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# Diverted Secondary Metabolism and Improved Resistance to European Corn Borer (*Ostrinia nubilalis*) in Maize (*Zea mays* L.) Transformed with Wheat Oxalate Oxidase

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An alteration in the secondary metabolism of maize (*Zea mays* L.) genetically modified with the wheat oxalate oxidase (*OxO*) gene was observed using HPLC and fluorescence microscopy. Phenolic concentrations in the *OxO* lines were significantly increased, but DIMBOA synthesis was reduced due to a diversion in the shikimate pathway leading to phenolic and hydroxamic acids. Ferulic acid exhibited the largest increase and accounted for 80.4% of the total soluble phenolics. Transcription of a 13-lipoxygenase gene, coding for a key enzyme involved in the regulation of secondary metabolism, was substantially higher in the *OxO* line than in the null line. To test whether the high levels of soluble phenolic acids, in particular ferulic acid, contributed to the insect resistance in the *OxO* maize, ferulic acid was administered in meridic diets to European corn borer (ECB). A significant negative correlation between ferulic acid concentration and ECB larval growth rate was found. Field testing during 2001 showed that *OxO* maize was more resistant to ECB, with leaf consumption and stalk-tunneling damage significantly reduced by 28−34 and 37−39%, respectively, on all of the *OxO* lines tested and confirming published 2000 findings.

**KEYWORDS: Maize; oxalate oxidase; European corn borer; genetic transformation; phenolics; secondary compounds**

## INTRODUCTION

European corn borer (ECB) (*Ostrinia nubilalis* H.; Lepidoptera: Pyrallidae) is a major insect pest in maize (*Zea mays* L.) production. The damage caused by ECB is not only from its direct feeding but also as a result of secondary invasions of other pathogens, such as *Fusarium* stalk-rot fungi, through ECB wound sites. The economic losses to maize production can reach 30% in certain areas (*1*).

Biotechnology provides opportunities to improve host plant resistance (HPR) to insects by introduction and regulation of specific genes. In our previous paper, a cereal gene, wheat *oxalate oxidase* (*OxO*), was introduced into maize lines to investigate effects of enhanced resistance to European corn borer herbivory (*2*). The wheat *OxO* gene (M63223), isolated from the wheat genomic clone gf-2.8, codes for an enzyme (EC 1.2.3.4) that catalyzes the oxidative conversion of oxalate into hydrogen peroxide ($H_2O_2$) and $CO_2$ (*3*). We demonstrated that in corn inbred lines transformed with the *OxO* gene under the control of a constitutive promoter, the steady-state $H_2O_2$ levels in leaf tissue were increased 2−3-fold, and these lines exhibited significantly improved resistance to insect feeding in laboratory and field evaluations (*2*). However, the resistance mechanism was unknown.

There is compelling evidence that $H_2O_2$ is involved in plant defense responses (*4*). First of all, $H_2O_2$ is a central signaling molecule that coordinates various defense responses, such as stress and wounding responses and pathogen defense, and regulates the cell cycle and cell death (*5*). Additionally, $H_2O_2$ may play a role in strengthening the plant cell wall, and a high level of $H_2O_2$ is toxic to pathogens (*6*) and insects (*2*). The roles of oxidative stress in disease (*7*, *8*), in heavy metals (*9*), in salts (*10*), and in drought (*11*) have received considerable attention within the past decade. In contrast, the oxidative responses in plants to insect herbivore feeding are only recently receiving attention (*12*−*14*).

A very early response in plants to insect attack is the transient production of reactive oxygen species (ROS) including $H_2O_2$

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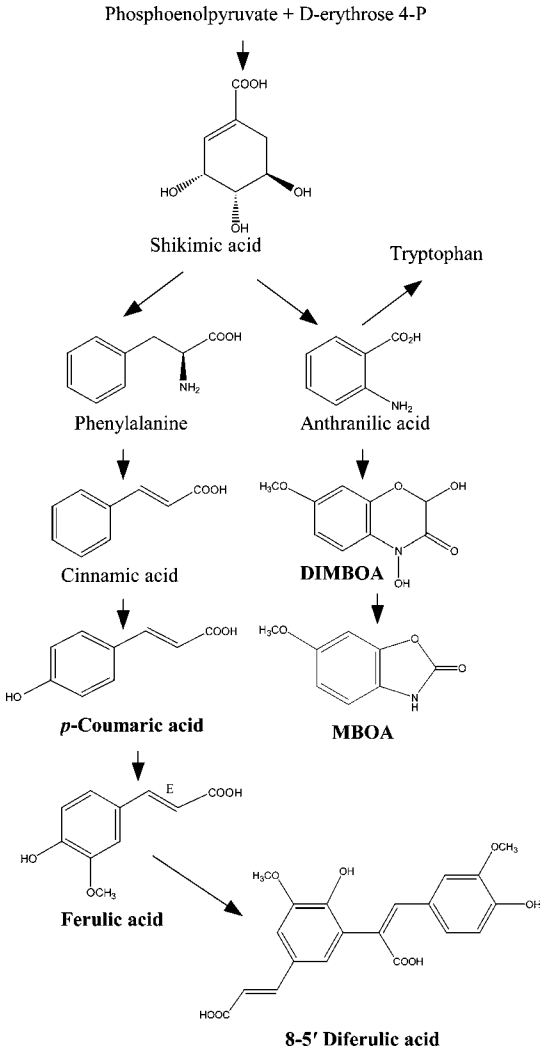


**Figure 1.** Shikimate biosynthetic pathway of phenolic acids in maize and the intermediates point with the synthesis of DIMBOA [based on Desai et al. (*21*), Frey et al. (*22*), and Rad et al. (*23*)].

