CONSTITUTIVE PROMOTERS AND *SCLEROTINIA* DISEASE RESISTANCE IN SUNFLOWER

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SUMMARY: Resistance to the fungal pathogen *Sclerotinia* is a trait of major importance for crops such as sunflower, canola, and soybean. However, genetic resistance is currently limited for breeding programs to counter the various forms of this fungal disease. We have focused on a transgenic approach to combat *Sclerotinia*. *Sclerotinia* disease in sunflower can be established at various developmental stages with the main targets being head, stem, and root tissues. We have developed constitutive promoters such as SCP1 and UCP3 to express *Sclerotinia* resistance genes. Addition of the 5'-untranslated leader (ω') of TMV downstream of the promoters significantly enhanced the promoter activity in sunflower tissues. The major toxic and pathogenic factor produced by *Sclerotinia* is oxalic acid which can be converted into H_2O_2 and CO_2 by oxalate oxidase. Over-expression of oxalate oxidase controlled by SCP1 significantly enhances resistance to *Sclerotinia* in sunflower.

INTRODUCTION

The fungal pathogen *Sclerotinia sclerotiorum* is worldwide in distribution and is pathogenic to more than 400 plant species at all developmental stages (1, 2). *Sclerotinia* synthesizes and excretes large amounts of the toxin oxalic acid into infected host tissues. Oxalate not only acidifies the plant tissues but also chelates Ca^{2+} from the cell wall rendering the stressed tissue susceptible to a battery of fungal-produced degradative enzymes. The synergistic action of the oxalate and cell wall degrading enzymes produced by *Sclerotinia* in the host cells appears to be a requirement for the infection (3, 4). *Sclerotinia* disease causes significant yield loses of crops including sunflower, canola, and soybean (1, 2).

Although *Sclerotinia* disease has been recognized for more than 100 years (1), little information on plant genetic resistance is available. Since oxalic acid is the main toxic and pathogenic factor, we have worked on a detoxification strategy to combat this disease using a wheat oxalate oxidase, which converts oxalate into H_2O_2 and CO_2. The potential impact of this enzyme is two fold: degrading *Sclerotinia* toxin oxalate and production of the defense-inducing molecule H_2O_2 (5), a by-product of the enzyme action on oxalate.

Sclerotinia disease can be established in several tissues of sunflower at all developmental stages with the main targets being root, basal and middle stems, and head tissues (Fig. 1). This fact suggests that resistance genes need to be constitutively expressed in order to efficiently meet the flexible challenge. Herein we report constitutive promoters to express *Sclerotinia* resistance genes in sunflower. The bioassay data demonstrate that SCP1 is adequate as a constitutive promoter for expressing oxalate oxidase and conferring enhanced *Sclerotinia* resistance in sunflower.

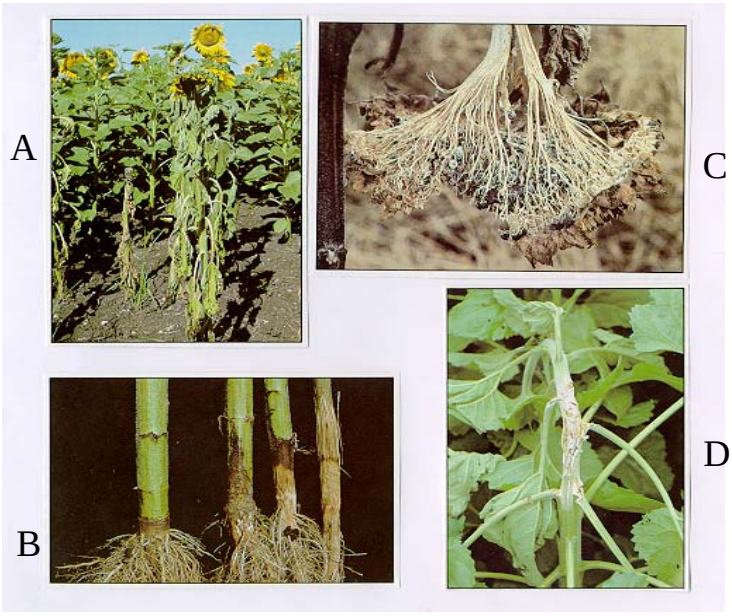

Figure 1. *Sclerotinia* diseases in sunflower. A, *Sclerotinia* wilt; B, basal stalk rot; C, head rot; and D, middle stalk rot.

