Oxalate Decarboxylase from *Collybia velutipes*

MOLECULAR CLONING AND ITS OVEREXPRESSION TO CONFER RESISTANCE TO FUNGAL INFECTION IN TRANSGENIC TOBACCO AND TOMATO*

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Oxalic acid is present as nutritional stress in many crop plants like Amaranth and Lathyrus. Oxalic acid has also been found to be involved in the attacking mechanism of several phytopathogenic fungi. A full-length cDNA for oxalate decarboxylase, an oxalate-catabolizing enzyme, was isolated by using 5′-rapid amplification of cDNA ends-polymerase chain reaction of a partial cDNA as cloned earlier from our laboratory (Mehta, A., and Datta, A. (1991) *J. Biol. Chem.* 266, 23548–23553). By screening a genomic library from *Collybia velutipes* with this cDNA as a probe, a genomic clone has been isolated. Sequence analyses and comparison of the genomic sequence with the cDNA sequence revealed that the cDNA is interrupted with 17 small introns. The cDNA has been successfully expressed in cytosol and vacuole of transgenic tobacco and tomato plants. The transgenic plants show normal phenotype, and the transferred trait is stably inherited to the next generation. The recombinant enzyme is partially glycosylated and shows oxalate decarboxylase activity *in vitro* as well as *in vivo*. Transgenic tobacco and tomato plants expressing oxalate decarboxylase show remarkable resistance to phytopathogenic fungus *Sclerotinia sclerotiorum* that utilizes oxalic acid during infestation. The result presented in the paper represents a novel approach to develop transgenic plants resistant to fungal infection.

Green leafy vegetables such as spinach, rhubarb, and amaranth are rich source of vitamins and minerals (1–3) but accumulate large amount of oxalate. Such vegetables when consumed in large quantities cause secondary hyperoxaluria where precipitation of oxalic acid as calcium oxalate leads to kidney stones and hypocalcemia (4, 5). *Lathyrus sativus*, a protein rich hardy legume that grows under extreme environmental conditions, is not edible due to the presence of a neurotoxin β-N-oxalyl-L-α,β-diaminopropionic acid. Oxalic acid is an essential precursor of the neurotoxin (6). β-N-Oxalyl-L-α,β-diaminopropionic acid acts as a metabolic antagonist of glutamic acid, which is involved in the transmission of nerve impulse in the brain (7).

Oxalic acid production by fungi has been associated with the pathogenesis of several plant pathogenic fungi, *e.g. Sclerotinia sclerotiorum*, *Sclerotinia rolfsii*, and *Sclerotinia ceptivorum* (8–10). *S. sclerotiorum* is an important plant pathogen that causes substantial loss in crop yield each year throughout the world (11). The fungus has a very wide host range (12) resulting in about 95% loss of economically important crops like oil seed rape, bean, tomato, and sunflower. Disease symptoms on individual plants are variable but often include watery soft rot of infected leaf and stem tissue. The exact role of oxalic acid during infection is not well understood. During the early stage of disease development and at advancing margins of the lesion, oxalic acid may work synergistically with pectolytic enzymes. Oxalic acid being a strong chelator may chelate calcium from the calcium pectate of host cell wall causing maceration of the host tissue. Oxalic acid also affects pH of the infected tissue favoring the activity of some cellulolytic enzymes (13). In another study, it has also been shown to inhibit the activity of host *o*-diphenol oxidase in apple fruits (14), suggested to be involved in plant defense (15). The importance of oxalic acid in pathogenesis has been suggested recently by studies on a mutant of *S. sclerotiorum*, specifically lacking the ability to synthesize oxalic acid. This mutant was found nonpathogenic in bioassays with *Phaseolus vulgaris* (16) and *Arabidopsis thaliana*. Moreover, Pseudomonad-like bacterial strains capable of degrading oxalic acid could prevent *S. sclerotiorum* infection in *A. thaliana* (17).

Introduction of a gene that can specifically degrade oxalic acid in the crop plants would have 2-fold advantages, first in reducing the nutritional stress and the second in conferring resistance to fungal pathogen. Oxalic acid is catabolized by two major pathways, *i.e.* decarboxylation and oxidation, and decarboxylation occurs either by activation of oxalic acid to oxalyl-CoA by means of oxalyl-CoA decarboxylase or directly to CO_2 and formic acid by oxalate decarboxylase. Oxalate oxidation has been detected in plants where oxalic acid is broken down to CO_2 and H_2O_2. We earlier reported purification and partial cDNA cloning of oxalate decarboxylase from *Collybia velutipes* (18). The enzyme has several advantages over other oxalate-degrading enzymes. First, OXDC[1] is specific to oxalate, and it catabolizes to formic acid (non-toxic organic acid) and CO_2 in a single step without requirement of a cofactor (18). Second, the enzyme is active at low pH which would be helpful as most of the oxalate is localized in plant cell vacuoles, where pH is low.

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[1] The abbreviations used are: OXDC, oxalate decarboxylase; RACE-PCR, rapid amplification of cDNA ends-polymerase chain reaction; BLAST, basic local alignment search tool; HPLC, high performance liquid chromatography; bp, base pair; WT, wild type; kb, kilobase pair; nt, nucleotide; PR, pathogenesis-related.

We report here the sequence of the complete cDNA and genomic clone encoding oxalate decarboxylase. The predicted amino acid sequence shows strong homology with hypothetical proteins from bacteria and a weaker homology with germin like proteins from plant. The cDNA is interrupted with 17 small introns. We have expressed the *OXDC* cDNA in *Escherichia coli*, which could not produce biologically active enzyme. However, it has been successfully expressed in tobacco and tomato, and as a result both plants showed resistance to infection by *S. sclerotiorium*. The resistance has been correlated with the induced expression of pathogenesis-related proteins in the transgenic plants by oxalate, and the possibility of catalytic product of oxalate as signal for induction of defense genes has been discussed.

EXPERIMENTAL PROCEDURES

cDNA Sequencing, 5′-RACE, and Cloning and Sequencing of Genomic DNA of Oxalate Decarboxylase—A cDNA encoding oxalate decarboxylase was cloned from *C. velutipes* (18). The entire sequence of both strands using deletion subclones was obtained by dideoxy chain termination method (19). The 5′ end of the transcript was determined by 5′-rapid amplification of cDNA ends and polymerase chain reaction (5′-RACE-PCR) according to the protocol of the manufacturer (Life Technologies, Inc.). Nested PCRs were carried out using 5′-oligonucleotides from the adapter and internal 3′-oligonucleotides from cDNA sequence; nested primers used were O10 (5′-TCACTGGTCTCATTCCA-GAGC-3′) and O06 (5′-TGAGGTACCGGTCGCAGTT-3′). The PCR products from the 5′-RACE-amplified cDNA were cloned in pBluescript KS II vector (pNOX5). The sequences of the clones were determined as described above. The 5′ sequence from a clone containing the largest insert was used to complete the cDNA. The subclone containing complete cDNA was named as pOXDC.

