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Full Length Research Paper

Unintended effects were investigated in antioxidant activity between genetically modified organisms and their nontransgenic control

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Other than the targeted approach on compositional analysis, non-targeted approaches on genomics, proteomics and metabolomics are developing to search for unintended effects with respect to genetically modified (GM) food safety assessments. Antioxidant activity system was closely related with plant growth and reproduction as well as human health. This study was to investigate some other potential unintended effects from a range of primary and secondary metabolites by comparison of antioxidant activity system between six pairs of GMOs and their nontransgenic control. Antioxidant activity system was explored in total phenolics, unsaturated fatty acids and oxido-reductase activity analysis (including peroxidase (POD), polyphenol oxidase (PPO), catalase activity (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR)). The results from oxido-reductase activity analysis indicated significant differences ($P < 0.05$) between GMOs and their nontransgenic control, except for a few enzymatic activities of several GM crops. The data of total phenolics and unsaturated fatty acids also showed significant differences ($P < 0.05$) between GMOs and their nontransgenic control. However, no obvious differences occurred among all tested maize samples or canola samples.

Key words: Unintended effects, antioxidant activity, genetically modified organisms, maize, canola.

INTRODUCTION

Concerns have been raised that the current approach of using targeted analyses (OECD, 1993; FAO/WHO, 1991, 2000) to compare the composition of genetically modified (GM) crops to their traditional nontransgenic control is biased (Millstone et al., 1999) and does not take into account the possibility of other unintended effects that could result directly or indirectly from the genetic modification. The potential occurrence of such "unintended effects" is currently one of the concerns being raised regarding the application of recombinant DNA techniques in the production of foods.

"Unintended effects" represent a statistically significant difference in the phenotype, response or composition of the GM plant compared with the parent from whom it is derived, but taking the expected effect of the target gene into account. Such comparisons should be made when GM and non-GM control are grown under the same regimes and environments. Whether the unintended effects are predictable or unpredictable, they may or may not prove to have relevance in terms of product safety, but should be taken into account when assessing safety.

Many unintended effects has been testified, such as adverse tuber tissue perturbations of potato (Turk and

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Abbreviations: GM, Genetically modified; **GMOs,** genetically modified organisms; **POD,** peroxidase; **PPO,** polyphenol oxidase; **CAT,** catalase; **SOD,** superoxide dismutase; **APX,** ascorbate peroxidase; **GR,** glutathione reductase

Smeekens, 1999), multiple metabolic changes (tocopherol, chlorophyll, fatty acids, phytoene) of canola (Shewmaker et al., 1999) and formation of unintended carotenoid derivatives (β-carotene, lutein, zeaxanthin) of "golden rice" (Ye et al., 2000). More unintended effects were explained in functional genomics (GMOCARE, 2003; Cheng et al., 2008), proteomics (Kubo, 2000; Zolla et al., 2008) and metabolomics (Shewmaker et al., 1999; Bovy et al., 2002; Le Gall et al., 2003a).

Antioxidant activity system was closely related with crop health. Reactive oxidative species (ROS), closely related to primary or secondary metabolic materials, are the primary mediators of oxidative damage in plants and mainly include superoxide radicals ($O_2^{\cdot-}$), H_2O_2 and hydroxyl radicals (•OH). These ROS can rapidly attack all types of biomolecules to cause membrane deterioration, lipid peroxidation and DNA mutation, leading to irreparable metabolic and structural dysfunctions and cell death. Even under optimal conditions, many metabolic processes produce ROS, which increases as a result of several abiotic or biotic stress conditions (Jimenez et al., 2002). The level of ROS *in vivo* depends upon the balance between their generation and the capacity to remove them. Major ROS-scavenging mechanisms of plants include superoxide reductase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR). The balance between SOD, APX, GR and CAT activities in cells is crucial for determining the steady-state level of superoxide radicals and hydrogen-peroxide (Sala and Lafuente, 2004). The oxidation process involves the degradation of polyunsaturated fatty acids (PUFA), vitamins and other tissue components and the generation of free radicals, which lead to the development of rancid odours and changes in colour and texture in food stuffs (Kanner, 1994).

