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Microarray Analysis of Genes that Respond to γ-Irradiation in *Arabidopsis*

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To reveal the signal transduction mechanism of the response to stress in the form of active oxygen species, we used a microarray system to analyze gene expression patterns 2 or 24 h after γ-irradiation of *Arabidopsis*. γ-Irradiation induces several signal transduction and metabolite genes. By analysis of *cis*-elements located on the promoter region of the γ-responsive genes, we have also found several *cis*-elements related to various signal transduction systems. We also analyzed the pleiotropic mutant *ttg1-1*, which has a dramatically altered physiological response to γ-irradiation. By comparing the gene expression patterns of wild-type (L*er*) and *ttg1-1* mutant plants after γ-irradiation, we identified various TTG1-regulated γ-response genes. Analysis of the *cis*-elements in the promoter region of the γ-responsive genes also revealed that the many transcription factors interacting with TTG1 protein (WD40 protein) are related to the γ-responsive gene expression.

KEYWORDS: **γ-Irradiation; active oxygen species; microarray; *Arabidopsis***

INTRODUCTION

Active oxygen species (AOS) are generated as byproducts of energy metabolism and the photophysiological reaction (*1*, *2*). Because they are also synthesized as key factors in stress response, developmental control, and defense responses (*3*−*7*), it is important to elucidate the signal transduction pathways of AOS stress responses. Generation of AOS upon exposure to strong light (*8*), UV irradiation (*9*), or chemical treatment alters the transcription of many signal transduction genes in ways that are not specific to the AOS stress response. Because the natural environment is devoid of high levels of γ-rays, *Arabidopsis* presumably has few sensors for γ-rays and few signal transduction systems that respond to them. Physiological analyses have revealed that γ-irradiation induces trichome development, ethylene synthesis, expansion of root radial cells, synthesis of anthocyanin and ascorbic acid, and development of root hairs (*10*, *11*). Treatment of plants with antioxidants or AOS-generating reagents before γ-irradiation has shown that these phenomena are responses to the generation of AOS (*10*, *11*). Microarray is an important tool for gene expression analysis. To know the gene expression profiles after γ-irradiation and for the analysis of signal transduction, we used the microarray system. To further focus our search for AOS response genes, we also analyzed the transcript patterns of the *transparent testa glabrous1-1* (*ttg1-1*) (WD-40) mutant. The *ttg1-1* gene encodes a WD-40 protein, and mutation of the *ttg1-1* locus results in the absence of trichomes from the stem, leaves, and sepals and blocks the production of anthocyanin and seed coat mucilage (*12*, *13*). These phenomena point to defects in the generation of, and defense against, AOS. Therefore, this WD-40 mutant is thought to have lost its AOS responsive gene networks, and its AOS signal transduction systems may fail to be activated. We compared the gene expression patterns of wild-type (L*er*; Landsberg *erecta* ecotype) and *ttg1-1* plants after γ-irradiation and then selected candidate AOS signaling genes. In addition, we analyzed the 5′-*cis*-element regions of the γ-response genes and identified several elements highly conserved among γ-irradiation- and AOS-induced genes as possible AOS responsive elements.

MATERIALS AND METHODS

***Arabidopsis* Strains and Growth Conditions.** The wild-type *Arabidopsis* strain that we used was the L*er* ecotype. The Arabidopsis Research Center (Ohio State University, Columbus, OH) provided seeds of the *ttg1-1* mutant. Sterilized seeds were sown on a 1% agar medium containing 0.5× Murashige and Skoog basal salts (Sigma), 2% sucrose, 100 mg L^{-1} inositol, 1 mg L^{-1} thiamine, 0.5 mg L^{-1} nicotinic acid, and 0.5 mg L^{-1} pyridoxine and were grown under continuous illumination (light intensity, 400−500 μW m^{-2} s^{-1}) at 21 °C.

γ-Irradiation. Eighteen days after germination, when the fifth leaf had expanded fully, plants growing on the agar medium were irradiated with γ-rays from a cobalt-60 source in a γ-cell irradiator (3.0 kGy h^{-1}, 1300 TBq; Nordion, Ontario, Canada). After irradiation, the plants were grown alongside unirradiated plants under the conditions described above.

Extraction and Quantification of L-Ascorbic Acid. L-Ascorbic acid was extracted and measured according to the methods of Beutler et al. by using an L-ascorbic acid analysis kit (Boehringer Mannheim). L-Ascorbic acid and oxidized ascorbic acids were extracted from whole plants with an extraction buffer containing the tetrazolium salt 3-(4,5-

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dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT). The fresh weight and number of plants were measured, and the material was crushed in a mortar with an aliquot of the extraction buffer. In the presence of the electron carrier 5-methylphenazinium methosulfate (PMS) at pH 3.5, L-ascorbic acid was reduced by MTT to formazan; the reduced substances in the cuvette were measured. Adding ascorbate oxidase (AAO) to the sample blank oxidatively removes only the L-ascorbate; the dehydroascorbate formed does not react with MTT or PMS. The optical density (OD) (at a wavelength of 578 nm) of the sample minus that of the sample blank is equivalent to the quantity of L-ascorbate in the sample. The concentration of MTT−formazan was the measured parameter.

Extraction and Measurement of Anthocyanin. Anthocyanin was extracted from entire plants by using an extraction solution of propanol: HCl:H_2O (18:1:81). Once we had determined their wet weight, the plants were crushed in a mortar with an aliquot of the extraction buffer. The extract was boiled for 3 min and incubated overnight at 25 °C. The ODs at 535 nm were measured. The difference between these two values was normalized according to the fresh weight.

RNA Isolation. Total RNA was isolated from whole plants by phenol extraction (Isogen-LS, Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions and purified twice by LiCl precipitation.

