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Gene transcriptomic profile in arabidopsis thaliana mediated by radiation-induced bystander effects

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► ***Original article***

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ABSTRACT

Background*:** The in vivo radiation-induced bystander effects (RIBE) at the developmental, genetic, and epigenetic levels have been well demonstrated using model plant Arabidopsis thaliana (A. thaliana). However, the mechanisms underlying RIBE in plants are not clear, especially lacking a comprehensive knowledge about the genes and biological pathways involved in the RIBE in plants. ***Materials and Methods*:** A high-density oligonucleotide probe-based cDNA microarray was used to analyze transcriptomic response in aerial leaf tissues of A. thaliana seedlings at 24 h after root exposure to 10 Gy of α-irradiation. ***Results: The root-localized irradiation resulted in up-regulated expressions of 238 genes and down-regulated expressions of 42 genes in bystander aerial tissues with a ≥ 2.0-fold difference and < 0.05 p-values. The high frequency of gene families for up-regulated expressions were glutathione S-transferases, cytochrome P450 enzyme, the ethylene response factor, and the multidrug and toxic compound extrusion efflux, and for down-regulated expressions was the later embryogenesis abundant protein. Moreover, there were 200 up-regulated genes and 183 down-regulated genes with 1.5–2.0-fold expression changes. ***Conclusion:*** In addition to the canonical IR-induced genes, some genes that are not previously linked to radiobiological effects were found to be involved in RIBE in plants.

Keywords: *Radiation-induced bystander effects, transcriptomic profile, cDNA microarray, Arabidopsis thaliana, α-particle irradiation.*

INTRODUCTION

The canonical radiation biology dogma describes the effects of ionizing radiation that are restricted to directly hit cells [1]. Radiation-induced bystander effects (RIBE), however, represent a paradigm shift in the understanding of the radiobiological effects, in which biological effects are induced in non-hit cells when their neighboring cells are irradiated [2]. The RIBE have been well demonstrated in single-cell culture models [1, 3-5], multi-cellular tissue models [6-11] and whole organisms [12-19].

Recently, the RIBE in model plant *Arabidopsis thaliana* (*A. thaliana*) have been well investigated in our research team [20-28]. After micro-beam irradiation of naked seed embryos and low-energy ion irradiation of intact seeds, some of the post-embryonic developmental phenotypes were significantly inhibited, which differentiate from the non-irradiated shoot apical meristem cells and root apical meristem cells [20, 21]. We also demonstrated long-distance bystander mutagenic effects with root-irradiation in growing seedlings and dormant seeds of *A. thaliana*. The root-localized irradiation resulted in an enhanced level of DNA damage, which further promoted the activity of the homologous recombination repair machinery characterized by the up-regulation of the *AtRAD54* gene expression and an increased homologous recombination frequency [22, 23].

Some changes in DNA epigenetic status, such as DNA methylation and transcriptional gene silencing (TGS), were also observed in the RIBE in plants [28]. In addition to the testing for various types of biological endpoints, the signal pathways of ROS [22, 24, 28] and Jasmonic acid (JA) [27, 29] have also been found to take part in the induction of RIBE. Although so, there is no yet a comprehensive knowledge about the molecular mechanisms underlying the RIBE in plants.

Transcriptomic profile can generate useful information about the differential expression of genes under various stress conditions [30], and can be analyzed using a high-throughput microarray that provides detailed information on a genome-wide scale with advantages in analysis speed, accuracy and comprehensiveness [31]. Recently, the microarray for transcriptomic profile has been widely applied for plant researches [32]. Here, we adopted the high-density oligonucleotide probe-based cDNA microarray to profile gene expressions in the bystander aerial tissues of *A. thaliana* seedlings after root exposure to α-irradiation. The resulting data from gene transcript arrays were further analyzed on multiple levels, including distribution and extent of transcriptional changes, the significance of gene up-regulation, the high frequency gene families, biological processes, and pathways.

MATERIALS AND METHODS

A. thaliana Lines and Plant Growth

The wild-type *A. thaliana* line (Columbia ecotype) was obtained from the NASC (Nottingham Arabidopsis Stock Center, UK).