In this study, many typical GMOs, including maize and canola were studied. Based on the materials, unintended effects were investigated in antioxidant activity system between GMOs and their nontransgenic control in the aspects of total phenolics, unsaturated fatty acids and oxido-reductase activity (POD, PPO, SOD, APX, GR and CAT).

MATERIALS AND METHODS

Bt11 maize, Bt176 maize, MS1/RF1 canola, MS8/RF3 canola and their nontransgenic control were from Syngenta Company. DAS-59122-7 maize and its nontransgenic control were kindly provided by DuPont Company. GT73 canola and its nontransgenic control were from Monsanto Company. The GM and nontransgenic control materials came from the same field, in order to get rid of growing conditions, years and other factors which could influence the results of comparisons.

Sample preparation of enzyme extracts

For the determination of antioxidant enzyme activities, whole grains were powdered. One gram FW of each material was homogenized in 2ml (for maize) or 3ml (for canola) 100 mM acetate buffer (pH 5.5) containing 8% (w/v) PVPP (Sigma, U.S.A.), 1 mM polyethylene glycol 6000 (Sigma, U.S.A.), 1 mM methyl sulfonyl fluoride benzene (PMSF) (Sigma, U.S.A.) and 0.01% (v/v) Triton X-100 at 4°C, were centrifuged at 12.000×*g* for 30 min and the supernatant was used for the POD and PPO assays. In the case of SOD and CAT activities, the seeds powder were homogenized in 2 ml (for maize) or 3 ml (for canola) 100 mM potassium phosphate buffer (pH 7.5) containing 5 mM dithiothreitol (DTT) (Sigma, U.S.A.) and 2% PVPP, at 4°C, were centrifuged at 12.000×*g* for 30 min and the supernatant was collected. Those powder was homogenized in 2 ml (for maize) or 3 ml (for canola) 0.05 M phosphate buffer (pH 7.5) containing 8% (w/v) PVPP at 4°C, were centrifuged at 12.000×*g* for 30 min and the supernatant was used for the APX and GR assays. The supernatant to be used for the enzymatic activity assay was stored at -80°C.

Oxido-reductase activity assay

A colorimetric assay for enzymatic activity was performed with a SECOMAM UVIKON spectrophotometer (French) and the stated materials were determinated of CAT (Havir and McHale, 1987), POD (Alscher et al., 1997), PPO (Chen, 2000), SOD (Giannopolitis and Ries, 1997; Prochazkova et al., 2001), APX (Venisse et al., 2001; Nakano and Asada, 1981) and GR (Chen et al., 2000). Each reaction was run in triplicate and all enzymatic activities were measured at 25°C.

For determination of CAT, the reaction mixture contained 3 ml 10 mM H_2O_2 (diluted with 50 mM phosphate buffer, pH 7.5) and 0.1 ml enzyme extract. Absorbance was recorded at 240 nm. The reaction was allowed to proceed for 4 min, beginning 30 s after adding the crude extract. The level of enzyme activity was expressed as Δ OD_{240}/min·mg protein. POD activity was assayed spectrophotometrically at 470 nm in a 2 ml reaction mixture, which contained 0.2 ml of crude extract and 2.5 ml 25 mM guaiacol (Sigma, U.S.A.) (diluted with 50 mM, pH 5.5 acetate buffer) and 0.2 ml 250 mM hydrogen peroxide. The reaction was allowed to proceed for 4 min, beginning 1 min after adding the crude extract to the substrate. The level of enzyme activity was expressed as ΔOD_{470}/min·mg protein. Each reaction was run in triplicate.