Genomic library of *C. velutipes* was prepared in lambda GEM12 vector. The genomic DNA was partially digested with *Sau*3AI and filled partially with dA and dG followed by ligation with *Xho*I half-site of the lambda GEM12 vector. The ligation mix was packaged *in vitro* using GigapackII packaging extract according to the manufacturer's instruction. The primary library was plated on *E. coli* LE392 cell. The library was screened with ^{32}P-labeled *OXDC* cDNA. Six clones were obtained and characterized with restriction enzyme digestion and Southern hybridization with ^{32}P-labeled *OXDC* cDNA. A 6.0-kb *Xho*I fragment encoding full-length OXDC was subcloned in pBluescript II KS vector (Stratagene, La Jolla, CA). Both strands of the insert were sequenced after generation of deletion subclones. The structural organization of the *OXDC* gene was determined by DNA sequence comparison between full-length cDNA and genomic DNA. Computer-assisted sequence analysis was done using PC/Gene sequence analysis package (Intelligenetics). Homology search of the amino acid sequence was carried out using BLAST (20) and Clustal W (21)

Expression in Plant—For the cytosolic expression of oxalate decarboxylase in plants, the cDNA was cloned in a binary vector pBI121 (CLONTECH) under transcriptional control of CaMV35S promoter and was named as pCOXA. The insert was obtained by *Eco*RI digestion, end-filled, and by *Bam*HI digestion of pOXDC. This fragment was ligated to *Bam*HI and *Ecl*136II double-digested pBI121 vector. To target the oxalate decarboxylase to plant vacuoles, another construct named pSOVA was made. OXDC cDNA was PCR-amplified using sense primer O13 (5′-CCGGATCCGATGTTCAACAACT-3′) and a antisense primer O14 (5′-GAAAGATCTAGGTTCACAGGACCAACA-3′) to incorporate *Bam*HI and *Bgl*II for in frame fusion with secretion and the vacuolar targeting sequence at the 5′ and 3′ end, respectively. The 1.4-kb PCR-amplified fragment was cloned in *Eco*RV site of pBlueScript II KS+ vector. The resulting plasmid was restricted with *Bgl*II and *Eco*RI, and the vector DNA containing the cDNA was gel-purified and ligated to a *Bgl*II, *Eco*RI fragment (50 bp) having the vacuolar targeting sequence of tobacco chitinase. The 50-bp fragment was obtained by digestion of pTUCH10 (25) with *Bgl*II and *Eco*RI. The resulting plasmid was called as pVOD. pVOD was digested with *Xba*I, end-filled with Klenow, and then further digested with *Bam*HI. The insert was purified and ligated downstream of the secretion signal sequence present in a plasmid pTRCH4 (23). The vector pTRCH4 was prepared by digestion with *Hin*dIII, made blunt by Klenow polymerase, and then digested with *Bcl*I. This construct was named pSOVR. The junctions were sequenced to confirm right fusions. The chimeric construct containing secretion and vacuolar targeting sequence was excised by digestion

with *Hin*dIII filled in and then digestion with *Bam*HI. The fragment was then ligated to *Bam*HI, *Ecl*136II double-digested pBI121 binary vector to yield pSOVA.

Plant Transformation—*Nicotiana tabacum* var. Petite Havana and Tomato var. Pusa Ruby seeds were surface-sterilized and germinated on Murashige and Skoog basal medium (24). The constructs pCOXA and pSOVA were mobilized in *Agrobacterium tumefaciens* strain LBA4404 by triparental mating (25) for transformation of tobacco,whereas pSOVA was introduced into *A. tumefaciens* strain EHA105 by electroporation (26) for tomato transformation. Transformation of *N. tabacum* was carried out using the *Agrobacterium*-mediated leaf disc transformation method (27). Tomato cotyledonary leaves were transformed by the method described below. The explants were precultured on feeder plates (MS medium supplemented with 0.1 mg/liter kinetin and 3 mg/liter 2,4-dichlorophenoxyacetic acid overlaid with 3–4 ml of tobacco suspension cell) for 24 h. The explants were inoculated with bacterium culture and co-cultivated for 48 h. The explants were transferred to selection medium (MS medium supplemented with 1 mg/liter zeatin and 50 mg/liter kanamycin). Shoots obtained were rooted on the medium containing 0.1 mg/liter indole butyric acid and 50 mg/liter kanamycin. All transformed plants were maintained in the growth chamber at 25 °C with 16-h photoperiod.

Molecular Analysis of Transgenic Plants—Total plant DNA was isolated from tobacco plants using cetyltrimethylammonium bromide method as described (28). PCR was carried out using plus and minus primers Crt1 (5′-CACCGACTACTGATCATGG-3′, downstream to ATG) and O08 (5′-GAACGAAAGTTCAGTTCACAG-3′, spanning stop codon), respectively, when COXA was used as template and plus and minus primer Ch1 (5′-CGTTTGCATTTCACCAG-3′) and Ch2 (5′-CGATTAT-AGTCGTGATCCC-3′), respectively, with SOVA DNA as template. Wild type (WT) DNA of tobacco was also amplified with both sets of primers. Plant protein extract was prepared as described (29). Crude protein extracts of COXA (10 μg), SOVA (5 μg), and WT plants were separated on a 10% SDS-polyacrylamide gel electrophoresis. The proteins were blotted onto nitrocellulose membrane by electrotransfer (30). The membrane was probed with affinity purified oxalate decarboxylase polyclonal antibody (18). The secondary antibody used was alkaline phosphatase-conjugated goat IgG (Bio-Rad). Oxalate decarboxylase assay was carried out by the method as described previously (18). Ten micrograms of crude protein extract was taken for assay. Each sample was assayed in triplicate, and the mean value was taken.

Localization of the recombinant enzymes in plant tissue was performed by immunogold labeling. Tissue was fixed in 2% glutaraldehyde and embedded in LR-white resin. Sections were incubated with OXDC-specific affinity purified antibody (18) diluted 1:100 overnight at 4 °C. After washing, the samples were incubated for 1 h at room temperature in goat anti-rabbit 10-nm gold complex (1:100, Amersham Pharmacia Biotech) and stained in 2% uranyl acetate and lead citrate.

Wilting Assay of Leaves—Photosynthetically active leaves (three each) from WT and transgenic plants were excised, and the petioles were immediately dipped in 20 mM oxalic acid solution (pH 4.0). Negative control was imposed by dipping the leaves in water (pH adjusted to 4.0 with hydrochloric acid). The leaves were incubated in a plant growth chamber for 24 h at 24 °C under 16-h photoperiod. The extent of wilting was recorded after 24 h.

