed symptoms of Verticillium wilt 5 weeks after planting and after 9 weeks 90% had symptoms. Control plants in which no *V. dahliae* was present in the soil displayed no symptoms at any time during the experiments. Of those plants treated with *V. dahliae* and one of the *Talaromyces* isolates, 14-34% had symptoms after 5 weeks and 65-70% had symptoms after 9 weeks. Paired comparisons at 5, 7 and 9 weeks of disease incidence indicated that the presence of any of the *Talaromyces* isolates in the soil resulted in a significantly lower amount of disease incidence ($P \leq 0.01$). However, the presence of *T. flavus* 32908 did not reduce the incidence of Verticillium wilt significantly more than the presence of the *T. flavus* glucose oxidase-deficient mutant GOH-1. Similarly the transformant *T. macrosporus* GO10 which secretes glucose oxidase was not significantly more effective than the wild-type parent *T. macrosporus* FRR2417 at reducing the incidence of Verticillium wilt in cotton ($P > 0.05$).

During the early stages of infection the presence of a *T. flavus* isolate in the soil (32908 or GOH-1) resulted in significantly lower levels ($P \leq 0.01$) of Verticillium wilt than when a *T. macrosporus* isolate (FRR2417 or GO10) was present in the soil. At this stage of infection (5 weeks post planting) an average of 15% of plants grown with *T. flavus* isolates were infected whereas at the same time 30% of plants grown with *T. macrosporus* isolates were infected. A difference in disease incidence was

Figure 3.6 Symptoms of Verticillium wilt in the leaves of cotton. **a**: Uninfected leaf; **b - f**: Progression of symptoms from initial yellowing of the leaf through to complete necrosis of the leaf.

a

b

c

d

e

f

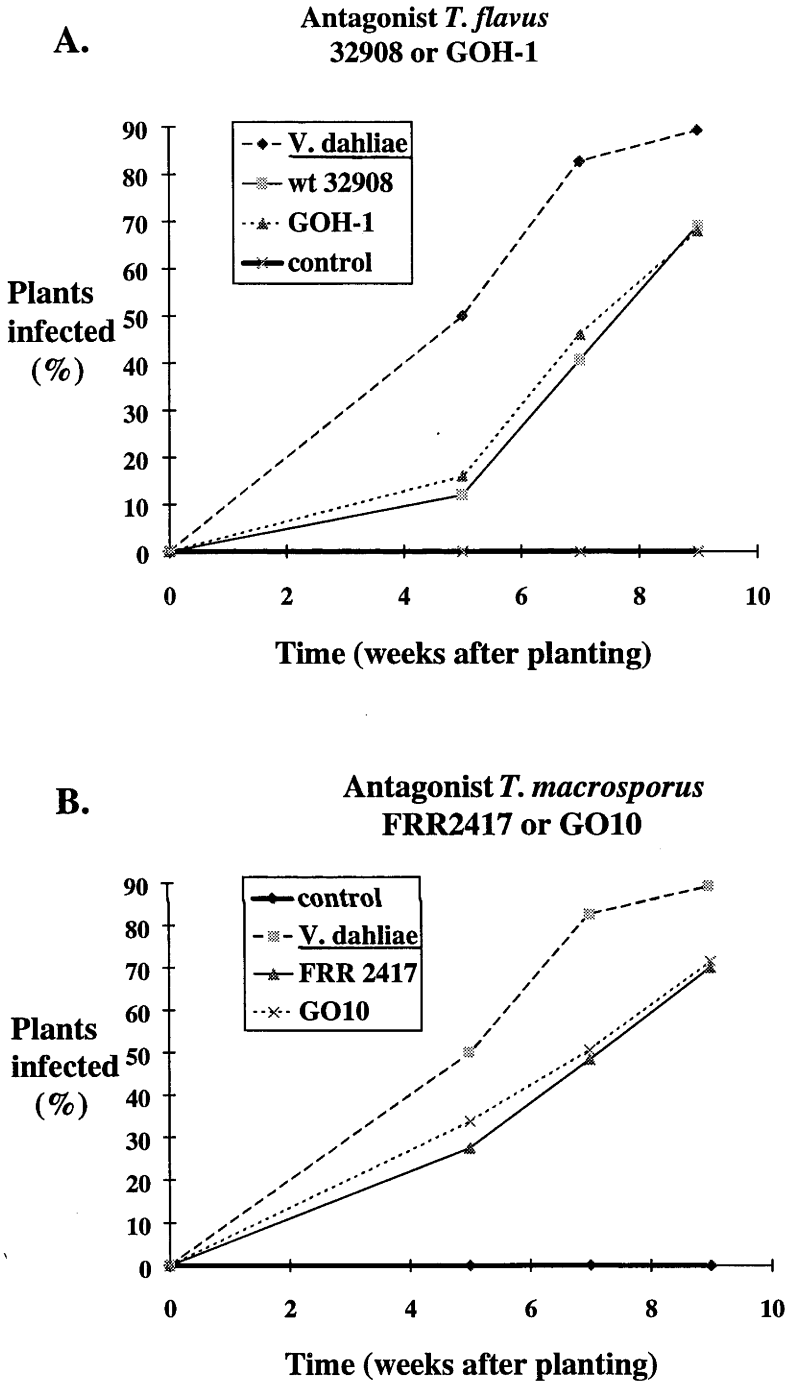


Figure 3.7 Relationship of percentage of cotton plants showing symptoms of Verticillium wilt (% of plants infected) to time (weeks after planting) when grown in soil containing different *Talaromyces* isolates. **Control**: no *V. dahliae* or *Talaromyces* in the soil. **V. dahliae**: Soil infested with only *V. dahliae*. **GOH-1, wt 32908, FRR 2417, GO10**: Soil infested with *V. dahliae* and either GOH-1, *T. flavus* 32908, *T. macrosporus* FRR2417 or GO10 (respectively). Maximum standard error is ± 8.4% of the values obtained.

still noticeable at 7 weeks (45% and 50% respectively) however by 9 weeks no significant difference in disease incidence was found ($P \leq 0.05$).

3.3.4 Parasitism of *V. dahliae* microsclerotia by *T. flavus*

To observe parasitism of *V. dahliae* microsclerotia by *T. flavus*, microsclerotia cut from agar plates were placed on fresh PD media and challenged with freshly growing *T. flavus* 32908. Observation after 8-12 days under a microscope revealed *T. flavus* hyphae growing over the microsclerotia but no penetration of the microsclerotia was observed.

3.3.5 Catalase activity in *T. flavus*, *T. macrosporus* and *V. dahliae*

As the enzyme catalase detoxifies hydrogen peroxide to oxygen and water, fungi such as *V. dahliae* which are particularly susceptible to hydrogen peroxide may have lower levels of catalase compared with other more hydrogen peroxide-tolerant fungi such as *T. flavus*. To investigate this question, cell free extracts prepared from freshly grown hyphal material of *V. dahliae* and each of four *Talaromyces* isolates (*T. flavus* 32908 and GOH-1, *T. macrosporus* FRR2417 and GOH-10) were prepared and assayed for catalase activity as described in Materials and Methods. In addition, catalase activity in cell free extracts prepared from *V. dahliae* previously grown on media containing a low concentration of glucose oxidase (5µg/ml) was determined. Higher concentrations of glucose oxidase in the growth media were toxic to *V. dahliae* and no growth occurred. Two extracts were prepared for each fungus and the experiment repeated once. The results are shown in Table 3.2.

Within the sensitivity of detection, no activity was detected in the control which contained only buffer and hydrogen peroxide. Activities ranging from 88 to 3292 units/µg of protein were obtained for the different fungal extracts (units as defined in the Materials and Methods). The highest level of catalase activity was obtained from *T. flavus* 32908, the level of activity was 2-3 fold lower in the *T. flavus* glucose oxidase-deficient mutant GOH-1. Wild-type *T. macrosporus* FRR2417 had substantially less catalase activity, approximately an eighth of that seen in *T. flavus* 32908. This activity was increased 2-3 fold in the glucose oxidase-producing transformant *T. macrosporus* GO10. The lowest level of catalase activity,

Fungal Strain	Catalase Activity (Units/μg protein)
Control	4 ± 8
***T. flavus* 32908**	3292 ± 79
GOH-1	1378 ± 34
***T. macrosporus* FRR 2417**	423 ± 16
GO10	1183 ± 36
V. dahliae	88 ± 18
V. dahliae **(on 5μg/ml glucose oxidase)**	195 ± 10

Table 3.2 Catalase activity in *V. dahliae* and different *Talaromyces* isolates. Values are the means ± the standard error. One unit is defined as that amount which will decompose 1.0 μmole of H_2O_2 per minute at pH 7 and 25°C.

88units/μg, was present in *V. dahliae*. Growth on media containing glucose oxidase increased *V. dahliae* catalase activity just over 2 fold, however the level of activity obtained is still low compared with that obtained for *T. flavus* 32908 and GO10 (17 and 6 fold lower respectively). Therefore compared with the *Talaromyces* isolates tested, *V. dahliae* does have a low level of catalase activity. Also, in *T. flavus* and *T. macrosporus*, catalase is induced by either glucose oxidase and/or its reaction products (hydrogen peroxide, glucono-δ-lactone and gluconic acid).

The different fungi were also grown in broth and the filtrates assayed for both catalase and peroxidase activity. No catalase or peroxidase activity was detected in any of the filtrates indicating that catalase and peroxidase are not secreted by any of these isolates. Measurement of peroxidase activity in the fungal extracts was not possible as the catalase present in extract quickly removed the hydrogen peroxide substrate needed to assess peroxidase levels. Purification of the extract to remove the catalase would be necessary to measure peroxidase levels accurately in these extracts.

3.4 DISCUSSION

Using the procedure developed in Chapter 2, transformation of *T. flavus* 32908 has been achieved. The transformation frequency obtained for *T. flavus* was much lower than that obtained for *T. macrosporus* (approximately 1 transformant/μg DNA compared with 20 transformants/μg DNA respectively) The reason for this is not known but is probably due to differences between the two species. Despite the low transformation frequency of *T. flavus*, the technique of targeted gene disruption was still used successfully to create the glucose oxidase-deficient *T. flavus* strain, GOH-1.

As only one glucose oxidase-deficient transformant was obtained, the frequency with which targeted transformation occurs in *T. flavus* cannot be determined. In other filamentous fungi the efficiency of targeted transformation varies widely. Low frequencies of 4-6% have been reported for *Magnaporthe grisea*, *Nectria haematococca*, *Giberella pulicaris* and *Cephalosporium acremonium* (Sweigard *et al.*, 1992; Stähl and Schäfer, 1992; Hohn and Desjardins, 1992; Hoskins *et al.*, 1990) Much higher frequencies of 50-95% have been obtained for *A. nidulans* and

Glomerella cingulata (Smith *et al.*, 1990; Mayorga and Timberlake, 1990; Rikkerink *et al.*, 1994). The occurrence of homologous integration in the only transformant obtained could therefore be attributed to chance, or may result from *T. flavus* being particularly amenable to targeted transformation. Alternatively, the glucose oxidase gene may influence the frequency of homologous recombination. Glucose oxidase expression, particularly under laboratory conditions where there is an abundant supply of glucose, could be detrimental to *T. flavus* as the fungus would need to maintain sufficient catalase to prevent toxicity from the hydrogen peroxide produced by glucose oxidase. Under these stressful conditions the glucose oxidase gene could be selected against and this may enhance selection for inactivation. Also, recombination hot spots have been identified in *S. cerevisiae* and may also exist in filamentous fungi (Voelkel-Meiman *et al.*, 1987). If glucose oxidase is situated at or near one of these places, efficient gene targeting could occur.

When used in *V. dahliae* inhibition assays *in vitro*, filtrate from the glucose oxidase-deficient mutant GOH-1 did not inhibit *V. dahliae* growth as effectively as filtrate from the wild-type parent *T. flavus* 32908. Also, filtrate from the glucose oxidase-producing transformant *T. macrosporus* GO10 inhibited *V. dahliae* growth, whereas filtrate from the wild-type *T. macrosporus* parent did not. This suggests that under these conditions glucose oxidase is responsible for a significant amount of the growth inhibition seen in *V. dahliae*. However in pot trials, the incidence of Verticillium wilt in cotton was the same when either *T. flavus* GOH-1 or *T. flavus* 32908 was used as a biocontrol agent. Similarly, no significant difference in wilt incidence was found between plants treated with either *T. macrosporus* GO10 or wild-type *T. macrosporus*. In this experiment therefore, the presence or absence of glucose oxidase in the biocontrol fungus has no effect on the incidence of Verticillium wilt in cotton. As glucose oxidase was found to inhibit *V. dahliae* growth *in vitro*, these results suggest that in this experiment insufficient glucose is present in the soil to allow production of inhibitory levels of hydrogen peroxide. Repetition of the experiment using soil with added glucose may answer this question.

The result obtained here is in contrast to that reported by Fravel and Roberts (1992) who found that a *T. flavus* variant which produced 2% of the level of glucose oxidase

produced by the wild-type isolate did not control Verticillium wilt of eggplant but the wild-type isolate did. This could be because, in comparison with the *T. flavus* parent, the variant may produce different compounds or different levels of compounds which have affected its biocontrol ability.

Other reasons could also account for the difference between their results and those reported here. More glucose may be present in the rhizosphere of the eggplants infected by Fravel and Roberts. This would allow a greater concentration of hydrogen peroxide to be produced which could inhibit *V. dahliae* and so reduce the incidence of Verticillium wilt. Greater glucose concentrations could occur either because eggplant secretes more glucose from the roots than cotton and/or because Fravel and Roberts used non-sterile soil. The presence of microorganisms in the soil environment may provide additional glucose.

More glucose oxidase may be present because the wild-type *T. flavus* isolate used by Fravel and Roberts (Tf-1) secretes more glucose oxidase into the soil than *T. flavus* 32908. However, this is unlikely because *in vitro*, 32908 secretes approximately four times the amount of glucose oxidase as Tf-1 (Kim *et al.*, 1990a). The size of the *Talaromyces* population in the rhizosphere would also influence the glucose oxidase concentration. However, as Fravel and Roberts used non-sterile soil, the *Talaromyces* soil population obtained in their experiments probably does not exceed the size of the *Talaromyces* population obtained in the experiments reported here. This is because competition from other soil microorganisms in non-sterile soil would result in a lower *T. flavus* population. Indeed an example of this effect may be illustrated in the experiments reported here. The presence of any *Talaromyces* isolate in the soil (glucose oxidase producing or not) was found to significantly decrease the incidence of Verticillium wilt. This is probably because *Talaromyces* colonizes the sterile soil more effectively than the slower growing *V. dahliae*. As a result the *V. dahliae* population in the soil is smaller and less disease occurs.

The decrease in wilt incidence observed in pot trials when any of the *Talaromyces* isolates was present in the soil could be due to competition as mentioned above and/or to *Talaromyces* producing other compounds inhibitory to *V. dahliae*. Both

species have been reported to produce mycotoxins, *T. flavus* produces vermicellin and *T. macrosporus*, duclauxin (Frisvad *et al.*, 1990). These and other secondary metabolites (mitorubin, mitorubrinic acid) produced by these species could inhibit *V. dahliae* growth. Results obtained here also indicate that *T. flavus* in particular produces other compounds which inhibit *V. dahliae* growth. Compared to *T. flavus* 32908, approximately 2.5 times more glucose oxidase activity was required in *T. macrosporus* culture filtrate to inhibit *V. dahliae* growth by 50% *in vitro*. As no catalases or peroxidases were found in the filtrate of these *T. flavus* or *T. macrosporus* isolates, this difference in toxicity is not due to increased decomposition of hydrogen peroxide in *T. macrosporus* GO10 filtrate. The presence of additional antifungal compounds in the *T. flavus* 32908 filtrate could account for this difference in inhibitory abilities. In addition, the filtrate from the *T. flavus* glucose oxidase-deficient-mutant GOH-1 was found to inhibit the growth of *V. dahliae* by approximately 20%. Diluting the filtrate with water was found to decrease growth inhibition, suggesting that other inhibitory compounds were present in the filtrate. However, as autoclaving the filtrate did not alter the effect on *V. dahliae* growth rate, it is unlikely these inhibitory compounds are proteins. The compounds identified by Madi *et al.* (1989) in *T. flavus* culture filtrate (cellulases, β 1-3 glucanase and chitinase) are unlikely to be responsible for the growth inhibition observed here. In pot trials, *T. flavus* isolates (32908 and GOH-1) were also found to reduce the incidence of Verticillium wilt more effectively than *T. macrosporus* isolates (FRR2417 and GO10) at the early stages of infection. This delay in Verticillium infection could also be attributed to *T. flavus* secreting other substances inhibitory to *V. dahliae*. Alternatively, *T. flavus* may grow faster than *T. macrosporus* in the soil and so compete better against *V. dahliae*. So, although glucose oxidase produced by *T. flavus* is predominantly responsible for the inhibition of *V. dahliae* growth *in vitro*, *in vivo*, under the conditions used here, other factors are more important in the biocontrol of Verticillium wilt in cotton.

In addition to antibiosis and competition, the antagonism of *V. dahliae* by *T. flavus* has also been attributed to parasitism. In particular, *T. flavus* has been shown to parasitise *V. dahliae* microsclerotia (Madi and Henis, 1992). The involvement of

glucose oxidase in this process is not known. Glucose oxidase alone does not affect microsclerotia (Fravel *et al.*, 1987), suggesting intact microsclerotia do not supply enough glucose to allow production of toxic quantities of hydrogen peroxide. However, as suggested by Kim *et al.* (1988), other food sources may supply the necessary glucose, in which case hydrogen peroxide may weaken microsclerotia, increasing their susceptibility to parasitism. Also, after *T. flavus* penetrates microsclerotia, the microsclerotia may provide glucose and thereby hasten their own death. Unfortunately, no parasitism of *V. dahliae* microsclerotia by *T. flavus* 32908 was observed under the conditions described here. This may have been because of the *T. flavus* and *V. dahliae* strains used, or because the conditions used were not favourable to parasitism. If the correct conditions could be achieved however, the availability of *T. flavus* 32908 and the corresponding glucose oxidase-deficient mutant GOH-1 would allow the role of glucose oxidase in this interaction to be investigated.

V. dahliae was found to have significantly less catalase activity than *T. flavus* or *T. macrosporus*. This probably accounts for the finding by Kim *et al.*, (1990b) that *V. dahliae* is particularly susceptible to hydrogen peroxide. Also, in *V. dahliae* and the *Talaromyces* species tested, catalase is induced by glucose oxidase and/or the enzyme's reaction products (hydrogen peroxide, glucono-δ-lactone and gluconic acid). In all cases, the highest activities were found in those isolates producing glucose oxidase or grown on glucose oxidase-containing media. Activity decreased 2-3 fold in all fungi when glucose oxidase was not present.

Similar results have been obtained in *A. niger*, where favourable conditions for glucose oxidase activity (high levels of oxygen and glucose) have been found to induce catalase. In *A. niger*, catalase was not induced when D-xylose was substituted for glucose or by gluconic acid or glucono-δ-lactone, but hydrogen peroxide alone did induce catalase (Witteveen *et al.*, 1993). Therefore, in *A. niger*, the hydrogen peroxide, not the glucose oxidase, is responsible for catalase induction. This could also be true in the fungi examined here. Presumably, this is because of the toxicity of the hydrogen peroxide to living organisms. When more glucose oxidase is produced by the fungus or is present in the surrounding environment under favourable

conditions, higher levels of catalase are required to detoxify the additional hydrogen peroxide produced by the glucose oxidase.

Despite the sensitivity of *V. dahliae* to hydrogen peroxide, the results presented here suggest in soil, glucose oxidase secreted by *T. flavus* has no significant role in the biocontrol of Verticillium wilt of cotton. As low concentrations of glucose oxidase inhibit *V. dahliae* growth *in vitro*, the inability the of glucose oxidase to control *V. dahliae* in soil, is presumably due to an inadequate supply of glucose. To confirm this possibility, the experiments need to be repeated in soil to which glucose has been added. The complex interaction between the soil microenvironment and biocontrol organisms also means it would be of interest to repeat these experiments in unsterilized soil. In addition, the potential of *T. flavus* to be useful as a biocontrol agent in the field must still be explored.

CHAPTER 4
EXPRESSION OF *T. FLAVUS* GLUCOSE OXIDASE IN TRANSGENIC PLANTS

4.1 INTRODUCTION

Hydrogen peroxide generated enzymatically by glucose oxidase has been shown to be toxic to *V. dahliae* at low concentrations *in vitro*. However, in the soil environment using cotton as the plant host, glucose oxidase was found to have no significant role in the biocontrol of Verticillium wilt by *T. flavus* (see Chapter 3). This is probably because there is insufficient glucose or secreted glucose oxidase in the soil to allow production of toxic quantities of hydrogen peroxide. The toxic properties of glucose oxidase and its enzymatic product hydrogen peroxide might however still be utilized effectively against *V. dahliae* if the enzyme were expressed in transgenic plants. In transgenic plants, expression of glucose oxidase could be targeted specifically to the plant's roots where the pathogen first invades the plant. Transgenic plants are therefore not encumbered by the problems of application and maintenance of the biocontrol agent (both to the soil and appropriate regions of a growing root system) that limits the use of biocontrol organisms in environmental situations.

Hydrogen peroxide has been implicated in natural plant pathogen defence mechanisms. When a pathogen attacks a resistant plant cell, one of the earliest responses is a metabolic oxidative burst which generates free radicals and hydrogen peroxide (reviewed in Dixon *et al.*, 1994). The hydrogen peroxide generated, in addition to being directly antimicrobial, has been shown to drive the oxidative cross-linking of cell wall structural proteins (Bradley *et al.*, 1992). This cross-linking, which is initiated about 5 minutes after pathogen inoculation, is completed within 10 to 20 minutes and appears to make the cell wall more resistant to pathogen digestion. Hydrogen peroxide is therefore involved in one of the earliest events in plant pathogen responses and so is quite a natural plant defence chemical. Augmentation of this response through the expression of glucose oxidase in plants may enhance the plant's defence capabilities.

Apostol *et al.* (1989) found that 24 hours after pathogen inoculation, soybean cells treated with the peroxide scavenger catalase, failed to accumulate antimicrobial compounds known as phytoalexins. This suggests that hydrogen peroxide may also be involved in signalling the nucleus for defence gene activation. However, other groups (Devlin and Gustine, 1992; Davis *et al.*, 1993) have shown that hydrogen peroxide is not always essential for bacterial induction of phytoalexin accumulation. Salicylic acid, one of the natural signal molecules which has been implicated in the activation of some plant defence responses, has been shown to increase hydrogen peroxide levels by 50-60% in plants (Chen *et al.*, 1993). It has been proposed that this increase in hydrogen peroxide level is responsible for some of the effects of salicylic acid in the phenomenon of systemic acquired resistance (where infection of plants by pathogens leads to enhanced resistance to subsequent attacks by the same or unrelated pathogens). As hydrogen peroxide is so intimately involved in plant defence, the production of glucose oxidase-mediated hydrogen peroxide in plants may also result in plants resistant to fungal infection because other defence mechanisms such as cell wall cross-linking and defence signal transduction may be stimulated.

The expression of glucose oxidase in plants involves some difficulties. Unlike the genes involved in the generation of the oxidative burst of hydrogen peroxide produced by plants in response to fungal attack, the genes being used in this study are likely to be expressed for extended periods of time, at least in some tissues. Long-term production of hydrogen peroxide by glucose oxidase could be toxic to the plant and even result in cell death or undesirable phenotypes such as slow growth and reduced fruit formation. If not toxic to the plant itself, those with increased levels of hydrogen peroxide in the root could make the root environment less amenable to colonization by mycorrhizal fungi. These fungi, which infect a plant's root system and then grow out into the surrounding soil where they provide an 'extended root system' for the plant, have been implicated in increasing the vigour and yield of many plants, including cotton (Allen *et al.*, 1992). Although the sensitivity of these fungi to hydrogen peroxide is not known, it would be undesirable if hydrogen peroxide produced by glucose oxidase inhibited mycorrhizal colonization of cotton roots. Also, as in the soil, availability of glucose may limit production of hydrogen peroxide

by glucose oxidase in plants. Plants also possess a range of peroxidases, which together with a poor supply of glucose could result in little effective hydrogen peroxide being produced in plants.

