

Minireview

Engineering *Sclerotinia sclerotiorum* Resistance in Oilseed Crops

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The fungal pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary is worldwide in distribution and pathogenic to more than 400 plant species. This disease causes significant yield losses of various important crops including sunflower, canola, and soybean. Applying fungicides and crop rotation are currently the major methods of controlling this disease. However, fungicide chemicals are expensive, not all environmentally safe, and not always effective. Few genetic sources of resistance to the pathogen are available to breeders. Therefore, farmers have a continuing demand for new approaches to control the disease. Biotechnology opens a new avenue to control this pathogen. Several strategies, including detoxification, defense activation, and fungal inhibition, have potential to engineer *Sclerotinia* resistance. This review summarizes the progress of transgenic *Sclerotinia* resistance in oilseed crops including sunflower, canola, and soybean.

Key words: detoxification, disease resistance, fungal inhibition, hydrogen peroxide, oxalate oxidase, *Sclerotinia sclerotiorum*, white mold.

INTRODUCTION

As the world's population continues to increase, food supplies must grow to meet nutritional requirements. One means of ensuring the stability and plentitude of the food supply is to mitigate crop losses caused by crop pathogens (Campbell et al., 2002). Oilseed crop improvement by conventional breeding is severely restricted by the availability of a rather limited gene pool and by the time scale of most breeding programs.

Agricultural biotechnology has the potential to develop a more-sustainable farming practice. Therefore, much attention has been directed towards the newly emerging technologies of plant cell and molecular biology that provide a powerful means to supplement and complement the traditional methods of plant improvement (Durante et al., 2002). Traditional breeding has been strengthened and streamlined with the advent of new molecular markers for rapid selection of desired traits. Transformation methodology has been developed for generating fertile transgenic oilseed crops. *Sclerotinia* disease is a common important fungal disease in oilseed crops. This review will use *Sclerotinia* disease as an

Abbreviations: GLP, germin-like protein; OA, oxalic acid; OXO, oxalate oxidase; OXO, oxalate oxidase gene; SCO, sunflower carbohydrate Oxidase; and TP, tachyplesin.

example to illustrate how agricultural biotechnology is being applied to a problem of importance to farmers.

***Sclerotinia sclerotiorum* DISEASE**

Sclerotinia sclerotiorum (Lib.) de Bary pathogen have been discovered and studied for more than 150 years. This fungus is a necrotrophic pathogen. There are four stages in its life cycle: sclerotia, apothecium, ascospore, and mycelium (Purdy, 1979). Infection of susceptible plants can occur from mycelium that originates from eruptive germination of sclerotia in soil. Hyphal germination of sclerotia causes infection by first invading nonliving organic matter and forming a mycelium, which is an intermediate necessity for mycelial infection. Apothecia can be developed from sclerotia and eject ascospores. Then ascospores become airborne and alight on nonliving or senescent plant parts, germinate, ramify the nonliving plant parts, and invasion of healthy plant parts. Ascospore may directly penetrate healthy host tissues and establish infection. Sunflower is the only crop that *Sclerotinia* consistently infects through the roots. As the fungus grows in and on tissues, sclerotia are formed; most are produced in the decayed stem pith and on the roots as the plant dies. *Sclerotinia* passes winter as sclerotia in the soil or in plant debris.

S. sclerotiorum is worldwide in distribution and is pathogenic to more than 400 plant species at various developmental stages (Purdy, 1979; Boland and Hall, 1994). A survey of the literature revealed more than 60 names used to refer to diseases caused by this fungal pathogen (Purdy, 1979). It is often called the "white mold", but it is better labeled *Sclerotinia* disease. The *Sclerotinia* disease is the most important fungal disease of sunflower. *Sclerotinia* wilt (root rot), middle stalk rot, and head rot are recognized in sunflower fields. Stem rot is the most significant *Sclerotinia* disease in soybean and canola (rapeseed or *Brassica napus*). Infection of oilseed plants can occur any time after seedling emergence. *Sclerotinia* disease can cause serious yield losses of crops including sunflower, canola, and soybean. The grain yield losses can be 100% (Purdy, 1979). The cost of annual crop losses of these three oilseed crops is more than 60 million dollars. The importance of this disease has led to significant investigation of the pathogen and the disease.