(*4*). Hydrogen peroxide generated in response to wounding can be detected at the wound site and in distal leaves within an hour of wounding (*15*). Activation of defense responses results in the accumulation of diverse secondary metabolites, which interfere with herbivore feeding, growth, fecundity, and fertility. An important signal compound for activating the secondary chemical defense response pathway in plants is jasmonic acid (JA) (*16*). Following wounding, JA accumulation is initiated via the octadecanoic acid pathway (*4*). The enzyme 13-lipoxygenase (13-*LOX*) (EC 1.13.11.12) is a key marker for this pathway (*17*, *18*). It has also been demonstrated that $H_2O_2$ is involved in lipid peroxide, salicylic acid (*19*), and ethylene (*20*) synthesis.

It is well documented that one of the major insect defenses in corn plants, hydroxamic acid [2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA)], and related compounds share intermediates in the shikimate pathway with the synthesis of phenolic acids, another major type of defense compound in corn (**Figure 1**) (*21*−*23*). We hypothesized that the increased $H_2O_2$-induced transcriptional activation may ultimately lead to the alteration of secondary metabolism in the biosynthetic pathway of phenolic acids and DIMBOA. The defense roles of DIMBOA and phenolic acids in corn plants have been reported in ECB (*24*−*26*), southwestern corn borer and sugarcane borer (*2*), maize weevil, *Sitophilus zeamais* (*27*, *28*), and maize stem borer

**Table 1.** Maize Transgenic Lines Tested in the Present Study in Insect Herbivory and Leaf Phenolic Assessments (Data for Leaf *OxO* Activity of CK44, CO286, and CO328 Previously Reported in Reference *2*)

| maize line | event (copy no.) | *OxO* zygosity | leaf *OxO* activity[a] (U × $10^{-4}$/mg of FW) |
|---|---|---|---|
| CK44 T8 (BC6S2) | 1E (3) | ++ | 5.7 ± 0.2 |
| CK44 null | | −− | 0 |
| CO286 T7 (BC6S1) | 1E (3) | +− | 1.8 ± 0.1 |
| CO286 null | | −− | 0 |
| CO328 T7 (BC6S1) | 1H (3) | +− | 3.9 ± 0.3 |
| CO328 null | | −− | 0 |
| B73 T4 (BC1S3) | 9A (2) | ++ | 2.9 ± 0.5 |
| B73 null | | −− | 0 |

[a] One unit (U) of *OxO* activity forms 1 nmol of $H_2O_2$/min from oxalate at pH 3 at 37 °C.

*Sesamia nonagrioides* (Lefebvre) (*29*). In maize leaves, ferulic acid (FA) and coumaric acid (*p*-CA) are the most prevalent phenolic acids (*28*−*30*). The dimer diferulic acid (DFA), present in at least four isomers (DFA 5′-5′, DFA 8′-5′, DFA 8-0-4, and DFA 8′-5′ benzofuran), is considered to be a key phytochemical indicator in plant resistance (*25*).

We report here altered profiles of hydroxamic acids and phenolic acids in leaves of *OxO* transgenic lines. Feeding bioassays with the major phenolic acid up-regulated in *OxO* maize provide direct evidence of involvement in *OxO* resistance. We also predicted changes in the octadecanoic acid signaling pathway and monitored the response of 13-*LOX* as an indicator in *OxO* and null lines. A final step was to demonstrate that *OxO* transformation consistently reduced insect damage to field-grown plants in two years using two transformation events.

## MATERIALS AND METHODS

**Plant Materials.** Wheat germin OXO gene isolated from genomic clone gf-2.8 in pGermin1 (*3*) was a 0.9 kb Sph1 fragment. It was blunt-ended and ligated into the Sma1 site of pCOR113 (*31*). With *Hin*dIII, approximately 200 bp of germin 5′-UTR was removed, and then this construct was religated to create pCOR113-ger. The nos′ 3 region was obtained from pAct1-D as a Sac1/Xba1 fragment, which was inserted in pCOR113-ger to create vector pAct-OXO. With the pAct-*OxO*, maize *OxO* transgenics were generated by particle bombardment of A188 × B73 maize embryogenic cultures. The primary lines with simple patterns of transgene insertion were outcrossed to four maize inbred genotypes (CK44, B73, CO286, and CO328). Lines maintaining stable *OxO* expression over several generations were selfed to obtain transgene homozygous, hemizygous, and null lines (*2*). Phenolic acids and hydroxamic acids were quantified by HPLC for the two independent transgenic events 1E and 9A in two inbred lines, CK44 and B73. The 2000 and 2001 insect field trials were done with event 1E in T7 CK44 progeny and T6 CO286 and event 1H in CO328 progeny (**Table 1**). For in vitro resistance assays and analyses of phenolics, maize plants were grown in a controlled chamber at 25 ± 1 °C with a 16:8 h (light/dark) photoperiod and light intensity of 300 $\mu$Einstein $m^{-2}$ $s^{-1}$ from cool white fluorescent tubes. Each transformed line and null control was arranged randomly in a growth chamber (20 plants per line). At stage V7 (the seventh leaf emerged), the middle parts of the sixth leaves were collected, wrapped in aluminum foil, and stored at −80 °C. For fluorescence microscopy tests, sample leaves were freshly prepared.