Microarray Analysis. Microarray analyses were carried out with a commercial analyzing kit (Agilent Oligo DNA microarray hybridization, Agilent Technologies, CA). The total RNA was labeled with a Fluorescent Direct Label Kit (Agilent). The cDNA array filters (21 560 base oligonucleotide probes), each corresponding to a different *Arabidopsis* gene on the basis of information in the TIGR database (http://www.tigr.org/tdb/tgi/agi/), were hybridized with the labeled and fragmented RNA (100 ng mL^{-1}) at 60 °C for 17 h under the standard conditions described by the manufacturer of the labeling kit, washed once with 0.5 × SSC containing 0.01% (w/v) SDS at 65 °C and once with 0.06 × SSC at 65 °C, and dried. The signals were monitored and calculated with a Scan Array 5000 Microarray Scanner (GSI Lumonics Co.,) and Quant Array software (GSI Lumonics Co.). We also analyzed the expression profiles of several genes with Northern blot analyses and confirmed the reproducibility of the results.

Cis-Element Analysis. The sequences of the 5′-flanking regions of the genes of interest were obtained from the MIPS database (http://mips.gsf.de/proj/thal/db/index.html), and 2 kb of the sequences was analyzed by a PLACE database signal scan search (http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html).

RESULTS AND DISUCUSSION

Physiological and Morphological Responses of γ-Irradiated *Arabidopsis*. To identify genes responsive to AOS stress, we gave plants a massive dose of γ-rays. γ-Irradiation causes the formation of AOS by water radiolysis, but because γ-irradiation is an abiotic stress, plants lack a specific signal transduction system to defend against it. Therefore, the genes that respond to γ-irradiation likely represent AOS reactive genes. We noted the synthesis of anthocyanin and ascorbic acid, development of trichomes, synthesis of ethylene, expansion of root radial cells, and development of root hairs in response to γ-irradiation (**Figure 1**). Biochemical and physiological analyses have revealed that the ascorbic acid and anthocyanin work as radical scavengers, whereas the AOS signals stimulate trichome and root hair formation (*11*). Because pretreatment with antioxidant suppresses these phenomena, the respective genes may be responsive to AOS (*10*, *11*). We determined that a dose of 2 kGy was optimal for observing these biological effects.

γ-Irradiation Responsive Genes of Wild-Type (L*er*) *Arabidopsis*. We extracted RNA from unirradiated and irradiated plants 2 and 24 h after irradiation with 2 kGy and analyzed the gene expression patterns by using a microarray system. Stress response genes can be divided into two types according to their expression times: early genes (expressed 1−2 h after stimulation) and late genes (expressed 2−24 h after stimulation) (*14*).

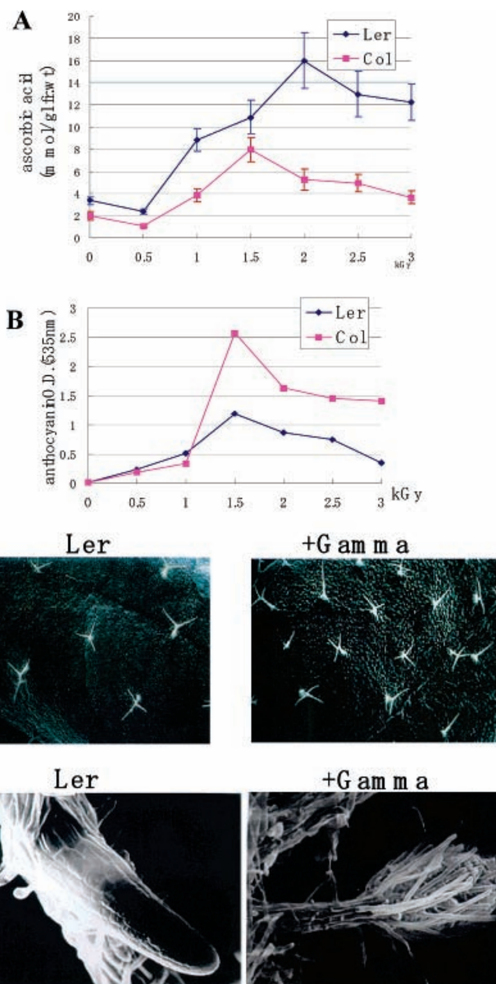

Figure 1. Physiological changes after γ-irradiation. (**A**) γ-Irradiation-induced ascorbic acid accumulation. The amount of ascorbic acid 4 days after irradiation is plotted against the dose of γ-irradiation. L*er*, Landsberg *erecta* ecotype. (**B**) γ-Irradiation-induced accumulation of anthocyanin. The amount of anthocyanin 4 days after irradiation is plotted against the dose of γ-irradiation. (**C**) Formation of trichome. Surfaces of leaves were observed 5 days after γ-irradiation (2 kGy). L*er*, Landsberg *erecta* (control, unirradiated); +Gamma, γ-irradiated Landsberg *erecta*. (**D**) Expansion of root radial cells and development of root hairs. Roots were observed 4 days after γ-irradiation (2 kGy).

Therefore, we analyzed gene transcripts 2 and 24 h after γ-irradiation. Because the unpaired electrons generated at the time of irradiation reacted with many biological molecules, an unstable energy condition was conserved over several days in these molecules. Therefore, AOS were generated continuously from these chemical reactions. We hybridized 21 560 base oligonucleotide probes with the extracted RNAs. Each oligonucleotide probe corresponded to a different gene in the TIGR database (http://www.tigr.org/tdb/tgi/agi/). The leading categories of genes whose transcription was upregulated in the 2 h after irradiation are shown in **Table 1**. The main genes activated were signal transduction genes and transcription factors (e.g., WRKY, myb, kinase, phosphatase, zinc finger protein, methyltransferase, and CDPK). There were also genes for metabolism (e.g., xyloglucan endotransglycosylase, and glutamyltransferase), cell rescue, defense, cell death and aging (e.g., nematode and drug and disease resistance proteins), and redox proteins (e.g., oxidoreductase). Additional upregulated genes were those for p450, NAD^{+}ADP-ribosyltransferase, BCS1, and pirin. The leading categories of genes whose transcription was upregulated