PPO activity was assayed spectrophotometrically at 420 nm in a reaction mixture which contained 0.1 ml of crude extract and 2.5 ml 50 mM acetate buffer, pH 5.5 and 0.25 ml 0.1 mM catechol. The reaction was allowed to proceed for 4 min, beginning 1 min after adding the crude extract to the substrate. The level of enzyme activity was expressed as ΔOD_{420}/min·mg protein. SOD activity was estimated by recording the decrease in optical density of nitro-blue tetrazolium (NBT) (Sigma, U.S.A.) dye by the enzyme. 3 ml of the reaction mixture contained 13 mM methionine, 75 µM nitroblue tetrazolium chloride, 10 µM EDTA, 50 mM phosphate buffer (pH 7.5) and 0.05 ml enzyme. Reaction was started by adding 2 µM riboflavine (Sigma, U.S.A.) and placing the tubes under 4000 LUX fluorescent lamp for 15 min. A complete reaction mixture without enzyme which gave the maximal colour, served as control. Reaction was stopped by switching off the light and putting the tubes into dark. A non-irradiated complete reaction mixture served as a blank. Absorbance was recorded at 560 nm and each reaction was run in triplicate. One unit of SOD was defined as the amount of enzyme that gave half-maximal inhibition. APX was assayed by recording the decrease in optical density due to ascorbic acid at 290 nm. The reaction mixture contained 2 ml 0.2 mM Tris-HCl (pH 7.8), 150 µl 5 mM ascorbic acid, 150 µl 10 mM H_2O_2 and 0.1 ml enzyme. The reaction was started with the addition of hydrogen peroxide. The level of enzyme activity was expressed as ΔOD_{290}/min·mg protein. GR was assayed as described by Schaedle and Bassham

with slight modifications as reaction mixture containing 4 ml 0.2 M Tris-HCl (pH 7.8, containing 3 mM EDTA), 50 µl 4 mM $NADPH_2$ (Sigma, U.S.A.), 300 µl 5 mM oxidized glutathione (c) (Sigma, U.S.A.). The oxidation of NADPH was recorded at 340 nm for 4 min after adding 50 µl enzymes extract. The level of enzyme activity was expressed as ΔOD_{340}/min·mg protein.

Total phenolics

Samples were extracted in 2% HCl in methanol for 24 h in the dark and at room temperature. The extracts were diluted with the same solvent used for extraction, to a suitable concentration for analysis. Total phenolics were measured according to the Folin-Ciocalteu reagent method (Singleton and Rossi, 1965).

Determination of fat content

The fat content was gravimetrically determined after extraction with anhydrous ether, according to the method of standards press of China (GB/T 5009.6-2003). The sample was homogenized with a food processor for 10 s and the procedure was repeated five different times to give a homogeneous sample. The samples (3.00 ± 0.05 g; W1) were weighed on a weighing paper and extracted with anhydrous ether in a Cable-extraction tube at 50°C for 12 h. The extract was filtered through a Whatman filter paper and collected in a round-bottom flask (W2) and evaporated under vacuum with a rotary evaporator. Once dried, the flask was cooled and reweighed (W3). The difference in weight (W3-W2) corresponded to the fat content of the sample: %Fat= (W3-W2)/W1×100. Determinations were performed in triplicate and the standard deviation (SD) was also determined.

Determination of fatty acids

Methyl esters of fatty acids from the seed were prepared by using a reaction mixture consisting of methanol, benzene, 2, 2-dimethoxypropane (Sigma, U.S.A.) and *n*-heptane (Sigma, U.S.A.), as described previously (Certel and Uslu, 2005; Garces and Mancha, 1993). The powder of seed (250 mg) was weighed into a glass tube and then 3 ml of reaction mixture and 2 ml *n*-heptane were added to the sample. Headspace of the tube was filled with carbon dioxide gas and it was covered with a teflon lid. The tube was shaken strongly and placed in a water bath at 80°C for 2 h. Then, the tube was allowed to reach room temperature until two phases formed. The upper phase (*n*-heptane, 1 µl), containing methyl esters of fatty acids, was injected into a gas chromatography (Agilent 6890)-mass spectroscopy (GC-MS).