The possibility of expressing the *T. flavus* glucose oxidase in plants is explored in this chapter. The glucose oxidase gene is engineered into different plant expression constructs and transformed into *Nicotiana tabacum* (tobacco) and *Gossypium hirsutum* (cotton). Expression of glucose oxidase in T_0 transgenic plants and T_1 (*N. tabacum* only) progeny is examined. Three different promoters (35S, TobRB7 and carrot root extensin) are used to drive glucose oxidase expression in transgenic plants. The pattern of gene expression determined by these promoters is also investigated by fusing each promoter to the GUS reporter gene and examining β-glucuronidase expression in transgenic tobacco plants.

4.2 MATERIALS AND METHODS

4.2.1 Gene constructions

4.2.1.1 Glucose oxidase plasmids

All DNA manipulations were performed using standard procedures (Sambrook *et al.*, 1989). A 2.2kb *PstI*/*Sac*I fragment containing the glucose oxidase ORF in addition to approximately 250bp of 5' and 150bp of 3' DNA was subcloned from the *T. flavus* 7.7kb *Eco*RI fragment previously isolated from the genomic library into pBluescript SK^- (Stratagene) to create pGO. The 4kb extensin *Eco*RI DNA fragment from pDC5A1 (Chen and Varner, 1985) was recloned into the *Eco*RI restriction site of pJKKm creating pDC5A2. Restriction mapping revealed a *Kpn*I site approximately 3kb downstream from the 5' *Eco*RI site of the extensin fragment. This 3kb *Kpn*I fragment from pDC5A2, was subcloned into pBluescript SK^- to create pEx. *In vitro* mutagenesis was carried out with either pGO or pEx to introduce restriction enzyme recognition sites around the ATG and 3' end of the putative secretion signal peptides of both genes. The following synthetic oligonucleotides were used to introduce the restriction enzyme sites:

GO1 $^{5'}$ CCTTGCCG**ACTAGT**AATGGTGT $^{3'}$

GO2 $^{5'}$ CCTACCTG**GATATC**CAACAGAT $^{3'}$

Ex1 $^{5'}$ TTGGTTGT**ACTAGT**CATGGGAA $^{3'}$

Ex2 $^{5'}$ CCACAGCT**GTTAAC**ACTTACTC $^{3'}$

The introduced restriction sites are in bold and the initiating ATG is underlined. Restriction digest analysis was used to confirm the presence of the correct mutations. The following plasmids were created: **pGO-1** (pGO mutated with oligonucleotide GO1 to introduce a *Spe*I site just prior to the initiating ATG of the *T. flavus* glucose oxidase gene); **pGO-2** (pGO mutated with oligonucleotide GO2 to introduce an *Eco*RV site at the 3' end of the putative *T. flavus* glucose oxidase signal peptide); **pEx-1** (pEx mutated with oligo Ex1 to introduce a *Spe*I site just prior to the initiating ATG of the carrot extensin gene) and **pEx-2** (pEx mutated with Ex1 and Ex2 to introduce a *Hpa*I site at the 3' end of the putative carrot root extensin signal peptide and a *Spe*I site just prior to the initiating ATG of the extensin gene).

Plasmid pEGGON (Appendix 1A)

The *SpeI/Sac*I DNA fragment from pGO-1 containing the glucose oxidase gene and 5' signal peptide was subcloned into pBluescript SK$^-$ to create pGO-1A. The *SpeI/Eco*RI fragment from pEx-1 was cloned into the *SpeI/Eco*RI restriction sites upstream of the glucose oxidase gene in pGO-1A to create pEGGO. The *Eco*RI/*Sac*I fragment from pEGGO (glucose oxidase gene and signal peptide fused to the extensin promoter) was then joined to a Nos terminator by subcloning into the *Eco*RI/*Sac*I restriction sites of pGN100 (Bogusz *et al.*, 1990).

Plasmid pEEGON (Appendix 1B)

The 2.2kb *PstI/Sac*I fragment from pGO-2 containing the glucose oxidase gene into which a *Eco*RV restriction site had been engineered, was subcloned into the *PstI/Sac*I restriction sites in pJKKm (Kirschman and Cramer, 1988). This plasmid was digested with *Eco*RV/*Eco*RI and the 1.9kb fragment containing the glucose oxidase gene cloned into pBluescript SK$^-$ to create pGO-2A. The 0.75kb *HpaI/Kpn*I DNA fragment from pEx-2 containing the extensin promoter and secretion signal peptide

was then subcloned into the *Eco*RV/*Eco*RI restriction sites upsteam of the glucose oxidase gene in pGO-2A to create pEEGO. The *Eco*RI/*Sac*I fragment from pEEGO containing the extensin promoter, signal peptide and glucose oxidase gene was subsequently subcloned into the *Eco*RI/*Sac*I restriction sites of pGN100 to create pEEGON.

Plasmid pTGGON (Appendix 1C)

The TobRB7 promoter, a gift from D. Last, CSIRO Division of Plant Industry was subcloned into the *Apa*I/*Sal*I sites of pBluescript SK$^-$ to create pTobRB7-2. The *Spe*I/*Eco*RI fragment from pTobRB7-2 containing the promoter was then cloned into the *Spe*I/*Eco*RI restriction sites of pEGGON, where it replaced the extensin promoter and created pTGGON.

Plasmid pTEGON (Appendix 1D)

This plasmid was created by replacing the *Spe*I/*Eco*RI extensin promoter fragment from pEEGON with the *Spe*I/*Eco*RI promoter fragment from pTobRB7-2.

Plasmid pSGGON (Appendix 1E)

The 35S promoter from 35SKNΔBam (J. Walker, unpublished) was removed by digestion with *Pst*I/*Bam*HI and subcloned into the *Pst*I/*Bam*HI restriction sites of pBluescript SK$^-$ creating p35S. Plasmid pSGGON was then created by replacing the *Spe*I/*Eco*RI extensin promoter fragment in pEGGON with the *Spe*I/*Eco*RI DNA fragment from p35S.

Plasmid pSEGON (Appendix 1F)

The *Eco*RI/*Spe*I extensin promoter fragment in pEEGON was replaced with the *Eco*RI/*Spe*I 35S promoter fragment from p35S.

4.2.1.2 Gus plasmids

Plasmid pEEGusN (Appendix 1G)

Plasmid $pGUS_{358\rightarrow S}$ was a gift from L. Farrell, CSIRO Division of Plant Industry. The *Sal*I/*Eco*RI fragment containing the modified Gus gene was recloned into pJKKm. This plasmid was then linearized with *Eco*RI, its ends blunted with the Klenow fragment of DNA polymerase I and the Gus gene excised by further

digesting the linearized plasmid DNA with *Hin*dIII. The fragment was then cloned into the *Hin*dIII/*Sma*I restriction sites of 35SKNΔBam to create pGUSN-1. To create pEEGusN the *Hpa*I/*Hin*dIII fragment from pEx-2 (extensin promoter and signal peptide) was cloned into the *Nco*I (3' overhang removed with mung bean nuclease)/*Hin*dIII restriction sites of pGUSN-1.

Plasmid pTEGusN (Appendix 1H)

The *Sal*I/*Spe*I extensin promoter fragment of pEEGusN was replaced with the *Spe*I/*Sal*I TobRB7 promoter fragment from pTobRB7-2.

Plasmid pEGusN (Appendix 1I)

The Gus gene was excised from $pGUS_{358\rightarrow S}$ the Klenow fragment of DNA polymerase I and the as an *Eco*RI/*Sal*I fragment and in order to destroy the *Eco*RI restriction site, the *Eco*RI end blunted by treatment with the Klenow fragment of DNA polymerase I before cloning into the *Bam*HI (also blunted with Klenow)/*Sal*I restriction sites of pBluescript SK^-. The *Sal*I/*Sac*I fragment containing the Gus gene was then excised from this plasmid, the *Sal*I end blunted by treatment with the Klenow fragment of DNA polymerase I and the fragment cloned into the *Spe*I(blunted with Klenow)/*Sac*I restriction sites of pEEGON (replacing the extensin signal peptide and glucose oxidase gene with the $Gus_{358\rightarrow S}$ gene).

Plasmid pTGusN (Appendix 1J)

The *Sal*I (end filled with Klenow)/*Sac*I GUS fragment from $pGUSN_{358\rightarrow S}$ (pBluescript) was cloned into the *Spe*I (blunted with Klenow)/*Sac*I restriction sites of pTEGON (replaced the extensin signal peptide and glucose oxidase gene).

Gene fusions occurring within an ORF were checked by dideoxy sequencing using a Pharmacia T7 sequencing kit to ensure the correct ORF was conserved. All of the above plasmids, with the exception of pEEGusN and pTEGusN, were linearized with *Eco*RI and cointegrated into the binary vector pTAB5 (Tabe *et al.*, submitted) in the opposite orientation to the selectable kanamycin gene. Plasmid pEEGusN was linearized with *Hin*dIII and its ends blunted with the Klenow fragment from DNA polymerase I. This plasmid was then ligated into the *Eco*RI restriction site in pTAB5 which has also been blunted by treatment with the Klenow fragment of DNA

polymerase I. Similarly, pTEGusN was linearized with *Sal*I, its ends blunted and the plasmid ligated into the blunted *Eco*RI restriction site of pTAB5. Both plasmids were introduced into the binary vector in an opposite orientation to the selectable kanamycin gene. Triparental mating was employed to transfer the binary vector constructs to the super-virulent disarmed *Agrobacterium tumefaciens* strain AGL1 (Lazo *et al.*, 1991) and constructions were verified by restriction enzyme analysis.

4.2.2 Plant transformations

Transformation of *N. tabacum* (*cv.* Wisconsin 38) using *A. tumefaciens* was performed as described by Ellis *et al.* (1987). Plants were subcultured every 6-8 weeks to fresh MS medium containing 3% sucrose and 0.8% agar. Seed from *Gossypium hirsutum* (*cv.* Coker 315) were surface sterilized as described in Chapter 3 and transformed as described in Cousins *et al.* (1991) with the following modifications. After 2 days co-cultivation with the appropriate *A. tumefaciens* strain each explant was transferred to callus initiation media containing 50mg/L kanamycin. Six weeks later callus was subcultured to the same media containing 25mg/L kanamycin. After a further six weeks, surviving callus was subcultured to solidified basal medium containing no hormones or antibiotics. Embryos formed 5-12 weeks later; large embryos forming roots were transferred to deep petri dishes containing Stewart and Hsu (1977) medium solidified with Phytogel and magnesium chloride. Seedlings were subsequently transferred into pots containing the same medium and finally into soil in pots in the glasshouse.

4.2.3 Analysis of plant tissue for the presence of glucose oxidase activity

Tobacco seeds (*cv.* Wisconsin 38 and transgenic derivatives) were surface sterilized by placing seeds in 70% ethanol for 1 minute and then transferring to a 10% bleach solution containing 1 drop/100ml of Tween 20 for 10 minutes. After washing five times with sterile distilled water seeds were placed on MS media containing 100µg/ml kanamycin and germinated in the light at 26°C. Glucose oxidase activity was assayed qualitatively by removing small pieces of plant tissue and submerging them in the KI/starch stain described in Chapter 2 for fungal spores. Tissue was incubated overnight at room temperature before being scored for activity. For quantitative glucose oxidase assays, leaf or root tissue removed from plants

propagated in tissue culture was homogenized with 0.1M Na_2PO_4 buffer (pH 6.0) in a mortar and pestle. Homogenate was poured into eppendorf tubes and centrifuged at 13000 rpm for 15 minutes. The supernatant was removed and incubated at 4°C for 2-3 hours before it was assayed for glucose oxidase activity using the method described in Chapter 2. Protein concentration was determined by the method of Bradford (1976) and measured using a Labsystems Multiskan Plus.

4.2.4 PCR reactions

4.2.4.1 Isolation of genomic DNA

Genomic DNA was isolated from young leaves of tobacco plants propagated in tissue culture. One leaf was homogenized in an eppendorf tube containing 300µl of grinding solution (comprising 1.25ml of TE3D buffer (0.02M Tris, 002M Na_2EDTA, 1% Nonadet P-40, 1.5% lithium dodecal sulphate and 1% sodium deoxycholate), 2.5ml of equilibrated phenol and 50µl of β-mercaptoethanol). After homogenization, 250µl of ammonium acetate/EDTA solution (3M ammonium acetate, 0.4mM Na_2EDTA, 0.18M NaOH) and 400µl of chloroform was added and the tube mixed for 20 minutes. The tube was centrifuged at 13000 rpm at 4°C for 10 minutes, the supernatant removed and DNA precipitated by the addition of 0.6 volumes of isopropanol. The pellet was resuspended in TE (10:1) and treated with Ribonuclease A (previously boiled), then extracted with phenol/chloroform, precipitated with 2 volumes of ethanol and resuspended in distilled water.

4.2.4.2 PCR conditions

The following oligonucleotide primers, which are complementary to two regions in the *T. flavus* glucose ORF (position 681 and 1513 with respect to the initiating ATG) were synthesized on an Applied Biosystems DNA synthesizer.

FMGO3A $^{5'}$ GCGGTCATCCTCGAGGTGTCTCTATG $^{3'}$

GOFM4 $^{5'}$ TACTCCTTCATAGCACCTTGGCTGGT $^{3'}$

PCR was carried out using either 250ng of tobacco genomic DNA or 10ng of pSEGON plasmid DNA and *Taq* DNA polymerase buffer (Promega); 2.5mM $MgCl_2$;

200μM each dNTP; 1μM each primer and 2.5 units of *Taq* DNA polymerase (Promega). The reaction consisted of 30 cycles:

cycle 1 = 5min @ 94°C, 2min @ 55°C, 2min @ 72°C (performed once)

cycle 2 = 1min @ 94°C, 1min @ 55°C, 2min @ 72°C (performed 29 times)

cycle 30 = 5min @ 30°C (performed once)

PCR reaction products were visualized by running the completed reaction on a 0.7% agarose gel.

4.2.5 Northern analysis

Total RNA was extracted from young tobacco leaves excised from plants being propagated in tissue culture using the method of Dolferus *et al.* (1994). Equal amounts (20μg) of total RNA was loaded on 1.1% agarose gels containing 2.2M formaldehyde in the presence of ethidium bromide. After electrophoresis the gels were transferred and UV cross-linked onto Hybond-N nylon membranes (Amersham). The plasmid pGO-1 which contained the entire *T. flavus* glucose oxidase ORF was used to generate a riboprobe to detect glucose oxidase RNA. This plasmid was linearized with *Eco*RI and an antisense ^{32}P-dUTP-labelled riboprobe was made using T3 polymerase and a Promega *in vitro* transcription kit. An antisense riboprobe corresponding to an *Arabidopsis* ubiquitin clone (Burke *et al.*, 1988) was used to quantitate the amount of tobacco RNA. RNA probe hybridizations and washing of filters was carried out as described in Dolferus *et al.* (1994). Filters were exposed to X-ray film at -80°C for 2-3 days. Analysis and quantitation of the hybridization signals were performed with a phosphoimager (Molecular Dynamics, Sunnyvale, CA). The size of the hybridising bands were estimated with reference to RNA size standards (BRL).

4.2.6 GUS histochemical assay

Roots were excised from plants grown in tissue culture and stained using X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid, Progen Industries) as substrate in a solution of 10mM sodium phosphate (pH 7.0), 0.5mM potassium ferricyanide, 0.5mM potassium ferrocyanide, 10mM EDTA (Jefferson, 1987). Tissue was vacuum

infiltrated for 30 seconds, incubated at 37°C in the dark for 14-16 hours and then washed four times in 10mM sodium phosphate buffer (pH 7.0). Stained tissue was photographed using either bright or dark field optics. For cell localization, stained tissue was fixed overnight (3% glutaraldehyde in 25mM sodium phosphate pH 7.0) and dehydrated through a graded ethanol series. Tissue was embedded in L. R. White (London Resin Co) under nitrogen at 60°C for several days before 2-3μm thick sections (Reichart ultramicrotome) were mounted in glycerol and photographed under dark field.

4.2.7 GUS fluorometric assay

Cell free extracts were prepared from tobacco roots and leaves as for glucose oxidase assays except a different extraction buffer was used (50mM Na_2HPO_4 (pH 7.0), 10mM Na_2EDTA, 0.1% Triton-X-100, 0.1% sodium lauryl sarcosine, 10mM β-mercaptoethanol). Quantitative GUS assays were performed essentially as described by Jefferson (1987) using 4-methylumbelliferyl β-D-glucuronide (MUG, Sigma) as substrate. Aliquots from each reaction mix were taken at 30 minute intervals and added to 3 volumes of 0.2M Na_2CO_3. Fluorescence of each sample was measured using a Labsystems Fluoroskan II and compared to concentration standards prepared with commercially available 4-methyl umbelliferone (MU) (Sigma). Protein concentration was determined by the method of Bradford (1976) and measured using a Labsystems Multiskan Plus. Gus activities were then extrapolated and expressed as the total number of pmoles 4-methyl umbelliferone produced/min/mg protein.

4.2.8 Wounding of tobacco roots

Roots from tobacco plants subcultured three weeks previously were cut from plants, wounded with forceps, placed in 50mM Na_2HPO_4 buffer (pH 7.0) and shaken gently in the dark for 48 hours. Alternatively, plants were subcultured onto soft agar (0.5%) for three weeks, then lifted, their roots wounded with forceps and the plant replaced in the pot for 48 hours before assaying for enzyme activity. Root pieces were assayed directly for glucose oxidase activity by submerging them in the KI stain previously described in Chapter 2. To determine β-glucuronidase activity, cell free extracts

were prepared from roots and assayed for β-glucuronidase activity as described above.

4.3 RESULTS

4.3.1 Construction of plasmids for the expression of *T. flavus* glucose oxidase in plants

The glucose oxidase gene isolated was of fungal origin and it was not known if the secretion signal peptide at the 5' end of the gene would correctly target the protein into the endoplasmic reticulum and subsequently into the default pathway required for secretion in plants. To obtain good glucose oxidase expression in plants, the presence of a functional signal peptide was thought to be important for two reasons. First, glucose oxidase is a glycosylated protein and lack of glycosylation caused by mistargeting of the protein to the cytoplasm could conceivably abolish or decrease enzyme activity. Second, because of the potential toxicity of the hydrogen peroxide produced by glucose oxidase, it would be desirable to have the enzyme excreted from the cell. The effect of replacing the fungal signal peptide with one from plants on glucose oxidase gene activity is not known. Also, the precise end point of the glucose oxidase signal peptide has not been experimentally determined so too much or too little of the glucose oxidase sequence may be deleted in the exchange of DNA. An error such as this could decrease gene expression and/or protein activity. In view of these potential problems, two different series of expression vectors were constructed. In the pGGO series, the glucose oxidase secretion signal peptide was retained and in the pEGO series, the fungal signal peptide was replaced with the secretion signal peptide from the carrot extensin gene (Chen and Varner, 1985) as described in Material and Methods. This plant signal peptide was used because a similar extensin signal peptide from tobacco has been shown to mediate the secretion of neomycin phosphotransferase II (*npt*II) gene product from tobacco protoplasts (De Loose *et al.*, 1991).

In order to successfully express the glucose oxidase gene successfully in plants, root specific or inducible gene expression may be necessary because of the potential toxicity of hydrogen peroxide to the plant, particularly in any tissue where

endogenous glucose levels may be high. The glucose oxidase gene and signal peptide from the pGGO and pEGO plasmids was therefore linked to three different promoters as described in Materials and Methods. The promoters used were the 35S promoter from cauliflower mosaic virus (CaMV), the TobRB7 promoter from tobacco (Yamamoto *et al.*, 1991) and the carrot root extensin promoter (Chen and Varner, 1985). Each of these promoters directs a different pattern of gene expression in plants. The 35S promoter constitutively expresses genes in most plant tissues (Odell *et al.*, 1985), TobRB7 directs constitutive, root specific gene expression (Yamamoto *et al.*, 1991) and the carrot root extensin promoter has been shown to be wound-inducible in carrot roots (Chen and Varner, 1985; Granell *et al.*, 1992).

As described in Materials and Methods, each construct was fused to a Nos 3' terminator and then cloned into the *Eco*RI site of the binary vector pTAB5. This vector (shown in Figure 4.1) contains a chimeric 35S/*npt*II gene coding for kanamycin resistance within the left and right T-DNA borders of the *Agrobacterium* Ti plasmid and a bacterial tetracycline resistance gene (Tabe *et al.*, submitted). Restriction enzyme analysis was performed on all constructs to confirm the correct integration and orientation of the glucose oxidase gene in the binary vector (results not shown). Constructs containing the glucose oxidase gene in the opposite orientation to that of the chimeric 35S-*npt*II gene were introduced into tobacco and cotton by way of *Agrobacterium*-mediated transformation as described in Materials and Methods. Diagrams of the expression cassettes of the plasmids, pEEGON (carrot root extensin promoter), pTEGON (pTobRB7 promoter) and pSEGON (35S promoter) are shown in Figure 4.2. These constructs all contain the extensin secretion signal peptide. The three constructs containing the glucose oxidase secretion signal peptide attached to the glucose oxidase gene (pGGO series) are also shown.