OXALIC ACID IS THE MAJOR PHYTOTOXIC AND PATHOGENICITY FACTOR

Secretions from pathogens are the primary functional and signal molecules for them to interact with their hosts. An intricate exchange of molecular signals and responses between the pathogen and host determines the pathogenicity. *S. sclerotiorum* synthesizes and secretes

millimolar concentrations of oxalic acid (OA) into infected host tissues (Maxwell and Lumsden 1970; Marciano et al., 1983; Godoy et al., 1990). This acid, acting as a mobile toxin, can cause a wilting syndrome in sunflower (Noyes and Hancock, 1981). OA not only acidifies the plant tissue but also chelates Ca^{++} from the cell wall, rendering the stressed tissue susceptible to a battery of fungal degradative enzymes (Lumsden, 1979). The acid also inhibits the activity of an *O*-diphenol oxidase (Ferrar and Walker, 1993), suppressing the oxidative burst (Cessna et al., 2000). Manual injection of oxalate, or of culture filtrate containing oxalate, into plants caused the development of *Sclerotinia* disease-like symptoms (Bateman and Beer, 1965; Noyes and Hancock, 1981). Mutants of *S. sclerotiorum* that are deficient in the ability to synthesize oxalate are nonpathogenic, whereas revertant strains that regain their oxalate biosynthetic capacity exhibit normal virulence (Godoy et al., 1990). This data clearly demonstrate that OA is a pathogenicity factor for *S. sclerotiorum*. This factor suppresses the hypersensitive response of the host plants (Cessna et al., 2000).

***Sclerotinia* DISEASE MANAGEMENT**

Strategies for combating *Sclerotinia* diseases include traditional technologies such as plant breeding and chemical applications. However, few genetic sources of resistance to the pathogen are available for breeders. Genetic studies demonstrated *Sclerotinia* disease is a quantitative trait (Gentzbittel et al., 1998; Zhao and Meng, 2003). The effective controls for *Sclerotinia* disease are to not plant on infested soil and to prevent build-up of sclerotia in soils. There are several chemicals that were used to control this disease. However, the chemicals are expensive, often not effective, and not all environmentally safe. Therefore, farmers have a demand for new approaches to manage *Sclerotinia* disease. Transgenic modification of crops provides a new technology to control *Sclerotinia* disease. The transgenic strategies include detoxification of the pathogenicity factor OA, activation of endogenous defense pathways, and inhibition of *S. sclerotiorum* growth by anti-fungal proteins.

OXALATE OXIDASE (OXO) CONFERS *Sclerotinia* RESISTANCE

A common strategy for combating the *Sclerotinia* pathogen is to degrade OA, the plant toxic and *Sclerotinia* pathogenicity factor secreted from the pathogen. There are three classes of known enzymes that can catabolize OA, namely oxalate oxidase (OXO) (EC 1.2.3.4) (Lane et al., 1991), oxalate decarboxylase (EC 4.1.1.2) (Mehta and Datta, 1991), and oxalyl-CoA decarboxylase (EC 4.1.1.8) (Lung et al., 1994). The

bacterial oxalyl-CoA decarboxylase gene could be used for oxalate degradation and engineering *Sclerotinia* resistance in plants (Dickman and Mitra, 1992). However, both fungal and bacterial oxalate decarboxylases convert oxalate into CO₂ and formic acid, which might have a toxic effect on plant cells. Therefore, scientists have been focusing on OXO, which releases CO₂ and H₂O₂ from O₂ and OA. This enzyme was first isolated and characterized from barley and wheat (Lane et al., 1993; Kotsira and Clonis, 1997). Wheat OXO, also known as germin, is the best-characterized member of the cupin family (Dunwell et al., 2000; Lane, 2000). Wheat germin is found to be an apoplastic, multimeric and glycosylated enzyme with extreme resistance to heat and chemical degradation by protease or H₂O₂ (Lane, 2000). Germin-like proteins (GLPs) have been isolated from many higher plants including both dicotyledonous and monocotyledonous species. These reported GLPs have high sequence identity to the wheat germin (Dunwell et al., 2000; Lane, 2002). However, only wheat, barley, maize, oat, rice, rye, and pine germins have been shown to have OXO activity (Dunwell et al., 2000; Lane, 2000).