The fatty acid composition of the oil was determined by using the GC-MS under the following operating conditions: chromatogram column (DB-5, Agilent), length, 60 m; column diameter, 0.25 mm; film thickness, 0.25 µm; carrier gas, helium; oven temperature, 35°C for 3 min, rising to 240°C at 8°C /min and held for 10 min; injector temperature, 240°C; scanning scope, 30 to 800 a.m.u.; ionization voltage, 70 eV; ionization electric current, 30 µA. A library search was carried out using the Wiley GC-MS Library. The percent fatty acid composition was calculated from the ratio of individual peak area to total definable peak area.

Statistics analysis

Experimental data were the mean ± S.E.M. of three replicates of the determinations for each sample. Statistics were calculated with the use of ANOVA. The term significant is used when $P < 0.05$ with the ANOVA.

RESULTS

Oxido-reductase activity assay

All the GMOs had significant differences from their non-transgenic control in PPO activity except Bt176 (Figure 1a). MS1/RF1 MS3/RF8 and Bt11 had significantly lower PPO activity values than their nontransgenic controls. On the other hand, the PPO activity values of GT73 and DAS-591122-7 were significantly higher than those of their nontransgenic control.

The POD activity values of MS1/RF1, Bt176 and Bt11 were significantly lower than those of their nontransgenic control (Figure 1b). However, GT73, MS3/RF8 and DAS-59122-7 did not differ from their nontransgenic controls in POD activity. Unlike Bt176 and DAS-59122-7, all the others showed significant differences from their nontransgenic control for SOD activity (Figure 1d). For GT73, MS1/RF1 and MS3/RF8, SOD activity values were significantly lower than those of their nontransgenic controls. On the other hand, the SOD activity values of Bt11 were significantly higher than its nontransgenic control.

All of the GMOs involved had significant differences from their nontransgenic control in GR activity (Figure 1c). GT73 and DAS-591122-7 had significantly higher GR activity values than their nontransgenic control. On the other hand, the GR activity values of MS1/RF1, MS3/RF8, Bt176 and Bt11 were significantly lower than those of their nontransgenic control. Figure 1e indicates that all of the GMOs involved had significant differences from their nontransgenic control in APX activity. For GT73, MS1/RF1, Bt176 and DAS-591122-7, APX activity values were significantly higher than those of their nontransgenic control. On the other hand, the APX activity values of Bt11 and MS3/RF8 were significantly lower than those of their nontransgenic control.

All of the GMOs involved had significant differences from their nontransgenic control in CAT activity (Figure 1f). Furthermore, GMOs had significantly lower CAT activity values than their nontransgenic control.

All the data in Figure 1 were consistent with (Guo et al., 2004, Tuna et al., 2008, Wu et al., 2004, Zhang and Wen, 2000, Zheng et al., 2008).

Total phenolics content and fat content

Other than Bt11, all the others had significant differences from their nontransgenic control in the values of total phenolics (Figure 2a). For GT73, MS1/RF1 and DAS-591122-7, the values were significantly higher than those of their nontransgenic control. On the other hand, Bt176 and MS3/RF8 had significantly lower total