4.3.2 Expression of *T. flavus* glucose oxidase in *Nicotiana tabacum*

For each of the six constructs, fifteen to twenty transgenic tobacco plants were regenerated. Glucose oxidase activity in transgenic plants was assayed qualitatively by submerging small pieces of plant tissue into a solution of glucose, potassium iodide (KI) and soluble starch. In transgenic plants expressing functional glucose

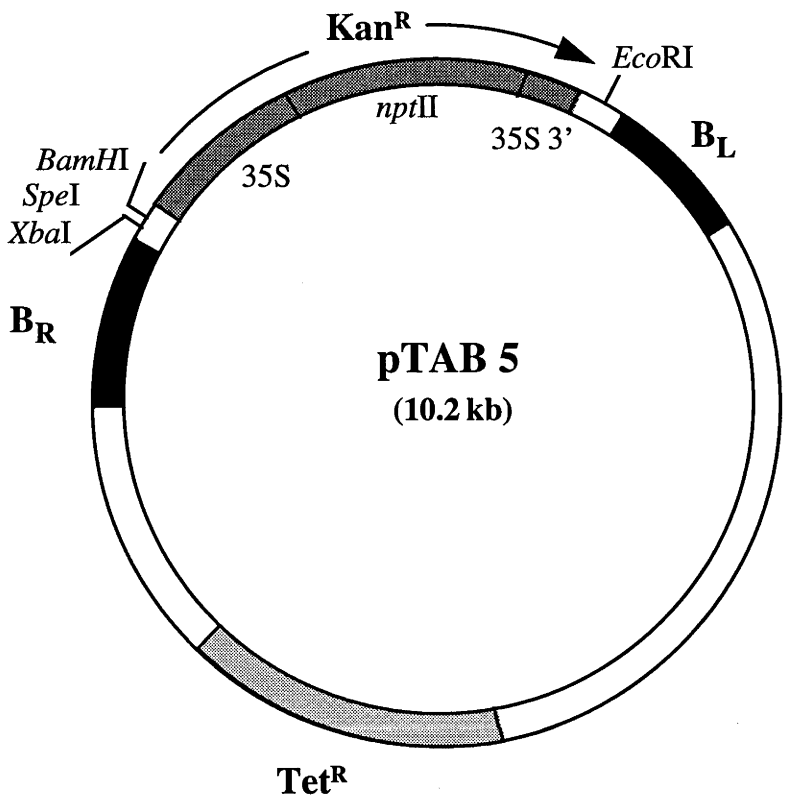
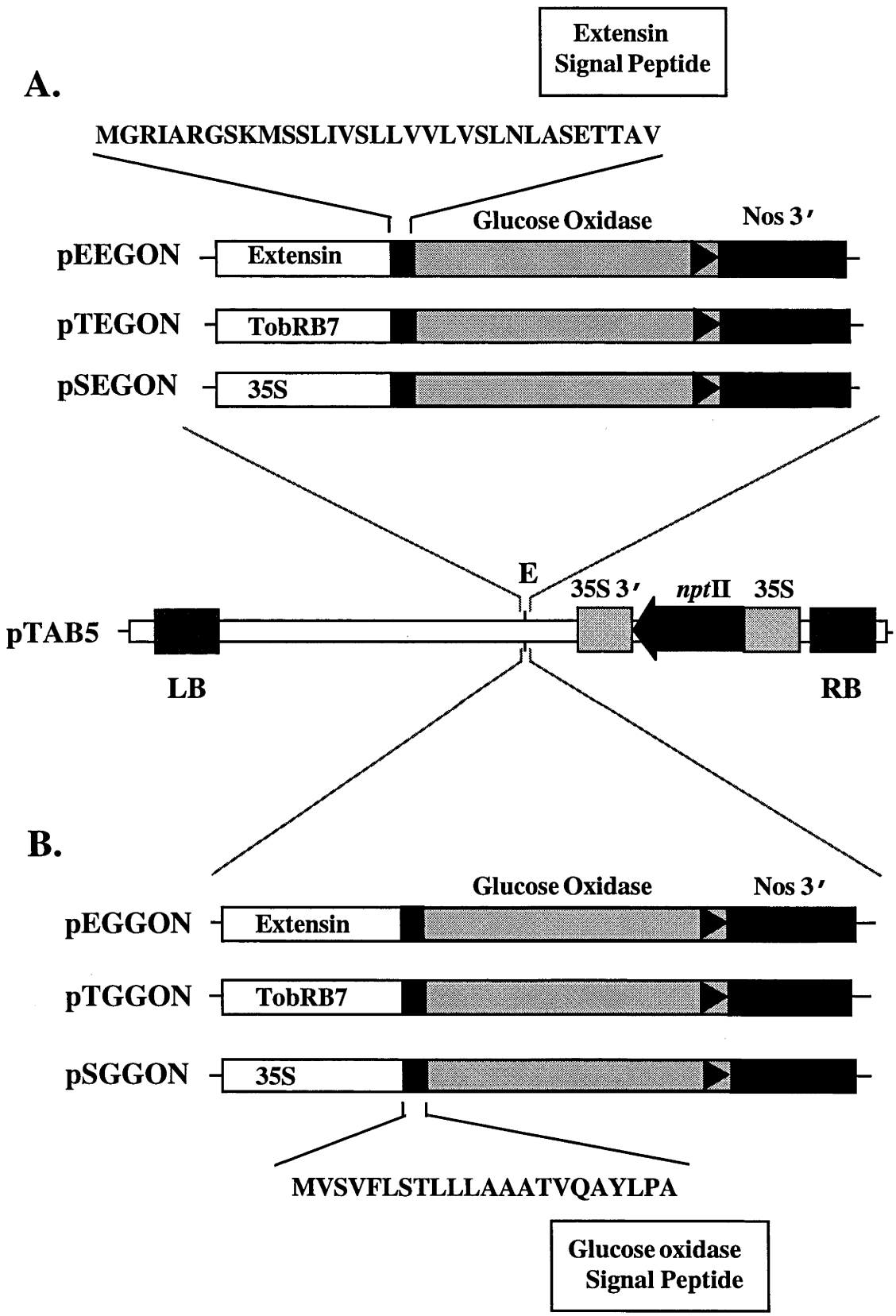


Figure 4.1 Schematic diagram of plasmid pTAB5. Black regions represent the right (B^R) and left (L^R) T-DNA borders from the *Agrobacterium* Ti plasmid. The chimeric kanamycin resistance gene shown in grey (Kan^R) consists of the Cauliflower mosaic virus (CaMV) 35S promoter (35S), the neomycin phosphotransferase gene from Tn 5 (*npt*II) and the CaMV 3' terminator sequence (35S 3'). Also shown is the gene for tetracycline resistance (Tet^R). Approximate size is given in kilobases (kb).

Figure 4.2 Glucose oxidase expression cassettes transformed into tobacco. Constructs contain the glucose oxidase gene fused in frame with the carrot root extensin signal peptide (**A**) or its own glucose oxidase signal peptide (**B**). Glucose oxidase was placed under control of either the carrot root extensin, TobRB7 or 35S promoter and the 3' end fused to the nopaline synthase (Nos) terminator sequence from *A. tumefaciens*. All constructs were cointegrated into the *Eco*RI site of the binary vector pTAB5 in an opposite orientation to the selectable kanamycin resistance gene. Abbreviations: E, *Eco*RI; *npt*II, neomycin phosphotransferase; LB, Left Border; RB, Right Border. Linear maps are not drawn to scale.


A.
Extensin
Signal Peptide
MGRIARGSKMSSLIVSLLVVLVSLNLASETTAV
Glucose Oxidase
Nos 3′
pEEGON
Extensin
pTEGON
TobRB7
pSEGON
35S
E
35S 3′
nptII
35S
pTAB5
LB
RB
B.
Glucose Oxidase
Nos 3′
pEGGON
Extensin
pTGGON
TobRB7
pSGGON
35S
MVSVFLSTLLLAAATVQAYLPA
Glucose oxidase
Signal Peptide

oxidase, hydrogen peroxide produced by glucose oxidase oxidises the KI to iodine (I_2). The I_2 then interacts with the starch to form a blue-black starch complex (Figure 4.3). The presence of glucose in the stain solution was not necessary for the formation of the blue-black colour but its presence did greatly increase speed of colour formation indicating that glucose is not totally limiting in plant tissue. A similar solution has been used by Olson and Varner (1993) and Schopfer (1994) to detect endogenous hydrogen peroxide in plant tissue. However, when tissue is completely submerged in stain solution (hypoxic conditions) no endogenous activity is detected. This stain solution can therefore be used under these conditions to specifically assay glucose oxidase activity.

As seen in Table 4.1, glucose oxidase activity could be detected in only three of the sixty tobacco plants obtained from transformation with constructs in which the glucose oxidase gene retained its own secretion signal peptide (pGGO series). As very weak staining was observed in all three transformants, the glucose oxidase activity in these transformants is probably very low. Many of the plants (58%) obtained from transformation with constructs containing the glucose oxidase gene attached to the extensin secretion signal peptide (pEGO series) did show glucose oxidase activity. In particular, 80% of the plants transformed with constructs in which the glucose oxidase gene was driven by the 35S and TobRB7 promoter showed some activity.

As the extensin promoter has been reported to be wound inducible (Chen and Varner, 1985; Granell *et al.*, 1992) roots were wounded as described in Materials and Methods and then submerged in stain solution. Only roots from the two weakly positive plants previously identified without prior wounding stained with KI. In both cases, no increase in staining intensity or decrease in staining time was observed indicating that the extensin promoter was not induced by the wounding procedures used here.

To quantitate the amount of glucose oxidase produced by individual transformants, crude root extracts from ten untransformed plants and sixty transgenic plants grown in tissue culture (ten plants for each of the six constructs) were assayed for

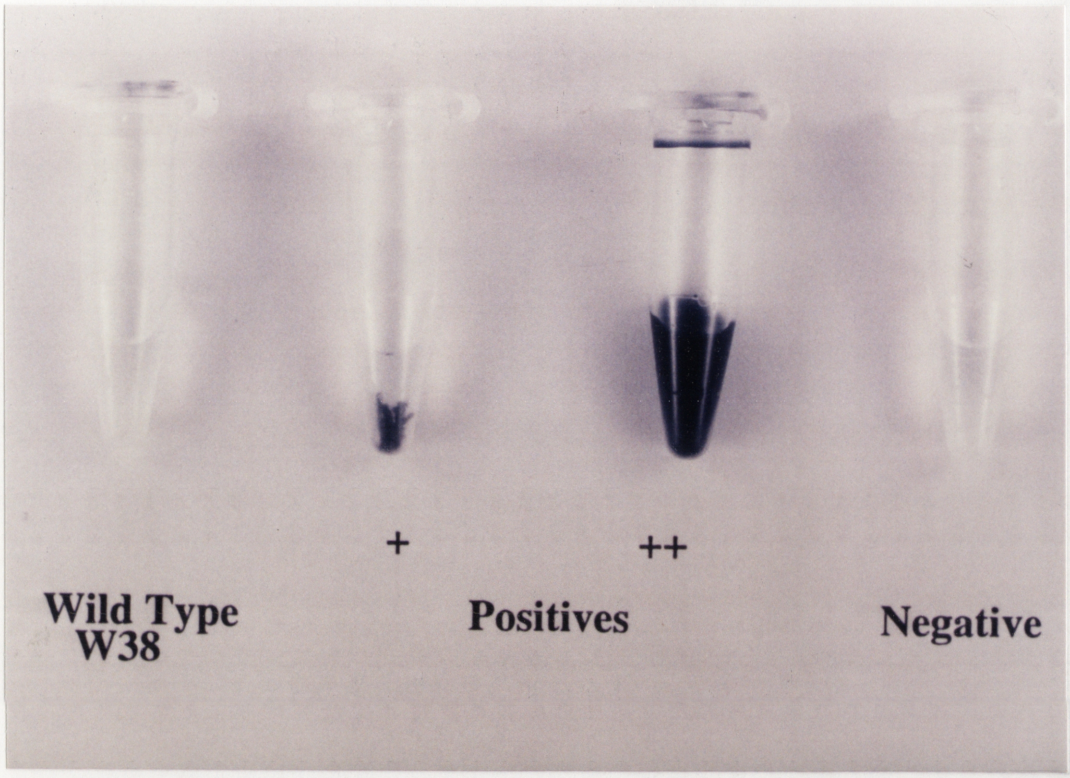


Figure 4.3 Detection of glucose oxidase activity in the roots of transgenic plants. Transgenic roots producing glucose oxidase cause the KI stain solution to turn a blue/black colour. Shown from left to right in stain solution, Untransformed W38 roots; Tobacco roots producing a small and large amount of glucose oxidase and Transgenic tobacco roots which fail to produce glucose oxidase.

Construct used to transform W38 tobacco	Number of positive transgenic plants in KI stain
pSEGON	13/15
pTEGON	11/15
pEEGON	2/15
pSGGON	3/20
pTGGON	0/20
pEGGON	0/20
Untransformed W38	0/5

Table 4.1 Glucose oxidase expression in transgenic tobacco transformed with different glucose oxidase expression constructs. All plants are believed to be independent transformants. Activity was determined qualitatively using a KI/starch solution. Each plant was tested three times, the results were identical each time.

production of hydrogen peroxide as described in Materials and Methods. Low levels of activity, 7-10 units/mg protein were detected in extracts from untransformed plants (units as defined in Chapter 2 Materials and Methods). As no activity was detected in the control experiment which contained all assay components except plant extract, this activity represents background levels of hydrogen peroxide present in root extract. The glucose oxidase activity detected in the best expressing transgenic plants is shown in Table 4.2. With the exception of the wild-type untransformed Wisconsin 38 (W38) control plants and SEGO-5 (a Kan^+/GO^- plant), all plants listed tested positive with KI stain. Some of those plants which stained only weakly with KI were found to produce only wild-type levels of hydrogen peroxide and are not included in the table. The glucose oxidase activity detected by the KI stain in these weak plants is thought to represent real transgene activity but the activity is too low to be distinguished from endogenous hydrogen peroxide production using the enzyme assay. The KI stain is therefore a more sensitive test to determine if the glucose oxidase gene is present and expressing in transgenic plants.

None of the root extracts from plants containing the pGGO constructs, including the KI staining lines, had levels of activity higher than that seen in wild-type untransformed roots. This confirms that the three positive transformants previously detected with KI stain had very low levels of glucose oxidase activity. A wide range of glucose oxidase activity was seen in root extracts from plants containing the pEGO constructs. The best activity, 6579 units/mg protein, is approximately 650 fold higher than wild-type roots. However this very high activity is dependent upon the presence of glucose in the assay buffer. In all plants expressing glucose oxidase, activity in root extracts decreased on average five to twenty fold when glucose was not present in the assay solution. The amount of glucose present in the root extract therefore limits the production of hydrogen peroxide by glucose oxidase.

In general, the highest glucose oxidase activity was seen in plants transformed with the glucose oxidase gene driven by the 35S promoter The level of activity in root extracts of these plants in the presence of glucose, is on average 100 fold higher than activity in wild-type roots. The TobRB7 promoter also gave good activity, but under the same conditions, extract activity was on average only 17 fold higher than wild-

	Glucose Oxidase Activity (Units/mg protein)		
Plant	**Root Extracts[a]**		**Leaf Extracts[b]**
	Plus glucose	**No glucose**	**Plus glucose**
Control Untransformed W38	0.2 ± 1 7.5 ± 3	0.0 ± 1 6.4 ± 3	0.3 24.5
SEGO-2	6578.6 ± 200	214.6 ±17	1238.2
SEGO-5	7.4 ± 2	8.4 ± 3	29.8
SEGO-9	140.6 ± 11	38.9 ± 3	146.6
SEGO-10	186.9 ± 13	22.2 ± 2	75.4
SEGO-12	469.9 ± 46	44.4 ± 3	244.8
SEGO-13	1942.5 ± 109	162.8 ± 6	965.0
SEGOS-18	255.3 ± 22	27.8 ± 2	69.3
SEGO-23	14.8 ± 3	13.9 ± 2	54.2
SEGO-24	13.0 ± 3	13.0 ± 2	986.7
SEGO-30	410.7 ± 30	42.6 ± 6	129.7
TEGO-2	135.1 ± 10	22.2 ± 2	*nd*
TEGO-9	229.4 ± 18	48.1 ± 4	*nd*
TEGO-10	373.7 ± 26	24.1 ± 2	*nd*
TEGO-15	283.1 ± 21	24.6 ± 3	*nd*
TEGO-17	207.2 ± 15	25.9 ± 3	*nd*
TEGO-18	142.5 ± 14	14.8 ± 2	*nd*
TEGO-19	27.8 ± 4	13.0 ± 2	*nd*
TEGO-20	20.4 ± 3	9.3 ± 3	*nd*
TEGO-21	111.0 ± 6	18.5 ± 2	*nd*
EEGO-11	11.1 ± 2	11.1 ± 3	*nd*
EEGO-20	29.6 ± 4	13.0 ± 2	*nd*

Table 4.2 Glucose oxidase activity in extracts prepared from roots or leaves of transgenic plants. All numbers represent independent transformants and refer to transformation with plasmids pSEGON, pTEGON and pEEGON. One unit of glucose oxidase activity is defined as the amount which will oxidise 1.0 µmole of β-D glucose to D-gluconic acid and H_2O_2 per minute at pH 5.1 and 25°C. **a** Average glucose oxidase activity and standard error calculated from duplicate assays on four clones of each plant. Control experiment contained all assay components except plant extract. Plus glucose and No glucose refers to inclusion or omission of 8% glucose in the assay buffer; **b** Results from a single experiment; *nd*: not determined.

type activity. Only two plants were found expressing the glucose oxidase gene under the control of the extensin promoter. In both plants very low levels of activity (just over wild-type levels) were detected.

Leaf extracts from plants containing pSEGON were also assayed for glucose oxidase activity (Table 4.2b). In general plants with high levels of glucose oxidase activity in the root extracts had high levels of glucose oxidase activity in the leaf extracts. The one exception was SEGO-24. Of the ten transformants tested, this plant has the second highest level of glucose oxidase activity in the leaf extracts, but almost no activity is detected in root extracts. This pattern of gene expression, which is abnormal for genes driven by a 35S promoter, is probably due to the site at which the gene integrated into the plant genome. Other elements present in the DNA surrounding the integrated gene could be influencing the pattern of glucose oxidase gene expression.

4.3.3 Altered phenotype as a result of the overproduction of glucose oxidase

In tissue culture, the two plants producing the most glucose oxidase (SEGO-2 and 13) grew noticeably slower than the other plants and produced fewer, finer roots. The plant with the highest activity (SEGO-2) was very stunted and grew very slowly (results not shown). As the degree of growth inhibition correlates with increasing levels of glucose oxidase activity, these effects are thought to be due to presence of the glucose oxidase gene. In particular, the results could reflect toxicity of the hydrogen peroxide produced by the protein.

Expression of glucose oxidase under control of the TobRB7 promoter in tobacco had no noticeable effect on plant growth. This is probably because significantly less glucose oxidase activity is present in the roots of these plants (less than one fifth activity) compared with that in SEGO-2 and SEGO-13 roots. Presumably insufficient hydrogen peroxide is produced in these roots to be toxic to the plant.

4.3.4 Analysis of glucose oxidase expression in T_1 progeny

To obtain plants for further analysis, plants expressing the glucose oxidase gene controlled by the 35S and TobRB7 promoter (pSEGON and pTEGON constructs) were potted in soil and transferred to the glasshouse where they were propagated

further. In soil, all plants appeared to grow normally and produced flowers and pollen. Seeds were obtained from all plants containing plasmid pTEGON. Only six of the ten plants containing the glucose oxidase gene controlled by the 35S promoter (pSEGON) produced seed (Table 4.3). Seed pods obtained from four of these six plants (SEGO-9, 10, 13 and 18) were small and contained few seeds (Figure 4.4). The other two plants (SEGO-5 and 24) produced seed pods similar in size and seed number to those produced by untransformed W38 tobacco plants.

Seeds from eleven of the transgenic plants (SEGO-9, 13, and 24; TEGO-2, 9, 10, 15, 17, 18, 19 and 21) were surface sterilized as described in Materials and Methods and germinated on agar medium. To detect glucose oxidase activity, seedlings from each plant were stained with KI solution (Figure 4.5). Some localization of gene expression is seen; staining occurs over the entire seedling in seedlings containing pSEGON (Figure 4.5c), but is confined to the root in seedlings containing pTEGON (Figure 4.5b). The pattern of gene expression determined by the promoters used in these constructs is investigated later in this chapter.

Thirty seedlings from each plant were randomly chosen and scored for glucose oxidase activity by staining with KI/starch solution (Table 4.4). It was not possible however, to determine whether seedlings were homozygous or hemizygous for the glucose oxidase gene using this method. Except for seedlings from plant SEGO-13, glucose oxidase activity was detected in approximately 75% of the seedlings from each plant (Table 4.4a). Approximately 96% (29/30) of the seedlings from SEGO-13 tested displayed glucose oxidase activity. Seeds were also germinated on medium containing kanamycin and scored for survival (Table 4.4b). Three percent of SEGO-13 seedlings and approximately 25% of the other seedlings germinated, bleached and later died. Therefore, in most plants the glucose oxidase and kanamycin resistance gene segregates in a Mendelian 3:1 ratio. The higher frequency of the glucose oxidase and kanamycin resistance gene in SEGO-13 progeny could result if the T-DNA has integrated into two different loci. In this situation 15/16 seedlings (approximately 94%) would be expected to have glucose oxidase activity. The occurrence of two different integration events is consistent with the high level of glucose oxidase activity found in SEGO-13 roots and leaves.

Plant	Seed Production
Untransformed W38	Yes
SEGO-2	No
SEGO-5	Yes
SEGO-9*	Few
SEGO-10	Few
SEGO-12	No
SEGO-13*	Few
SEGO-18	Few
SEGO-23	No
SEGO-24*	Yes
SEGO-30	No

Table 4.3 Seed production in transgenic tobacco plants transformed with pSEGON. Progeny of plants marked with an asterisk were analysed for glucose oxidase activity.

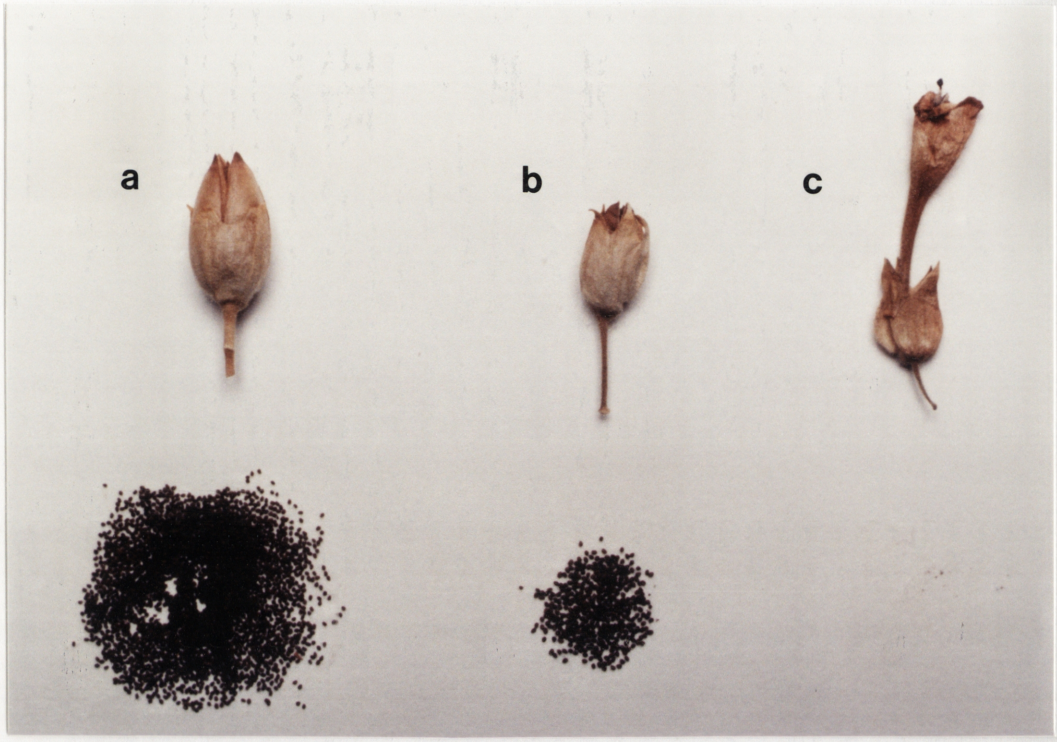


Figure 4.4 Seed pods from untransformed and transgenic tobacco plants expressing glucose oxidase under control of the 35S promoter. **a**: Untransformed W38; **b**: SEGO-13; **c**: SEGO-23.

Figure 4.5 The effect of different promoters on localisation of glucose oxidase activity in ten day-old T_1 seedlings. Seedlings were stained for 45 minutes in KI stain solution. **a**: Untransformed W38 seedling. **b**: Seedling from a plant transformed with pTEGON (TobRB7 promoter). **c**: Seedling from a plant transformed with pSEGON (35S promoter).

Plant	Percentage of T_1 seedlings producing glucose oxidase.[a] (of 30 scored)	Percentage of kanamycin resistant T_1 seedlings.[b] (of 30 scored)
SEGO-9	77	73
SEGO-13	97	97
SEGO-24	73	83
TEGO-2	80	83
TEGO-9	73	70
TEGO-10	67	77
TEGO-15	67	83
TEGO-17	83	70
TEGO-18	67	73
TEGO-19	80	70
TEGO-21	73	83

Table 4.4 Inheritance of the glucose oxidase and kanamycin resistance gene in T_1 transgenic tobacco seeds. **a**: Seedlings were stained with KI/starch solution to determine glucose oxidase activity. **b**: Seedlings were germinated on media containing 100μg/ml kanamycin to determine presence of *npt*II (kanamycin resistance) gene.

4.3.5 Molecular analysis of *Nicotiana tabacum* transformed with pSGGON

As no significant glucose oxidase activity could be detected in any of the plants transformed with pSGGON, PCR was used to confirm that the glucose oxidase gene had stably integrated into the genome of transformants created with this vector. Specific primers were designed to amplify a central 800bp region of the coding region of the *T. flavus* glucose oxidase gene sequence and PCR was carried out as described in Materials and Methods. The expected 800bp fragment amplified in DNA from all eleven pSGGON transformants analysed and in control plasmid DNA containing the *T. flavus* glucose oxidase gene. No amplification was seen when no DNA was added to the reaction mixture or when DNA extracted from untransformed W38 tobacco plants was added to the reaction (results not shown).