**Soluble Phenolic Quantification by HPLC Analysis.** The extraction method of soluble phenolics was adapted from that of Weidner (*32*) and Bily (*33*) with modifications. Frozen maize leaves were collected in 50 mL flasks with 5 g per flask and 5 replicates per test. To extract soluble phenolic compounds, 30 mL of 80% methanol was added to each flask. The leaf samples soaked in methanol were homogenized with a Polytron mixer (Brinkman, Westbury, NY), shaken in darkness for 1 h at 220 rpm, and then centrifuged for 10 min at

1000*g*. The supernatants were collected, and the pellets were extracted twice more using the same method. Pooled supernatants were evaporated to 30 mL in a Savant SpeedVac (Bio101, Vista, CA) at 45 °C to remove most of the methanol and thus facilitate subsequent liquid−liquid extraction. The solutions were acidified to pH 2 using 2 N HCl and filtered through Whatman no. 1 paper. Free phenolic compounds were extracted into an equal volume (30 mL) of ethyl acetate (EtOAc). The aqueous fractions were divided into two equal parts (10 mL per part) to extract ester- and ether-conjugated phenolics. One part was treated with 10 mL of 2 N NaOH and digested under a nitrogen atmosphere in darkness for 3 h to hydrolyze the esterified phenolic acids; pH was then adjusted to 2 with 2 N HCl. The other part was combined with 10 mL of 2 N HCl and incubated at 4 °C for 1 h to release ether-conjugated phenolic acids; the pH was then adjusted to 2 with 1 N NaOH. EtOAc (20 mL) was added to each of the two parts to extract the freed phenolic acids. The above three fractions were evaporated under vacuum. All dried samples were dissolved in 3 mL of methanol and stored at −18 °C until HPLC analysis.

HPLC analysis followed the method of Bily (*33*). A Hewlett-Packard (Agilent, Palo Alto, CA) ChemStation series1100 LC with a diode array detector (DAD) was used. Separations were obtained with a Waters YMC ODS-AM narrow-bore column (100 × 2 mm i.d.; 3 μm particle size). The mobile phase system consisted of acetonitrile (solvent A) and 0.05% aqueous trifluoroacetic acid buffer (pH 2.4) (solvent B). The gradient was as follows: initial conditions 10:90 (A/B), 30:70 in 3.5 min, 32:68 in 6.5 min, 100:0 in 4 min, hold at 100:0 for 4.5 min, and then returned to the initial conditions 10:90 (A/B) by 3 min. The elution profiles were monitored at 325 and 280 nm. Standard compounds *p*-CA and FA were purchased (Sigma, St. Louis, MO), whereas 5-5 DFA was synthesized by G. H. N. Towers Laboratory (University of British Columbia, Vancouver, Canada). Sample extracts and different concentrations of standards were filtered through a 0.22 μm PTFE membrane (Chromatographic Specialties, Brockville, ON) and injected into the HPLC column. The mobile phase rate was 0.3 mL/min, and the total analysis was completed in 21.5 min. The peak identity of each phenolic was confirmed by retention time and UV spectrum comparisons with the commercial standard solutions and known spectra.

**DIMBOA Quantification by HPLC Analysis.** The method for extraction of DIMBOA was from Melanson (*34*). Sample leaves stored at −80 °C were removed and immediately treated with liquid nitrogen. The frozen leaves were ground to a fine powder, and 1.0 g was put into each of five flasks. Once the ground leaves had warmed to room temperature, 5 mL of water was added and then incubated at room temperature for 1 h to allow the aglycones to be released. After centrifugation, the supernatant was collected and extracted four times using an equal volume of EtOAc. Pooled extracts were evaporated under vacuum with a Savant SpeedVac (Bio101, Vista, CA). The solid residue was dissolved in 1.0 mL of MeOH for HPLC analysis. Analyses were performed using a Hewlett-Packard ChemStation series 1100 HPLC equipped with a Waters YMC ODS-AM narrow-bore column (100 × 2 mm i.d.; 3 μm particle size). The mobile phase system of methanol (solvent A) and trifluoroacetic acid (0.05%) in water (pH 2.4; solvent B) was used as follows: initial conditions 25:75 (A/B), changing to 40:60 in 10 min, and then to 100:0 in 5 min; 100% methanol is held for 2 min and then returned to initial conditions after 3 min. The column was allowed to re-equilibrate for 5 min. The mobile phase flow rate was 0.2 mL/min, and the total analysis time was 25 min. The sample injection volume was 4 μL, and the elution profiles were monitored at 210 and 265 nm. DIMBOA eluted at 4.25 min and MBOA at 7.25 min. Peak identities were confirmed by matching retention time and absorbance spectra with purified standards.