Oxalic Acid Measurement—Four leaves from wild type and transgenic plants grown under controlled environment were dried at 40 °C until constant weight was obtained. Dried leaves were mixed by crushing and divided into three parts. Each aliquot was ground to fine powder in a mortar pestle. Duplicate sample of 10 mg of powder was extracted for 10 min in boiling 250 mM H_2SO_4. Samples were cooled to room temperature, and the debris was removed by centrifugation. The supernatant was filtered with a 0.2-μm filter before loading on the column. Triplicate measurements were made from each sample. The organic acid content was analyzed on HPX 87-H column (Bio-Rad) by HPLC (31).

Pathogenesis Assay of Transgenic Plants with S. sclerotiorum—A carrot isolate of the *S. sclerotiorum* was obtained from Indian type culture collection IARI, New Delhi, India. The fungus was maintained on potato/dextrose/agar (PDA, 20% potato, 2% dextrose, and 1.5% agar) slants. Infection was carried out by the mycelium agar disc method on detached leaf (16). Five leaves from 10 independent transgenic plants (3 from COXA and 7 from SOVA with different copy number) and a WT plant were excised from 1-month-old hardened plants. Five leaves each from two transgenic and control tomato plants were taken. The leaves were kept in a 90-mm Petri dish containing wet Whatman No. 3 filter paper disc. Mycelial agar plug of 3-mm diameter was punched from growing margins of a 4-day-old *S. sclerotiorum* culture grown on PDA.

Fig. 1. **Analysis of *OXDC* gene.** *A,* hydropathy profile of predicted amino acid oxalate decarboxylase. *Solid line* indicates the N-terminal hydrophobic stretch characteristic of a secretion signal. *B,* alignment of amino acid sequence of hypothetical proteins from *B. subtilis* and *Synechocystis* with OXDC predicted amino acid sequence. *C,* alignment of OXDC predicted amino acid sequence with germin-like proteins from *Arabidopsis*, tomato, and rice. The *shaded areas* indicate the identical amino acids. *D,* nucleotide and predicted amino acid sequence of *OXDC* gene of *C. velutipes*. Coding and non-coding regions are shown in *upper* and *lowercase letters,* respectively. The sequence starts with transcription start site (+1). Single letter codes for amino acids are given *below* the coding region (the *asterisk* indicates stop codon). All introns are *numbered* and indicated by *dotted underlines.* The putative polyadenylation signal is *underlined,* and the site of polyadenylation in the cDNA is indicated by an *arrowhead.*

The mycelial agar disc was applied on the adaxial surface of the leaf. Uninoculated PDA disc was applied on leaves as negative control. The plates were kept under 16-h photoperiod and 100% humidity. The disease symptoms were observed every 24 h, over a period of 1 week. This experiment was repeated three times under similar conditions.

RESULTS

Structure of C. velutipes Oxalate Decarboxylase Gene—Oxalate decarboxylase (*OXDC*) cDNA was cloned from *C. velutipes* as described earlier (18). After sequence analysis, the 5′ end of

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arabidopsis  --MFCLFVIPS---LSSDSDPLQDFCVGDLKASASINGFPCKSAVSASDFFYSGLGGPLD  55
tomato       MVIMTLLAINSDKACAGDPDMLQDVCVADLTSTLKLNGFTCKNMFSAADFSSMVISKPGA  71
oryzae       -VLLSFLLLPF-----SSMALTQDFCVADLTCSDTPAGYPCKASVGAGDFAYHGLAAAGN  63
oxdc         VILKNFRAKNP---AAWSHIPAQQLYIFPSEPPADNQPDVSPQGTVPLPYSFNFSSVEP  297
                 :  :               .    *:. :          .   .  .      :.

arabidopsis  TSNPNGVTVAPANVLTFPGLNTLGISMNNVELAPGGVNPPHLHPRATEVGTVIEGSVFVG  115
tomato       TNNMFGSLVTGANVMAIPGLNTLGVSMARIDYAPGGINPPHLHPRATEMIYVLQGELDVG  131
oryzae       TSNLIKAAVTPAFVGQFPGVNGLGISAAKLDIAVGGVVPLHTHPAASELLFVTQGTVAAG  123
oxdc         TQYSGGTAKIADSTTTFN--ISVAIAVAEVTVEPGALRELHWHPTEDEWTFFISGNARVT  355
                *.           .      :     :.::   .:     *.:   * **    *   . .*   .

arabidopsis  FLS-TNNTLFSKVLNAGEAFVIPRGLVHFQWNVGQVKARMITAFNSQLPGAVVLPSTLFG  174
tomato       FIT-TANVLVSKHIVQGEVFVFPRGLVHFQKNNGHMPAAVIAGFNSQLAGTQSIATTLFA  190
oryzae       FITSSSNTVYTRTLYAGDIMVFPQGLLHYQYNAGQSAAVALVAFSGPNPGLQITDYALFA  183
oxdc         IFA-AQSVASTFDYQGGDIAYVPASMGHYVENIGNTTLTYLEVFNTDRFADVSLSQWLAL  414
                :::: :  ..  :       *:     .*  .:  *:    * **:      :   *.      .  *

arabidopsis  SKPE--IPNAVLTRAFRTDDTTVQNLKSKFAV--  204
tomato       ATPAPGVPNDILAQAFQIGTMQVQQIKAKFAPRN  224
oryzae       NN----LPSAIVEKVTFLDDAQVKKLKSVLGGSG  213
oxdc         TPPSVVQAHLNLDDETLAELKQFATKATVVGPVN  448
                .   :             .        :  ..
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D

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atccatcgcatttccgATGTTCAACAACTTCCAACGTCTGCTCACTGTCATCCTTCTCTC  60        gagctcgactactttgtagTTATCCTGAAGAACTTCAGAGCCAAGAATCCCGCCGCATGG  1320
                M  F  N  N  F  Q  R  L  L  T  V  I  L  L  S                        ................)  I  L  K  N  F  R  A  K  N  P  A  A  W

CGGTTTTACCGCTGGAGTGCCTTTGGCGTCCACCACGACGGGAACTGGAACTGCGACCGG  120       TCTCACATACCTGCTCAACAGCTATACATCTTCCCTAGTGgtgcgtttcctcttcctctt  1380
 G  F  T  A  G  V  P  L  A  S  T  T  T  G  T  G  T  A  T  G            S  H  I  P  A  Q  Q  L  Y  I  F  P  S  E(...................