To ensure that the glucose oxidase gene in these transformants was being properly transcribed, a Northern analysis was performed. Total RNA prepared from the leaves of eleven pSGGON and nine pSEGON transformants was hybridized with an antisense riboprobe generated from the *T. flavus* glucose oxidase gene. Figure 4.6 shows a typical hybridization pattern obtained for RNA from ten plants transformed with either pSGGON or pSEGON.

Several hybridizing bands are seen in most transgenic plants analysed but no hybridizing bands are seen in untransformed W38 RNA or transformant SGGO-19 RNA. The predominant hybridizing band has a predicted size of approximately 2000nt, which is in agreement with the expected size for the glucose oxidase message as predicted from the gene sequence. Several larger messages (approximately 4400nt and 6500nt) are also seen in some of the transformants, which are probably caused by transcription termination occurring at different sites more distant from the glucose oxidase polyadenylation site.

Levels of hybridizing RNA were quantified and standardised by comparison with hybridization to a *Arabidopsis* ubiquitin probe (Table 4.5). Although the level of message varies from transformant to transformant, on average there is little difference in glucose oxidase mRNA levels in plants transformed with pSEGON or pSGGON (2.0 and 2.5 respectively). Of those plants transformed with pSEGON,

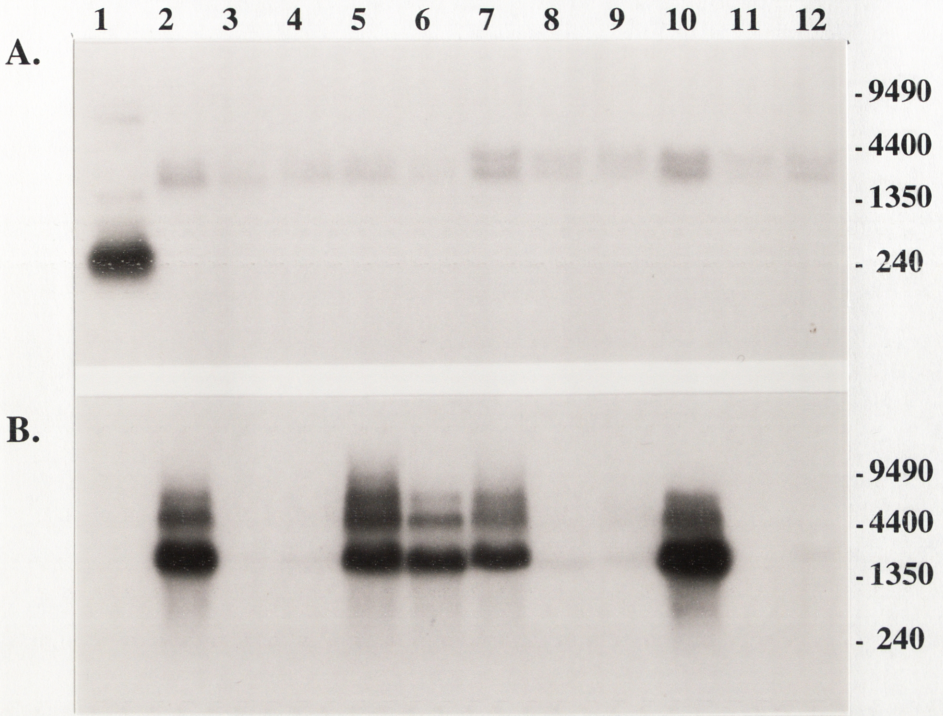


Figure 4.6 Northern analysis of glucose oxidase gene expression in plants transformed with either pSEGON or pSGGON. Total RNA was isolated from the leaves of plants and hybridized with **A**: A ^{32}P-labelled *Arabidopsis* ubiquitin riboprobe or **B**: A ^{32}P-labelled riboprobe corresponding to the ORF of the *T. flavus* glucose oxidase gene. Lane 1, BRL ladder; lane 2, SGGO-15; lane 3, SGGO-19; lane 4, SGGO-20; lane 5, SGGO-1; lane 6, SGGO-18; lane 7, SEGO-13; lane 8, SEGO-5; lane 9, SEGO-12; lane 10, SEGO-2; lane 11, Untransformed W38; lane 12, SEGO-23. The position of the BRL size standards is indicated in nucleotides.

Plant	RNA Level
Untransformed W38	0.00
SEGO-2	6.73
SEGO-5	0.55
SEGO-9	2.34
SEGO-12	0.82
SEGO-10	0.85
SEGO-13	3.15
SEGO-18	0.82
SEGO-23	0.75
SEGO-24	2.34
SGGO-1	4.97
SGGO-2	6.00
SGGO-3	0.03
SGGO-4	1.03
SGGO-5	0.84
SGGO-15	5.47
SGGO-18	3.71
SGGO-19	0.48
SGGO-20	0.60
SGGO-21	1.80
SGGO-26	0.84

Table 4.5 Relative levels of glucose oxidase gene transcript in leaves of transgenic tobacco plants previously transformed with either pSEGON (35S promoter and extensin signal peptide) or pSGGON (35S promoter and glucose oxidase signal peptide). All plants are believed to be independent transformants. Levels of RNA are relative to ubiquitin transcript abundance.

plants producing the most glucose oxidase mRNA (SEGO-2, 13 and 24) have the highest levels of glucose oxidase activity in leaf extracts (Table 4.2b). The three plants transformed with pSGGON producing the most glucose oxidase mRNA (SGGO-1, 2 and 15) were the three plants whose roots stained very weakly with KI/starch stain solution.

4.3.6 Expression of the GUS gene driven by the TobRB7 and carrot root extensin promoters in the roots of *Nicotiana tabacum*

4.3.6.1 Construction of plasmids

Although the KI stain could be used to grossly examine the pattern of glucose oxidase gene expression, the stain is too soluble to be used to study gene expression at a cellular level. Therefore, to compare the pattern of expression and strength of the TobRB7 and carrot root extensin promoters, use was made of the β-glucuronidase (GUS) reporter gene. As described in Materials and Methods, each promoter and the extensin signal peptide sequence were fused to the GUS gene and Nos 3' terminator. As GUS enzymatic activity is severely inhibited due to N-linked glycosylation when it is targeted to the endoplasmic reticulum, the modified $Gus_{358\rightarrow S}$ gene (Farrell and Beachy, 1990) was used. This gene has the cryptic N-linked glycosylation site in the GUS gene destroyed and can therefore be used for secretory studies. These plasmids, called pTEGusN (TobRB7 promoter) and pEEGusN (Extensin promoter), are shown in Figure 4.7. Control plasmids were also constructed in which the promoters were fused directly to the $GUS_{358\rightarrow S}$ gene and Nos terminator. These plasmids which do not contain the extensin signal peptide were called pTGusN and pEGusN (Figure 4.7).

Each of the four constructs was cloned into the *Eco*RI site of the binary vector pTAB5 and restriction enzyme analysis performed to determine the orientation of the GUS gene in the binary vector. Constructs containing the GUS gene in the opposite orientation to that of the 35S-*npt*II gene were introduced into tobacco by *Agrobacterium*-mediated transformation. Twelve to fifteen primary transformants were selected for each construct.

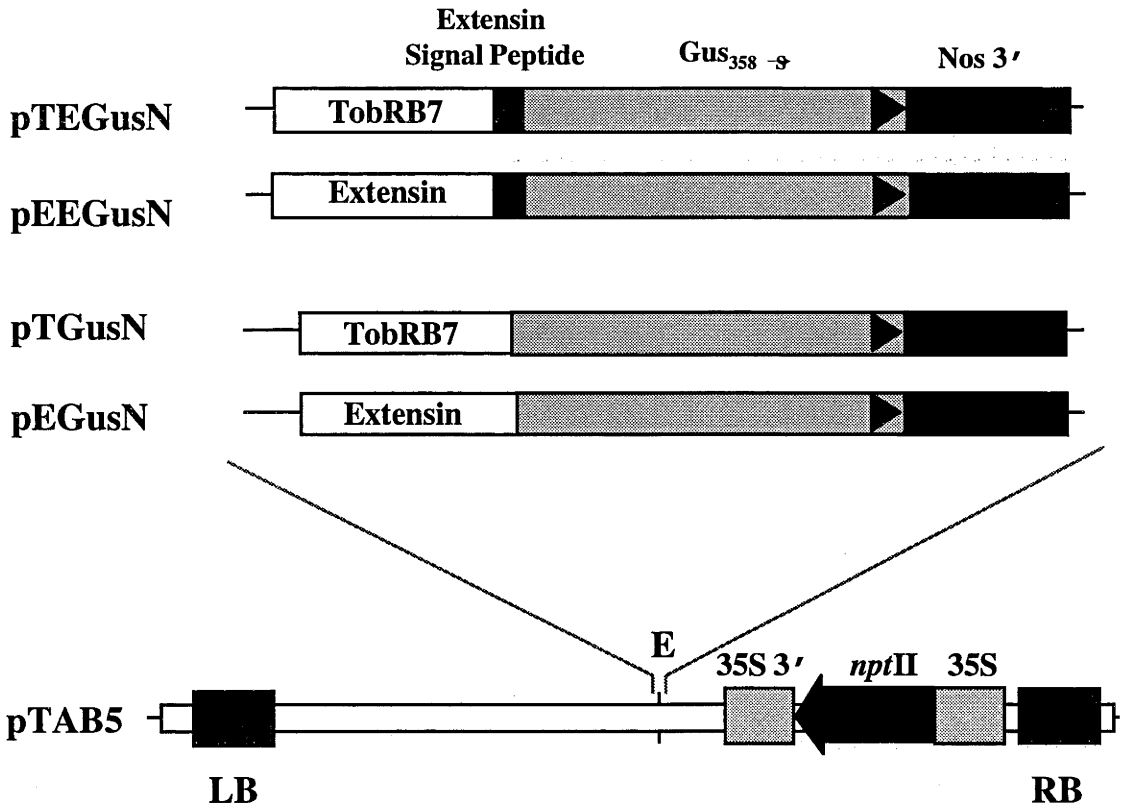


Figure 4.7 Chimeric gene fusions used to determine TobRB7 and carrot root extensin promoter activity in transgenic plants. Promoters were fused to the GUS reporter gene and the nopaline synthase (Nos) terminator region from *A. tumefaciens.* Plasmids pTEGusN and pEEGusN have the carrot extensin signal peptide fused in frame to the 5' end of the GUS gene. All constructs were cointegrated into the *EcoRI* site of the binary vector pTAB5 in an indirect orientation to the selectable kanamycin resistance gene. Abbreviations: E, *Eco*RI; *npt*II, neomycin phosphotransferase; LB, Left Border; RB, Right Border. Linear maps are not drawn to scale.

4.3.6.2 Strength and localization of GUS activity in *Nicotiana tabacum* roots

Extracts prepared from the roots of primary transformants were assayed for β-glucuronidase activity by the fluorometric method using MUG as the substrate (Table 4.6). Of the plants containing the GUS gene fused to the extensin signal peptide, GUS activity was detected in only four of the thirty plants assayed (15 plants per construct). The level of activity in three of these plants is very low, only two to five fold that seen in untransformed tobacco. Higher activity, approximately 25 fold wild-type levels, is observed in plant EE-2. A much higher level of activity, 12 to 900 fold wild-type level, is seen in plants transformed with pTGusN. On average, activity 3 fold lower than that seen in pTGusN is seen in plants transformed with pEGusN (1.6 to 90 fold wild-type levels). So when plants are grown in tissue culture the extensin promoter generally results in weaker gene expression in the roots than the TobRB7 promoter and fusion of the extensin signal peptide to the 5' end of the GUS gene significantly decreases GUS activity.

Localization of GUS activity in the roots of either untransformed plants or plants transformed with one of the four GUS constructs was determined by staining fresh root tissue in the chromogenic GUS substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc). Roots from transgenic tobacco plants expressing the GUS gene under the control of the 35S promoter (Bogusz *et al.*, 1990) were also stained with X-Gluc so the pattern of GUS expression determined by the TobRB7 and extensin promoters could be compared to GUS expression controlled by the 35S promoter. Transverse sectioning of all roots through the region of maturation was performed and GUS staining observed under dark field to examine the cellular location of GUS expression in this region (Figure 4.8 b, d, f, and h). With the exception of the controls (Figure 4.8 a& b), photographs of roots from transgenic plants expressing the highest levels of GUS are shown in Figure 4.8. Roots from three different plants for each line were analysed. The photographs are a typical representation of what was seen in all sections except Figure 4.8f (TobRB7 transverse section). In TobRB7 plants expressing lower levels of GUS, expression in the outer cortex cells was much weaker.

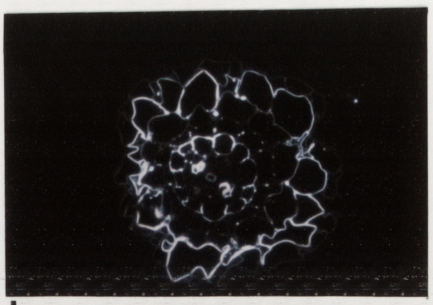
Plant	MU produced (pmol/min/mg protein)
Untransformed W38	0.6 ± 0.1
TE-2	2.1 ± 0.3
TE-5	1.5 ± 0.2
EE-2	18.7 ± 2
EE-6	3.5 ± 0.3
T-1	625.6 ± 84
T-2	50.5 ± 6
T-3	24.1 ± 2
T-5	58.1 ± 4
T-7	52.6 ± 5
T-8	150.0 ± 12
T-12	93.1 ± 10
T-17	8.1 ± 0.7
T-19	36.6 ± 4
E-2	90.2 ± 8
E-5	56.9 ± 5
E-6	14.5 ± 2
E-9	1.6 ± 0.2
E-14	21.0 ± 4
E-18	56.4 ± 8

Table 4.6 β-Glucuronidase activity in crude root extracts from transgenic tobacco plants. Values are the means ± the standard error (from three independent experiments). **TE-**: plants transformed with pTEGusN; **EE-**: plants transformed with pEEGusN; **T-**: plants transformed with pTGusN; **E-**: plants transformed with pEGusN. **MU**: 4-methyl umbelliferone, the fluorescent compound produced when 4-methyl-umbelliferyl glucuronide is cleaved by β-glucuronidase.

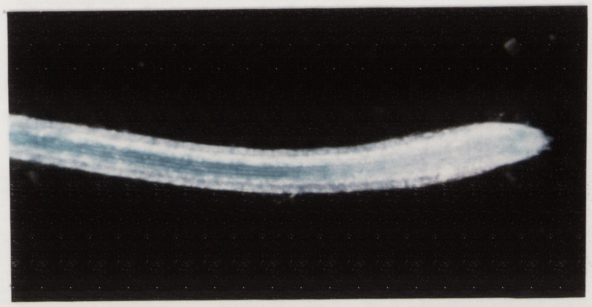
Figure 4.8 Histochemical localisation of GUS activity in tobacco roots when expression is directed by either the carrot root extensin, TobRB7 or 35S promoter. Roots were stained with X-Gluc, sectioned if necessary and photographed using either bright or dark field optics. Under bright field, crystals of the GUS product appear blue, under dark field the crystals appear pink. All transverse sections (b, d, f, h) were taken from the region of maturation.

a & b Untransformed W38 root.
c & d Root from a plant transformed with pEGusN (carrot root extensin promoter).
e & f Root from a plant transformed with pTGusN (TobRB7 promoter).
g & h Root from a plant transformed with 35SGus (35S promoter).

a



b



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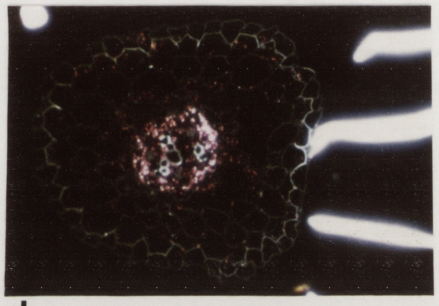
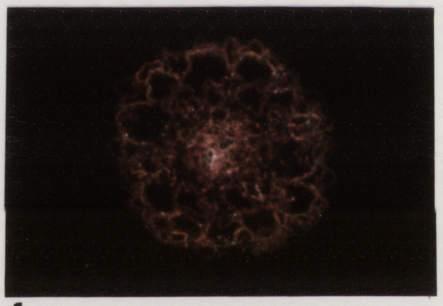


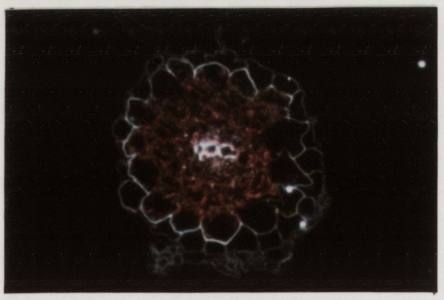
d

e



f

g



h

As roots from plants transformed with the GUS gene fused to the extensin signal peptide stained very faintly or not at all, it was not possible to determine the localization of GUS activity in these plants. As expected, no GUS activity was seen in untransformed W38 roots (Figure 4.8a & b). When the GUS gene is driven by the carrot root extensin promoter GUS expression is observed in all regions of the root, although the intensity of staining suggests gene expression is lower in the region of elongation (Figure 4.8c). Transverse sectioning through the region of maturation shows expression in this area to be predominantly located in the phloem and endodermal cells (Figure 4.8d). The TobRB7 promoter drives GUS expression in all regions of the root except for the root tip and root cap (Figure 4.8e). In the region of maturation the enzyme is present in all root cells except the older xylem cells (Figure 4.8f). This pattern of gene expression observed for TobRB7 is similar to that found by Yamamoto *et al.* (1991), however expression is not confined to the central cylinder region as they report. This could be because the region of root sectioned here is older than that sectioned by Yamamoto and coworkers or because plants expressing very high levels of β-glucuronidase were photographed. The 35S promoter also drives GUS expression in all regions of the root but is particularly strong in the root cap and meristematic region (Figure 4.8g). Expression is confined to the vascular tissue and the inner cortex cells of the root in the region sectioned (Figure 4.8h).

4.3.6.3 Induction of the carrot root extensin promoter

As the carrot root extensin promoter has been reported to be wound-inducible in carrot roots (Chen and Varner, 1985; Granell *et al.*, 1992), various treatments as described in Materials and Methods were used to try and increase β-glucuronidase expression in plants containing the extensin promoter fused to the GUS gene. Also, in a separate experiment, plants were sprayed with 10mM salicylic acid or water (control) 48 hours prior to assaying roots to determine if this treatment would induce the extensin promoter. No increase in β-glucuronidase activity was seen in root extracts of plants transformed with either pEEGusN or pEGusN following any of these treatments.

4.3.7 Expression of glucose oxidase in *Gossypium hirsutum*

Plasmids pTEGON (glucose oxidase gene driven by the TobRB7 promoter and extensin signal peptide), pEEGON (glucose oxidase gene driven by the extensin promoter and extensin signal peptide) and pSEGON (glucose oxidase gene driven by the CaMV 35S promoter and extensin signal peptide) were separately transformed into *G. hirsutum cv.* Coker by way of *Agrobacterium* mediated transformation. Calli were selected from each of the transformations on kanamycin-containing medium and regenerated into plants as described in Materials and Methods.

Thirty-four putatively independent transgenic plants were regenerated after transformation of cotton with pTEGON. Approximately 50% of the callus derived from tissue transformed with pEEGON died 3-4 months after the transformation; however sixteen putative transformants were still regenerated. Callus derived from tissue transformed with pSEGON also regenerated poorly and from three independent transformation experiments only four putatively independent transgenic plantlets were regenerated. Roots from several clones of all putative transgenics were tested for glucose oxidase activity in KI/starch stain solution before being transferred to pots in the glasshouse. Twenty-three of the thirty-four lines regenerated after transformation with pTEGON and three of the sixteen lines regenerated after transformation with pEEGON showed glucose oxidase activity (Table 4.7). However, none of the plantlets regenerated after transformation with pSEGON displayed glucose oxidase activity. As the 35S promoter has been shown to drive gene expression in cotton callus (Lyon *et al.*, 1993), callus thought to be transformed with pSEGON was also tested for glucose oxidase activity by submerging it in KI stain. None of forty individual callus lines stained tested positive for glucose oxidase activity.

Transgenic plants were placed into different groups depending on the time taken for the KI/starch stain solution to completely change colour after the addition of three 2 cm pieces of root to 250µl of solution. Group one roots completely changed the colour of the stain solution within one hour of roots being added, Group two within four hours, Group three overnight and Group four never completely changed the colour of the solution although some staining of solution and roots was observed.

	Regenerated cotton lines producing glucose oxidase.	Total number of plants in group.
Plants containing pTEGON		
Group 1	**T-53^{S}**, **T-76^{S}**, T-78, T-97	4
Group 2	**T-2^{S}**, T-10, T-12, T-13*, **T-24^{S}**, T-52*, **T-71^{S}**, T-77, **T-90^{+}**, **T-92^{S}**, T-105	11
Group 3	**T-5^{+}**, T-11, **T-14^{S}**, T-16, **T-68^{S}**	5
Group 4	**T-48^{S}**, T-99, T-19	3
Plants containing pEEGON		
Group 1	-	0
Group 2	-	0
Group 3	-	0
Group 4	E-42, E-60, E-91	3

Table 4.7 Regenerated cotton lines producing glucose oxidase. Each line is believed to represent an independent transformation event. Cotton lines were placed into different groups based upon the time taken for three 2cm roots to completely change the colour of 250µl of KI/starch solution. In Group one, colour change was complete within one hour of roots being added to the stain solution, in Group two within four hours, in Group three, overnight and Group four never completely changed the colour of the solution but some staining and/or staining of roots was observed. Seeds have been collected from lines in bold.

^{S} selfed lines from which seeds have been obtained
$^{+}$ male sterile lines, seeds obtained by crossing to *cv.* Coker 315
* completely sterile lines, no seeds obtained
Other lines are still flowering and forming seed

Glucose oxidase activity ranging from weak to strong is seen in cotton plants transformed with pTEGON (Table 4.7). Similar activity was seen in roots from tobacco plants transformed with pTEGON. Only very weak activity is seen in cotton plants transformed with pEEGON, which is also consistent with the low expression levels seen in tobacco plants transformed with pEEGON. The lack of expression observed in pSEGON transformed cotton and the difficulty experienced in obtaining such regenerated plants is thought to be because cotton, unlike tobacco, regenerates from callus and constitutive expression of the glucose oxidase gene inhibits the plant regeneration process. The few plants that were obtained are probably either transgenic only for the *npt*II (kanamycin resistance) gene or are 'escape' plants which are not transformed and have survived antibiotic selection.

The twenty-three pTEGON transgenic cotton lines which expressed glucose oxidase and the sixteen putative EEGON transgenic lines were propagated further in the glasshouse. Seed has been obtained from eleven of the twenty-three TEGON lines (two lines were male sterile and were crossed to *cv.* Coker 315 to obtain seed) and two lines are known to be completely sterile (Table 4.7). The remainder of the lines are still flowering and forming seed.

Seeds from six of the TEGO lines were germinated in vermiculite and roots from the seedling placed in KI stain to test for glucose oxidase activity. Glucose oxidase activity was detected in approximately 75% of the seedlings from four of the lines, indicating that in these lines, as in most of the tobacco plants examined, the glucose oxidase gene is segregating in a Mendelian 3:1 ratio (Table 4.8). In one of the other lines (T-68) 95% of the seedlings were positive for glucose oxidase; so more than one integration event probably occurred in this line. The sixth line (T-48) had very few progeny with glucose oxidase activity; the reason for this is unclear.