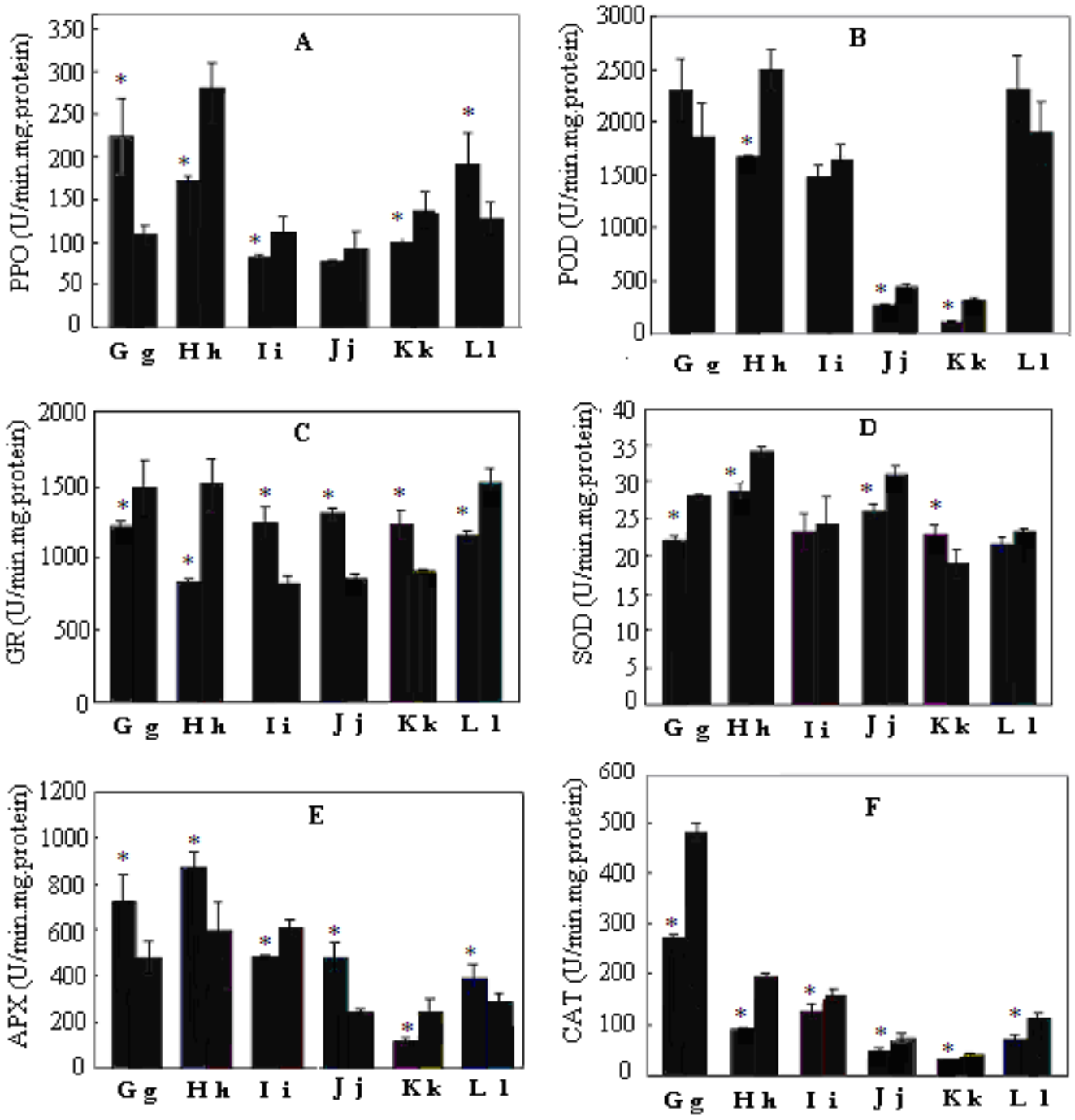

Figure 1. The activities of antioxidant enzymes of six kinds of GMOs and their nontransgenic control. (PPO, A; POD, B; GR, C; SOD, D; APX, E; CAT, F). G: GT73; H: MS1/RF1; I: MS8/RF3; J: Bt176; K: Bt11, L: DAS-591122-7; g, h, i, j, k, l are respectively the nontransgenic control of GT73, MS1/RF1, MS3/RF8, Bt176, Bt11 and DAS-591122-7. The values are the average and standard deviation of triplicates. Bars with * are significantly different at the $P < 0.05$ level according to *t*-test.

phenoics content than their nontransgenic control. The data of total phenolics content in this study is in accordance with the data in literature (Simić et al., 2004, Vuorela et al., 2003).