TACCTCAACCGCCGCAGAGCCCAGCGCGACTGTCCCCTTCGCCAGCACTGATCCCAACCC  180       cccctgaccgtgacacggctcaacatgtaacatagAACCTCCTGCGGACAACCAGCCGGA  1440
 T  S  T  A  A  E  P  S  A  T  V  P  F  A  S  T  D  P  N  P           ..intron-11......................)  P  P  A  D  N  Q  P  D

CGTGCTCTGGAATGAGACCAGTGACCCAGCGCTTGTAAAGCCAGAGAGGAACCAGCTTGG  240       CCCCGTTAGCCCACAGGGGACGGTCCCCTTCCATATTCATTCAACTTCTCCTCTGTCGA  1500
 V  L  W  N  E  T  S  D  P  A  L  V  K  P  E  R  N  Q  L  G           P  V  S  P  Q  G  T  V  P  L  P  Y  S  F  N  F  S  S  V  K

TGCGACAATCCAAGGACCGGATAATCTGCCTATAGACCTTCAGAATCCGGACTTGCTCGC  300       GCCGACGCAGTATTCCGGTGGGACAGCGAAGATTGCAGATTCCACGACGTTCAACATTTC  1560
 A  T  I  Q  G  P  D  N  L  P  I  D  L  Q  N  P  D  L  L  A           P  T  Q  Y  S  G  G  T  A  K  I  A  D  S  T  T  F  N  I  S

CCCACCGACTACTGATCATGGCTTTGTgtgagtttttgtatactagtactggctgggtat  360       CGTCGCTATCGCCGTGGCCGAGGTTACAGTTGAGCCTGGTGCTTTGAGgtgggtttctcg  1620
 P  P  T  T  D  H  G  F  V  (..............................               V  A  I  A  V  A  E  V  T  V  E  P  G  A  L  R  (...........

gtgattgaagaggtttttacagCGGTAATGCGAAGTGGCCATTCAGCTTCAGCAAGCAGCG  420      tagtggcttgcacgttctaaatctgacccttggtatcaagAGAGCTGCACTGGCATCCG  1680
..intron-1..........)  G  N  A  K  W  P  F  S  F  S  K  Q  R           ......intron-12.........................)  E  L  H  W  H  P

 CTGCAGACGGGTGGCTGGGCTCGGCAGCAGAATGgtgggttgtcttcgttgatttgggg  480       ACTGAGGACGAGTGGACATTCTTCATgtatgtgtactttcggagatgccaagatgtataa  1740
 L  Q  T  G  G  W  A  R  Q  Q  N  E(...........................               T  E  D  E  W  T  F  F  I  (........intron-13............

tgctttggacgctgacggatcgttcaagAGGTCGTTTTGCCTCTCGCGACTAgtaagcta  540       taactcggttttacagCTCTGGAAACGCGAGGGTGACAATTTTCGCTGCGCAGAGTGTAGC  1800
..intron-2.................)  V  V  L  P  L  A  T  N(...........            ...............)  S  G  N  A  R  V  T  I  F  A  A  Q  S  V  A

aaoctttacttagcttggctcgacaaagcataaacatatcgaacagATCTCGCTTGCACAA  600       CTCTACGTTTGATTACCAAGGTGGTGATATCGgtgagtacatttagtctgagcgaagtgc  1860
...............intron-3..................)  L  A  C  T  N           S  T  F  D  Y  Q  G  G  D  I  A(............intron-14.......

ATATGCGTCTTGAAGCAGGCGCTATCAGgtactatggttcgcatcctgtcgcgtcaagag  660       ttagtgaagctgactgccgacagCTTATGTTCCTGCATCTATGGgtaccattttatcttc  1920
 M  R  L  E  A  G  A  I  R(...............................               .....................)  Y  V  P  A  S  M  G(..............

tttgaccgctctttcttacacagGGAGCTGCATTGGCACAAGAACGCTGAGgtacgtctg  720       ctccatcttgcctgaatctttctgacatattttactacagGCCATTATGTAGAGAACAT  1980
..intron-4..........)  E  L  H  W  H  K  N  A  E(........               ....intron-15..........................)  H  Y  V  E  N  I

tctgacccagtcaatgtttcttcgaacttagactttccctagTGGGCATATGTTCTGAA  780       TGGAAACACGACTTTGACTTATCTGGAGGTCTTCAATACCGgtgcgttatacaacaatgc  2040
..............intron-5..................)W  A  Y  V  L  K           G  N  T  T  L  T  Y  L  E  V  F  N  T  D(...............

GgtgctgaaaactgtgtatgcaatttcgtcgtgagactgagaatatcgtcatgGGGTCT  840       gcgttggttcttctgtctcatgtgtatcatgcagACCGTTTTGCTGGATGTCAGTCTAAGTCA  2100
 (..............intron-6......................)G  S           .........intron-16..........)  R  F  A  D  V  S  L  S  Q

ACCCAAATCTCAGCTGTCGATAACGAAGGGCGCAATTATATTTCCACCGTCgtgagtgta  900       GgtatgtttgttgtagcatatacggagttatatactgagcgagtgcaacagTGGCTGGCGT  2160
 T  Q  I  S  A  V  D  N  E  G  R  N  Y  I  S  T  V  (........               (.............intron-17............)W  L  A  L

cttgcacatatactttattagacttatttactgtgaccgacttttagGGCCCTGGTGATT  960       TAACACCTCCGAGTGTCGTGCAGGCGCACCTGAACTGGACGACGAGACACTTGCGGAGC  2220
..............intron-7.........................)G  P  G  D  L           T  P  P  S  V  V  Q  A  H  L  N  L  D  D  E  T  L  A  E  L

TGTGGTACTTCCCACCAGGCATTCCTCACTCGCTACAAGCGACAGCCGATGATCCAGAAG  1020     TCAAGCAGTTTGCGACCAAGGCGACTGTTGTTGGTCCTGTGAACTGAactttcgttcctt  2280
 W  Y  F  P  P  G  I  P  H  S  L  Q  A  T  A  D  D  P  E  G            K  Q  F  A  T  K  A  T  V  V  G  P  V  N  *

GCTCAGAGTTCATCTTAgtatgcattactcattcttttggtcatgacaagtggctgaag  1080      taaactcatcaaattatcattggaattctatgtagatgttgtaatcaatgcagttcttcg  2340
 S  E  F  I  L  (...................intron-8...............               &

aaagtcttttttgcgaagGTCTTTGATTCAGGCGCCTTCAATGACGACGGTACATTCTTGg  1140    gctatcccgttgaagaggatatattgatgataaggcattatgggcaagttcattattcgaat  2400
.................)V  F  D  S  G  A  F  N  D  D  G  T  F  L  (           aaacttaactgagatcagcctcgctgcaaacggagcggtttcctttttacggcggatatga  2460

tatgagatctctctcttcctacgtgtcatagaactcaacatattcagCTCACTGACTGG  1200      tttcctcccggccggtcgcttcgttgctctcctccatcctttggctagggcacattctcg  2520
..............intron-9...................)L  T  D  W           ttccaccgtatggtggttgttattggacttggcgaggacccgtagcaagttcaaggccttc  2580