Pictures are from "Sunflower: Production and Management" (Compiled and edited by Marcia P. McMullen, 1985).

RESULTS AND DISCUSSION

Sclerotinia Disease Resistance Gene

The synergistic action of the oxalate and cell wall degrading enzymes in the host cells appears to be a requirement for *Sclerotinia* infection (3,4). A common strategy to combat this disease is detoxification using oxalate-degrading enzymes, such as barley oxalate oxidase (4). Craig Hastings and Sean Coughlan isolated a wheat oxalate oxidase cDNA (6) using PCR for engineering *Sclerotinia* resistance in sunflower.

Constitutive Promoter

Sclerotinia diseases can be established in several tissues of sunflower (Fig. 1). This fact suggests that resistance genes need to be constitutively expressed. The expression pattern and level of a transgene are predominantly controlled by the promoter, and the activity of a promoter is tightly regulated by elaborate complexes of proteins that assemble on DNA. Most important is the interaction of TATA- binding proteins with activators and/or repressors that interact with upstream *cis*-acting element (7).

To identify constitutive promoters, we generated SCP1 and UCP3 promoters by cloning upstream sequences of known strong constitutive promoters such as maize Ubi-1 (8) to the 5' end of a synthetic core promoter (SynCore) (9). In a sunflower transgenic callus assay, SCP1 and UCP3 promoters expressed GUS at very higher levels. In order to confirm these two promoters are constitutive, we transformed them into sunflower and tobacco using oxalate oxidase or NPT II respectively as reporter genes. As shown in Figure 2A and 2B, SCP1 and UCP3 direct transgene expression in various tissues that include the main *Sclerotinia*-preferred tissues, root, stem, corolla tube, and receptacle tissues. In addition, SCP1 and UCP3 maintained strong activity in these tissues that were from various developmental stages.