Table 1. Expression Data of Induced and Suppressed Genes after γ-Irradiations in Normal (L*er*) Plants[a]

Induced Gene

putative ID	ratio	accession no. of TIGR ID	putative ID	ratio	accession no. of TIGR ID
			2 h		
WRKY family transcription factor	27.44	At1g80840	phosphate-induced (phi-1) protein	13.82	At1g35140
embryonic abundant protein-like	27.24	At3g54150	nematode resistance protein-like protein	13.40	At3g55840
cytochrome p450 family	22.53	At5g57220	multidrug resistance P-glycoprotein	13.38	At3g62150
NAD+ ADP-ribosyltransferase	22.27	At4g02390	calcium-dependent protein kinase	13.36	At5g66210
BCS1 protein-like protein	22.17	At3g50930	similar to pirin-like protein	13.14	At2g43120
FAD-linked oxidoreductase family	21.36	At1g26380	putative phospholipase	12.81	At2g39400
xyloglucan endotransglycosylase (TCH4)	20.34	At5g57560	putative γ-glutamyltransferase	12.70	At4g39640
O-methyltransferase 1, putative	18.13	At1g21130	disease resistance protein (TIR-NBS-LRR class)	12.69	At5g41750
putative nematode-resistance protein	17.03	At2g40000	WRKY family transcription factor	12.55	At2g30250
calcium-binding protein-like	16.08	At4g20780	leucine rich repeat protein family	12.28	At1g33600
protein kinase, putative	15.94	At3g25250	expressed protein	12.05	At1g69890
monooxygenase family	15.65	At2g29720	receptor-like protein kinase-like	12.00	At5g25930
Rad51-like protein	15.38	At5g20850	putative protein kinase	11.83	At2g30360
putative thymidine kinase	14.36	At3g07800	myb-like protein	11.71	At5g03780
salt-tolerance zinc finger protein	13.97	At1g27730	growth factor like protein	11.47	At4g12720
			24 h		
L-ascorbate oxidase	13.08	At4g39830	aquaporin (plasma membrane intrinsic protein 1B)	8.38	At2g45960
Avr9 elicitor response like protein	11.39	At4g26940	ethylene responsive element binding factor 2 (ATERF2)	8.14	At5g47220
14-3-3 proteing F14chi (grf1)	11.12	At4g09000	fibrillarin 1 (AtFib1)	7.87	At5g52470
homeodomain transcription factor (ATHB-6)	11.11	At2g22430	phospholipase like protein	7.66	At4g38560
glycoprotein (EP1)	10.89	At1g78850	H^+-transporting ATPase 16K chain P2, vacuolar	7.47	At4g38920
sucrose-phosphate synthase-like protein	10.23	At5g20280	translation factor EF-1 α-like protein	7.14	At4g22780
E2, ubiquitin-conjugating enzyme	9.76	At5g56150	ABC transporter	7.11	At3g13080
pathogenesis-related PR-1-like protein	9.31	At2g14610	NAC domain protein	7.10	At1g01720
glutathione S-transferase (GST6)	8.72	At2g47730	WRKY type DNA binding protein	6.83	At2g25000
disease resistance protein-like	8.55	At3g44480			

Supressed Gene

putative ID	ratio	accession no. of TIGR ID	putative ID	ratio	accession no. of TIGR ID
			2 h		
serine threonine protein kinase	0.12	At5g45820	putative AP2 domain transcription factor	0.27	At2g44940
putative lipid transfer protein	0.16	At2g15050	polysaccharide lyase family 1 (pectate lyase)	0.27	At4g24780
expansin, putative	0.17	At2g40610	protein phosphatase 2C (PP2C)	0.27	At5g59220
receptor-like protein kinase (ATR1), putative	0.18	At5g60890	homeobox-leucine zipper protein ATHB-12	0.27	At3g61890
myrosinase-binding protein homologue, putative	0.20	At1g52040	myb-related transcription factor mixta, putative	0.27	At1g18710
putative steroid sulfotransferase	0.21	At2g03750	auxin-induced protein-like	0.28	At4g38840
putative auxin-regulated protein	0.22	At2g46690	copper chaperone (CCH)-related	0.28	At4g08570
expressed protein	0.23	At1g19670	water stress-induced protein, putative	0.28	At1g56600
glutathione transferase, putative	0.24	At3g03190	myb-related transcription factor (mixta), putative	0.29	At5g61420
9-*cis*-epoxycarotenoid dioxygenase	0.24	At4g19170	cytochrome p450, putative	0.29	At3g30180
similar to Dr4 (protease inhibitor)	0.25	At1g73325	expansin, putative	0.29	At2g03090
AP2 domain containing protein	0.25	At5g25390	transcription factor TINY	0.29	At5g25810
expansin, putative	0.25	At2g37640	putative auxin-induced protein	0.29	At4g38860
polygalacturonase, putative	0.26	At1g60590	CONSTANS B-box zinc finger family protein	0.30	At1g25440
3-isopropylmalate dehydratase-like protein	0.26	At3g58990	putative auxin-regulated protein	0.30	At2g21210
			24 h		
DC1.2 homologue-like protein	0.10	At5g62360	germin-like protein	0.18	At5g20630
prx10 peroxidase-like protein	0.11	At5g15180	acyltransferase	0.18	At5g23940
microtubule-associated motor-like	0.13	At5g60930	zeaxanthin epoxidase precursor	0.18	At5g67030
Myb transcription factor homologue (ATR1)	0.14	At5g60890	formamidase-like protein	0.18	At4g37550
β-carotene hydroxylase	0.14	At5g52570	sugar transporter like protein	0.20	At4g36670
NADPH:protochlorophyllide oxidoreductase A	0.14	At5g54190	bZIP DNA-binding protein-like	0.22	At5g15830
subtilisin-like serine protease	0.15	At5g44530	NAM-like protein (no apical meristem)	0.22	At3g29035
E2, ubiquitin-conjugating enzyme 17 (UBC17)	0.16	At4g36410	peptide transporter PTR2-B	0.23	At1g32450
ethylene responsive element binding factor-like	0.17	At5g61590	fatty acid elongase-like protein	0.23	At5g04530
β-galactosidase (emb CAB64740.1)	0.17	At5g56870			

[a] Wild-type (L*er*) *Arabidopsis* genes whose transcription was up- or downregulated 2 or 24 h after γ-irradiation. Ratio: intensity of signal from γ-irradiated plants divided by that from unirradiated plants (for upregulated genes) or vice versa (for downregulated genes).