4.4 DISCUSSION

The *T. flavus* glucose oxidase gene has been successfully expressed in both tobacco and cotton plants. To obtain high levels of glucose oxidase activity in plants it was necessary to replace the glucose oxidase secretion signal peptide with one from the carrot root extensin gene. Why the replacement was necessary to obtain good

Plant	Number of T_1 seedlings expressing glucose oxidase	Expected number of positive plants for 3:1 segregation
T-53	9/11	8.25
T-76(1)	15/20	15
T-76(2)	13/19	14.25
T-2	9/15	11.25
T-14	21/24	18
***T-68**	19/20	15
***T-48**	4/15	11.25

Table 4.8 Inheritance of the glucose oxidase gene in T_1 transgenic cotton seedlings. Seedlings were stained with KI/starch solution to determine glucose oxidase activity. Asterisks indicate those lines in which the glucose oxidase gene is not segregating in a Mendelian 3:1 ratio.

activity is not clear as although the extensin signal sequence is eleven amino acids longer than the glucose oxidase signal peptide (see Figure 4.2), both sequences should function as signal peptides according to the consensus rules of Van Heijne (1985). As an extensive literature search did not reveal any other instances in which fungal genes with signal peptides have been expressed in plants, it is not possible to compare this result with others of a similar nature. However, findings that plant signal peptides function in fungi (Rothstein *et al.*, 1984; Tague and Chrispeels, 1987) suggest that the result obtained here is unexpected.

Abundant glucose oxidase mRNA was isolated from plants transformed with plasmid pSGGON so the presence of the glucose oxidase signal peptide probably does not affect transcription of the glucose oxidase gene or stability of the corresponding mRNA. Low levels of glucose oxidase activity were detected in the three pSGGON transformed plants which produced the largest amounts of RNA, so in these plants a small amount of glucose oxidase mRNA was correctly translated and processed.

Inefficient translation initiation of the glucose oxidase mRNA in these plants could account for the low levels of glucose oxidase activity observed. Sequences flanking the initiating ATG influence how efficiently the 40S ribosome begins translation at this site. If the sequence surrounding the initiating ATG in pSGGON is suboptimum compared to the corresponding sequence in pSEGON, this could result in poor translation of the glucose oxidase gene in pSGGON transgenic plants. The consensus sequence for efficient initiation of translation in plants is TAAACAATGGGCT (Joshi 1987). When compared to this sequence (Figure 4.9), both pSGGON and pSEGON have the G immediately following the ATG conserved, the *T. flavus* glucose oxidase sequence has the A at -1 conserved and the extensin signal peptide sequence after modification by site directed mutagenesis has the A at -4 conserved (both numbers are with respect to the initiation codon). Both sequences therefore share little similarity to the consensus sequence so it is unlikely that inefficient recognition of the initiating ATG is the cause of poor glucose oxidase activity in pSGGON transformed plants.

Plant consensus sequence	**TAAACAATGGCT**
T. flavus sequence	ATCGA**A**ATG**G**TG
Extensin sequence	CT**A**GTCATG**G**GA

Figure 4.9 Comparison of sequences surrounding the initiating ATG of the *T. flavus* glucose oxidase gene and the carrot root extensin gene (after modification by site-directed mutagenesis) to the plant consensus sequence determined by Joshi (1987). The initiating ATG is underlined and letters in bold share identity with the plant consensus sequence.

Low levels of glucose oxidase activity in pSGGON plants may also result if the glucose oxidase signal peptide itself functions incorrectly and causes rapid degradation of the glucose oxidase protein or production of a protein with very little activity. It has been proposed that the signal peptide on a newly synthesized polypeptide protrudes from the ribosome and interacts with a signal recognition particle and a receptor in the endoplasmic reticulum (ER) membrane. Interaction with these structures anchors the ribosome complex and nascent protein chain to the ER membrane. The signal recognition particle is then released and translocation machinery moves the protein through the ER membrane at which time the signal peptide is cleaved from the nascent protein by an endopeptidase contained within the ER (Calmels *et al.*, 1991; High, 1992).

Several reasons could therefore account for the glucose oxidase signal peptide not operating properly in plants. It may not efficiently recognize and interact with the plant signal recognition particle so little of the protein would enter the ER and Golgi system and very low levels of functional glucose oxidase would occur. Also, the fungal signal peptide may be cleaved inefficiently or at the wrong place in plants. This could result in the glucose oxidase protein not folding or glycosylating properly and glucose oxidase enzyme activity may be eliminated or severely decreased. In future, antibodies to *T. flavus* glucose oxidase could be raised and Western analysis of both pSGGON and pSEGON plants performed. This would reveal the size and amount of protein present which would indicate whether glucose oxidase is degraded or non-glycosylated in pSGGON plants.

The level of hydrogen peroxide in root extracts from plants producing glucose oxidase was on average 17 to 100 fold higher than in wild-type root extracts. When glucose was not included in the assay solution, activity in root extracts decreased 5-20 fold. Staining of transgenic roots expressing glucose oxidase with KI/starch solution also took much longer when glucose was not included in the solution. This may suggest that hydrogen peroxide produced by glucose oxidase in plants will be severely limited by the amount of glucose in the plant. However in a plant, utilization of glucose by glucose oxidase could result in more glucose being produced by invertases.

Invertases (β-D-Fructofuranoside fructohydrolases EC 3.2.1.26) cleave sucrose into glucose and fructose. The substrate for invertase, sucrose, is the prime product of photosynthesis and being the major form in which carbon is translocated in plants it should be present in most plant tissues. Invertases are widely distributed in plants with most plant tissues analysed found to contain multiple forms of invertases located in various subcellular compartments (Ap Rees, 1988). Soluble invertases which have pH optima ranging from pH7.5 to pH4.5 are known to be located in the vacuole (Leigh *et al.*, 1979) and the cytosol (Karuppiah *et al.*, 1989; Fahrendorf and Beck, 1990). Insoluble invertase which has a pH optimum of between 4.0 and 5.5 is often associated with the apoplast or cell walls (Fahrendorf and Beck, 1990). Invertase activity is usually high in tissues which are undergoing rapid growth and development such as root tips, developing cotyledons, and growing callus cultures (reviewed by Avigad, 1982). In addition, invertase activity can increase quickly (within one hour) after wounding or pathogen infection. (Matsushita and Uritani, 1974; Billett *et al.*, 1977; Krishnan and Pueppke, 1988; Sturm and Chrispeels, 1990).

The data suggests that glucose should not be limiting in plants. Although the precise cellular location of glucose oxidase in the plant is not known, it is probable that the enzyme resides in the vacuole or apoplast of the cell due to the presence of a secretion signal peptide at the 5' end of the gene. In both of these locations there are invertases which function at an acceptable pH for glucose oxidase activity (pH 4.0 - 7.0). It is however difficult to determine what effect the gluconic acid produced by glucose oxidase would have on invertase activity or at what concentration the fructose generated by the invertase would feed back to inhibit invertase activity. The high invertase activity in tissues such as root tips and at sites of emerging secondary roots could be particularly beneficial in obtaining resistance to Verticillium wilt as these regions are prominent sites of Verticillium infection (Gerik and Huisman, 1988). Also the rapid increase in invertases in response to pathogen infection could be beneficial as it would provide glucose to glucose oxidase when the production of hydrogen peroxide is most required.

In tissue culture, transformed plants producing high levels of glucose oxidase-mediated hydrogen peroxide (55 to 870 fold wild-type levels) grew slowly and

produced fewer, finer roots than untransformed plants or plants producing lower levels of hydrogen peroxide. This phenotypic effect was not noticeable when the affected plants were grown in the soil. As plant tissues can take up externally applied sucrose (Lucas and Madore, 1988) the poor growth of the highly expressing transformants in tissue culture is thought to be caused by the abundance of sucrose present in the tobacco tissue culture media. Conversion of the sucrose to glucose and fructose by invertases present in the apoplast would provide sufficient glucose and large amounts of hydrogen peroxide which may slow plant growth, could be generated. Alternatively, the large concentration of gluconic acid produced by glucose oxidase could adversely affect the invertase and/or other enzymes present. This too could result in slow plant growth. In both instances the tissue culture step in the transformation process selects against plants producing high levels of glucose oxidase. It may therefore be possible to generate plants producing even higher levels of glucose oxidase if a different sugar was used in the tissue culture medium.

Seeds from most of the plants expressing the glucose oxidase gene under the control of the 35S promoter did not develop normally. However, seed development in all plants transformed with the glucose oxidase gene fused to the root specific promoter, TobRB7, proceeded normally. This suggests that the phenotype associated with 35S-glucose oxidase gene expression is not caused by random insertion of the T-DNA into essential genes but by glucose oxidase expression in the developing seed. This is substantiated by the following observations. Of the ten SEGO transgenic plants analysed, only SEGO-5, which had no detectable glucose oxidase activity, and SEGO-24, which had an abnormal pattern of gene expression, produced wild-type sized seed pods containing abundant seeds. Within the remaining eight SEGO transgenic plants, there was some correlation between the severity of this phenotype and the levels of hydrogen peroxide observed in root and leaf extracts of these plants. Most plants which failed to form seeds (SEGO 2, 12 & 30) had higher levels of glucose oxidase activity than those that formed a few seeds (SEGO 9, 10 & 18). The two exceptions (SEGO 13 & 23) could be explained if, due to the integration site of the T-DNA, the pattern of glucose oxidase gene expression was different in these plants.

Analysis of glucose oxidase activity in T_1 seedlings from SEGO-9 and 13 indicated that in both lines the glucose oxidase gene was segregating in a Mendelian fashion. Therefore, it is unlikely that poor seed production in these plants is due to the absence of a glucose oxidase homozygous class from the population. However, other reasons could account for small pods and poor seed formation. More glucose could be present in the developing seed possibly as a result of increased invertase activity. This may result in an increased concentration of hydrogen peroxide which is toxic to the seed. Alternatively the developing seeds may be particularly susceptible to hydrogen peroxide at this stage. Turley and Trelease (1990) found that catalase activity in developing cotton seeds did not significantly increase until approximately 35 days post anthesis. If a similar delay in catalase or peroxidase production occurs in tobacco seed development, the young tissue may be particularly susceptible to damage caused by glucose oxidase-generated hydrogen peroxide.

When used to drive glucose oxidase gene expression in transgenic plants the extensin promoter resulted in plants with very low levels of glucose oxidase activity. However, in the absence of a signal peptide, the extensin promoter fused to $GUS_{358\rightarrow S}$ resulted in β-glucuronidase activity on average only 3 fold lower than activity in extracts from plants transformed with $GUS_{358\rightarrow S}$ fused to the TobRB7 promoter. This suggests the extensin promoter is not as weak as the glucose oxidase activity data indicates. In protoplasts the carrot extensin promoter has been reported to drive high levels of GUS expression (6-7 fold 35S promoter) (Granell *et al.*, 1992). This very high expression is induced by various stimuli, including wounding. Cell culturing has been found to induce other extensin genes (reviewed by Showalter, 1993) so it is possible the carrot extensin promoter is also induced during tissue culture. Therefore in tissue culture, particularly during regeneration, plants transformed with pEEGON may have been producing large amounts of glucose oxidase. In this situation the large amount of hydrogen peroxide produced by glucose oxidase could be toxic to plants. Only those plants producing very small amounts of glucose oxidase would survive, which corresponds to what is seen here in both the tobacco and cotton plants. This theory also explains the observation that approximately 50% of the cotton callus derived from tissue transformed with

pEEGON died 3-4 months after transformation. The failure of wounding to induce GUS activity in plants transformed with pEGusN may be because the plants examined were being propagated in an artificial tissue culture environment. To examine this possibility, seed from this line is being generated. Induction studies can be repeated on progeny plants grown in soil.

Fusion of the extensin signal peptide and either the TobRB7 or extensin promoter to the $GUS_{358\rightarrow S}$ reporter gene resulted in very low GUS expression levels so it was not possible to analyse GUS activity in these plants. However, in the absence of the extensin signal peptide GUS activity up to 900 fold higher than wild-type levels was observed. The reason for this drop in GUS activity upon fusion of the gene to the extensin signal peptide is not clear but other workers (Denecke *et al.*, 1990) have also observed a significant drop in GUS activity (30 fold) when the gene is fused to a signal peptide. If the signal peptide was not properly removed this could account for the drop in activity. Also, the modified $GUS_{358\rightarrow S}$ gene used here is reported to produce only 64% of wild-type GUS activity (Farrell and Beachy, 1990) and the two promoters used (TobRB7 and carrot extensin) are not particularly strong. In combination these factors could result in the very low levels of GUS activity seen here.

Localization of GUS activity in roots of plants transformed with either pTGusN, pEGusN or p35SGUS indicates that the tissue specificity of each promoter in tobacco roots is different. However, none of the promoters examined is ideal for expressing the glucose oxidase gene in plants to obtain resistance to Verticillium wilt. Expression is very low in plants where the glucose oxidase gene is driven by the carrot extensin promoter and higher levels of expression are probably required for disease resistance. It was not possible to regenerate transgenic cotton when the 35S promoter was used to drive glucose oxidase expression and the transgenic tobacco transformed with glucose oxidase controlled by the 35S promoter either failed to produce seed or produced seed very poorly. The TobRB7 promoter does not express in the root tip. This could be a disadvantage when the TobRB7 promoter is used to drive glucose oxidase expression in plants in order to obtain resistance to Verticillium wilt because this region of the root is often the site of *V. dahliae*

infection. Lack of gene expression in this region would increase the likelihood that the attacking pathogen would successfully infect the plant. It would therefore be of benefit to examine other promoters for more suitable expression patterns (for example in the root tip and endodermis/cortex of the root) and use these to express glucose oxidase in plants.

4.4.1 Future directions

Twenty-three cotton plants expressing glucose oxidase under control of the TobRB7 promoter have been generated and expression of glucose oxidase in the next generation of six of these lines confirmed by KI staining. Seeds will be generated from the remaining transgenic cotton plants and the inheritance pattern of the glucose oxidase gene in these plants will be analysed. Southern analysis of each line will be performed to determine how many copies of the T-DNA are present in each line. This information is important if the gene is to be backcrossed to commercial varieties as lines containing only one integration event are the most suitable for this process. Seedlings expressing glucose oxidase will be grown to the next generation and putative homozygotes selected by examining glucose oxidase production in T_2 seedlings. Plants homozygous for the glucose oxidase gene can then be tested for increased tolerance to Verticillium wilt in the field. However, accurate evaluation of Verticillium wilt resistance in transgenic plants could be difficult to obtain as the incidence of wilt is influenced to a large degree by environmental factors such as temperature and rainfall (see Chapter 1). Such parameters are neither controllable nor predictable so testing over several seasons may be needed to evaluate the wilt resistance of the plants. To obtain some indication of the ability of these plants to resist fungal disease, bioassays such as those described in the next chapter could be performed in growth cabinets where control of environmental conditions is possible.

CHAPTER 5
TRANSGENIC TOBACCO PLANTS EXPRESSING GLUCOSE OXIDASE SHOW INCREASED RESISTANCE TO *RHIZOCTONIA SOLANI*

5.1 INTRODUCTION

Australian isolates of *V. dahliae* do not infect tobacco, so the glucose oxidase-producing transgenic tobacco plants generated in Chapter 4 could not be tested for increased resistance to Verticillium wilt. Another soil borne fungus, *Rhizoctonia solani*, was also found to be susceptible to low concentrations of hydrogen peroxide by Kim *et al.* (1990b). This fungus infects many plant species including tobacco and cotton. It usually invades the hypocotyl of young seedlings where it decays stem tissue eventually causing the seedling to collapse. The pathogen is not a major problem in agriculture as it is readily controlled by fungicides, or as cool wet conditions early in growing seasons have been found to favour disease development, losses can be greatly reduced by delayed planting (Lucas, 1965; Hillocks, 1994).

An Australian isolate of *R. solani* originally isolated from cotton was found to infect tobacco *cv.* Wisconsin 38 under favourable conditions. This isolate was tested for its tolerance to glucose oxidase and found to be moderately sensitive to the enzyme. To determine if plants expressing glucose oxidase are resistant to *R. solani* infection, T_1 tobacco seedlings expressing the *T. flavus* glucose oxidase gene under control of either the 35S or TobRB7 promoter were tested for increased ability to survive in sand infested with the *R. solani* isolate.

5.2 MATERIALS AND METHODS

5.2.1 Fungal isolates

A *R. solani* isolate previously isolated from cotton was kindly supplied by Michael Priest, NSW Department of Agriculture, Rydalmere, Australia. The *T. flavus* and *V. dahliae* strains used are those described in Chapters 2 and 3.

5.2.2 *In vitro* inhibition assays

T. flavus filtrate was prepared as described in Chapter 3. *A. niger* glucose oxidase solutions were prepared with *A. niger* glucose oxidase purchased from Sigma. *R. solani* was grown in the dark in liquid medium (potato dextrose broth, Difco) at 26°C for 6 days on a rotary shaker (80 rpm). Mycelia were harvested by filtering though two layers of autoclaved Whatman #1 filter paper and then homogenized in an eppendorf tube using a hand-held glass grinder (previously sterilized). An equal volume of mycelial suspension was added to filtrate solution in microtitre wells to achieve an initial OD of approximately 0.05. The remainder of the experiment and calculation of a glucose oxidase IC_{50} for *R. solani* was carried out as described in Chapter 3.

5.2.3 *R. solani* infection trials

Tobacco seeds (*cv.* Wisconsin 38 and transgenic derivatives) were surface sterilized and germinated as described in Chapter 4. *R. solani* was grown at 26°C on potato dextrose agar for 7 days. Mycelia were removed from plates with a spatula and blended with sterile nutrient solution (Hoagland No 2 solution (Hewitt, 1966) and 0.5% glucose) for 30 seconds in a Waring blender. Mycelial fragments were filtered through 2mm nylon mesh before being counted with a 'Weber Scientific' counting chamber. Sand (300ml) was previously dispensed into containers (13cm by 11cm) lined with two layers of muslin cloth and autoclaved twice. *R. solani* mycelial fragments were mixed with nutrient solution and the solution poured evenly over sand in the containers (4×10^8 mycelial fragments/container). Eighteen day-old tobacco seedlings were removed from agar and planted directly into the sand (20/container). Containers were covered with clingwrap and placed in an 'Environ Air' growth cabinet for 5-6 weeks (24°C, 12 hours light; 20°C, 12 hours dark). Seedlings were removed from sand, washed in water and blotted dry on blotting paper. After weighing, a small amount of tissue was placed in KI/starch stain (see Chapter 2) to test for glucose oxidase production.

5.3 RESULTS

5.3.1 Effect of glucose oxidase on the growth of *R. solani*

Glucose oxidase was examined *in vitro* for its ability to inhibit the growth of an Australian isolate of *R. solani*. Different concentrations of *A. niger* glucose oxidase suspended in potato dextrose broth and different amounts of filtrate from wild-type *T. flavus* 32908 and the *T. flavus* glucose oxidase-deficient mutant GOH-1 were inoculated with *R. solani* and fungal growth was monitored spectrophotometrically using a microtitre plate reader. Duplicates were performed for each experiment and the experiment was repeated twice.

As shown in Figure 5.1, filtrate from *T. flavus* 32908 broth and broth containing *A. niger* glucose oxidase were found to significantly inhibit *R. solani* growth. The growth profile of *R. solani* in PD broth alone, PD broth plus gluconic acid or in filtrate from GOH-1 was very similar, indicating that, as with *V. dahliae*, it is the hydrogen peroxide produced by glucose oxidase that is toxic to *R. solani*. Growth inhibition curves for *R. solani* were calculated 26 hours after the start of fungal growth (Figure 5.2). Approximately 4.4µg/ml of *A. niger* glucose oxidase and the equivalent of 4.1µg/ml of *A. niger* glucose oxidase in *T. flavus* 32908 filtrate was required to inhibit *R. solani* growth by 50% (IC_{50}). These IC_{50}values are approximately twice that determined for *V. dahliae* isolates (2.2µg/ml).

5.3.2 Infection of transgenic tobacco plants expressing glucose oxidase with *R. solani*

Seedlings from several of the tobacco plants found to express glucose oxidase (SEGO-9, 13, 24 and TEGO-9, 10, 21) and seedlings from the 35SGUS (control) were surface sterilized and germinated on growth media containing kanamycin. Eighteen days later, seedlings either homozygous or hemizygous for the kanamycin resistance gene were transferred to sand infested with *R. solani* to determine their susceptibility to fungal attack. As a control, seedlings were also transferred to sand containing no *R. solani*. The sand in both experiments was previously moistened with sterilized Hoagland's solution containing 0.5% glucose. The presence of glucose in the solution was necessary to promote fungal infection; in its absence no infection occurred even in control tobacco plants when *R. solani* concentrations of

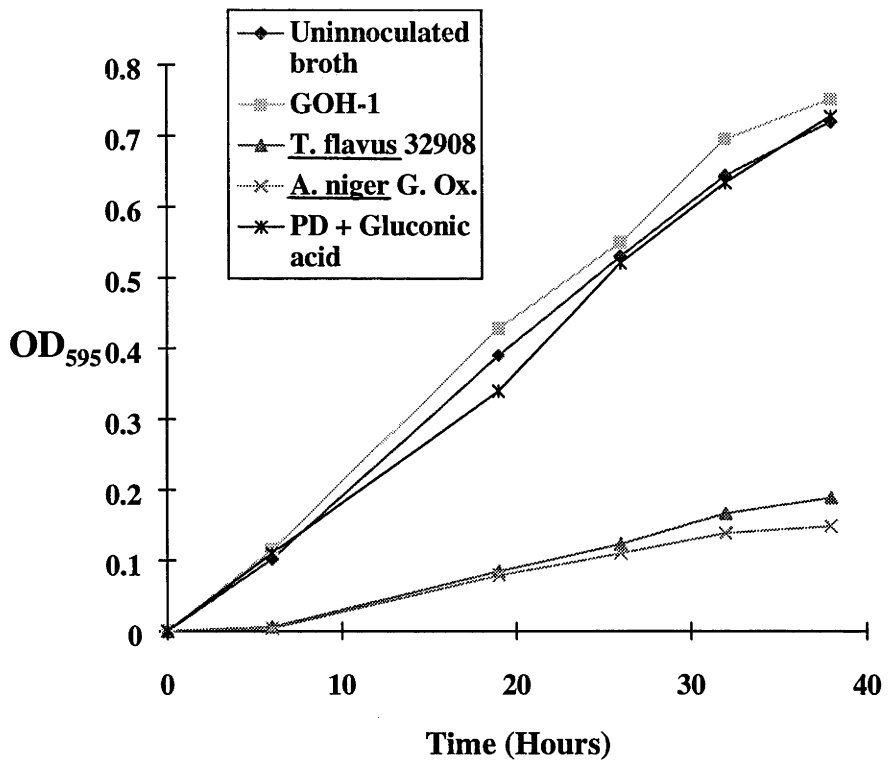


Figure 5.1 *In vitro* inhibition of *R. solani* growth. Either *A. niger* glucose oxidase or culture filtrate from *T. flavus* 32908 or GOH-1 were incorporated into growth medium and growth of *R. solani* monitored spectrophotometrically over 38 hours. All growth media was pH 5.2 at the start of the experiment. The glucose oxidase concentration in media containing *A. niger* glucose oxidase or *T. flavus* 32908 filtrate was 0.01 units/ml where one unit equals that amount which oxidises 1.0 μmole of β-D glucose to D gluconic acid and H_2O_2 per minute at pH 5.1 and 25°C. Maximum standard error was ± 7.5% of the values obtained (omitted from the graph for the sake of clarity).