**Specimen Preparation for Fluorescence Microscopy.** Sample leaves were freshly collected from plants grown in a chamber with controlled environment as described above. The middle of the leaves (0.5 cm wide) with midrib veins was isolated and immediately frozen using Tissue-Tek O.C.T. compounds (Miles Inc.) at −20 °C. The tissue was freshly sectioned with a microtome at −20 °C. To locate soluble phenolics, the cryosections (nominally 8 μm) were gently moved to slides and mounted in glycerol. The images were taken at an excitation wavelength of 360 nm (UV) and an exposure of 23 μs, using a 10× objective.

**RNA Hybridization and Northern Blot Analysis.** Northern blot of 13-*LOX* (2.8 kb in length) transcription was conducted in CK 44 *OxO* homozygous and the corresponding null line. The transcript was measured in tissue of leaf 6 isolated from V7 plants (leaf 7 emerging) throughout the 24 h photoperiod. The collected leaf tissues were frozen in liquid nitrogen and ground to powder. Total RNA was isolated using TRIZOL reagent (GibcoBRL, Grand Island, NY). About 15−20 μg of total RNA was separated by 1% formaldehyde agarose gel electrophoresis, and Northern transfers were obtained by blotting RNA onto Magnacharge nylon transfer membrane (Osmonics Inc.). Equal loading of RNA was confirmed by ethidium bromide staining of the gel. Hybridization was performed under high-stringency conditions (0.1× SSC, 68 °C) using a random-primed $^{32}$P-labeled 0.8 kb maize EST fragment (BG320094), with 98% nucleotide similarity to the terminal 3′ region (2131−3016 bp) of a maize 13-lipoxygenase transcript (ZmLox11, AR700671).

**Meridic Diet Test.** A 4-day feeding assay was performed to test the effects of FA on ECB larval growth using the bioassay method presented in Ewete (*35*). Commercially available FA (Sigma) was used. Meridic diet was made from agar, methylparaben solution, propionic−phosphoric acid, formaldehyde, and fumagillin (*36*). On the basis of the total concentration of FA in *OxO* transgenic maize leaves, the experimental treatments were designed as control with vehicle ethanol, FA 30, 100, 300, 1000, and 3000 μg/g of fresh weight of diet (FW). Compounds dissolved in ethanol were incorporated into the diet before the agar was solidified. Ethanol concentration was 0.5% in all diets. The bioassay was conducted under the same environmental conditions as the ECB was maintained. Second-instar larvae reared on the defined culture diet were exposed on each treatment and negative controls, 20 larvae per vial. Each larva was weighed and transferred to individual glass vial (5 dram) provided with an appropriately treated or control diet cube with 20 larvae per treatment and control. The vials were plugged with cotton and kept in a growth chamber at 28/19 °C (day/night), 85% relative humidity (RH), and an 18:6 h (light/dark) photoperiod. On day 4, the larva and diet were weighed before incubation. Two nutritional indices were calculated on a wet weight basis according to the methods from Ewete (*35*):

$$\text{relative growth rate (RGR)} = (\text{final wt} - \text{initial wt})/[(\text{final wt} - \text{initial wt})/2]$$

$$\text{conversion efficiency of ingested food (ECI)} = (\text{larval wt gained}/\text{ingested food wt}) \times 100$$

**Insect Field Trials.** The insect field trial was conducted in 2001 according to the results from our laboratory in 2000 (*2*). Egg masses of a bivoltine strain of the European maize borer were obtained from French Agricultural Research (Lamberton, MN). These egg masses were maintained in a controlled chamber at 80% RH with an 18 (26 °C):6 (19 °C) h photoperiod in the laboratory until the black head stage of development. The test plants were grown at a randomized block design with 15 plants per row, 1 row per replicate, and 3 replicates per test ($n$ = 45 plants for each line). Feeding assay was conducted at midwhorl stage (40 cm in height) of maize plants and at black head stage of ECB eggs. Egg masses were manually placed in the plant whorl using long forceps with 2 egg masses per plant and 25 eggs per mass. Leaf damage was evaluated 8 weeks after infestation following the 1−9 scale method (from no damage to most leaves with long lesions) developed by Guthrie et al. (*37*). Damage extent on stalks was recorded after mature plants were harvested and the stalk was split with a knife. The cumulative lengths of borer tunnels were measured in a 90 cm section of stalk, which was isolated at 45 cm above and below the primary ear node.

**Statistical Analysis.** All of the data were subjected to either analysis of variance (ANOVA) or linear regression analysis using Microsoft Excel-General vsn. 1.71 1997−2000 Analysis-it Software Ltd. (*38*).