2−24 h after irradiation were those for metabolism (e.g., sucrose-phosphate synthase, phospholipase), transcription factors (e.g., AtHB-6, NAC, and WRKY), cellular communication/signal transduction (e.g., 14-3-3, ABC transporter, and AtERF2), cell rescue, defense, cell death and aging (e.g., disease resistance protein), and transport facilitation proteins (e.g., aquaporin, H^+-ATPase). Additional upregulated genes were those for glycoprotein EP1, glutathione S-transferase (GST6), fibrillarin 1, and EF-1. Therefore, AOS induces factors involved in the stress response, intra- and extracellular transport, and supplemental energy systems.

The genes whose transcription was downregulated early after γ-irradiation fell mainly into the functional categories of signal transduction and transcription factors (e.g., serine threonine protein kinase, phosphatase, homeobox-leucine zipper protein, zinc finger protein, myb, TINY). There were also genes for metabolism (e.g., polygalacturonase, isopropylmalate dehydratase, and polysaccharide lyase), cell rescue, defense, cell

death and aging (e.g., water stress-induced protein), and transport facilitation proteins (e.g., lipid transfer protein, copper chaperone). Additional downregulated genes were those for expansin, auxin-regulated gene, and protease inhibitor. Genes downregulated late after γ-irradiation were those for metabolism (e.g., PRX10, β-carotene hydroxylase, and fatty acid elongase), transcription factors (e.g., ATR1, bZIP binding protein), cellular communication/signal transduction (e.g., ethylene responsive element binding factor), and cell growth, division, and DNA synthesis. AOS stress induced the expression of genes responsive to stress (e.g., heat shock, disease, and wounding) and light (e.g., light-induced, circadian, photosynthetic), those involved in energy metabolism (e.g., sugar transport, glucose regulation, and carbohydrate catalysis), and many transcription factors (e.g., myb, zinc finger factors, NAM, CDPK, WRKY, and MADS) and suppressed those involved in carbohydrate metabolism, cell growth and division, and DNA synthesis (**Table 1**).

γ-Irradiation Responsive Genes of the *ttg1-1* Mutant. The *ttg1-1* mutant lacks the phenomena characteristic of AOS responses and shows reduced viability after γ-irradiation. To identify changes in the expression patterns of genes potentially involved in responses to AOS stress, we used a microarray assay to study γ-irradiated (2 kGy) *ttg1-1* mutant plants. We then compared the expression patterns of this mutant with those of wild-type (L*er*) plants to reveal AOS responsive genes whose control was associated with WD-40 signal transduction (**Table 2**). Transcriptome analysis of *ttg1-1* revealed that 88% of γ-induced or -suppressed genes were common to L*er* and *ttg1-1*, but there were differences in up- and downregulation patterns. Genes upregulated early after irradiation in L*er* but not in *ttg1-1* plants were those for cytochrome P450, P-glycoprotein, pirin, myb, zinc finger protein, AtRad17, and copine. Genes downregulated early in L*er* but not in *ttg1-1* plants were those for myrosinase-binding protein, steroid sulfotransferase, 3-isopropylmalate dehydratase, photo protein, ARR6, bHLH protein, and En/pm-like transposon protein. Genes upregulated early in *ttg1-1* but not in L*er* plants were those for ribonucleotide reductase, anthocyanin acyltransferase, salt tolerance zinc finger protein, and cinnamoyl CoA reductase. Genes downregulated early in *ttg1-1* but not in L*er* plants were those for dehydrin, myb, lipid transfer, and bHLH protein. Genes upregulated late in L*er* but not in *ttg1-1* plants were those for Avr9 elicitor response protein, 14-3-3 homeo domain transcription factors, ubiquitin conjugating enzyme, aquaporin, ethylene responsive element binding factor, fibrillarin 1, and EF-1. Genes downregulated late in L*er* but not in *ttg1-1* plants were those for PRX10 peroxidase, microtubule-associated motor protein, ubiquitin conjugating enzyme, and formamidase. Genes upregulated late in *ttg1-1* but not in L*er* plants were those for myb, NAM, protein kinase, and calcium binding protein. Genes downregulated late in *ttg1-1* but not in L*er* plants were those for transcriptional activator, phytocyanin-related protein, lipid-transfer protein, RCP2, ZIP2, and xyloglucan endotransglycosylase.

Therefore, WD-40 protein may modulate the AOS response of genes for the stress response, the internal transport system of the cell, the special homeostasis and metabolite system, and antioxidant proteins. These signal transduction systems likely control many types AOS responsive genes, including those resistant to, as well as those regulated by, AOS. Because the viability of the *ttg1-1* mutant after γ-irradiation is dramatically lower than that of L*er* plants (*11*), putative *ttg1-1* protein-regulated signal transduction genes are included among the AOS resistance and response genes.

Genes whose transcription rates were upregulated more frequently in *ttg1-1* plants than in L*er* plants included those for transcription factors (e.g., NAC, WRKY), cellular communication/signal transduction (e.g., ABC transporter, peptide transporter), and disease resistance proteins. Those downregulated more frequently in *ttg1-1* plants included those for metabolism (e.g., β-1,3-glucanase-4), transcription factors (e.g., myb, ring finger proteins), and cellular communication/signal transduction (e.g., MADS box gene).