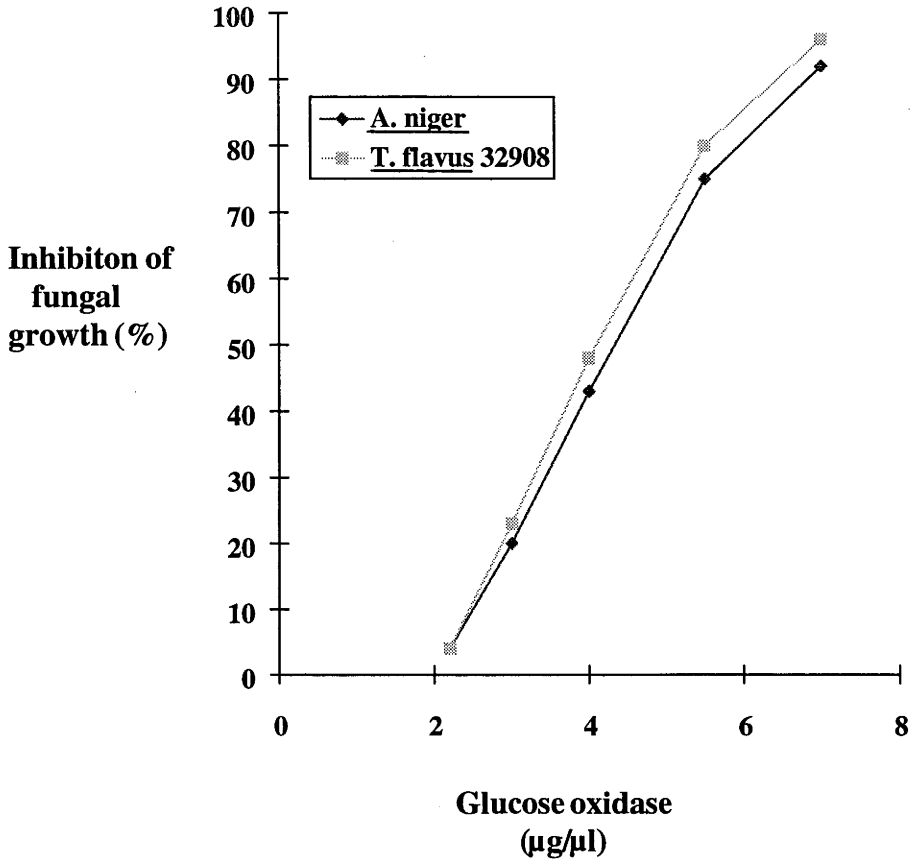


Figure 5.2 Growth inhibiton of *R. solani* in different amounts of *T. flavus* culture filtrate or different concentrations of *A. niger* glucose oxidase. The amount of glucose oxidase in the culture filtrate was determined by comparison to standard concentrations of *A. niger* glucose oxidase (Sigma).

4×10^9 propagules/container were used. After 38 days seedlings were assessed for fungal infection and surviving seedlings tested for glucose oxidase activity with KI/starch solution. Confirmation of the infecting organism was not possible as seedlings were too small to allow reisolation of the fungus. However, symptoms typical of *R. solani* infection (rotting of hypocotyl and slow growth) were observed only when seedlings were grown in sand infested with *R. solani* (Figure 5.3).

All surviving seedlings except those from 35GUS tested positive for glucose oxidase activity. When *R. solani* was not present in the sand, 85-100% of all the seedlings survived. When grown in infested sand, only 45% of 35SGUS seedlings survived whereas 65-100% of seedlings producing glucose oxidase survived (Table 5.1; Figure 5.4). The 35SGUS seedlings grown in infested sand weighed on average 47% less than 35SGUS seedlings grown under control conditions (Table 5.2). Little difference in average seedling weight was observed between glucose oxidase expressing seedlings grown in infested or uninfested sand.

Of the glucose oxidase-producing seedlings tested, those from TEGO-21 seemed to be the most susceptible to fungal infection. Only 65% of the seedlings survived when grown in infested sand and the weight of these seedlings on average was slightly lower (80%) than the weight of TEGO-21 seedlings grown under control conditions. No significant difference in weight and percentage survival was found among the other glucose oxidase-producing seedlings tested. As TEGO-21 produces the least amount of glucose oxidase among the transformants tested, this suggests there is a correlation between the level of glucose oxidase activity and resistance to *R. solani.*

5.4 DISCUSSION

In vitro, growth of *R. solani* is inhibited by low concentrations of glucose oxidase. As with *V. dahliae*, it is the hydrogen peroxide produced by the glucose oxidase which is predominantly responsible for *R. solani* growth inhibition. However, compared with *V. dahliae*, approximately twice as much glucose oxidase is required to inhibit *R. solani* growth by 50% (IC_{50} 2.2µg/ml and 4.4µg/ml respectively). This

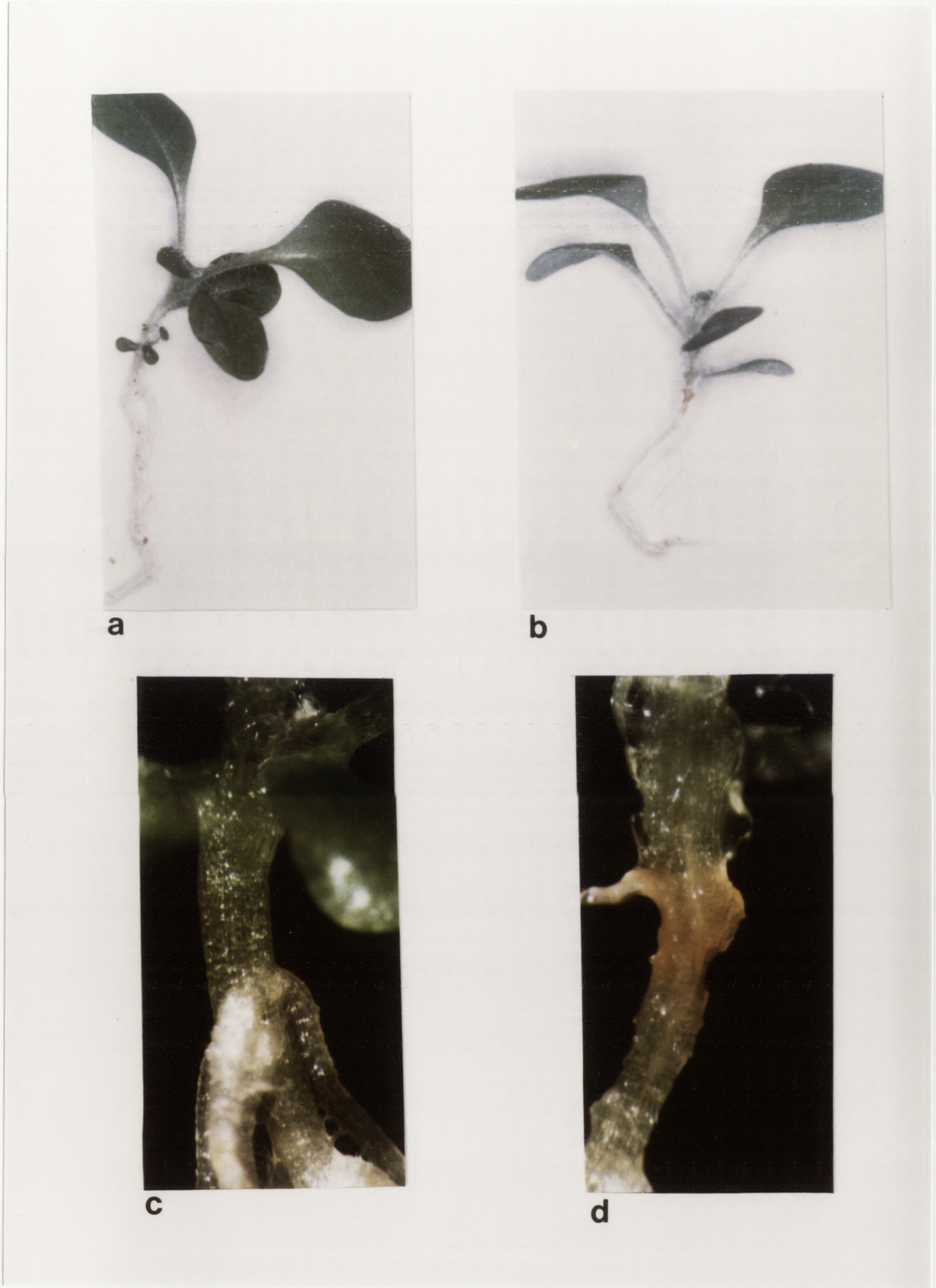


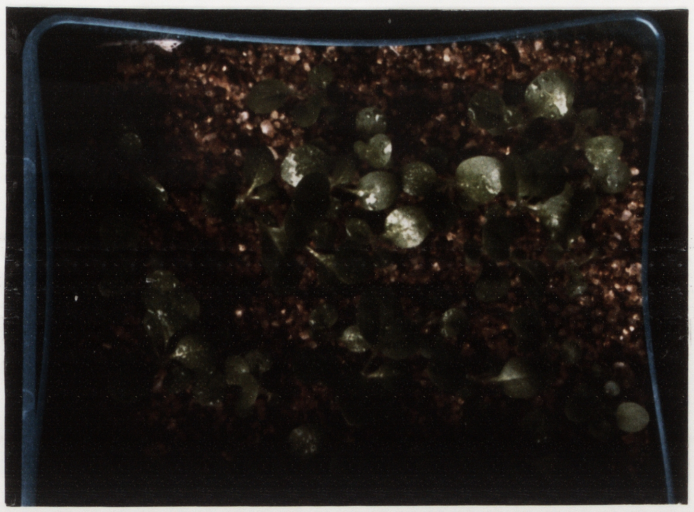
Figure 5.3 Symptoms of *R. solani* infection on tobacco seedlings. **a:** Seedling grown in uninfested sand. **b:** Seedling grown in infested sand - note the browning of the hypocotyl. **c:** The hypocotyl from the seedling shown in a. **d:** The hypocotyl from the seedling shown in b.

	Control (No *R. solani*)		4.0×10^8 *R. solani* propagules/tray	
Seedlings	**Number of surviving seedlings**	**Percentage**	**Number of surviving seedlings**	**Percentage**
35SGUS	18/20	90	9/20	45
SEGO-9	17/20	85	19/20	95
SEGO-13	17/20	85	20/20	100
SEGO-24	19/20	95	18/20	90
TEGO-9	17/20	85	18/20	90
TEGO-10	20/20	100	20/20	100
TEGO-21	20/20	100	13/20	65

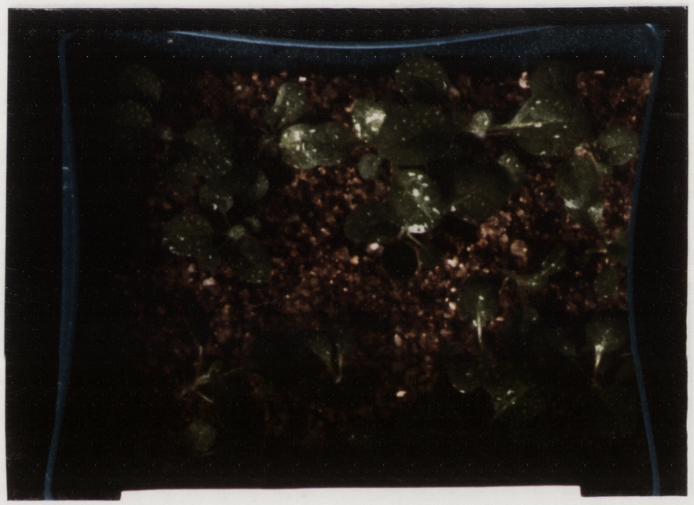
Table 5.1 Percentage survival of different transgenic glucose oxidase-producing seedlings 38 days after growth in sand infested with *R. solani*. Twenty seedlings from each transgenic plant were used in the experiment.

Figure 5.4 Growth of glucose oxidase-producing seedlings (TEGO-10) in sand either infested or uninfested with *R. solani*. Pictures were taken 38 days after transfer of seedlings to sand. **a**: 35SGUS (top) and TEGO-10 seedlings (bottom) growing in uninfested sand. **b**: TEGO-10 seedlings and **c**: 35SGUS seedlings. In both b & c seedlings are growing in sand infested with *R. solani* (4×10^8 propagules/tray).

a



b



c

	Average weight of surviving seedlings (g)		
Seedlings	**Control (No *R. solani*)**	**4.0×10^8 *R. solani* propagules/tray**	**Infected seedlings weight as a percentage of control seedlings weight**
35SGUS	0.089	0.047	53
SEGO-9	0.081	0.082	101
SEGO-13	0.082	0.090	110
SEGO-24	0.110	0.103	94
TEGO-9	0.086	0.094	109
TEGO-10	0.120	0.110	92
TEGO-21	0.085	0.068	80

Table 5.2 Average fresh weight of surviving transgenic seedlings 38 days after growth in sand either infested or uninfested with *R. solani* (4×10^8 propagules/tray). Twenty seedlings from each transgenic line were planted in the experiment.

result in not in agreement with that of Kim *et al.* (1990b) who found that *R. solani* was approximately four fold more sensitive to hydrogen peroxide than *V. dahliae*. As Kim *et al.* used the same *T. flavus* 32908 isolate as used here, the difference between their results and those obtained here may be due to differences in the *R. solani* isolates used, particularly as *R. solani* is known to be a very diverse species (Carling and Sumner, 1992). Differences between the *V. dahliae* isolates examined may also account for the disparate results obtained here. Although little variability in glucose oxidase sensitivity was observed between the two *V. dahliae* isolates examined in Chapter 3, these isolates were both of Australian origin whereas the isolate used by Kim and coworkers was isolated from American soil. This isolate may therefore be quite distinct from the Australian *V. dahliae* population with respect to hydrogen peroxide sensitivity.

Preliminary experiments indicate that expression of glucose oxidase in transgenic tobacco correlates with increased ability to resist *R. solani* infection. Compared with control seedlings, T_1 transgenic tobacco seedlings expressing glucose oxidase under control of either the 35S or TobRB7 promoter weighed more and showed an increased ability to survive in sand infested with *R. solani.* Seedlings from TEGO-21, the plant producing the least amount of glucose oxidase (approximately 4 fold wild-type levels of hydrogen peroxide in the absence of glucose) were the most susceptible to pathogen attack. Transgenic seedlings producing larger amounts of glucose oxidase showed greater resistance to infection by *R. solani*, indicating that higher levels of glucose oxidase correlate with increased disease resistance.

The mechanism by which glucose oxidase increases plant resistance to fungal attack is not clear. As *R. solani* is sensitive to low concentrations of hydrogen peroxide, hydrogen peroxide produced by glucose oxidase may be directly responsible for killing the fungal pathogen. Also, as mentioned in Chapter 4, increased disease resistance could result if hydrogen peroxide generated by glucose oxidase stimulates cell wall cross-linking and cell signalling involved in plant defence. As *R. solani* invades at the soil level, specifically attacking the hypocotyl of seedlings, and plants specifically expressing glucose oxidase in the roots were resistant to *R. solani* infection, this may suggest that hydrogen peroxide is acting to stimulate other plant

defence mechanisms. However, the expression pattern of TobRB7 promoter in this region was not closely examined and as the hypocotyl is very close to the roots, TobRB7 may express in this area or very close to it. As hydrogen peroxide can diffuse across membranes, expression of glucose oxidase very close to the hypocotyl may be adequate to protect the region against fungal attack. Some indication of which mechanism(s) are responsible for disease resistance in these transgenic plants may be gained if a pathogen not susceptible to hydrogen peroxide was used to infect the tobacco plants expressing glucose oxidase.

Unfortunately it was not possible to infect tobacco seedlings with *R. solani* (even at concentrations of 4×10^9 propagules container) unless 0.5% glucose was included in the nutrient solution used to moisten the sand. Presumably, the presence of glucose was necessary either to stress seedlings and make them more susceptible to *Rhizoctonia* infection and/or to promote rapid growth of the *Rhizoctonia* population. This would increase the inoculum concentration and occurrence of disease. However, glucose in the root environment could be taken up by the plant and allow glucose oxidase to produce larger quantities of hydrogen peroxide. Therefore, if naturally occurring glucose limits glucose oxidase activity in plants, the abundance of glucose in the root environment could be responsible for glucose oxidase-mediated disease resistance. The extent to which the exogenous glucose is involved in the observed disease resistance cannot be determined from these experiments. A method of inducing the *R. solani* isolate to infect tobacco without the use of exogenous glucose, or an alternative fungal pathogen, is needed to determine if the transgenic plants grown naturally are resistant to fungal infection.

5.4.1 Future directions

T_2 seedlings homozygous for the glucose oxidase gene will be generated and plants from these seeds tested for increased resistance to fungal infection. As the glucose oxidase gene will not be segregating among this population, infection trials with these plants will give a better indication of the level of glucose oxidase expression required for resistance to fungal infection. Other fungal pathogens are being tested for their ability to infect tobacco. If tobacco pathogens either susceptible or non-susceptible to glucose oxidase can be identified, the transgenic seedlings used here

could be tested for increased resistance to these pathogens. In particular, use of pathogens non-susceptible to glucose oxidase may help further elucidate how hydrogen peroxide produced by glucose oxidase is involved in generating fungal disease resistance.

CHAPTER 6
CONCLUSIONS

Using molecular techniques, this study has examined the role of glucose oxidase in biocontrol of Verticillium wilt by *T. flavus* and how glucose oxidase may be used in agriculture to help control Verticillium wilt in cotton. In order to do this the *T. flavus* glucose oxidase gene was cloned and characterized. At a molecular level this gene was found to be most similar to *A. niger* glucose oxidase. The two genes are 64% similar at a nucleotide level whereas the two proteins are 64% identical (77% similar), the same length (605aa) and both have an N-terminal secretion signal sequence as well as either seven or eight potential glycosylation sites (*T. flavus* and *A. niger* protein sequences respectively). Like the *A. niger* glucose oxidase sequence, the *T. flavus* glucose oxidase sequence has significant similarity to a group of enzymes known as GMC oxidoreductases. Based on this sequence similarity, *T. flavus* glucose oxidase is included in this class of enzymes.

The *T. flavus* promoter contained sequences characteristic of a functional fungal gene including a TATAA box at -122 (with respect to the initiating ATG) and a 50bp pyrimidine rich region 12 to 63 bases upstream of the coding region. No consensus AATAAA polyadenylation signal was found in the 3' untranslated region. A possible AAATA polyadenylation site was identified 162bp downstream of the stop codon although the importance of this sequence needs to be established by functional analysis. Confirmation that the gene cloned was a functional glucose oxidase gene was obtained by successfully expressing the putative glucose oxidase gene in *T. macrosporus,* a species of *Talaromyces* which does not naturally produce glucose oxidase.

Cloning the *T. flavus* glucose oxidase gene allowed the construction of several types of variants which were subsequently used to investigate the importance of glucose oxidase in the biocontrol properties of *T. flavus*. The first type of variant was created using *T. macrosporus*. Transformation of the *T. flavus* glucose oxidase gene into this species created glucose oxidase-producing *T. macrosporus* isolates. The second type was a glucose oxidase-deficient *T. flavus* isolate. This was created using a mutated,

non-functional *T. flavus* glucose oxidase gene and gene replacement techniques. Experiments performed *in vitro* with these mutant isolates and their respective wild-type parents showed *T. flavus* glucose oxidase (or more specifically the hydrogen peroxide that glucose oxidase produced) is predominantly responsible for the *V. dahliae* growth inhibition caused by *T. flavus* under these conditions. However, in pot trials the presence or absence of the glucose oxidase gene in either *T. flavus* or *T. macrosporus* had no effect on the incidence of Verticillium wilt in cotton. Therefore, under these conditions, glucose oxidase does not play a significant role in the biocontrol of *V. dahliae* by *T. flavus*.

The discrepancy between the *in vitro* and *in vivo* results is attributed to the difference in glucose availability in the two experiments. *In vitro*, glucose is readily available so large amounts of toxic hydrogen peroxide are produced and *V. dahliae* growth is inhibited. In the soil, glucose is much less prevalent so the amount of hydrogen peroxide produced is not sufficient to inhibit *V. dahliae* growth. As the soil trials undertaken here were performed in artificial conditions, further experiments (ideally in the field) are necessary to confirm that glucose oxidase does not play a significant role in the biocontrol of *V. dahliae* by *T. flavus* in the field. However, these experiments are difficult to perform given that the occurrence of Verticillium wilt is dependent on specific environmental conditions and that release of genetically modified fungal organisms has not yet been approved because of containment difficulties.

Additional experiments indicated that *V. dahliae* has significantly less catalase activity than *T. flavus* and *T. macrosporus*. This is consistent with the findings of Kim *et al.* (1990b) who found that *V. dahliae* is particularly sensitive to small amounts of hydrogen peroxide. However, despite this sensitivity to hydrogen peroxide, it is concluded that it is not feasible to control Verticillium wilt in cotton through manipulation of glucose oxidase levels in the biocontrol organism *T. flavus*. This decision is based on the results of the soil trials together with consideration of the intrinsic problems involved with use of biocontrol agents in the field (application and maintenance of the biocontrol organism at both the required concentration and

correct location in the soil) and the difficulty associated with releasing genetically engineered fungal organisms in the field.

Expressing glucose oxidase in plants is another possible way of effectively utilizing this enzyme against *V. dahliae*. This method is particularly attractive as recent findings suggest hydrogen peroxide is involved in natural plant defence responses. Expression of glucose oxidase in plants may therefore be successful against *V. dahliae* not only because this pathogen is particularly susceptible to hydrogen peroxide but also because hydrogen peroxide generated by glucose oxidase may stimulate plant defence cell signalling and natural plant defence mechanisms such as cell wall thickening.

Work outlined in this thesis has established that it is possible to express the *T. flavus* glucose oxidase gene in plants although it was necessary to replace the fungal signal peptide with one from plants to generate functional protein. The reason for the required replacement of the signal peptide not clear. Northern analysis of plants transformed with the unaltered glucose oxidase gene shows that this gene is efficiently transcribed in transgenic plants. The fungal signal peptide therefore interferes with translation or processing of the glucose oxidase protein. In future, Western analysis could be performed on these plants. This would indicate whether the plants produce glucose oxidase protein and if so whether the protein is the expected size. Such information may help determine at which level the fungal signal peptide is not functioning.

Three different promoters were used to express the glucose oxidase gene in plants. Of these promoters (extensin, TobRB7 and 35S), the TobRB7 and 35S promoters resulted in successful expression in tobacco and the TobRB7 promoter resulted in successful expression in cotton. Only very poor glucose oxidase activity was obtained in both tobacco and cotton plants transformed with the glucose oxidase gene driven by the extensin promoter. The reason for this is not clear. Promoter-Gus fusion experiments with the extensin promoter in tobacco, revealed that the extensin promoter drives moderate expression in plant roots. Higher glucose oxidase levels than those obtained would therefore be expected in plants transformed with pEEGON

(extensin driven glucose oxidase). As extensin promoters have been shown to be strongly induced by external stimuli such as wounding or ethylene, it is proposed that during the tissue culture process the extensin promoter was induced. Under these circumstances, toxic levels of hydrogen peroxide could be produced, killing all plants except those expressing glucose oxidase weakly. This would account for the results observed here.

Other observations also indicated that overexpression of glucose oxidase in plants was toxic to the plant. Tobacco plants with very high levels of glucose oxidase activity were stunted and grew poorly when grown in tissue culture. As these plants grew normally when transferred to soil, this phenotype is thought to reflect toxicity caused by excess production of hydrogen peroxide in tissue culture. Excess production would occur in tissue culture because the growth media has a high concentration of sucrose. Plant invertases acting on the sucrose could generate glucose, which would be utilized by glucose oxidase to create hydrogen peroxide. In soil, significantly less glucose is available therefore much less hydrogen peroxide would be produced allowing the plant to grow normally. The other unusual phenotype observed, lack of seed production by many of the tobacco plants transformed with pSEGON (35S driven glucose oxidase) is also attributed to hydrogen peroxide toxicity. Presumably, developing seeds are either more sensitive to hydrogen peroxide or glucose is more abundant during seed development so toxic quantities of hydrogen peroxide are produced.

As mentioned above, the 35S promoter was used successfully in tobacco to drive glucose oxidase gene expression yet when the same expression vector was transformed into cotton no plants expressing glucose oxidase were regenerated. This is attributed to the different ways in which transgenic tobacco and cotton plants regenerate. Cotton, unlike tobacco, regenerates from cotton cells which undergo somatic embryogenesis. As the 35S promoter has been shown to function in both embryogenic and non-embryogenic callus it is proposed that glucose oxidase expression together with the abundant supply of glucose in the tissue culture media, resulted in hydrogen peroxide levels which were either toxic to or inhibited the regeneration process in transformed cells.