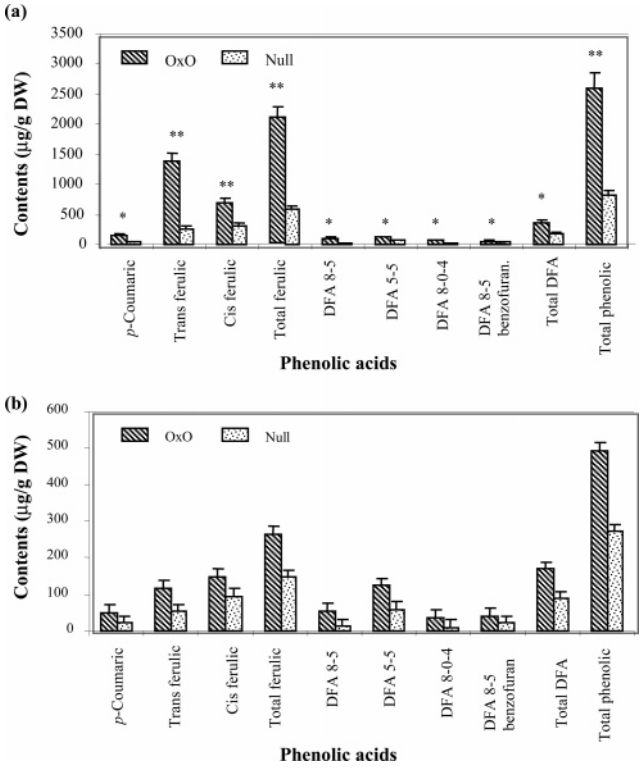


**Figure 2.** HPLC profiles of soluble phenolic acids derived from leaves of CK44 (**a**) and B73 (**b**) *OxO* transgenic lines and the corresponding null lines. Leaf extracts were obtained from leaf 6 of V7 plants. Contents of total FA and DFA were calculated by their isomers. Total phenolic content was the sum of *p*-CA, total FA, and total DFA (FA, ferulic acid; CA, coumaric acid; DFA, diferulic acid; DFA 5′-5′, DFA 8′-5′, DFA 8-0-4, and DFA 8′-5′ benzofuran, four isomers of DFA; DW, dry weight; **, significant at $p < 0.01$; *, significant at $p < 0.05$).

## RESULTS

**Concentrations of Soluble Phenolic Acids in Null and OxO Transformed Maize.** Three phenolic acids, *p*-CA, FA, and DFA, and their various isomers were examined. FA included two isomers, *trans*-FA and *cis*-FA, and DFA included four isomers, DFA 5-5, DFA 8-5, DFA 8-0-4, and DFA 8-5 benzofuran. Three different forms, free, soluble ester-bound,- and soluble glycoside-bound phenolics, were analyzed to determine the differential, respective contributions.

Two independent transformation events and their corresponding null controls were investigated for the analysis of soluble phenolic concentration. One was transgenic event 1E (transgene copy # = 3) in the eighth generation of back-cross and selfing in CK44 (*OxO* activity = $5.7 \times 10^{-4}$ unit/mg of FW). The second was transgenic event 9A (transgene copy # = 2) in the fourth generation of back-cross and selfing in B73 (*OxO* activity = $2.9 \times 10^{-4}$ unit/mg of FW). The two *OxO* lines were designated homozygous for the transgene (+ +) and null for the transgene (− −) (**Table 1**). The two independent events gave similar phytochemcial results (**Figure 2**). CK44 with event 1E had significantly higher levels of both total phenolics and each individual compound than its corresponding null line ($p < 0.01$ or $p < 0.05$) (**Figure 2a**). The HPLC analysis of soluble phenolic contents showed that total phenolic acids in the *OxO* line were 3.2-fold greater than in the null line. FA, *p*-CA, and DFA in the *OxO* line were 3.6, 2.8, and 2.0 times higher than in the corresponding null line, respectively. The highest level was recorded for FA content (2091 μg/g of DW), which accounted for 80.4% of total soluble phenolics. Total DFA was 365 μg/g of DW, whereas a lower level occurred with *p*-CA (144 μg/g). It was obvious that FA was the most prominent intracellular soluble phenolic acid for transgenic *OxO* maize lines. *trans*-FA (1388 μg/g) was 2.0-fold higher than *cis*-FA (703 μg/g). Among four DFA isomers, DFA 5-5 had the highest concentration (120 μg/g), followed by DFA 8-5 (112 μg/g), DFA 8-0-4 (72 μg/g), and DFA 8-5 benzofuran (62 μg/g). Free phenolic acids in CK44 homozygous line accounted for 92.5% of total soluble phenolics. In contrast, the ester conjugate was 6.5%, whereas the ether conjugate was only 1.0% (**Table 2**). B73 with event 9A produced similarly altered profiles in phenolic concentrations between the transgenic line and null line as were noted for event 1A. The levels of total phenolic acids and each individual compound were higher in transgenic lines than in the null lines (**Figure 2b**). Free phenolic acids in the B73 *OxO* homozygous line accounted for 77.1%, whereas ester- and ether-bound phenolics were only 8.5 and 1.0%, respectively (data not shown). Most of the soluble phenolic acids in *OxO* transgenic maize lines were present in free forms.

**Table 2.** Contents of Free, Ester-Bound, and Ether-Bound Phenolics in Leaf Tissue of CK44 *OxO* Homozygous Line (Event 1E) and Percentages in Total Soluble Phenolics

| | free PA | | ester-bound PA | | ether-bound PA | | |
|---|---|---|---|---|---|---|---|
| phenolic[a] | concn (μg/g of DW) | % | concn (μg/g of DW) | % | concn (μg/g of DW) | % | total PA (μg/g of DW) |
| FA | 1969 ± 107 | 94.2 | 106.5 ± 32 | 5.1 | 15.1 ± 5 | 0.7 | 2091 ± 200 |
| DFA | 334.4 ± 3 | 91.6 | 27.3 ± 9 | 7.5 | 2.9 ± 1 | 0.8 | 365.1 ± 56 |
| *p*-CA | 100.8 ± 12 | 69.3 | 37.0 ± 10 | 25.6 | 7.3 ± 7 | 5.1 | 144.5 ± 32 |
| total PA | 2404 ± 154 | 92.5 | 170.7 ± 47 | 6.5 | 25.3 ± 5 | 1.0 | 2600 ± 267 |

[a] PA, phenolic acid; FA, ferulic acid; CA, coumaric acid; DFA, diferulic acid.