CTTTCGCATGTTCCAATGGAAGGtggcccaaccccacttttgatcaagtattggtatactaa  1260    attacacctacaccctggatcgtcactaattgccgcggagttgactctggaagcgatagacg  2640
L  S  H  V  P  M  E  V(...................intron-10.........               ttagcccgacacgataccctca  2662
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FIG. 1—*continued*

FIG. 2. **Molecular analysis of representative transgenic *N. tabacum* lines.** WT, non-transformed plants (*C*), tobacco plants transformed with pCOXA, and *S* tobacco plants transformed with pSOVA (*A*) PCR amplification of transgene using genomic DNA from COXA (*left panel*) and SOVA (*right panel*) transgenic lines with primers Crt1:O08 and Ch1:Ch2, respectively. A single band of 1.1 and 1.6 kb was seen in all COXA and SOVA plants, respectively. No amplification was seen in wild type (*WT*) plant. Molecular weight markers (*M*) in kb are indicated on the *right*. *B,* Western analysis of soluble leaf proteins of tobacco; 15 μg of total soluble protein was separated on SDS-polyacrylamide gel electrophoresis and visualized with affinity purified polyclonal antibody raised against OXDC and a standard alkaline phosphatase immunoassay. Bands of 54–58 kDa are seen in most of the lines except in WT. The *right-most lane* contains purified OXDC from *C. velutipes*. Position of molecular markers (kDa) is shown at the *right*. *C,* OXDC activity, measured as micromole of CO_2 liberated in 30 min at 37 °C. Mean value of triplicate measurements are presented. *D,* 10 μg of total RNA from WT (*lane 1*), COXA (*lane 2*), and SOVA (*lane 3*) plants was separated on 1.5% agarose gel and subjected to Northern analysis using pOXDC cDNA. *Left panel* is the ethidium bromide-stained gel representing equal loading of RNA, and *right panel* shows hybridizing bands indicated by an *arrow*. *E,* immunogold localization of OXDC in transgenic tobacco plant transformed with pSOVA with OXDC-specific antibody. *C*, cytoplasm; *V*, central vacuole.
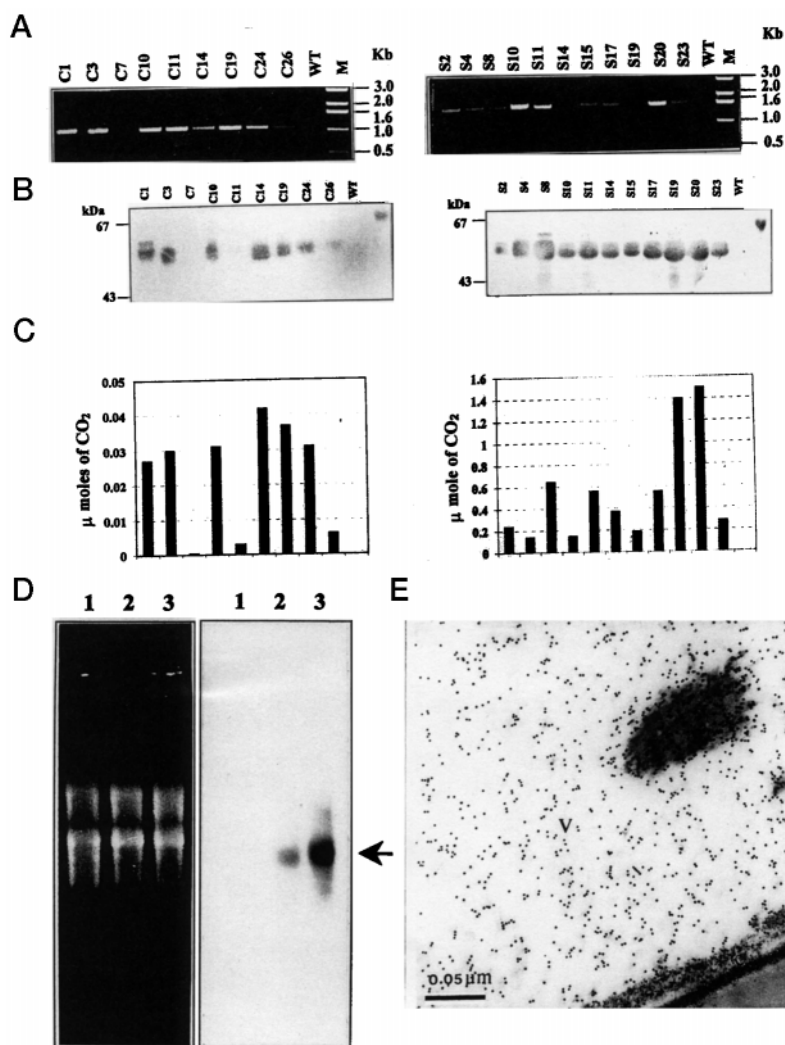
the sequence was found to be missing in the subclone. To obtain full length of the transcript, we used 5′-RACE-PCR. The complete cDNA was subcloned and named as pOXDC. The transcription start site as determined by 5′-RACE is an adenine residue present at 15 bp upstream of ATG. The message contains an open reading frame that encodes a 448-amino acid polypeptide with a predicted molecular mass of 50 kDa (which corresponds to the deglycosylated form of native OXDC purified from *C. velutipes*) and four potential *N*-linked glycosylation sites (18). The cDNA contains a putative polyadenylation signal ATCAAA at 15 bp upstream of the poly(A) tail. This sequence is similar to the poly(A) signal (AATCAA) of *LIP* gene from *Phanerochaete chrysosporium* (32). Hydropathy analysis indicated one prominent hydrophobic segment of 30 amino acid residues in length characteristic of a secretion signal (Fig. 1*A*). Data base search of the deduced OXDC amino acid sequence using BLAST revealed a strong homology with two *Bacillus subtilis* open reading frames (GenBank™ accession numbers Z99120 and AF027868 (10e-111)) and a *Synechocystis* sp. open reading frame (GenBank™ accession numbers D90907 open reading frames (10e-100)). In addition, OXDC also showed weaker homology to germin like proteins from barley, *Arabidopsis*, *Brassica napus*, tomato, and rice. Multiple alignment of OXDC with bacterial and plant protein sequence is presented in Fig. 1, *B* and *C,* respectively. To study the organization of the *OXDC* gene a genomic library of *C. velutipes* was prepared in λGEM12 vector. Six clones were obtained by screening the library with ³²P-labeled OXDC cDNA. The clones were mapped

by Southern blot hybridization, and a 6-kb fragment containing the complete gene was subcloned. The sequence determined is presented in Fig. 1*D*. The *OXDC* gene spans 2.4 kb. On comparison of the DNA sequences from cDNA and genomic DNA, we found that it contains 18 exon and 17 introns. All the introns contain conserved 5′ and 3′ splice junctions. The intron sizes vary from 40 to 60 bp, and the exon sizes exhibit a wider range with the smallest exon of 18 nt (E6) and the largest of 300 nt (E1).