Donaldson et al. (2001) generated transgenic soybean plants by *Agrobacterium*-mediated transformation with the wheat OXO gene (*gf-2.8*). Transgenic soybean homozygous for 35S-*gf-2.8* produced an approx. 130-kDa protein with OXO activity. OXO activity was prominent in cell walls proximal to the site of pathogen attack. The transgenics greatly reduced disease progression and lesion length following cotyledon and stem inoculation with *S. sclerotiorum*, indicating that OXO conferred resistance to the stem rot (Donaldson et al., 2001). Furthermore, they characterize the response of the OXO-transgenic soybean plants to *Sclerotinia* pathogens under field conditions and the agronomic performance of the transgenics under noninfected conditions. Field tests were conducted at three sites infested with *Sclerotinia* in Ontario and Quebec spanning 3 years. The field bioassay consistently demonstrated that OXO-transgenic soybeans exhibited significantly enhanced resistance against *Sclerotinia*. In noninfested trials, no significant differences were found between the transgenic and the parental lines for seed yield, maturity, seed weight, seed protein, and oil content. OXO provided *Sclerotinia* resistance equivalent to the best commercial cultivars in a *Sclerotinia* susceptible background (Cober et al., 2003).

Wheat OXO-transgenic sunflower plants were also generated by *Agrobacterium*-mediated transformation under a constitutive promoter SCP1 (Lu et al., 2000; Sclonge et al., 2000). The *Sclerotinia*-induced lesions in transgenic sunflower leaves were significantly smaller than those in the control leaves (Hu et al., 2003). The lesion sizes in the transgenic leaves were inversely related to the endogenous levels of OXO activity. In a petiole infection assay, three days after inoculation, *Sclerotinia* infection spread into the stem of untransformed control plants, but the lesions were limited

to the petioles of transgenic plants. Ten days after inoculation, the lesion length in the OXO-transgenic stems was about six-fold smaller than that in the untransformed controls. While *Sclerotinia* spread to the head tissue of the control plants within two weeks, the lesions on the transgenic plants were primarily confined to the main stem. These results demonstrated that OXO confers resistance against *Sclerotinia* in transgenic sunflower plants (Hu et al., 2003). The greenhouse and field bioassays also demonstrated that OXO-transgenic lines showed significantly enhanced resistance against mid-stalk rot, root rot, and head rot (Lu et al., 1998; Sclonge et al., 2000). When crossing the transgenic line with natural *Sclerotinia* resistance sunflower lines, the hybrids carrying the OXO transgene were more resistant to *Sclerotinia* than the corresponding non-transgenic isogenic hybrids. Thus, it should be possible to combine the transgenic lines with natural resistance to provide a higher level of *Sclerotinia* resistance than the currently available commercial hybrids (Bazzalo et al., 2000).

Barley OXO-transgenic canola plants exhibited enhanced OXO activity and were tolerant of exogenously supplied OA (Thompson et al., 1995). Interestingly, over-expression of wheat germin in transgenic hybrid poplars conferred enhanced resistance to the OA-generating pathogen, *Septoria musiva* (Liang et al., 2001). Of further interest is the fact that OXO-transgenic maize exhibited enhanced insect resistance (Ramputh et al., 2002). These results suggest that H₂O₂-generating enzymes such as OXO have potential utility in engineering resistance to a spectrum of pathogens and pests in plants. In addition, OXO has commercial significance in analyzing oxalate levels of blood plasma and urine and in human gene therapy (Dunwell, 1998; Dunwell et al., 2000).

OXO EVOKES DEFENSE RESPONSES

Various studies on the highly conserved family of GLPs have revealed that GLPs may have important roles in plant development and responsiveness to abiotic and biotic stresses (Dunwell et al., 2000; Lane, 2002). For example, germins are highly expressed during seed germination of wheat and barley and in the response of mature leaves to pathogen attacks (Zhang et al., 1995; Berna and Bernier, 1999). It has been shown that a specific pathogen-responsive OXO transcript is found in the wall of barley mesophyll cells 6 h after inoculation with powdery mildew (Zhou et al., 1998). Recently, it was reported that barley germin and, by inference, the related GLPs, represent a new group of extracellular manganese-containing enzymes with both OXO and superoxide dismutase activities (Woo et al., 2000). Two GLPs have been identified as SOD, but they have no OXO activity (Bernier and Berna, 2001). Superoxide dismutase activity can also lead to H₂O₂ production.