The localization and distribution of phenolic compounds photographically recorded directly using a fluorescence microscope further confirmed that the above results in soluble phenolic concentrations (figures not shown). Apparent differences in blue fluorescence were observed between the *OxO* and null lines. The leaf sections from CK44 homozygous line produced a bright blue color. In contrast, less fluorescence was seen from the two leaf sections of the null line. In addition, a fluorescent halo outside the cutting section was observed. This was from compounds that were diffusing out after sectioning. The *OxO* line section gave a very bright halo. The observed difference in fluorescence suggested that the contents of soluble phenolics differed between *OxO* and its null lines. The brighter blue color emitted from *OxO* maize leaf sections suggested that the contents of soluble phenolic compounds in *OxO* maize leaf tissue were much higher than in null line tissue. The microscopy results visually confirmed the conclusions from HPLC.

**Concentrations of DIMBOA and MBOA. The** maize CK44 and B73 transgenic lines were also used for the analysis of DIMBOA and MBOA contents. Significant differences in DIMBOA and MBOA contents were observed between the CK44 transgenic and null lines. Surprisingly, unlike soluble phenolic acids, which were increased in *OxO* lines, the levels of DIMBOA and MBOA were substantially lower in the transgenics (**Figure 3**). DIMBOA and MBOA concentrations of the CK44 line (event1E) were 41.4 and 6.7 μg/g, which were 10.6- and 4.1-fold lower than the null line, respectively. The reduction in DIMBOA concentrations was significant ($p < 0.01$). Event 9A in B73 also significantly reduced ($p < 0.01$) the DIMBOA concentration.

**Effect of *OxO* on 13-*LOX* Transcription.** The transcription of 13-*LOX* was measured throughout the 24 h photoperiod by Northern blot analysis (**Figure 4**). In the null line, *LOX* transcript

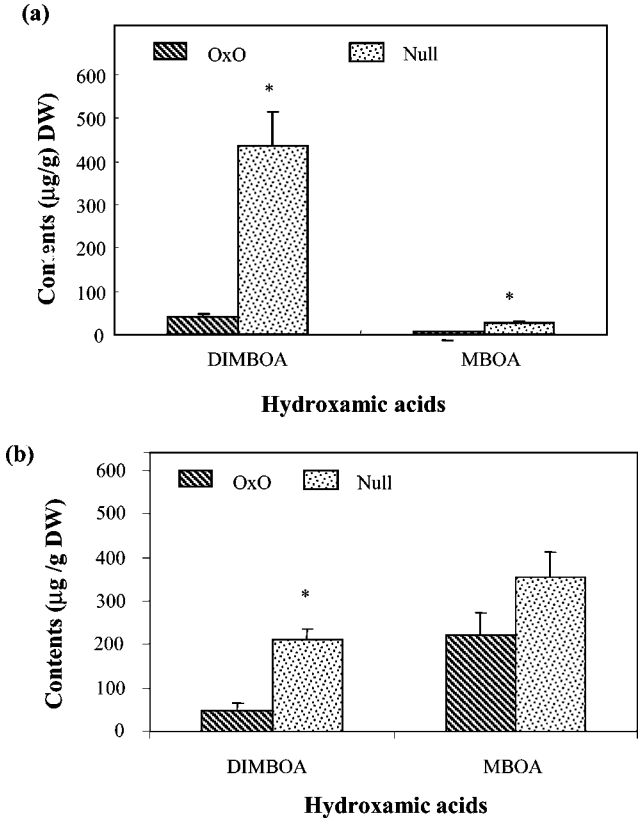


**Figure 3.** HPLC profiles of DIMBOA and MBOA derived from leaves of maize CK44 (event 1E) (**a**) and B73 (event 9A) (**b**) *OxO* homozygous lines and their corresponding null lines. Leaf extracts were obtained from the mid region of leaf 6 of V7 plants (DW, dry weight; **, significant at $p < 0.01$).

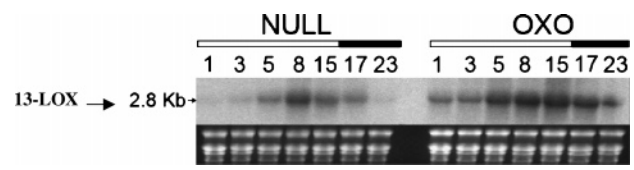


**Figure 4.** Northern blots of 13-lipoxygenase (2.8 kb in length) transcription in CK 44 *OxO* homozygous (event 1E) and the corresponding null line. The transcript was measured throughout the 24 h photoperiod (L/D = 16 h/8 h) using leaf tissue isolated from leaf 6 of V7 plants. Lanes 1, 2, 3, 4, 5, 6, and 7 referred to 1, 3, 5, 8, 15, 17, and 23 h after beginning of lights-on.

accumulated during the first 8 h of light and then diminished at the end of the light phase. By the end of the dark period, *LOX* was not detectable. This observation suggested that the *LOX* accumulation was photoperiod-regulated. In contrast, *LOX* transcript in the *OxO* line was detected throughout the 24 h photoperiod during both light and dark periods. *LOX* transcript in the *OxO* line was substantially higher than that in the null line at all time points investigated.