All the GMOs had significant differences from their nontransgenic control in the values of fat content except Bt176 (Figure 2b). For GT73, MS1/RF1, Bt11 and DAS-591122-7, the values were significantly higher than those of their nontransgenic control. On the other hand, MS3/RF8 had significantly lower fat content than its nontransgenic control. All of the data in Table 1 were in agreement with the literature (Wanasundara and Shahidi,

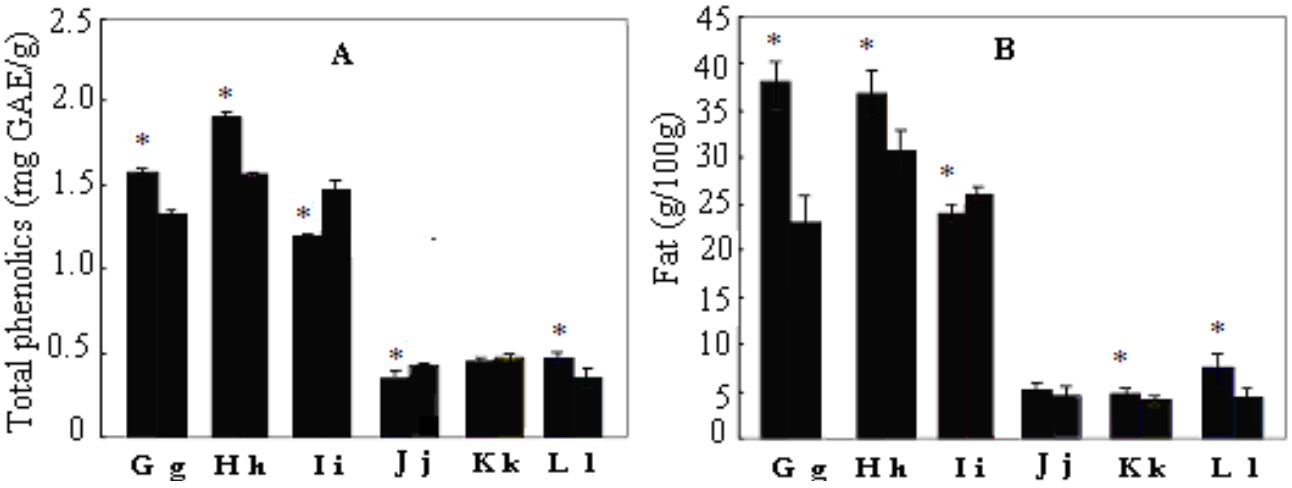

Figure 2. The total phenolics (A) and fat content (B) of six kinds of GMOs and their nontransgenic control. G: GT73; H: MS1/RF1; I: MS3/RF8; J: Bt176; K: Bt11; L: DAS-591122-7; g, h, i, j, k, l are respectively their nontransgenic control of GT73, MS1/RF1, MS3/RF8, Bt176, Bt11 and DAS-591122-7. The values are the average and standard deviation of triplicates. Bars with * are significantly different at the $P < 0.05$ level according to *t*-test.

1994, Hui et al., 2006).

Determination of fatty acids

Table 1 indicates the components of fatty acids of six couples of GMOs and their nontransgenic controls. Differences existed in both varieties and values of fatty acid composition between GMO and their nontransgenic controls. And double bond (DBI), reflecting unsaturation of fatty acids, existed significant differences between GMOs and their nontransgenic control except MS1/RF1 and its nontransgenic control. The DBI of GMOs was lower than those of their nontransgenic control except MS3/RF8 and DAS-59122-7. All of the data in Table 1 were in agreement with the literature (Wanasundara and Shahidi, 1994, Hui et al., 2006).