The glucose oxidase gene was correctly expressed in the progeny of transgenic cotton and tobacco plants and in most instances the glucose oxidase gene segregated in a Mendelian fashion. Due to the length of time required to regenerate transgenic cotton it was not possible to conduct trials to determine if expression of the glucose oxidase gene in cotton correlated with increased resistance to Verticillium wilt. However, preliminary infection trials were conducted with some of the T_1 tobacco seedlings expressing glucose oxidase and the fungus *R. solani* which causes damping off in both tobacco and cotton seedlings. Earlier experiments showed that compared with the *V. dahliae* isolated tested, twice as much hydrogen peroxide was required to inhibit growth of this fungus by 50%. Promising results were obtained with the seedlings expressing glucose oxidase showing significantly increased resistance to *R. solani* infection. However, in these infection trials it was necessary to include glucose in the sand to stimulate *R. solani* infection. Without this glucose in the sand, inadequate amounts of hydrogen peroxide for disease protection may have been produced by glucose oxidase in the plants. This is suggested by the observation that plants strongly expressing glucose oxidase grow poorly in tissue culture yet normally in soil. It indicates that the glucose concentration in the soil limits production of hydrogen peroxide by glucose oxidase in transgenic plants, however it is not known whether the low glucose concentration in the soil limits disease resistance in these plants. Further experiments, preferably in the field, are necessary to determine this. These experiments will be conducted in the future when the transgenic cotton expressing glucose oxidase is tested for increased resistance to Verticillium wilt. If glucose availability is a problem, possible solutions would be to use an alternative glucose oxidase gene such as that from the red alga, *I. flaccidum*, which is not specific for glucose . This may be easier than the alternative of manipulating plant invertase levels.

Although cotton expressing glucose oxidase has been obtained, optimum expression has not been obtained. Promoter-Gus fusion studies in tobacco with the TobRB7 promoter used to express glucose oxidase in cotton shows that this promoter does not drive gene expression in the root tip. Expression in this area could be crucial if Verticillium resistance is to be generated as this is the region of the plant at which the pathogen often penetrates. This problem may be addressed in future by obtaining

promoters with the required strength and expression pattern and using these to express the glucose oxidase gene in cotton.

Also of interest, is the potential of plants expressing glucose oxidase to be resistant to other fungal diseases. In cotton, another fungal disease, Fusarium wilt which is caused by the pathogen *Fusarium oxysporum*, is beginning to cause severe losses in some growing regions. Preliminary tests have shown that, like *V. dahliae*, growth of *F. oxysporum* is inhibited by low concentrations of hydrogen peroxide. Therefore, if cotton plants have increased resistance to Verticillium wilt, they may also have increased resistance to Fusarium wilt. This is assuming that hydrogen peroxide generated by glucose oxidase is acting directly to kill the fungal pathogen and stop or slow pathogen infection. Recent research has lead to proposals that implicate hydrogen peroxide in several aspects of the plant defence response to pathogen invasion. Shah (1994) recently reported that transgenic potatoes expressing *A. niger* glucose oxidase show reduced disease symptoms when inoculated with *Erwinia caratovora* and *Phytophthora infestans*. This report, and the results of this thesis, suggest that an improvement in plant disease resistance may be obtained if glucose oxidase is expressed alone or in combination with other defence genes in plants. One or several mechanisms may be involved in generating resistance and such resistance may not be dependent on the pathogen being particularly susceptible to hydrogen peroxide. This is of interest, not only because of the potential benefits to agriculture, but in addition transgenic plants expressing glucose oxidase may be used as tools to investigate the role of hydrogen peroxide in the plant defence response.

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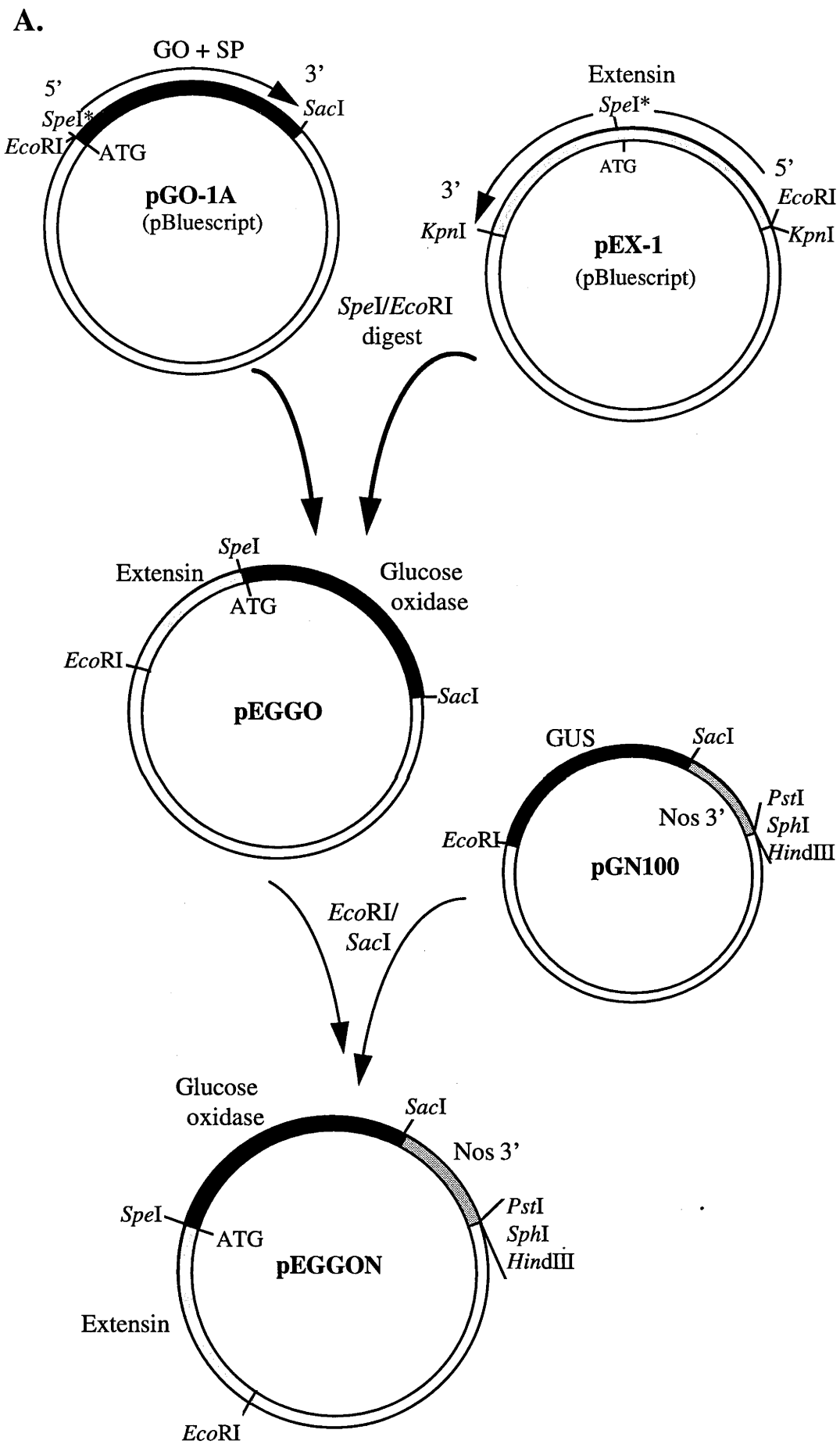
Appendix 1

Construction of glucose oxidase (**A-F**) and Gus expression vectors (**G-J**).

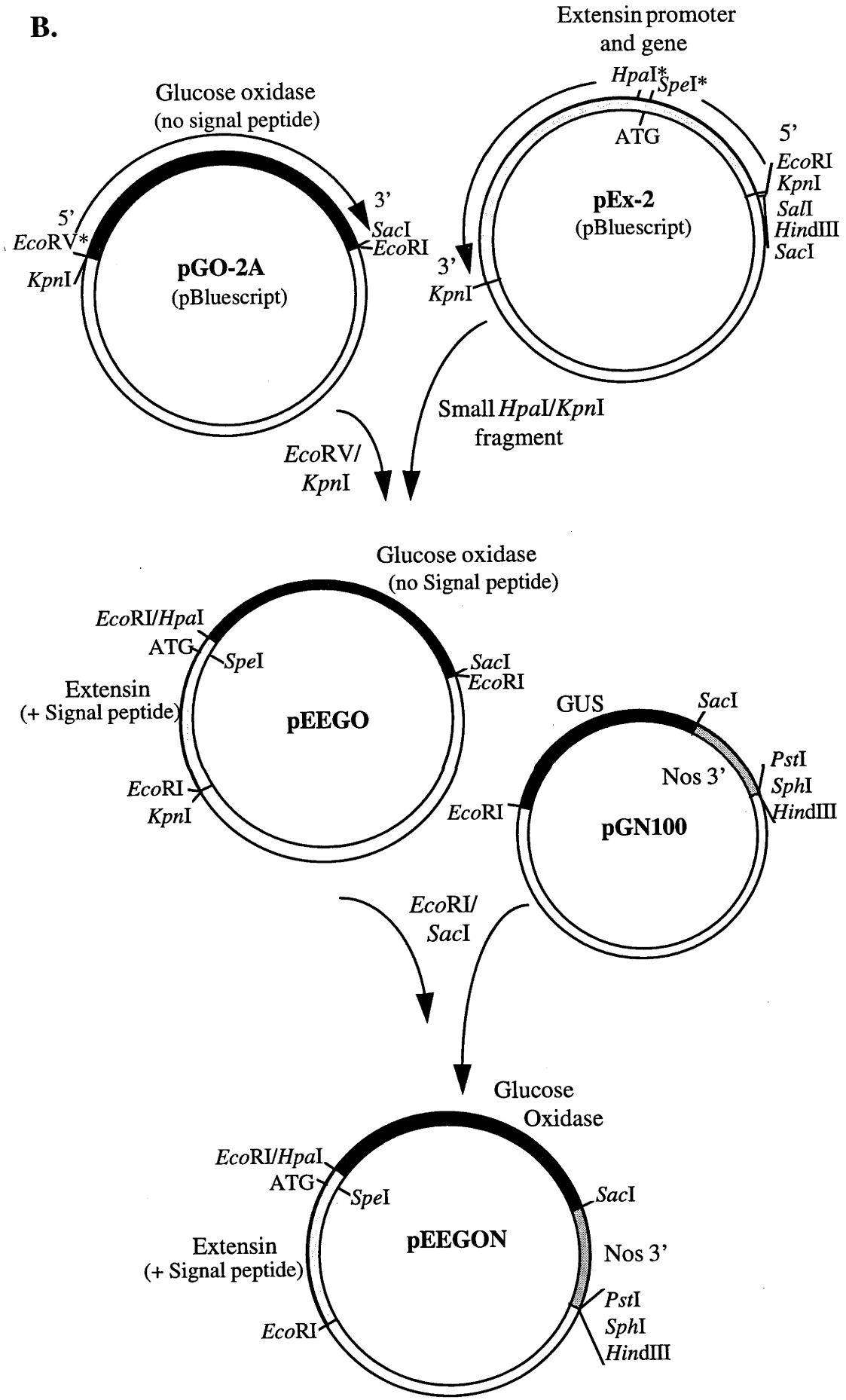
* restriction enzyme sites introduced by site directed mutagenesis.

+ restriction enzyme sites destroyed by subcloning step.

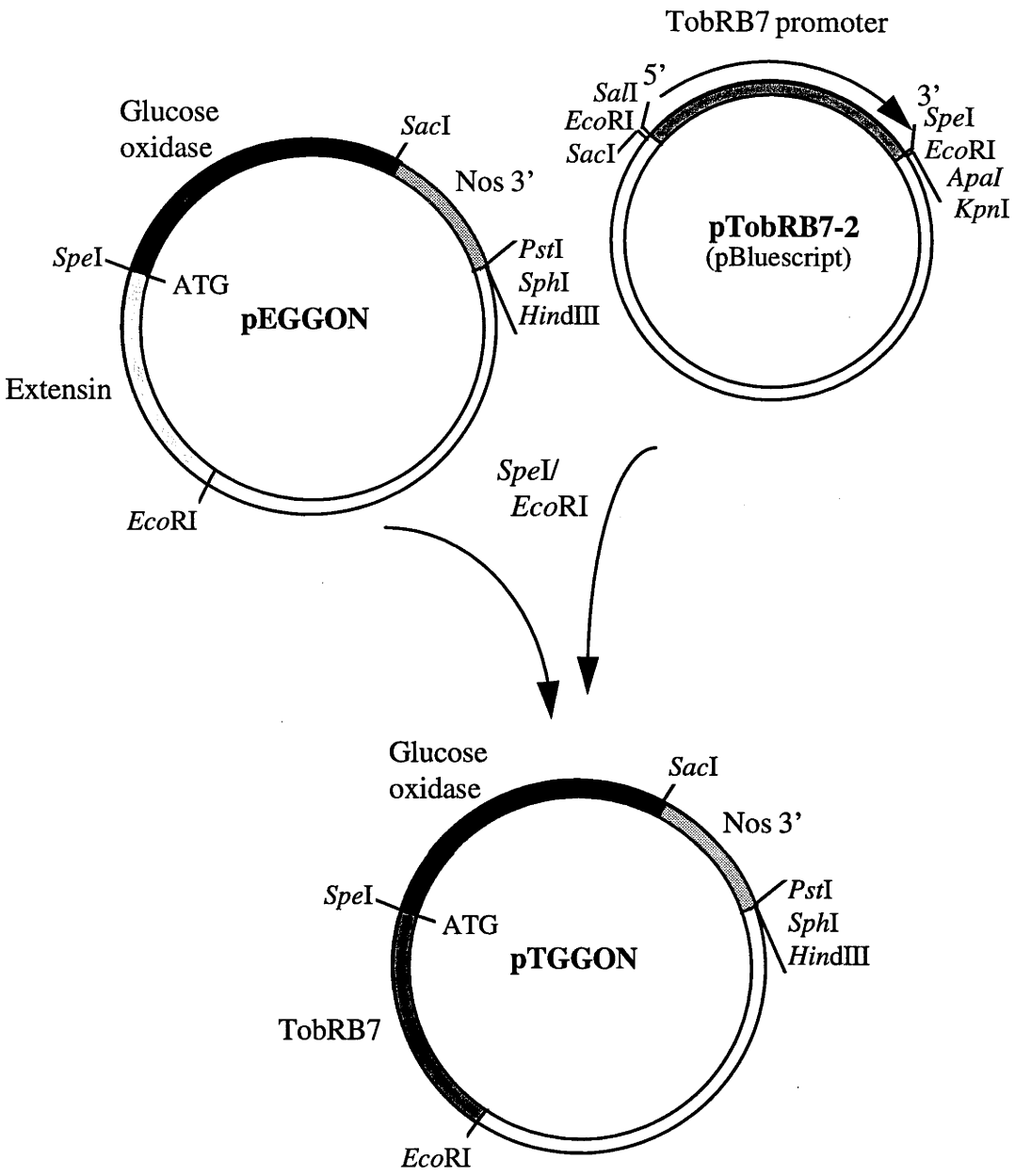
npt II neomycin phosphotransferase (kanamycin resistance)


A.
GO + SP
5'
3'
SpeI*
SacI
EcoRI
ATG
pGO-1A
(pBluescript)
Extensin
SpeI*
ATG
5'
3'
EcoRI
KpnI
KpnI
pEX-1
(pBluescript)
SpeI/EcoRI
digest
SpeI
Extensin
ATG
Glucose
oxidase
EcoRI
SacI
pEGGO
GUS
SacI
PstI
Nos 3'
SphI
EcoRI
HindIII
pGN100
EcoRI/
SacI
Glucose
oxidase
SacI
Nos 3'
SpeI
ATG
PstI
SphI
HindIII
pEGGON
Extensin
EcoRI

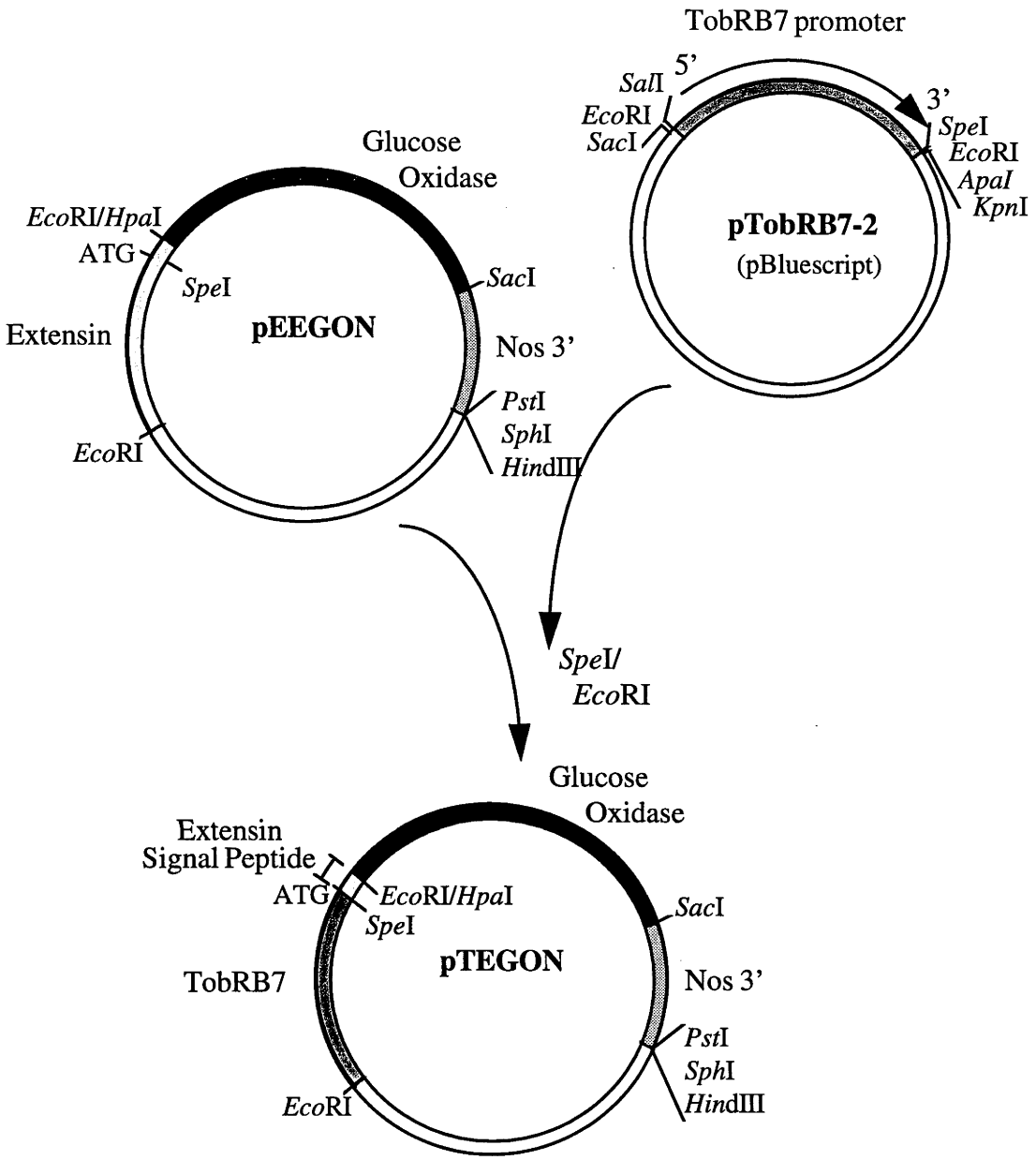
B.


Extensin promoter
and gene
HpaI*
SpeI*
ATG
5'
EcoRI
KpnI
SalI
HindIII
SacI
pEx-2
(pBluescript)
3'
KpnI
Glucose oxidase
(no signal peptide)
5'
EcoRV*
KpnI
pGO-2A
(pBluescript)
3'
SacI
EcoRI
Small HpaI/KpnI
fragment
EcoRV/
KpnI
Glucose oxidase
(no Signal peptide)
EcoRI/HpaI
ATG
SpeI
Extensin
(+ Signal peptide)
pEEGO
SacI
EcoRI
EcoRI
KpnI
GUS
SacI
PstI
Nos 3'
SphI
HindIII
EcoRI
pGN100
EcoRI/
SacI
Glucose
Oxidase
EcoRI/HpaI
ATG
SpeI
SacI
Extensin
(+ Signal peptide)
pEEGON
Nos 3'
PstI
SphI
HindIII
EcoRI

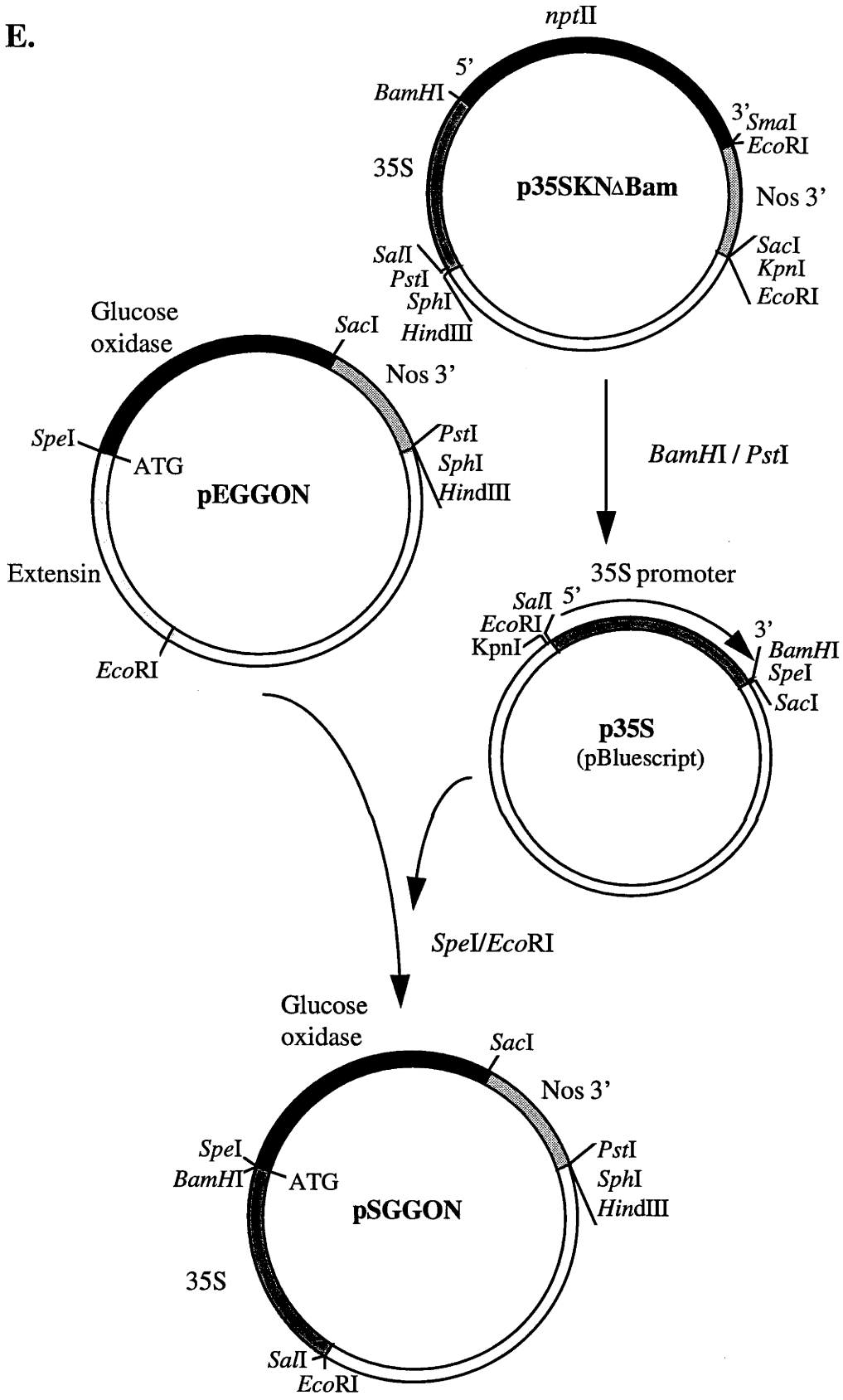
C.