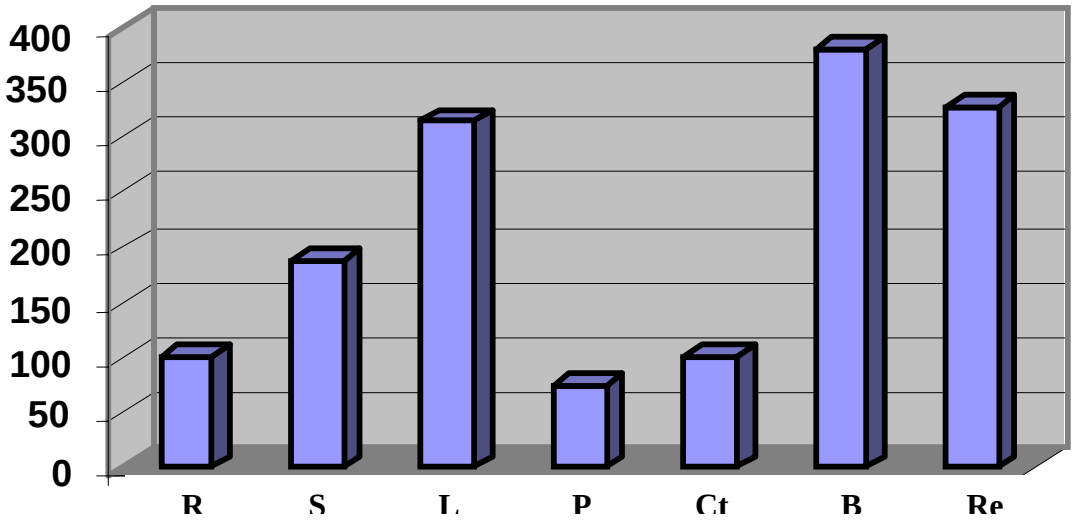

Figure 2A. SCP1 promoter activities in various transgenic parts including root, stems, and head tissues. Activities were expressed as relative activity (%) to its activity in root tissue (100%). Oxalate oxidase was used as a reporter gene and its activity was measured by a colorimetric assay (4). R, root; S, stem; L, leaf; P, petiole; Ct, corolla tube; B, bracts; and re, receptacle.

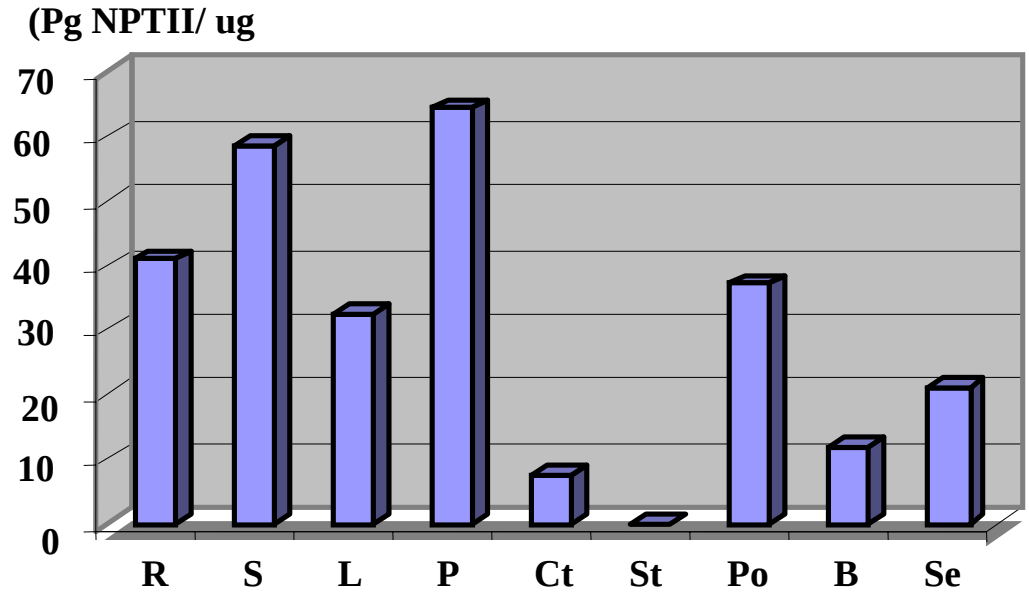

Figure 2B. UCP3 promoter activity in various transgenic tobacco tissues including root, stems, and head parts. NPTII was used as a reporter gene and NPTII protein was detected by ELISA. R, root; S, stem; L, leaf; P, petiole; Ct, corolla tube; St, stigma; Po, pollen; B, bracts; and Se, seed.

Omega' Element Increase Promoter Activity in Sunflower

It has been reported that the 5'-untranslated leader sequence (ω') of TMV significantly increased promoter activities in plant tissue (10); but no data has yet been reported from sunflower. As shown in Figure 3, omega' increased SCP1 and UCP1 promoter activity by 2-8 fold in transgenic sunflower callus.