**Leaf Consumption and Stalk Tunneling of ECB in Field-Grown Plants.** To confirm the insect field trial result completed with *OxO* and null lines in 2000 (*2*), a second field trial was completed in 2001 with event 1E in seventh generation of CK44 *OxO* transgenic progeny and sixth generation of CO286 and CO328 *OxO* transgenic progeny. The three *OxO* lines were designated homozygous for the transgene (++), hemizygous for the transgene (+−), and null for the transgene (− −). The field trials showed significantly lower leaf feeding rating on all of the *OxO* lines tested in 2001 (**Table 3**). The field trial results

**Table 3.** Field Rating and Stalk Tunneling from ECB Infestation in *OxO* Transgenic Maize Lines and Their Null Lines in 2001[a]

| maize line (event/copy no.) | leaf damage rating (1–9) | stalk tunneling (cm) |
|---|---|---|
| CK44 homozygous line1E (3) | 2.2 ± 0.1 d | 13.4 ± 0.9 d |
| CK44 null line | 3.0 ± 0.1 b | 18.3 ± 1.0 c |
| CO286 hemizygous line1E (3) | 2.8 ± 0.2 bc | 21.8 ± 1.6 b |
| CO286 null line | 3.6 ± 0.1 a | 31.4 ± 1.3 a |
| CO328 hemizygous line1H (3) | 2.4 ± 0.1 d | 19.2 ± 1.5 bc |
| CO328 null line | 3.4 ± 0.2 a | 31.1 ± 2.1 a |

[a] Leaf damage rating (1–9) was evaluated 8 weeks (*n*, 45 plants for each line). Damage on stalks was recorded after mature plants were harvested (45 plants for each line). The cumulative lengths of borer tunnels were measured in a 90 cm section of stalk, isolated at 45 cm above and below the primary ear node. Means followed by the same letter within a column are not significantly different (SNK test, $p < 0.05$). Note that data from 2000 is available in ref *2*.

in 2001 consistently showed significant differences between *OxO* and the null lines, although there was slightly less damage observed in both leaf consumption and stalk tunneling in all lines in 2001. The leaf damage, recorded 8 weeks after field infestation, were significantly reduced by 21−27% in the CO328 hemizygous, CO286 hemizygous, and CK44 homozygous compared to the damage in the null lines. Stalk-tunneling damage, measured at plant harvest, was also significantly decreased in all *OxO* transgenic lines. Reductions in stalk tunneling ranged from 27 to 38% among the three *OxO* transgenic lines. This suggested that the presence of the *OxO* gene significantly reduced ECB damage on leaves and stalks in all cases.

**Effects of Ferulic Acid in Meridic Diets on ECB Larvae.** When ECB second-instar larvae were fed the meridic diet containing pure FA, their growth was significantly reduced with increasing FA concentrations. Linear regression analysis showed a negative correlation between FA concentration and ECB relative growth rate (RGR) ($r = -0.5$, $p < 0.001$) (**Figure 5a**). Higher FA concentrations were associated with lower RGR. This trend in artificial diet studies was consistent with the observation that the higher levels of FA in *OxO* homozygous lines were correlated with increased ECB resistance. In addition, the efficiency of conversion of ingested food dropped as the FA concentration was increased (**Figure 5b**).

## DISCUSSION

Transformation of maize with the wheat *OxO* gene regulated by a constitutive rice actin promoter pAct1-D induced oxalate oxidase enzymatic activity and elevated $H_2O_2$ levels in leaf tissue (*2*). In the present study, alteration of secondary metabolism was observed in two biosynthetic pathways, leading to the enhancement of the phenolic acid synthesis pathway, which resulted in a significant increase in all soluble phenolic acid contents, especially free phenolics. Hydroxamic acid biosynthesis in this pathway shares certain intermediates with the phenolic biosynthesis pathway (*21*−*23*). Generally, free phenolic acid content in maize is low in comparison to those phenolic acids that are conjugated through ester linkages to sugars (*24*). However, in the *OxO* maize, free phenolics accounted for 92.5% of soluble phenolics, which was 14-fold higher than the ester-bound products. In particular, free ferulic acid was 18-fold higher than its esterified coupling products. The result suggested that high contents of free phenolics were the most significant change.

The presence of the wheat *OxO* gene significantly reduced ECB damage to leaves and stalk in all genotypes and years.

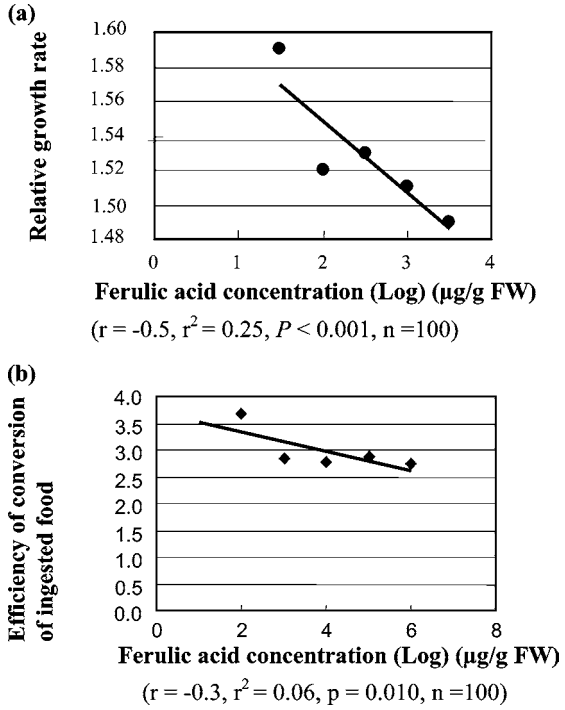


**Figure 5.** Linear regression analysis of effects of FA-supplemented diet on larval growth of ECB: (**a**) relative growth rate (RGR); (**b**) efficiency of conversion of ingested food (FA, ferulic acid).