Analysis of the 5′-Upstream Region of Wild-Type (L*er*) *Arabidopsis* Genes. To identify regulatory elements common among γ-irradiation response genes and to gain insight regarding possible early transcriptional regulators of stress, we used the PLACE (Plant *cis*-Acting Regulatory DNA Elements) database to search the 5′-*cis*-element regions of the γ-response genes. We found that signal transduction (e.g., ARF, CATATGG-SAUR, ERE, myb, Ry repeat, WB box), stress response (e.g., DRE, ELRE, and LTRE), light inducible (e.g., CACGTG motif, CCA1, evening element, and T-BOX), carbohydrate metabolism regulation (e.g., ACGTA box, prolamine box, SURE, TATC-CAOS-AMY, and TGACGTVM-AMY), storage protein controlling (e.g., CANBNNAPA, SEF1 motif), and other (GT-1, L1 box, and pyrimidine box) elements were involved in regulating the expression of both up- and downregulated γ-response genes in L*er* plants (**Table 3**). Many of these *cis*-elements are consistent with those of AOS reactive genes. Therefore, we theorize that the AOS signal affects transcription factors common among multiple signal transduction systems and/or many similar systems. In addition, upregulation specific elements included storage protein gene-controlling (e.g., CERE-GLU box), transcription-controlling (e.g., OCTEMER motif), and light inducible (e.g., SV40 core enhancer) elements; elements specific to downregulated genes were hormonal signal transduction elements (e.g., ABRE, AUXRE) (**Table 3**). Therefore, genes whose expression was specifically up- or downregulated in wild-type *Arabidopsis* exposed to γ-rays included those for histones, storage protein metabolism, the light−response network, and hormonal signal transduction.

Analysis of the 5′-Upstream Regions of *ttg1-1* Mutants. We analyzed the 5′-*cis*-element regions of the genes that showed different expression patterns between wild-type (L*er*) and WD-40 mutant (*ttg1-1*) *Arabidopsis* to identify AOS stress responsive WD-40-related element(s) (**Table 4**). Elements common to genes up- and downregulated in L*er* but not in *ttg1-1* plants included those for signal transduction (ARF, myb, and WB box), stress response (DRE, elicitor responsive element, and LTRE), light inducible genes (CCA1 binding site, evening element, and GT-1 motif), carbohydrate metabolism regulation (prolamine box), storage protein control [SEF1 motif, (CA)n element], and others (hexamer motif, L1 box, and pyrimidine box). Upregulation specific elements in L*er* plants included the Rbcs consensus, RY repeat, SURE, and TATCCA elements, and downregulation specific elements in L*er* plants were the NDE element, PAL box, Q element, Reα, and Z-DNA forming sequence. These 5′-*cis*-elements are candidate binding sites for WD-40-regulated AOS stress responsive genes. Therefore, genes involved in the auxin response, stress response (low temperature, drought, and elicitor), light signaling, carbohydrate metabolism (amylase, prolamine, and sucrose), storage protein regulation (pyrimidine, napin), and transcription control genes were revealed as candidate responders to the AOS signal. These findings agree with those from the gene analyses. Therefore, we hypothesize that AOS stress induces signal transduction

Table 2. Expression Data of Induced and Suppressed Genes after γ-Irradiation in *ttg1-1* Mutants[a]

Induced Gene

putative ID	ratio	accession no. of TIGR ID	putative ID	ratio	accession no. of TIGR ID
		2 h			
patatin, putative	33.82	At2g26560	nematode resistance protein-like protein	15.12	At3g55840
embryonic abundant protein-like	26.23	At3g54150	putative protein	15.08	At3g45730
WRKY family transcription factor	25.33	At1g80940	putative nematode resistance protein	15.03	At2g40000
FAD-linked oxidoreductase family	22.30	Atlg26380	Rad51-like protein	14.95	At5g20850
O-methyltransferase 1, putative	21.75	At1g21120	ribonucleotide reductase small subunit	14.90	At3g27060
putative phospholipase	21.60	At2g39400	calcium-binding protein-like	14.48	At5g39670
calcium-binding protein-like	20.29	At4g20790	leucine rich repeat protein family	14.38	At1g33600
monooxygenase family	18.67	At2g29720	myb-like protein	14.33	At5g03780
BCSI protein-like protein	18.31	At3g50930	NaCl inducible calmodulin-like	13.90	At5g49480
protein kinase, putative	17.79	At3g25250	putative γ-glutamyltransferase	13.56	At4g39640
O-methyltransferase 1, putative	17.40	At1g21130	xvloglucan endotransglycosylase, putative	13.53	At4g30280
xyloglucan endotransglycosylase (TCH4)	17.33	At5g57560	anthocyanin 5-aromatic acyltransferase	13.42	At5g61160
calmodulin-like protein	16.99	At2g41100	calcium-dependent protein kinase	13.13	At5g66210
O-methyltransferase 1, putative	16.55	At1g21110	disease resistance protein (TIR-NBS class)	13.06	At1g72900
NAD + ADP-ribosyltransferase	16.42	At4g02390	O-methyltransferase 1, putative	13.04	At1g21100
WRKY family transcription factor	15.32	At4g23810			
		24 h			
NAD + ADP-riibosyltransferase	7.59	At4g02390	receptor like protein kinase	3.00	At5g01540
nucleoid DNA-binding protein cnd4l-like protein	7.31	At5g10760	NAM (no apical meristem)-like protein	2.92	At2g17040
myb-like protein	7.29	At5g03780	putative calmodulin	2.79	At3g51920
NAM (no apical meristem)-like protein	5.26	At5g18270	heat-shock protein	2.74	At5g52640
putative L-ascorbate. oxidase	5.08	At4g39830	ABC transporter, putative	2.73	At3g13080
NaCl inducible, calmodulin-like	4.55	At5g49480	glutaredoxin, putative	2.72	At1g28480
putative calcium-binding protein	3.83	At3g47480	cytochrome P450, putative	2.66	At3g26210
calcium-binding protein (CaBP-22)	3.53	At2g41090	putative WRKY type DNA binding protein	2.62	At2g25000
peptide transporter	3.45	At5g46050	xyloglucan endo-1,4-β-D-glucanaw (XTR-6)	2.61	At4g25810
putative protein kinase	3.38	At2g30360	receptor like protein kinase	2.59	At5g60270