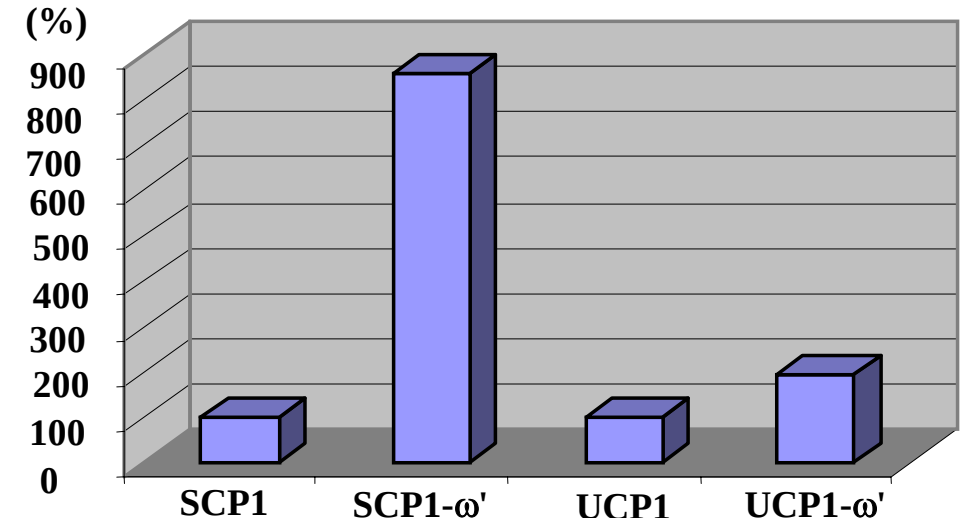

Figure 3. Effects of ω' on SCP1 and UCP1 activities in transgenic sunflower callus. GUS was used as a reporter gene and its activity was measured using GUS-Light™ kit (Tropix, Bedford, MA).

SCP1-ω'-Oxalate Oxidase-Transgenic Sunflower Leaves Exhibited Enhanced Tolerance to Oxalic Acid

The SCP1-ω'::oxalate oxidase-transgenic sunflowers expressed oxox activity in various tissues (Fig. 2A). In order to understand the effect of overexpressing oxox on the tolerance of sunflower plants to oxalic acid, we carried out an *in vitro* assay. As indicated in Figure 4, the oxox-transgenic leaves exhibited enhanced tolerance to oxalic acid (8 mM) compared to non-transformed SMF leaves.

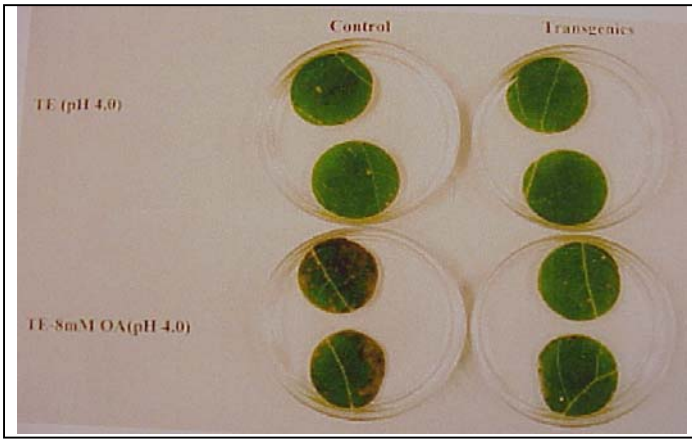

Figure 4. Tolerance of oxox-transgenic leaves to oxalic acid. Mature leaves from 60-day-old oxox-transgenic plants and SMF3 were floated on TE (pH 4.0) and 8 mM oxalic acid (OA)-TE (pH 4.0) solutions for 36 h. The control leaves were from the non-transformed SMF3 and showed lesions, but the oxox-transgenic leaves did not exhibit lesions.

Overexpression of Oxalate Oxidase in Sunflower Conferred Enhanced Resistance to *Sclerotinia*

As shown in Figure 5 and 6, the oxox-transgenic sunflower leaf and stem exhibited smaller lesions than the non-transformed SMF3 after inoculation with *Sclerotinia* mycelia (Fig. 5 and 6). The oxox activities in oxox-transgenic tissues were more than 500-fold higher than that in the non-transformed SMF3 plants (Fig. 2A). The whole transgenic plants were healthier than the non-transformed SMF3 plants three weeks after inoculation (11 and Scelonge et al., 15th International Sunflower Conference). These results showed the efficiency of expressing oxox with SCP1 promoter and the efficacy of oxox in combating *Sclerotinia* disease.