DISCUSSION

It is necessary to point out that in general, stress stimulation had significant effect on the activities of resistance related enzymes (Zhao et al., 2005). The effects of various environmental stresses on plants were known to be mediated, at least partially, by an enhanced generation of ROS (Alscher et al., 1997). These activities of enzymes increased in response to different types of stress, both biotic and abiotic (Rivero et al., 2001). PPOs ((EC 1.14.18.1 or EC 1.10.3.2)) catalyze the O_2-dependent oxidation of mono- and o-diphenols to o-diquinones, highly reactive intermediates whose secondary reactions are believed to be responsible for the oxidative browning which accompanies plant senescence, wounding and responses to pathogens. Transgenic plants with suppressed PPO levels exhibit increased water stress tolerance (Thipyapong et al., 2004). The enzymes SOD and POD are involved in the detoxification of $O_2^{\bullet -}$ and H_2O_2, respectively thereby preventing the formation of OH· radicals. And APX and GR are important components of the ascorbate-glutathione cycle response for the removal of H_2O_2 in different cellular compartments (Jimenez et al., 2002). On the other hand, plants contained a complex antioxidant system to detoxify ROS, which includes carotenoids, ascorbate, glutathione, tocopherols, anthocyannin pigments and those enzymes (Lopez-Gomez et al., 2006). It was reported that overexpression of ANS (anthocyanidin synthase) in a rice mutant resulted in novel transgenic rice with a mixture of flavonoids and an enhanced antioxidant potential (Ambavaram et al., 2007). However, in many cases it may be difficult to decide whether a response is adaptive or adverse. Enzyme induction, for instance, may be present in some situations as an adaptive response without any biological significance; sometimes it may be beneficial that it leads to more rapid metabolism and elimination of potentially toxic compounds or it may be a truly adverse response in that it may lead to increases in reactive intermediates and thus potentiate toxic effects (Dybing et al., 2002). Besides those enzymes, the unsaturation of fatty acids plays an important role in tolerance of plants to environmental stresses. Researches indicated that GM oilseed rape and soybeans with altered fatty acid profiles, for example, have already undergone regulatory review. Further advances in genomic sciences promise the discovery of new genes conferring desirable characteristics to crops that may fundamentally alter a crop's metabolic functions, promising further nutritional enhancement and resistance to abiotic stresses. It is important that we should continue to proactively assess whether current

Table 1. The fatty acids composition of six kinds of GMOs and their nontransgenic control.

Material	Composition of fatty acid (area/%)							
	16:0	18:0	18:1	18:2	18:3	20:0	20:1	DB
GT73	3.91±0.18*	1.97±0.08*	58.62±2.86	18.59±0.83*	7.54±0.27*	0.58±0.03	2.92±0.11*	19.02±0.91*
Nontransgenic control	4.55±0.20	1.76±0.08	60.73±2.99	20.89±1.01	10.77±0.49	/	1.30±0.06	21.57±0.97
MS1/RF1	5.09±0.25	2.04±0.10*	61.90±2.99*	22.10±1.00*	7.66±0.36*	/	1.28±0.07	18.26±0.83
Nontransgenic control	5.03±0.22	1.81±0.09	58.40±2.80	23.67±1.12	8.11±0.39	0.45±0.02	1.28±0.05	18.02±0.88
MS8/RF3	4.56±0.32*	2.15±0.08	62.33±1.65	22.86±0.89	7.51±0.36*	/	0.59±0.04*	22.81±0.87*
Nontransgenic control	4.86±0.36	2.05±0.07	63.46±1.87	23.26±0.96	5.87±0.25	/	0.50±0.03	21.09±0.83
Bt176	15.55±0.69	3.27±0.13*	28.60±1.25*	49.72±2.49*	1.03±0.03	0.48±0.09	/	6.79±0.29*
Nontransgenic control	15.33±0.58	2.72±0.14	30.71±0.35	50.74±1.00	/	/	/	7.32±0.30
Bt11	22.75±1.11*	2.50±0.19*	38.82±1.90*	37.18±1.58*	/	/	/	4.48±0.18*
Nontransgenic control	19.73±0.28	2.27±0.10	34.78±0.98	41.85±2.05	0.80±0.03	0.71±0.02	/	5.32±0.20
DAS-59122-7	17.15±0.68*	2.66±0.12	43.42±2.08*	31.01±1.29*	1.48±0.06*	0.94±0.05*	0.61±0.02*	7.71±0.31*
Nontransgenic control	19.44±0.88	2.56±0.16	46.88±2.28	25.82±1.16	1.36±0.05	1.14±0.04	0.72±0.03	4.46±0.18