Supressed Gene

putative ID	ratio	accession no. of TIGR ID	putative ID	ratio	accession no. of TIGR ID
		2 h			
dehydrin RABIS-like protein (sp P30185)	0.12	At5g66400	9-*cis*-epoxycarotenoid dioxygenase	0.25	Al4g19170
myb family transcription factor	0.17	At1g01520	putative auxin-regulated protein	0.25	At2g46690
kinesin-related protein	0.19	At2g37420	serine/threonine protein kinase-like protein	0.26	At5g10930
polygalacturanase, putative	0.19	At1g60590	putative AP2 domain transcription factor	0.26	At2g44940
δ-l-pyrroline 5-carboxylase synthetase (P5C1)	0.21	At2g39800	putative ADP-glucose pyrophosphorylase	0.21	At2g21590
expansin, putative	0.21	At2g03090	β-expansin pollen allergen protein	0.28	At4g28250
CONSTANS B-box zinc finger family protein	0.21	At1g25440	myb-related transcription factor mixta	0.28	Atlg18710
AP2 domain containing protein	0.21	At5g25390	putative protein kinase	0. 28	At3g14370
receptor-like protein kinasc(ATR1)	0.21	At5g60890	auxin-regulated protein	0.29	At2g25625
expansion, putative	0.22	At2g40610	zinc finger-like protein	0.29	At3g58070
late embryogenesis abundant protein	0.22	At1g52690	bHLH protein	0.3	At4g17880
copper chaperone (CCIII)-related	0.23	At4g08570	pathogenesis-related protein- like	0.3	At5g26130
protein phosphatase 2C (PP2C)	0.24	At5g59220	myb-related transcription factor (mixts)	0.3	At5g61420
cytochrome p450, putative	0.24	At3g30180	putative cytochrome P450	0.3	At2g46660
putative lipid transfer protein	0.25	At2g15050			
		24 h			
acyltransferase	0.11	At5g23940	putative zinc transporter ZIP2-like	0.25	At5g59520
DC1.2 homologue-like protein	0.17	At5g62360	putative xyloglucan endotransglycosylase	0.25	At5g03210
NADPH:protochlorophyllide oxidoreductase A	0.17	At5g54190	thioredoxin-like	0.25	At5g06690
ethylene responsive element binding factor-like	0.19	At5g61590	xylosidase	0.26	At5g49360
β-gulactosidase (emb CAB64740.1)	0.19	At5g56870	seed imbitition prolein-like	0.26	At5g20250
phytocyanin-related protein-like	0.22	At5g25090	conglutin γ-like protein	0.27	At5g19120
putative nonspecific lipid-transfer protein precursor	0.22	At2g13820	ferritin 1 precursor	0.27	At5g01600
germin-like protein	0.24	At5g20630	GDSL-motif lipase-hydrolase-like protein	0.27	At5g45950
root cap protein 2-like protein	0.24	At5g54370	subtilisin-like serine protease	0.28	At5g44530
Myb transcription factor homologue (ATR1)	0.24	At5g60890	pectin methylesterase-like	0.28	At5g47500

Table 2. Continued

L*er*–*ttg-1* (Induced Gene)

putative ID	ratio	accession no. of TIGR ID	putative ID	ratio	accession no. of TIGR ID
		2 h			
cytochrome p450 family	22.53	Agt5g57220	zinc finger protein ATZF1	10.13	At1g08930
multidrug resistance P-glycoprotein	13.38	At3g62150	CHP-rich zinc finger protein	9.42	At5g40590
similar to pirin-like protein	13.14	At2g13120	AtRAD17 (dbj|BAA90479.1)	7.91	At5g66130
myb-like protein	11.71	At5g03780	copine-like protein	7.89	At5g6l900
		24 h			
Avr9 elicitor response-like protein	11.39	At4g26940	aquaporin (plasma membrane intrinsic protein 1 B)	8.38	At2g45960
14-3-3 protein GF14chi (grf1)	11.12	At4g09000	ethylene responsive element binding factor 2 (ATERF2)	8.14	At5g47220
homeodomain transcription factor (ATHB-6)	11.11	At2g22430	fibrillarin 1 (AtFibl)	7.87	At5g52470
E2. ubiquitin-conjugating enzyme, putative	9.76	At5g56150	translation factor EF-1 α-like protein	7.14	At4g22780

L*er*–*agl-l* (Suppressed Gene)

putative ID	ratio	accession no. of TIGR ID	putative ID	ratio	accession no. of TIGR ID
		2 h			
myrosinase-binding protein homologue	0.2	At1g52040	response regulator 6 (ARR6)	0.34	At5g62960
putative steroid sulfotransferase	0.21	At2g03750	bHLH protein	0.35	At5g65640
3-isopropylmalate dehydratase-like protein	0.26	At3g58990	bHLH protein	0.35	At5g15160
photosystem II oxygen-evolving complex 23	0.33	At2g30790	En/Spm-like transposon protein	0.35	At1g49450
		24 h			
prx 10 peroxidase-like protein	0.11	At5g15170	E2, ubiquitin-conjugating enzyme 17 (UBC17)	0.16	At4g36410
microtubule-associated motor-like	0.13	At5g60930	formamidase-like protein	0.18	At5g20630

ttg1-1–L*er* (Induced Gene)

putative ID	ratio	accession no. of TIGR ID	putative ID	ratio	accession no. of TIGR ID
		2 h			
ribonucleotide reductase small subunit	14.90	At3g27060	salt-tolerance zinc finger protein	11.95	At1g27730
anthocyanin 5-aromatic acryltransferase	13.42	At3g27060	cinnamoyl CoA reductase	9.57	At5g14700
		24 h			
myb-like protein	7.29	At5g03780	protein kinase, putative	3.92	At5g25250
NAM (no apical meristem)-like protein	5.26	At5g18270	calcium binding protein (CaBP-22)	3.53	At2g41090