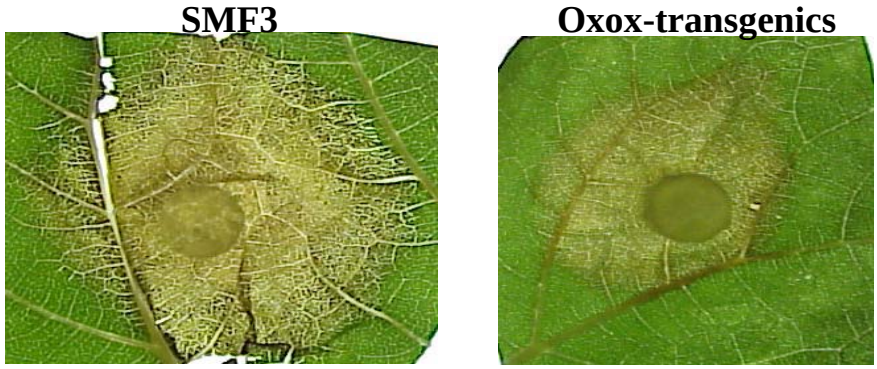

Figure 5. Detached leaf *Sclerotinia* infection experiment. Untransformed SMF3 and oxox-transgenic leaves were harvested from 6-week-old plants. The leaves were inoculated with 2-day-old *Sclerotinia* mycelium with PDA medium. Pictures were taken 24 hours after inoculation.

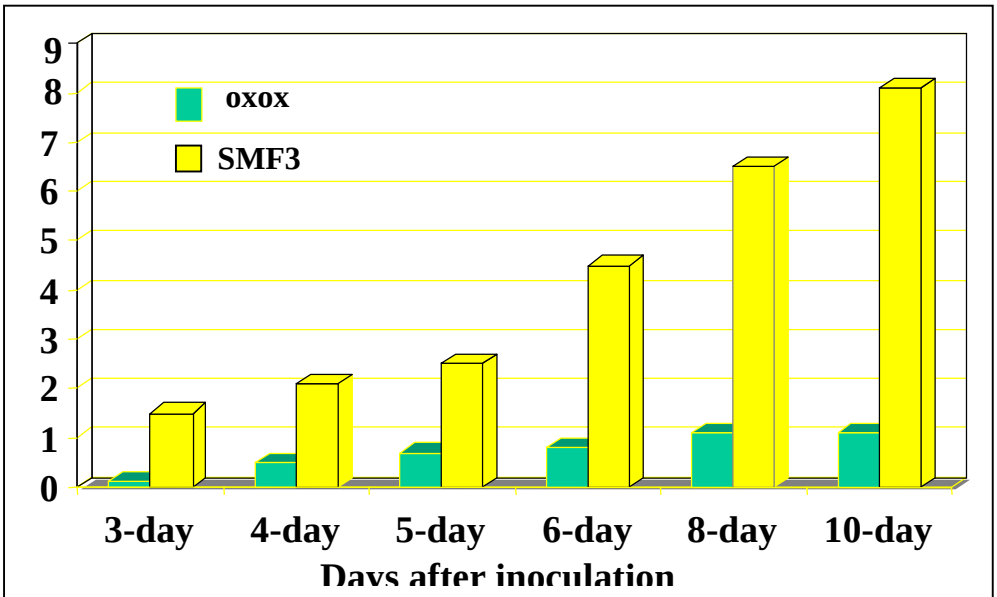

Figure 6. *Sclerotinia* middle stalk trial in greenhouse. Three petioles of every plant were inoculated by active growing mycelium. Lesion size (cM) on stem was recorded from 3 to 10 days after inoculation.

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