The significance of OXO is potentially two-fold for combating *Sclerotinia* disease: degrading the *Sclerotinia* toxin OA and producing the defense-inducing molecule H_2O_2 . Sunflower has very low germin-like OXO activity and is a host of the OA-generating *Sclerotinia* pathogen (Purdy, 1979); therefore, OXO-transgenic sunflowers are suitable for studying the biological significance of OXO enzyme in plant-pathogen interactions. We (Hu et al., 2003) demonstrated that transgenic sunflower leaf tissues could degrade exogenous OA and generate H_2O_2 . Hypersensitive response-like lesion mimicry was developed in the transgenic leaves expressing a high level of OXO, and the lesion development was closely associated with elevated levels of H_2O_2 , salicylic acid (SA), and defense gene expression. Activation of defense genes was also observed in the transgenic leaves that had a low level of OXO expression and had no visible lesions, indicating that defense gene activation may not be dependent on HR-like cell death. A mRNA profiling study revealed a number of differentially expressed genes in the OXO-transgenic sunflower plants. Among them, three defensive protein genes were characterized, including PR5, a sunflower carbohydrate oxidase, and a defensin. Expression of these genes was dramatically up-regulated in the leaves of uninfected OXO-transgenics, which showed elevated levels of SA and H_2O_2 . Their expression was also significantly induced in the untransformed leaves by treatment with SA, jasmonic acid (JA), or H_2O_2 . These observations suggest that H_2O_2 generated by OXO reacting with endogenous substrates directly, or possibly through SA (León et al., 1995) or JA, can trigger the expression of these defense genes.

It has been demonstrated that PR5-like proteins have *in vitro* antifungal activity against a variety of fungi, including *Phytophthora infestans*, *Candida albicans*, *Neurospora crassa*, and *Trichoderma reesei* (Vigers et al., 1991; Hu and Reddy, 1997). Plant defensin inhibits growth of a broad range of fungi at micromolar concentrations by inhibiting hyphal elongation or slowing down hyphal extension (Broekaert et al., 1995; Terras et al., 1995). The sunflower carbohydrate oxidase (SCO) has high homology with a reported sunflower SCO that has antifungal activity and confers resistance to fungal pathogens when expressed in transgenic *Arabidopsis* (Stuiver et al., 2000). SCO also has high homology to the berberine bridge enzyme (Dittrich and Kutchan, 1991), a key enzyme in the benzophenanthridine alkaloid pathway that generates antifungal compounds in poppies (Dittrich and Kutchan, 1991). Remarkably, both the berberine bridge enzyme and carbohydrate oxidase can generate H_2O_2 (Dittrich and Kutchan, 1991; Stuiver et al., 2000), indicating that OXO-induced SCO may further elevate the H_2O_2 level in OXO-transgenic leaves. Although the alkaloid biosynthetic pathway may not exist in sunflower plants, the potential antifungal and carbohydrate oxidase activities of SCO suggest its importance in the sunflower defense system.

The efficacy of constitutively expressed OXO in enhancing the *Sclerotinia* resistance of sunflowers may therefore be a consequence of multiple mechanisms (Greenberg et al., 1994; Hammond-Kosack and Jones, 1996; Lamb and Dixon, 1997; Lane, 2002). It may result from metabolism of endogenous substrate(s) to form H_2O_2 , which in turn triggers increased defense gene expression and enhanced sensitivity to subsequent pathogen attacks (Chamnongpol et al., 1998). Degradation of OA produced by *Sclerotinia* may reduce the damage that this pathogen causes in the plant tissues (Lumsden, 1979; Noyes and Hancock, 1981; Godoy et al., 1990), thereby slowing the advance of this necrotrophic fungus. Metabolism of OA may allow also a more rapid or powerful modification of plant defenses by counteracting the OA-mediated suppression of the oxidative burst (Cessna et al., 2000). Cell wall reinforcement (Schweizer et al., 1999) and direct antifungal activity of H_2O_2 (Lane, 1994; Zhang et al., 1995) may also play a role. It is likely that constitutive expression of OXO may confer enhanced resistance to other OA-generating pathogens, such as *Cristulariella pyramidalis* (Kurian and Stelzig, 1979) and *Septoria musiva* (Liang et al., 2001). Our results suggest that H_2O_2 -generating enzymes such as OXO have potential utility for engineering resistance to a spectrum of pathogens in plants. These important roles of H_2O_2 have attracted molecular pathologists' interest in manipulating the H_2O_2 level by overexpressing an H_2O_2 -generating enzyme, such as glucose oxidase (Wu et al., 1995; Kazan et al., 1998), to combat diseases in plants.