ttg1-1–L*er* (Suppressed Gene)

putative ID	ratio	accession no. of TIGR ID	putative ID	ratio	accession no. of TIGR ID
		2 h			
dehydrin RAB18-like protein (sp P30185)	0.12	At5g66400	putative-lipid transfer protein	0.25	At2g15050
myh family transcription factor	0.17	At1g01520	bHLH protein	0.35	At5g65640
		24 h			
transcriptional activator	0.07	At5g6l620	root cap protein 2-like protein	0.24	At5g54370
phytocyanin-related protein	0.22	At5g25090	putative zinc transporter ZIP2	0.25	At5g59520
liquid-transfer protein precursor	0.22	At2gl3820	xyloglucan endotransglycosylase	0.25	At4g03210

[a] WD-40 mutant (*ttg1-1*) *Arabidopsis* genes whose transcription was induced or suppressed 2 or 24 h after γ-irradiation. Ratio: intensity of signal from γ-irradiated plants divided by that from unirradiated plants (for induced genes) or vice versa (for suppressed genes). L*er*–*ttg1-1* (induced): genes upregulated only in L*er* plants. L*er*–*ttg1-1* (suppressed): genes downregulated only in L*er* plants.

systems of the stress response, light response, carbohydrate metabolism, and protein storage pathways.

Microarray analyses revealed that AOS stress induces genes associated with stress response, light signaling, supplemental energy, and many transcription factors and suppresses those involved in carbohydrate metabolism, cell growth and division, and DNA synthesis. These candidate AOS response genes include many nonspecific stress response and housekeeping genes. Therefore, to select candidates more precisely, we performed further analyses and evaluations. The physiological reactions induced by AOS were categorized into two types: (i) those prompted by defense systems against oxidation of the biological object and (ii) those indicating inappropriate stimulation or overstimulation of AOS-regulated systems. With regard

Table 3. Highly Conserved 5′-*cis*-Element Sequences of Induced and Suppressed Genes after γ-Irradiations in Normal (L*er*) Plants[a]

category	*cis*-element
common	
signal transduction	ARF
	CATATGGSAUR
	ERE
	Myb
	Ry repeat
	WB box
stress response	DRE
	ELRE
	LTRE
light response	CACGTG motif
	CCA1
	evening element
	TBOX
carbohydrate metabolism	ACGTABOX
	prolamine box
	SURE
	TATCCAOSAMY
storage protein regulation	TGACGTVMAMY
	CANBNNAPA
others	SEF1 motif
	GT-1
	L1box
	pyrimidine box
induced gene specific	
storage protein regulatory element	CEREGLUbox
transcriptionfactory	OCTEMERmotif
light inclucible element	SV40core enhancer
	Rbcs consensus
carbohydrate metabolism	SURE
	TATTCA element
suppressed gene specific	
signal transduction	PAL box
	Q (quantitative) element
light response	Reα and β
hormonal signal transduction	NDE element
transcription regulation	Z-DNA forming sequence
hormonal signal transduction	ABRE
	AUXRE

[a] 5′-*cis*-Element sequences highly conserved between genes of wild-type (L*er*) *Arabidopsis* whose expression was either induced or suppressed after γ-irradiation.

Table 4. Highly Conserved 5′-*cis*-Element Sequences of Induced and Suppressed Genes after γ-Irradiations in *ttg1-1* Mutants[a]

category	*cis*-element
common	
signal transduction	CATATGGSAUR
stress response	ELRE
light response	CACGTG motif
	TBOX
carbohydrate metabolism	ACGTABOX
	TATCCAOSAMY
	TGACGTVMAMY
storage protein regulation	CANBNNAPA
others	L1 box
L*er*–*ttg1-1* (common)	
signal transduction	ARF
	RY repeat
	Myb
	WB box
strss response	DRE
	ERE
	LTRE
light rsponse	CCA1 binding site
	evening element
	GT1
carbohydrate metabolism	prolamine box
induced gene specific	
storage protein regulatory element	CEREGLU box
transcription factor	OCTEMER motif
light inclucible element	SV40core enhancer
L*er*–*ttg1-1* (induced)	
light response	Rbcs consensus
carbohydrate metabolism	SURE
	TATCCA element
suppressed gene specific	
hormonal signal transduction	ABRE
	AUXRE
L*er*–*ttg1-1* (suppressed)	PAL box
signal transduction	Q (quantitative) element
light response	Reα and β
hormonal signal transduction	NDE element
transcription regulation	Z-DNA forming sequence

[a] 5′-*cis*-Element sequences highly conserved between genes of WD-40 mutant (*ttg1-1*) *Arabidopsis* whose transcription was induced or suppressed after γ-irradiation. L*er*–*ttg1-1* (common), elements common to up- and downregulated genes in wild-type (L*er*) but not *ttg1-1* plants. L*er*–*ttg1-1* (induced), 5′-*cis*-element of upregulated genes found only in L*er* plants. L*er*–*ttg1-1* (suppressed), 5′-*cis*-element of downregulated genes found only in L*er* plants.

to participants in AOS defense systems, the synthesis of low molecular weight antioxidants (e.g., ascorbic acid, glutathione S-transferase, and anthocyanin) has been reported (*15*−*18*). Induction of genes for stress response, signal transduction, biosynthesis, transporter, and supplemental energy is thought to indicate activation of the defense system. AOS-regulated systems affect trichome differentiation, leaf development, root development, and disease resistance (*3*, *6*, *10*, *19*). The induction of genes for transcription factors, signal transduction molecules, and metabolites is thought to indicate inappropriate stimulation or overstimulation of AOS-regulated systems.