Plant-expressed OXDC Is Enzymatically Active—Tobacco leaf discs were transformed with constructs pCOXA and pSOVA for cytosolic expression and for vacuolar targeting of OXDC, respectively. Tomato plants were transformed only with the pSOVA. In both the cases, OXDC cDNA was under control of CaMV35S promoter, and kanamycin was the selection marker. The regenerated tobacco plants were rooted in kanamycin-containing medium, and about 35 transgenic tobacco and 10 tomato plants were screened for the presence and expression of the transgene. The transgenic tobacco plants showed normal phenotype and were fertile. The transformed tobacco plants were confirmed by polymerase chain reaction as explained below. PCR was carried out using a plus primer designed from 5′ end and a minus primer spanning the stop codon of OXDC cDNA using genomic DNA from pCOXA-transformed plants as template. A single band of 1.1 kb was obtained in all the COXA transgenic plants (Fig. 2*A, left panel*). A plus primer Ch1 from the secretion signal sequence and a minus primer Ch2 from vacuolar targeting sequence were used with
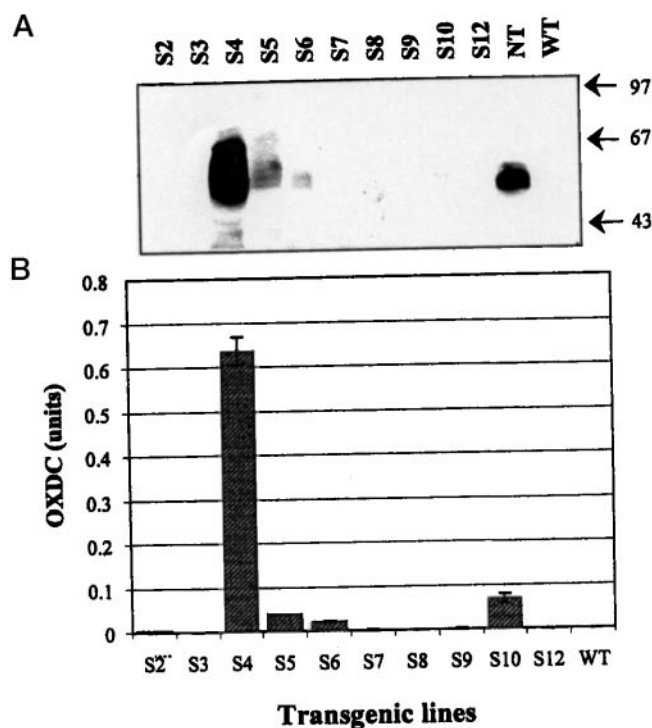
FIG. 3. **Analysis of OXDC expression in transgenic tomato plants.** *A,* the immunoblot analysis of independent transgenic lines (*S2–S12*). Protein molecular mass markers are given at the *right column* in kDa. *B, in vitro* activity of OXDC in the transgenic lines. Mean values of triplicate are presented. *NT*, pOXDC from transgenic tobacco plant; *WT*, wild type tomato plant.

FIG. 4. ***In vivo* activity of oxalate decarboxylase.** Wilting of excised leaves from WT and transgenic COXA and SOVA plants after 24 h. Excised leaves were immediately dipped in the oxalic acid solution, a negative control was kept with WT leaf in water (pH 4.0).

pSOVA-transformed plant genomic DNA as template. A band of 1.6 kb was observed in all transgenic tobacco plants (Fig. 2*A, right panel*), confirming the presence of transferred DNA in the regenerated plants. We also carried out genomic Southern hybridization with *OXDC* cDNA as probe and found that only transgenic plants gave hybridizing bands (data not shown). Western blot analysis of crude protein extract from pCOXA- and pSOVA-transformed plants showed three bands of 54, 56, and 58 kDa (Fig. 2*B*). No immunoreactive band was observed with wild type protein extracts establishing the specificity of the bands. The protein crude extract was also assayed for oxalate decarboxylase activity; all the plants showing detectable band showed decarboxylation of oxalic acid *in vitro* (Fig. 2*C*). The pH optimum and stability of the recombinant enzyme in crude extracts showed no changes when compared with the purified *C. velutipes* OXDC (data not shown). Furthermore, expression level was found about 5-fold higher in the case of SOVA as compared with COXA transgenic plants. This was correlated with difference in the transcript levels (Fig. 2*D*). To assess if the OXDC expressed from pSOVA was correctly targeted to vacuoles, we performed immunoelectron microscopy. Our results show that the protein was successfully targeted to vacuoles in addition to its expression in cytoplasm (Fig. 2*E*).

The transgenic tomato plants also showed the presence of transgene, which was analyzed by PCR and Southern hybridization (data not shown). Out of the 10 transgenic lines, 7 plants growing on kanamycin showed the presence of intact gene which were further analyzed for its expression. Four plants showed expressed protein on the Western blot that was similar to the product obtained in tobacco (Fig. 3*A*). The expressed enzyme showed oxalate decarboxylase activity (Fig. 3*B*) which varied according to the level of protein.