Double bond (DB) = (1×% monenes + 2×% dienes +3×% trienes) / (% saturated fatty acid). Values are the average and standard deviation of triplicates. Data followed by star are significantly different at the $P < 0.05$ level according to ANOVA.

approaches to safety assessment are appropriate also for future GM crop products with more complex traits (König et al., 2004).

The results from oxido-reductase activity analysis indicated significant differences ($P < 0.05$) between GMOs and their nontransgenic control, except for a few enzymatic activities of several GM fatty acids also showed significant differences ($P < 0.05$) between GMOs and their nontransgenic control. Only significant differences which would lie outside the range of natural variation would be investigated further. However, no obvious differences occurred among all tested maize samples or canola samples. The correct interpretation of the data generated by such analytical techniques relies heavily on the availability of adequate comparative data on and used as crop specific "benchmarks" to compare the crops. The data of total phenolics and unsaturated profile of the GM crop. To analytically determine all possibilities of unintended effects is a huge work and will be faced with many technical challenges. A further challenge is to determine the real significance of any unintended effect on consumer health. Unintended effects do not automatically imply a health hazard. Ideally, only those parameters that fall outside the range of natural variation would be considered further in safety assessment. However, there is a lack of information on the natural variation within and between given plant cultivars

for all the parameters that may now be measured.

A large proportion of the genes in any plant genome encode enzymes of primary and specialized (secondary) metabolism. For safety assessment of GMOs, there were some studies which indicated that those inserted DNA construct, exert indirectly on secondary metabolism or unintended effects via genomic disequilibrium on one or more traits. An example would be a DNA insertion event which alters a key enzyme used across multiple metabolic pathways (Williams and Davis, 2005). Now, the problem is, how much is the accepted range of the variability and what variations may be unacceptable? These questions do not seem to be completely clarified in the near future. Predictable and unpredictable unintended effects may or may not prove to have relevance in terms of safety, but must be taken into account when assessing risk of GMOs (Cellini et al., 2004). When the substantial equivalence of GMOs with their nontransgenic control is analyzed, the antioxidant activity has to be taken into consideration besides protein, oil, fibre, ash, moisture, amino acids and fatty acids.

Connotation and scope of potential unintended effects were being enriched. There were some other new methods applied to study the potential unintended effects, these methods including Wavelength Dispersive x-ray fluorescence (WD XRF) spectrometry method (Jastrzebska et al., 2003), visible (VIS) and near infrared (NIR) spectroscopy combined with multivariate analysis (Xie et al., 2007), gas chromatography/mass spectrometry (GC/MS), high performance liquid chromatography (HPLC) (König et al., 2004). When these methods are applied, some factors such as weather station, planting station, gene flow, etc. would be well explored, resulting in the significant difference between GMOs and their nontransgenic control. For example, it was reported that in contiguous cornfields, 100 m distance was sufficient to achieve 0.05% outcrossing consistently (Goggi et al., 2006). A further problem, which was discussed time after time, was the comparison between different studies, especially the parameters of analysis such as units and bases of standardization of substances.

In conclusion, when people study guidance on how to tailor the test strategy to the potential unintended effects from the genetic modification, we hope that the anti-oxidant system be taken into account.

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