Desikan et al. described the *Arabidopsis* transcriptome in regard to another oxidative stress (H_2O_2 stress) (*20*). To distinguish the γ-irradiation specific gene expression pattern and the AOS response gene expression pattern, we compared the results of our microarray analysis with the results of H_2O_2 stress (**Table 5**). We also compared the transcription patterns of WD-40 mutant plants and those exposed to H_2O_2 stress. The inability of the WD-40 mutant (*ttg1-1*) to respond to AOS stress indicates that WD-40 is a key protein in the control of AOS responses. Therefore, we screened the genes specifically upregulated in wild-type (L*er*) *Arabidopsis* as the AOS-related signal transduction genes and those specifically downregulated in L*er* plants as the WD-40-related ones. H_2O_2 stress induced AOS responses similar to those induced by γ-irradiation. Of the genes screened, those for the homeo domain transcription factor, ethylene responsive element binding factor, PRX10 peroxidase, blue copper-binding protein, mitochondrial uncoupling F1 factor, cellulose synthase, calmodulin, CDPK, MAPK, phospholipase, ring finger proteins, heat shock proteins, myb, syntaxin, extensin, and receptor-like protein kinase were AOS responsive genes common to both types of stimulation (**Table 5**). Therefore, proteins involved in redox reactions, the MAPK cascade, ethylene response, WD-40 related pathway, heat shock reaction, and calcium signal transduction, as well as other transcription factors (e.g., homeo box, ring finger), may have roles in the response to AOS.

We performed 5′-*cis*-element analyses to reveal the signal transduction pathway of AOS stimulation. By comparing all of the above results for L*er* plants, WD-40 mutants, and plants exposed to H_2O_2, we found that signal transduction (ARF, myb, and WB box), stress response (DRE, elicitor responsive element, and LTRE), light inducible response (CCA1 binding site, evening element, and GT-1 motif), carbohydrate metabolism

Table 5. Comparison of Expression Data of Induced and Suppressed Genes after γ-Irradiations and H_2O_2 Treatment in Normal (L*er*) Plants[a]

category	putative ID
induced genes (common)	
cellular organzation and biogenesis	blue copper-binding protein
	putative mitochondrial uncoupling protein
	cellulose synthase catalytic subunit
signal transduction	calmodulin
	calcium-dependent protein kinase
metabolism	cytochrome P450
	PAL1
	GST6
	monodehydroascorbate reductase
transcription	phospholipase-like protein
	heat shock transcription factor
	ring Zn finger protein
	myb-related transcription factor
protein destination and transport	syntaxin
cell rescue/defense	lipoxygenase
	peroxidase
γ-irradiation specific	
signal transduction	ethylene responsive element binding factor
metabolism	14-3-3 protein
	sucrose-phosphate synthase
	glycoprotein
	L-ascorbate oxidase
transcription	homeodomain transcription factor
	NAC domain protein
	WRKY
protein destination and transport	ubiquitin-conjugating enzyme
	aquapolin
	H^+-transporting ATPase
	ABC transporter
cell rescue/defense	Avr9 elicitor response protein
	PR-1
	GST6
H_2O_2 specific	
cellular organzation and biogenesis	lipid transfer protein
	ras GTP-binding protein
signal transduction	histidine kinase
	try phosphatase
metabolism	nitrite reductase
	stearoyl acyl carrier protein
transcription	DREB2A
	ethylene responsive element binding factor (EREBP-4)
protein destination and transport	12S cruciferin seed storage protein
cell rescue/defense	salt inducible calcium-binding protein
supressed genes (common)	
metabolism	
cellular organzation and biogenesis	berberine bridge enzyme-like protein
	putative pectinesterase
energy/chloroplast located	lipid transfer protein
	extensin homologue
protein distribution/destination/	chloroplast 50S ribosomal protein
transport/cell rescue/defense	chloroplast protease-like protein
	p-glycoprotein
	receptor-like protein kinase
γ-irradiation specific	
signal transduction	serine protease
	acyltransferase
metabolism	β-carrotene hydroxylase
	β-galactosidase
	zeaxanthin epoxidase
	formidase
transcription	NAM
	bZIP
protein destination and transport	peptide transporter

[a] Comparison of AOS and WD-40 responsive wild-type (L*er*) plant genes whose transcription was up- or downregulated after γ-irradiation or H_2O_2 treatment.

regulation (prolamine box), storage protein gene-controlling [SEF1 motif, (CA)n] elements, and others (e.g., hexamer motif, L1 box, and pyrimidine box) remained candidates for involvement in AOS responsive reactions. Therefore, myb, the WRKY-related pathway, redox, stress, and light−response systems and the MAPK cascade remained as potential AOS response systems. These results were similar to those obtained for γ-irradiated rice (Kishimoto, personal communication). After γ-irradiation of rice, signal transduction (e.g., Ser/Thr protein kinase), stress response (e.g., salt- or aluminum-induced), and

redox (e.g., peroxidase) pathways were induced, and the transcription of light-induced, pathogenesis-related, and cell growth proteins was suppressed. Our 5′-*cis*-element analyses indicated that myb, DRE, WRKY, and other factors were common to both rice and *Arabidopsis*, thereby confirming a pattern common to monocots and dicots.

In taking an overall look at all the available information, we can hypothesize that the genes involved in redox reactions (e.g., peroxidase, copper-binding protein), signal transduction (e.g., myb, WRKY, homeo box, and ring finger proteins), the MAPK cascade (e.g., MAPK), the calcium−calmodulin cascade (e.g. calmodulin, CDPK), stress responses (e.g., heat shock, salt stress), and light-induced responses are the keys to the AOS response. AOS-associated energy and/or electrons might oxidize biological material or be taken up by electron-accepting systems such as the photosystem. Therefore, redox and light inducible signal transduction mechanisms would be activated and would, in turn, induce the next level of gene network systems, comprising signal transduction factors (e.g., myb, WRKY, homeo box, and ring finger proteins), the MAPK cascade, the calcium−calmodulin cascade, and the stress response pathway. Next, we plan to further analyze individual candidate genes.

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