Clearly, the increase in soluble phenolic contents negatively corresponded with lower insect damage. To test if this was a direct toxicological relationship, the bioassay of ECB growth as a function of dietary FA content showed that ECB larva growth was reduced as the FA concentration was increased. The experiment demonstrated the sensitivity of ECB to FA, but significant effects were found at higher concentrations than that measured in *OxO* maize. The HPLC analysis of *OxO* maize was a crude total leaf measurement, whereas fluorescent microscope observations showed that most of the free FA was located in the epidermis and parenchyma, the preferred feeding substrate for the larvae. Contents in the tissues where the insects were feeding may be much higher than the global HPLC data suggested. Because of the increased toughness associated with higher DFA levels, the resistance engendered by *OxO* transformation appears to be based more on mechanical resistance than current commercial varieties transformed with the synthetic *cry1Ab* gene, which rely on a toxic mechanism. How this may influence the evolution of adaptation in pest insect species such as the European corn borer depends on a number of factors. Less toxic defenses may slow the onset of insect adaptation, especially if inheritance is recessive (*39*).

DIMBOA is known as a defensive phytochemical, and its role in resistance to the ECB has been well demonstrated (*23*, *40*−*43*). In plant tissue, DIMBOA is stored in the vacuole as an inactive glycoside (*44*). Following wounding from insect attack, a glycosidase is released, which removes the sugar from the DIMBOA glycoside to yield the toxic aglycone DIMBOA. Subsequently, its degradation product, 6-methoxybenzoxazolin-3-one (MBOA), is produced spontaneously without enzyme involvement. In maize, enzymatic release of DIMBOA is completed in 30 min after insect attack (*23*), and from previous studies in our laboratory, the release of the aglycones is completed after 1 h of incubation of freshly ground leaf samples in water at room temperature. Conversely to the phenolics, the biosynthesis of the hydroxamic acid DIMBOA was reduced, and the DIMBOA level dropped, possibly due to diversion of metabolism to the phenolics. The obviously reduced DIMBOA level in *OxO* maize suggested that hydroxamic acids DIMBOA and MBOA were not the phytochemical defense factors in the *OxO* maize lines and that another novel defense mechanism is operating.

Northern blot analysis of 13-*LOX* showed that transcription was significantly up-regulated in *OxO* maize compared to null lines. Plant lipoxygenases are considered to be involved in the biosynthesis of lipid-derived signaling molecules, which elevate octadecanoic acid pathway activity and jasmonic acid (JA) synthesis (*45*). JA is a powerful inducer of proteinase inhibitors (*46*) and enzymes of secondary metabolism (*47*). It is significant that in plants with antisense reduction of *LOX* transcription, wound-induced accumulation of JA was absent (*48*) and antisense-reduced *LOX* expression increased herbivore performance by decreasing defense response gene activity (*18*). These studies clearly indicate the significance of the *OxO*-induced *LOX* activity in the defense response of maize. In the case of *OxO* maize, phenolic acid metabolism is clearly up-regulated. Because phenolic acids and hydroxamic acids share precursors up to shikimic acid, we suggest the enzymes up-regulated are those following shikimate in the phenolic acid pathway. A similar example of metabolic alteration of secondary metabolism was obtained in transformed *Brassica napus* cv. Westar with the tryptophan decarboxylase (TDC) gene (*49*). In this case, diversion of metabolism to tryptamine reduced indole glucosinolate accumulation.

A highly relevant recent paper by Lou and Baldwin (*14*) examined the use of antisense RNA technology to silence the endogenous *OxO* gene in *Nicotiana attenuate*. They found that defensive responses such as diterpene glycosides and proteinase inhibitors were reduced and that two native herbivorous insect larvae performed significantly better on the transformed foliage as compared to controls. These results are clear confirmation of the role of *OxO* in establishing insect defensive responses in another plant.

The hyperacidification of insect gut from high contents of free phenolic acids is a direct way for secondary compounds to discourage insects from consuming tissue because ECB maintains a basic gut for optimal protease activity (*42*). Alternatively, $H_2O_2$ produced by *OxO* has direct effects on ECB growth (*2*), and the combination of high FA and $H_2O_2$ may be significant but has not yet been tested.

The alteration of metabolic pathways that was observed in this study was not entirely predictable. The present study newly focuses our attention on the level of metabolic precursors and expression of enzymes in the biosynthesis of hydroxamic and hydroxycinnamic acids in the shikimic acid pathway. This will help us to understand the metabolic networks that regulate the shikimic acid pathway and, more importantly, the redirection of the flow of the key defense metabolites in genetic engineering.

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