Transgenic Tobacco Plants Could Degrade Oxalic Acid in Vivo—To test the ability of transgenic plants to degrade oxalic acid *in vivo*, oxalic acid wilting test was carried out. Excised wild type plant leaves wilt rapidly (2 h) in oxalic acid due to xylem embolism (33). Leaves from COXA transgenic plants showed delayed wilting (12 h), whereas SOVA plant leaves did not wilt in the presence of oxalic acid even after 3 days (Fig. 4). This result demonstrated that the plants with high expression of OXDC are resistant to wilting induced by oxalic acid. This result could also be correlated with the higher level of expression of the oxalate decarboxylase in SOVA plants. To further show that the OXDC could degrade oxalate *in vivo*, total oxalate content was analyzed in transgenic and control plants by HPLC. SOVA transgenic lines S19 and S20 showed more than 60% decrease in the level of total oxalate in comparison to the control plants, whereas COXA line C14 could show only marginal (15%) decrease as expected (Table I).
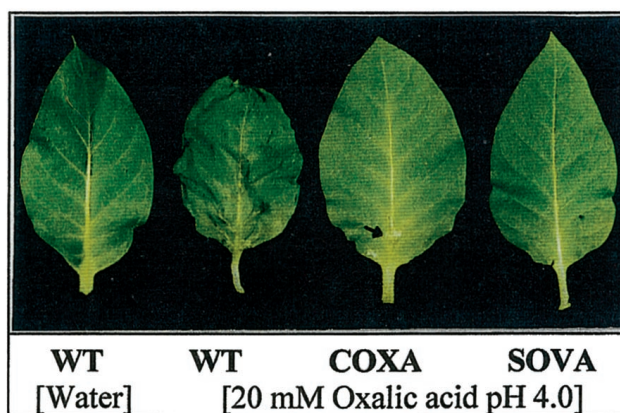
OXDC Expressing Tobacco and Tomato Plants Are Resistant to Fungal Infestation—Production of plants with the ability to degrade oxalic acid and exhibiting low oxalate levels promises to be a good model system to study pathogenesis. Since tobacco and tomato are natural hosts of *S. sclerotiorum* (11), we tested the ability of the fungus to infect leaves from control and transgenic plants. Five plants each from COXA and SOVA transgenic tobacco and two from SOVA transgenic tomato were selected for this assay based on different copy number and levels of expression. Leaves were considered as diseased when symptoms of water soaking, browning of tissue, and lesion formation appeared. The agar plug containing *S. sclerotiorum* was applied randomly near the center of the detached leaves. Controls showed typical water soaking and rotting surrounded by chlorotic halo. Both tobacco and tomato transgenic leaves show remarkable resistance to the pathogen infection (Fig. 5 and Fig. 6*A*) in comparison to the wild type leaves. No significant difference was observed between COXA and SOVA tobacco plants. The level of resistance was more pronounced in tobacco transgenics than tomato. Fig. 6*B* shows the disease development in transgenic tomato plants. As apparent, the disease spread is faster and more pronounced in control plants than transgenics, which is statistically significant. To test for the stability of the transferred trait in the next generation, T_1 seeds of tobacco were germinated on kanamycin and were tested for expression of the enzyme by Western blot. All plants showed expression of the enzyme. These T_1 plants were also tested for fungal infection and were found resistant (data not shown). These results show that OXDC expression is stable and is functionally inherited at least to the next generation.

It was of interest to study the mechanism involved in the

TABLE I
Level of oxalate in leaves of transgenic tobacco lines

Data are mean of six samples and duplicate measurements for each sample. C, tobacco plants transformed with pCOXA; S, tobacco plants transformed with pSOVA; and WT, non-transformed tobacco plant.

Plant sample	Oxalate content	Decrease
	g / 100g	*%*
WT	1.132 ± 0.120	
C14	0.954 ± 0.066	15.70
S19	0.430 ± 0.075	62.00
S20	0.440 ± 0.037	61.10
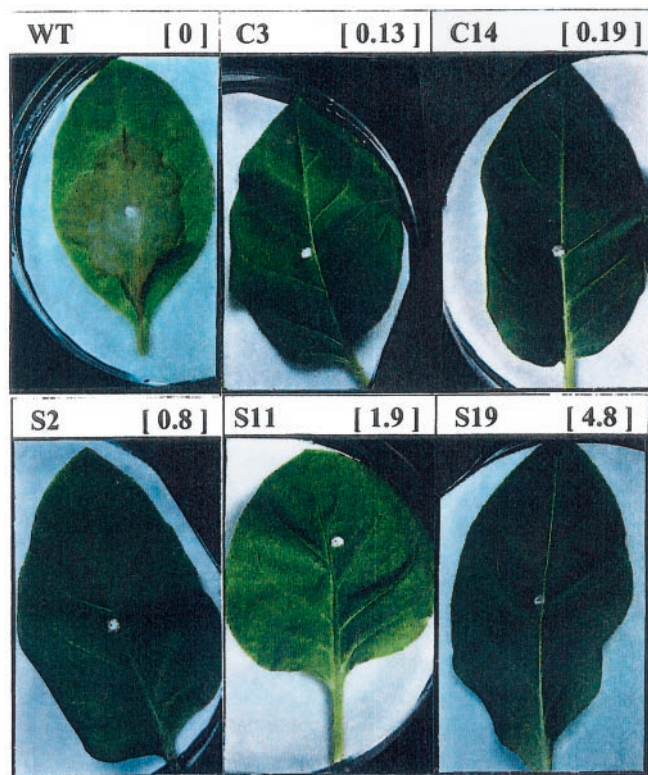
FIG. 5. **Pathogenesis assay of transgenic plants.** Transgenic tobacco plants expressing OXDC show resistance to infestation by *S. sclerotiorum*. Disease symptoms after 6 days. One leaf from each T_0 plant is presented; the name of transgenic plant and specific activity (units/mg) of OXDC is given *above* each leaf at *left* and *right side,* respectively. *C* denotes leaves from COXA transgenic plants, and *S* for leaves from SOVA transgenic plants. *WT*, leaf from wild type plant.

FIG. 6. *A, OXDC* overexpression in transgenic tomato plants. *OXDC* overexpression in transgenic tomato plants leads to resistance to infestation by *S. sclerotiorum*. The *left* and *right panels* showing the lesion formation after 2 and 5 days of inoculation, respectively. S4 and S6 independent transgenic tomato plants transformed with pSOVA and express OXDC. Control, non-transformed, or plants carrying the same cassette do not express OXDC. The diseased leaves show growth of the fungi, lesion formation, and yellowing. *B,* time kinetics of disease development in tomato as measured by size of the lesion. *t* test showed significant difference between control and transgenic plants. Data points represent the mean of lesion sizes from five leaves.

resistance; and thus, we tested the level of pathogenesis-related gene expression in tobacco transgenics as well as control plants. The leaves were treated with either oxalic acid or water. Treatment of leaves of transgenic plants with water showed no expression of in C3 and S20 lines, whereas expression was observed in S2 (data not shown). A weak expression of PR-3 and basic chitinase was also observed in other plants. This result suggested that the transgenic plants do not have significantly elevated level of PR proteins except in S2. However, expression of all the tested PR proteins was induced in transgenic plants after treatment with oxalic acid (data not shown). No induction was observed in the WT plants except a faint band of PR-1.