TobRB7 promoter
5'
3'
SalI
EcoRI
SacI
SpeI
EcoRI
ApaI
KpnI
pTobRB7-2
(pBluescript)
Glucose
oxidase
SacI
Nos 3'
SpeI
ATG
PstI
SphI
HindIII
pEGGON
Extensin
EcoRI
SpeI/
EcoRI
Glucose
oxidase
SacI
Nos 3'
SpeI
ATG
PstI
SphI
HindIII
pTGGON
TobRB7
EcoRI

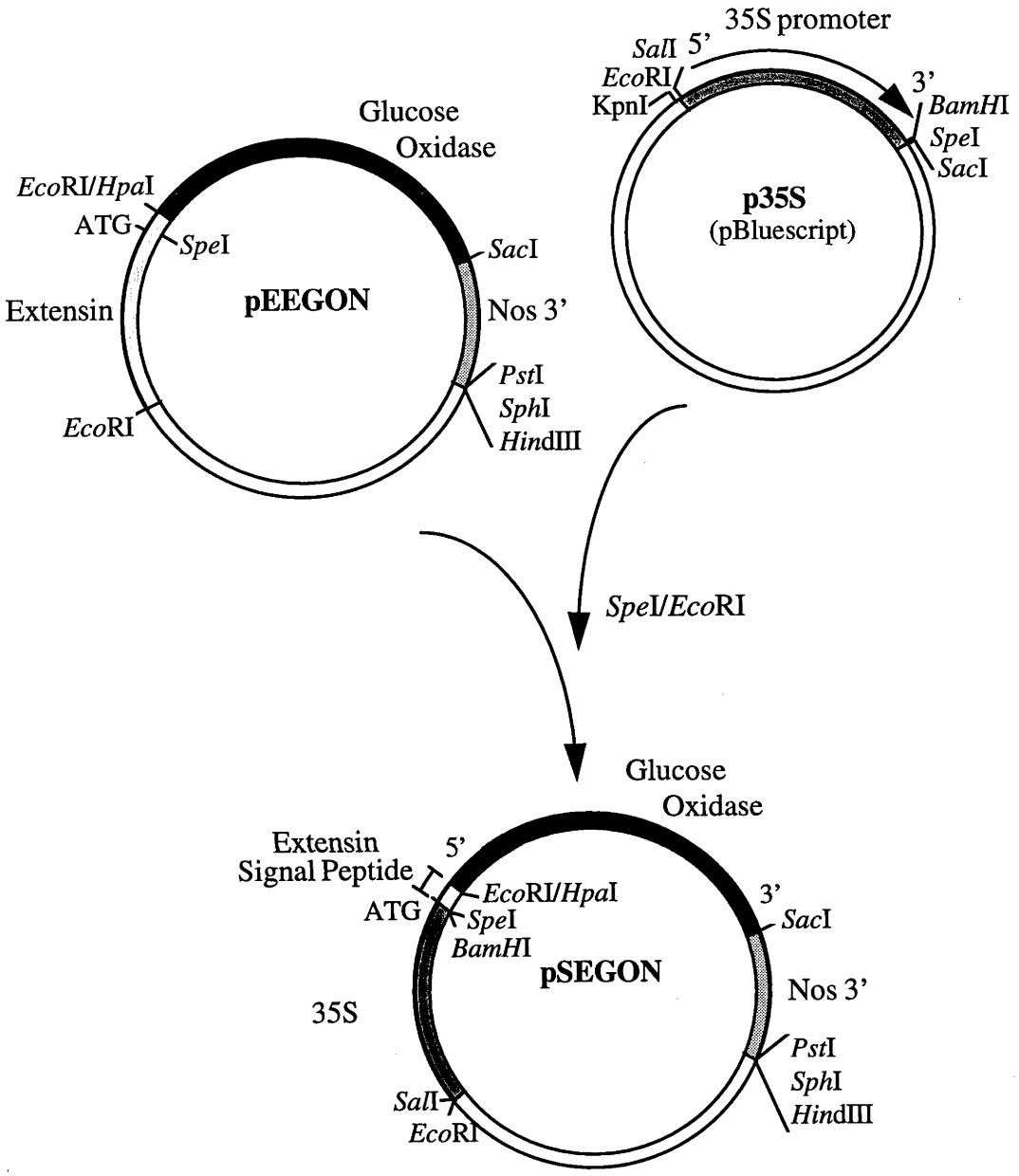
D.


TobRB7 promoter
5'
SalI
EcoRI
SacI
3'
SpeI
EcoRI
ApaI
KpnI
pTobRB7-2
(pBluescript)
Glucose
Oxidase
EcoRI/HpaI
ATG
SpeI
SacI
Extensin
pEEGON
Nos 3'
PstI
SphI
HindIII
EcoRI
SpeI/
EcoRI
Glucose
Oxidase
Extensin
Signal Peptide
ATG
EcoRI/HpaI
SpeI
SacI
TobRB7
pTEGON
Nos 3'
PstI
SphI
HindIII
EcoRI

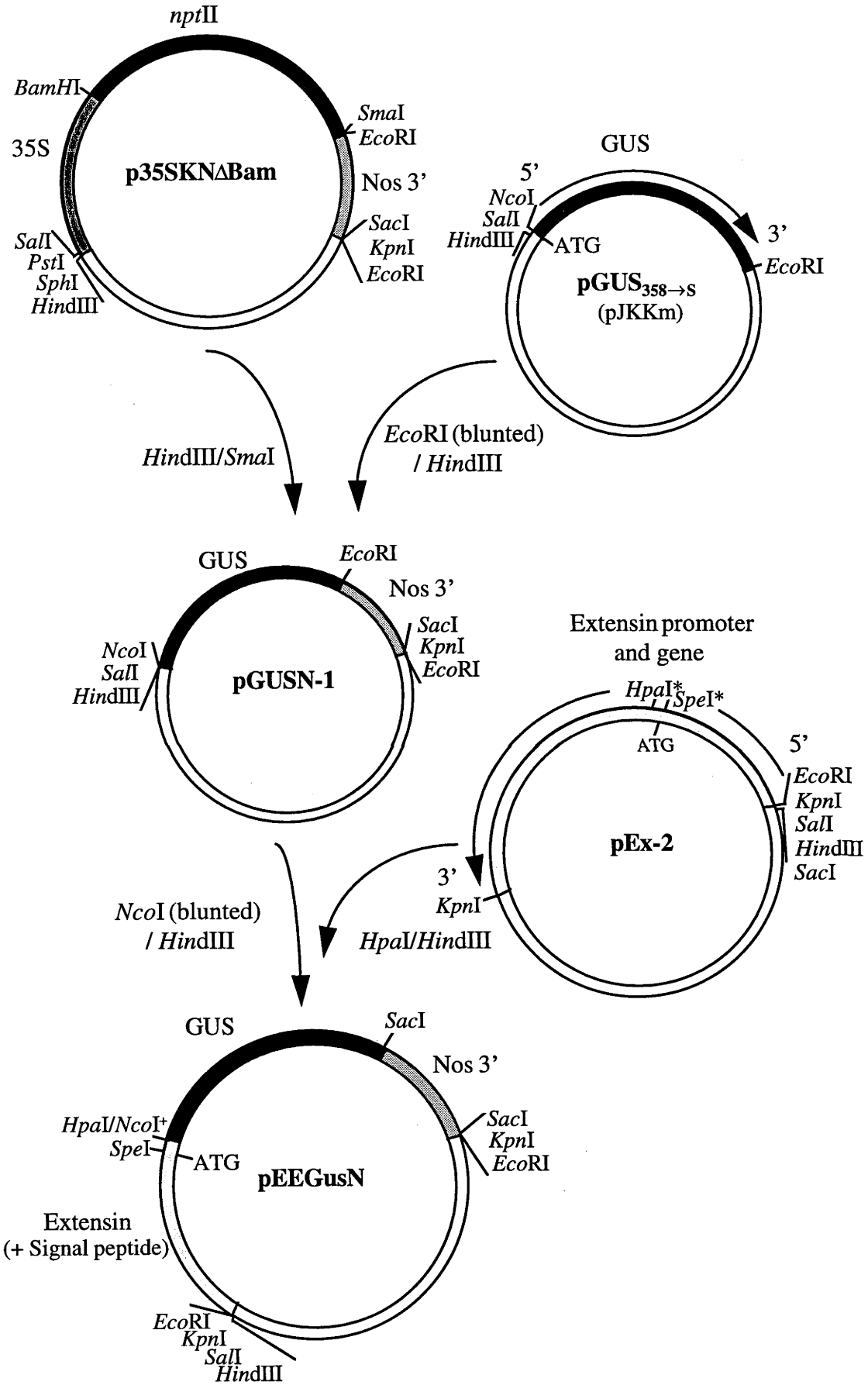
E.


nptII
5'
BamHI
3'
SmaI
EcoRI
35S
p35SKNΔBam
Nos 3'
SacI
KpnI
EcoRI
SalI
PstI
SphI
HindIII
Glucose oxidase
SacI
Nos 3'
SpeI
ATG
pEGGON
PstI
SphI
HindIII
Extensin
EcoRI
BamHI / PstI
35S promoter
SalI
5'
EcoRI
KpnI
3'
BamHI
SpeI
SacI
p35S
(pBluescript)
SpeI/EcoRI
Glucose oxidase
SacI
Nos 3'
SpeI
BamHI
ATG
pSGGON
PstI
SphI
HindIII
35S
SalI
EcoRI

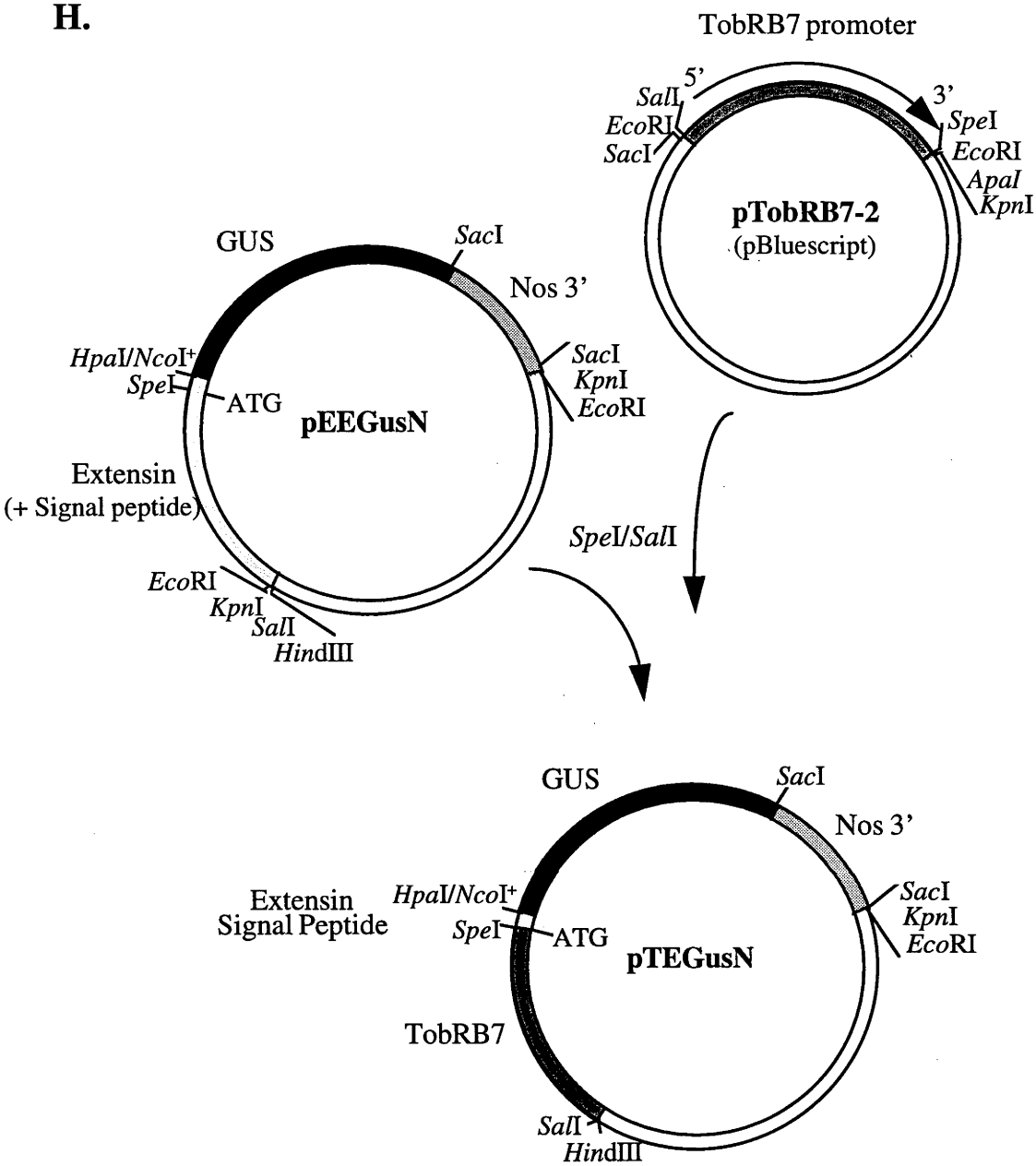
F.


35S promoter
SalI 5'
EcoRI
KpnI
3'
BamHI
SpeI
SacI
p35S
(pBluescript)
Glucose
Oxidase
EcoRI/HpaI
ATG
SpeI
SacI
Extensin
pEEGON
Nos 3'
PstI
SphI
HindIII
EcoRI
SpeI/EcoRI
Glucose
Oxidase
Extensin
Signal Peptide
5'
EcoRI/HpaI
ATG
SpeI
BamHI
3'
SacI
pSEGON
Nos 3'
35S
PstI
SphI
HindIII
SalI
EcoRI

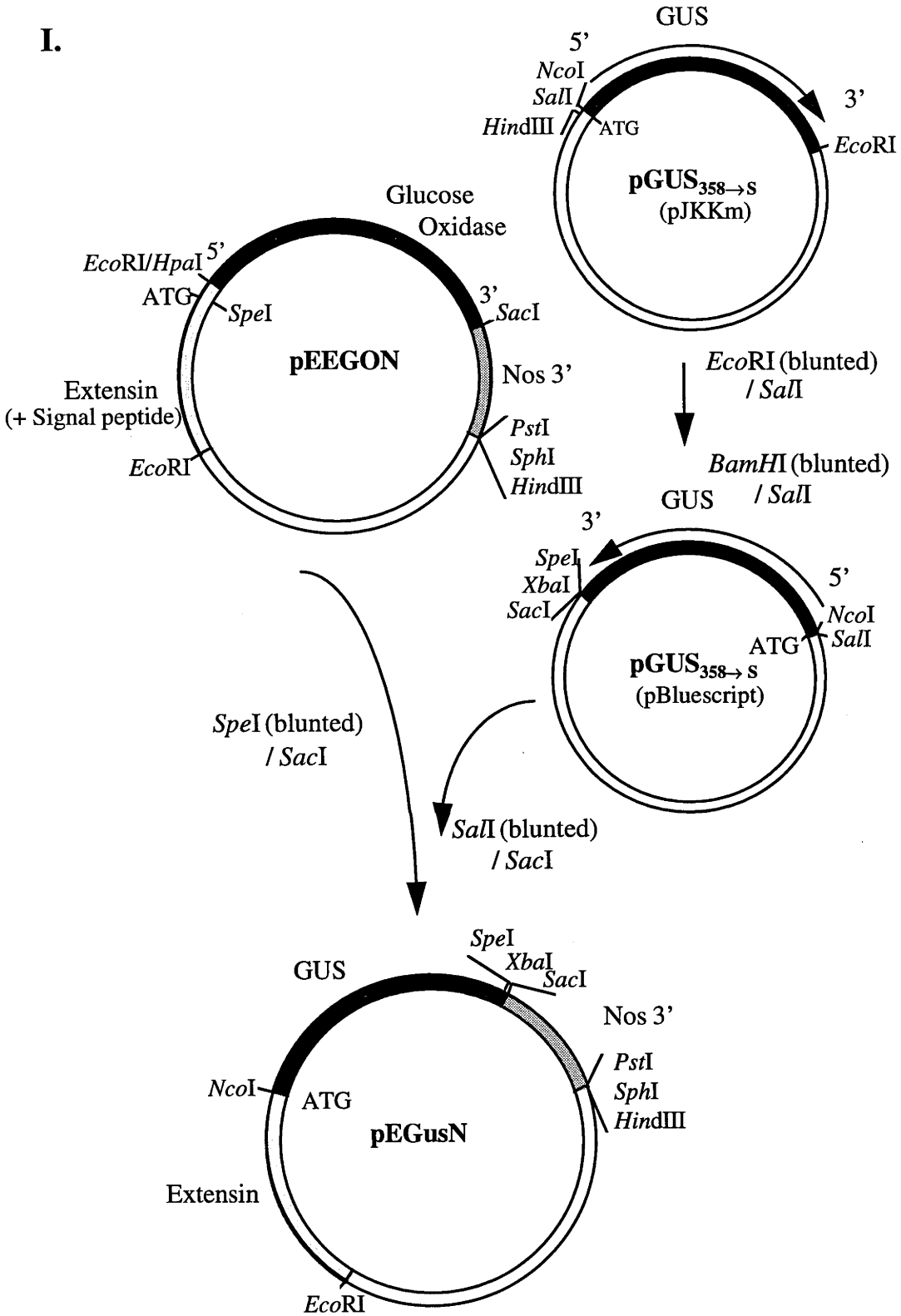
G.


nptII
BamHI
35S
p35SKNΔBam
SmaI
EcoRI
Nos 3'
SacI
KpnI
EcoRI
SalI
PstI
SphI
HindIII
GUS
5'
NcoI
SalI
HindIII
ATG
pGUS358→S
(pJKKm)
3'
EcoRI
HindIII/SmaI
EcoRI (blunted)
/ HindIII
GUS
EcoRI
Nos 3'
SacI
KpnI
EcoRI
NcoI
SalI
HindIII
pGUSN-1
Extensin promoter
and gene
HpaI*
SpeI*
ATG
5'
EcoRI
KpnI
SalI
HindIII
SacI
pEx-2
3'
KpnI
NcoI (blunted)
/ HindIII
HpaI/HindIII
GUS
SacI
Nos 3'
HpaI/NcoI+
SpeI
ATG
pEEGusN
SacI
KpnI
EcoRI
Extensin
(+ Signal peptide)
EcoRI
KpnI
SalI
HindIII

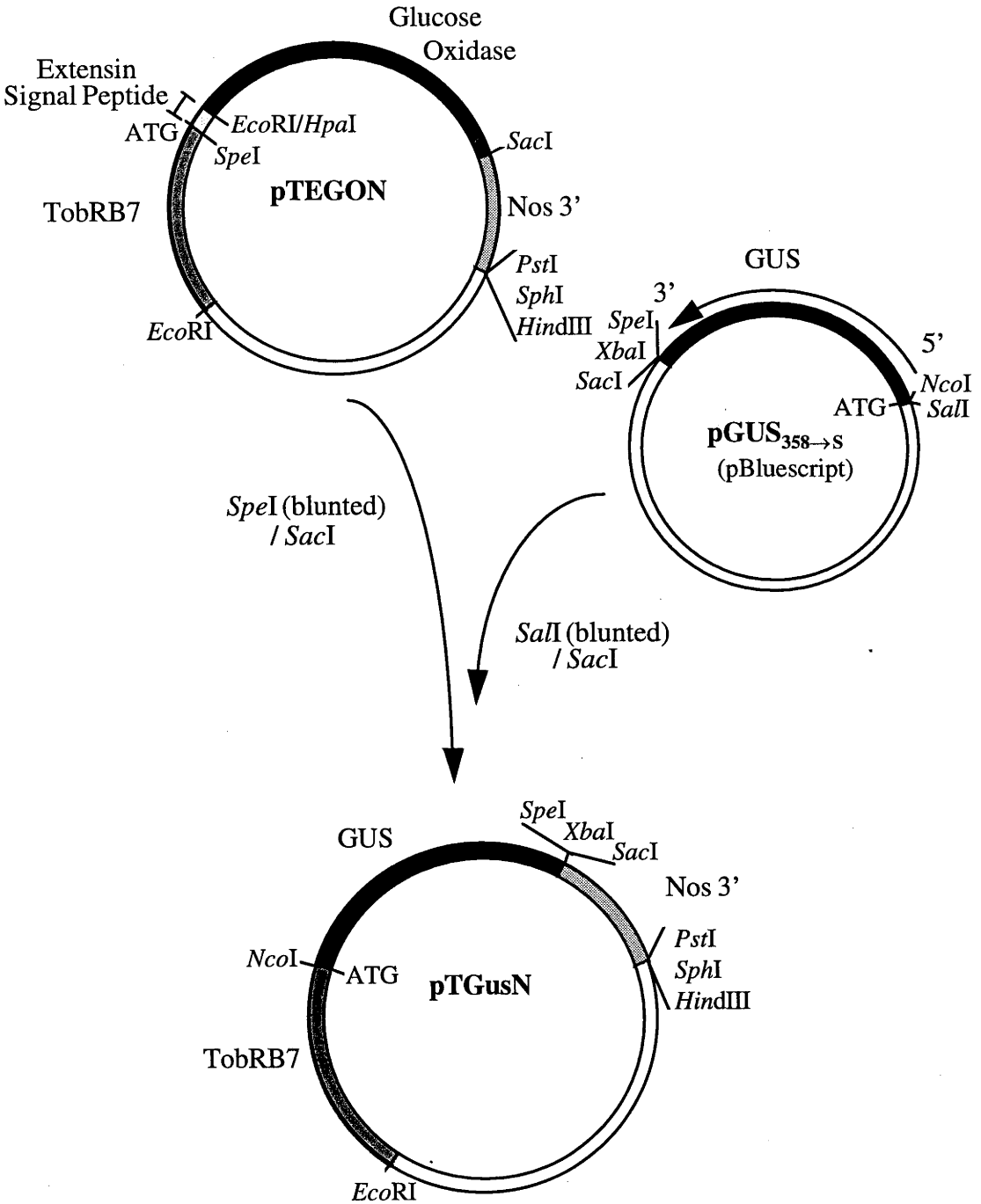
H.


TobRB7 promoter
5'
3'
SalI
EcoRI
SacI
SpeI
EcoRI
ApaI
KpnI
pTobRB7-2
(pBluescript)
GUS
SacI
Nos 3'
HpaI/NcoI+
SpeI
ATG
pEEGusN
SacI
KpnI
EcoRI
Extensin
(+ Signal peptide)
SpeI/SalI
EcoRI
KpnI
SalI
HindIII
GUS
SacI
Nos 3'
Extensin
Signal Peptide
HpaI/NcoI+
SpeI
ATG
pTEGusN
SacI
KpnI
EcoRI
TobRB7
SalI
HindIII

I.


GUS
5'
NcoI
SalI
HindIII
ATG
3'
EcoRI
pGUS358→S
(pJKKm)
Glucose
Oxidase
EcoRI/HpaI
5'
ATG
SpeI
3'
SacI
pEEGON
Nos 3'
Extensin
(+ Signal peptide)
PstI
SphI
HindIII
EcoRI
EcoRI (blunted)
/ SalI
BamHI (blunted)
/ SalI
GUS
3'
SpeI
XbaI
SacI
5'
NcoI
SalI
ATG
pGUS358→S
(pBluescript)
SpeI (blunted)
/ SacI
SalI (blunted)
/ SacI
SpeI
XbaI
SacI
GUS
Nos 3'
PstI
SphI
HindIII
NcoI
ATG
pEGusN
Extensin
EcoRI

J.


Glucose
Oxidase
Extensin
Signal Peptide
ATG
EcoRI/HpaI
SpeI
SacI
pTEGON
TobRB7
Nos 3'
PstI
SphI
HindIII
EcoRI
GUS
3'
SpeI
XbaI
SacI
5'
NcoI
SalI
ATG
pGUS358→S
(pBluescript)
SpeI (blunted)
/ SacI
SalI (blunted)
/ SacI
SpeI
XbaI
SacI
GUS
Nos 3'
PstI
SphI
HindIII
NcoI
ATG
pTGusN
TobRB7
EcoRI