ANTIFUNGAL PROTEIN APPROACH

Over-expression of antimicrobial peptides or proteins in crop plants is a most common and promising strategy to combat bacterial and fungal pathogens. A number of antimicrobial peptides have shown relative broad-spectrum activity against different pathogens (Rao, 1995). We evaluated a number of antifungal peptides with regard to their anti-*Sclerotinia* activity, stability, and toxicity in sunflower tissues. Of those candidates, tachyplestin (TP) was the most promising. TP is a potent antimicrobial peptide isolated from the hemocytes of the horseshoe crab, *Tachypleus tridentatus*. Previous studies have shown that the 17-residue peptide has an intrinsic amphipathic structure conferred by two antiparallel beta-sheets held rigidly by two disulfide bonds (Rao, 1995). Activity assays using natural and synthetic membranes, and conformational measurements, highlight the subtle influence and variability of the amino acid side-chain properties on peptide structure (Rao, 1999). TP completely inhibited the germination of *Sclerotinia* ascospores at a concentration that had only minor toxic effects on sunflower protoplasts. Notably, TP was relatively stable in the intercellular fluids of leaf, stem,

root, and floral tissues. TP-transgenic sunflower calli showed inhibitory effects on the *Sclerotinia* mycelium growth. These results indicate that TP is a potent anti-*Sclerotinia* peptide for engineering *Sclerotinia* resistance in oilseed crops (Lu, 2002).

Grison et al (1996) introduced a hybrid endochitinase gene under a constitutive promoter by *Agrobacterium*-mediated transformation into a winter-type oilseed rape (*Brassica napus* var. *oleifera*) inbred line. Progeny from transformed plants were challenged using three different fungal pathogens (*Cylindrosporium concentricum*, *Leptosphaeria maculans*, and *S. sclerotiorum*) in field trials at two different geographical locations. These plants exhibited an increased tolerance to these diseases when compared to the non-transgenic parental plants. Over-expression of the chitinase gene also conferred enhanced resistance to other fungal pathogens (Broglie et al., 1993).

Phenolics have important roles in plant disease resistance as well (Métraux and Raskin, 1993). Prats et al. (2003) analyzed disease symptoms and total soluble phenolic content in four sunflower (*Helianthus annuus* L.) lines with different resistance levels (from highly susceptible to resistant) to head rot caused by *S. sclerotiorum*. They found the amount of phenolic compounds depended on the sunflower line, the time after inoculation, and the tissue. Higher constitutive and induced phenolic content and phenylalanine ammonia-lyase activity were present in the most resistant line; these differences correlated with the absence/presence of disease symptoms. Therefore, manipulation of phenolic compound levels in oilseed crops may provide enhanced *Sclerotinia* resistance.

BIOSAFETY OF OXO-TRANSGENIC CROPS

Biotechnology is bringing about remarkable progress in the molecular breeding and development of new plant varieties exhibiting high quality and high yield, for example, those with excellent pest and disease resistance. The first commercial introduction of transgenes into field crops occurred in 1996. In the U.S.A, transgenic corn, soybean, and cotton accounted for more than half the fields. However, public acceptance of crops produced using biotechnology methods varies and regulatory agencies scrutinize new products closely. Some people seem to feel uncomfortable with biotechnology, frequently because the lack of sufficient information (Bruening, 2000; Hino, 2002). For sunflowers, a major concern is the potential transgene escape from a transgenic crop to its wild relatives. Burke and Rieseberg (2003) highlighted an important point concerning the potential escape of transgenes inserted into genetically modified crops. They examine the transgene that confers resistance to *S. sclerotiorum* in sunflowers (*Helianthus annuus* L.). An OXO transgene

was backcrossed into wild sunflower, and the resulting plants were grown in containment cages at field sites in Indiana, North Dakota, and California, U.S.A. The presence of the OXO transgene promoted resistance to *S. sclerotiorum* but did not significantly affect seed production and reproductive output. They demonstrated that it is not the transfer of genes per se that is important, but rather their contribution to the relative fitness of the new host plants that impacts the persistence of the transgenes in the wild populations (Heritage, 2003). Such studies will be important in demonstrating the safety of these new technologies.

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