DISCUSSION

Oxalic acid is present as nutritional stress in several nutritionally important plants, and it is also suggested to be involved in the pathogenesis of phytopathogenic fungi. We asked the question in the present study whether overexpression of oxalate-catabolizing enzyme, oxalate decarboxylase, in plants can remove the nutritional stress and also make plant resistant to fungal infection. Toward this we have isolated and characterized the oxalate decarboxylase gene, and we studied its expression in heterologous systems. Cloning of the partially cDNA was reported earlier from our laboratory (18). We obtained full-length cDNA using 5′-RACE-PCR. By using the complete cDNA, genomic clone was isolated from genomic library of *C. velutipes*. Sequence analysis of the genomic clone showed that it has all the structural features of fungal genes with some unique features. The OXDC is unique in containing 17 introns in the span of 2.4 kb as the maximum number reported in fungi is 12 from *Tranetes vesicolor* (34). Moreover, two exons of OXDC are of 18 and 21 nt in length; it is interesting to note that the smallest exon reported so far is from rat troponin gene which is unique in containing 7 out of 15 exons that are shorter than 20 nt. Significance of such large number of small introns is not well understood. A correlation has been

inferred between the density of intron and developmental complexity by phylogenetic analysis (35). Cloning of more genes from this organism will probably give some insight into the phylogenetic status of *C. velutipes*. Homology search of predicted amino acid sequence shows 48 and 29% homology with *Bacillus* and *Synechocystis* hypothetical proteins, respectively. The multiple alignment of these sequences revealed 3 domains of strong amino acid identity. Moreover, the amino acid sequence also shows a weaker homology with germin-like proteins from plants, which is confined to C terminus of the OXDC protein. Multiple alignment of OXDC with germins also shows a similar domain of amino acid identity from amino acids to amino acids. Since the germin is an oxalate oxidase (36), the homologous stretch may indicate the oxalate recognition/catalytic domain.

We expressed the OXDC in *E. coli,* but the enzyme was functionally inactive (data not shown). The results suggested that prokaryotic system is not conducive for the correct folding of the enzyme. However, failure of the LacZ-OXDC fusion product to show OXDC activity may also suggest that glycosylation may be important for its activity. Genes of agronomic importance such as those that confer resistance to pathogens and improve nutritional quality have been isolated from plants and other organisms. The efficacy of theses genes in improving the agronomic performance of plant is usually tested in transgenic tobacco. In this studies, we have introduced *OXDC* gene in transgenic tobacco plants. A large number of independent transformants have been obtained that are fertile. The transgenic plants express high levels of functional enzyme. On immunoblot analysis, more than one band was obtained, and the smallest band corresponds to the deglycosylated form of native OXDC from *C. velutipes*. Hence, the multiple bands could be due to partial and differential glycosylation of the enzyme in plant. This was further confirmed by digestion of the crude protein with endo-β-N-acetylglucosaminidase H, which resulted in a single product of 54 kDa, the de-glycosylated form of OXDC (data not shown). The recombinant enzyme showed no change with respect to the stability and pH optimum, whereas it showed higher K_m (5.0 mM) values as compared with native OXDC (4.5 mM) which could be due to the presence of interfering proteins in crude protein extracts. The level of enzyme in the SOVA plants was found to be higher than COXA which can be correlated to the level of transcript in these plants. It could be due to the fusion of tobacco chitinase secretion signal sequence at the N terminus of OXDC, which may result in higher stability of the transcript than COXA, which contains the fungal sequence at the 5′ end. Electron microscopy confirmed that the enzyme was successfully targeted to vacuoles, and therefore stabilization of the enzyme in vacuole cannot be ruled out. Although the tobacco chitinase targeting sequence has been shown to target completely secretory protein to vacuole in the heterologous system, the presence of the OXDC partly in cytosol could be due to overloading of the secretion pathway. Wilting assay strongly suggested that the enzyme is active *in vivo,* and the transgenic plants can resist the wilting caused by oxalic acid. The SOVA plant leaves were found more resistant to wilting than the COXA plant which possibly could be due lower level of expression of the enzyme in COXA plant. Results from the HPLC analysis of oxalate content suggested that most of the oxalate is degraded in plants where the enzyme was targeted to vacuole. The remaining amount of oxalate may be required by the plant as oxalate may play some role in cation balance (36). These results strongly suggested that transfer of *OXDC* gene to plants with high oxalate content will significantly reduce the nutritional stress.

The role of oxalate during the infection process is not well understood. Our preliminary bioassay with the detached leaves showed that the transgenic plants are completely protected from the infection by *Sclerotinia*. This result suggested that the plants with the ability to degrade oxalic acid can resist the infection and established that oxalate is an important determinant of pathogenesis. To understand the mechanism involved in resistance, we studied the levels of different classes of the pathogenesis-related (PR) genes, as PR gene expression is a marker of the defense activation during the systemic acquired resistance, which is induced by salicylic acid. These proteins have been shown to have antimicrobial activity *in vitro*. Some of these PR proteins can individually impart resistance to fungal pathogen in transgenic plants (38), and it has been demonstrated that the constitutive expression of chitinase and glucanase genes together in transgenic tobacco plants confers higher levels of resistance to fungus *Rhizoctonia solani* than either gene alone. (39, 40). Induction of PR genes by generation of H_2O_2 in transgenic potato plants expressing glucose oxidase have been found to be resistant to *Phytopthora infestans* (41). Our results showed that oxalic acid by itself can induce the expression of PR-1 and PR-P, Q proteins in wild type (data not shown), whereas the expression of all the PR proteins tested can be induced 2–10-fold in transgenic plants after treatment with oxalic acid. Induction of PR gene expression by oxalate in transgenic plants that degrade oxalate to formic acid and CO_2 is interesting as neither of these compounds have been reported earlier as signal for defense gene induction. Further studies with endogenous salicylic acid level in these plants will also suggest the pathway involved in the activation.

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Additions and Corrections

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DECAY, a novel *Drosophila* caspase related to mammalian caspase-3 and caspase-7.

Loretta Dorstyn, Stuart H. Read, Leonie M. Quinn, Helena Richardson, and Sharad Kumar

The cDNA clone LP3492 from Berkeley Genome Project that was used to derive the *decay* sequence reported in this paper contained a possible cloning artifact leading to the deletion of 2 bp in the 5′ region. In the correct DECAY protein sequence (GenBank™ accession no. AF130469), the first 5 amino acids (MPPRS) reported in our paper are replaced by 26 residues (MDDTDFSLFGQKNKHKKDKADATKIA). Because the entire caspase domain of the DECAY protein lies carboxyl to the amended sequence, and because the molecule used in our study is both biologically and biochemically functional, we believe that the different amino terminal sequence is unlikely to have affected the results and central conclusions reported in the paper.

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Oxalate decarboxylase from *Collybia velutipes*: molecular cloning and its overexpression to confer resistance to fungal infection in transgenic tobacco and tomato.

Meenu Kesarwani, Mohammad Azam, K. Natarajan, Anuradha Mehta, and Asis Datta

The name of Dr. Autar K. Mattoo was omitted from the acknowledgment. The corrected acknowledgment is shown below:

Acknowledgment—We thank Dr. J. M. Neuhaus (Germany) for providing the constructs pTUCH10 and pTRCH4 and Dr. Autar K. Mattoo (United States Department of Agriculture) for valuable suggestions on standardizing tomato transformation based on work in his laboratory.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.