A. thaliana seeds were soaked in water and incubated in the dark at 4°C for two days before they were sown on growth medium (1× Murashige and Skoog (MS) mineral salts, 0.8% agar [w/v], and 1% sucrose [w/v]) in square petri dishes that were then placed in a growth chamber in a vertical orientation. The growth chamber was maintained at 22°C, with an illumination of approximately 100 μM m^2 s^{-1} and a 16-h light/8-h dark cycle.

Root-Localized Irradiation with α-Particles

α-particles emited from a ^{241}Am source with an activity of 7.4 MBq in the Rotate-Adjustable α -particle Source Facility. Root-localized irradiation of *A. thaliana* was performed as described previously [22], and is also shown schematically in figure 1. In the present study, the 7-day-old seedlings were used for root exposure to α- particles. The average energy of α - particles measured 3.3 MeV, and the particles were delivered at a dose rate of α-particles of 1.51 $cGys^{-1}$.

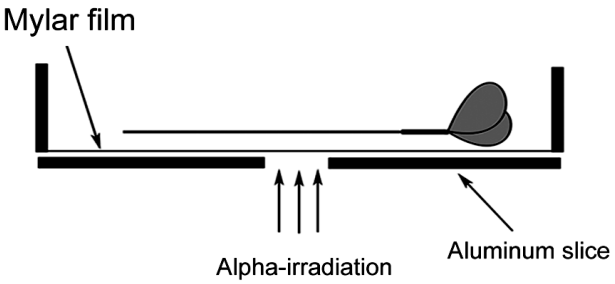

Figure 1. Schematics of the root-localized irradiation of A. thaliana with α-particles.

Microarray Analysis

Three control groups (N1-N3) and three irradiated groups (P1-P3) were sampled and frozen in liquid nitrogen at 24 h after root irradiation, and total RNA from aerial tissues was extracted using the Trizol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The quantity and quality of isolated total RNA were assessed by spectrophotometry and gel electrophoresis, respectively. All the microarray experiments were performed using Agilent *Arabidopsis* (V4; 4 × 44k) microarrays by CapitalBio Corp. (CapitalBio Corp., Beijing, China). Microarrays were scanned with a LuxScan™ 10K confocal laser scanner (CapitalBio Corp.), and the resulting images were analyzed with SpotData software (CapitalBio Corp.). Spots with fewer than 50% of the signal pixels that exceeded the local background value for both channels (Cy3 and Cy5), plus two standard deviations of the local background, were removed from the analysis. This step further ensured that spots with characteristic doughnut shapes that are

often encountered on microarrays would not be part of the subsequent analysis. An intensity-dependent program (LOWESS) in the R language package was used to normalize the ratio values. Identification of differentially expressed genes and determination of statistical significance were performed using GeneSpring GX software (Agilent Technologies Inc, Santa Clara, CA, USA). These genes were cluster analyzed using the Cluster 3.0 software (University of Tokyo, Tokyo, Japan). Genes that had ≥2-fold expression change (*P*-value < 0.05) and that were annotated in the TAIR database were selected for Gene ontology (GO) analysis and pathway analysis using the Molecule Annotation System (CB-MAS_3.0, http:// bioinfo.capitalbio. com/mas3/).

Real-time PCR analysis

The total RNAs were extracted using the Trizol reagent (Invitrogen, USA) according to manufacturer's protocols 24 h after root-localized irradiation. Total RNA was reverse transcribed using Transcript One-Step gDNA removal and the cDNA synthesis Supermix kit (Transgen Biotech, China) according to manufacturer's protocols. The qRT-PCR was conducted with the ABI StepOne plus system (Applied Biosystemst, Carlsbad, CA) according to the manufacturer's instructions. Each PCR reaction (20 µl) contained 10 µl 2× real-time PCR Mix, 0.2 µM of each primer and cDNA. The qRT-PCR was performed under the following conditions: 95°C for 10 sec for one cycle; 95°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec for 40 cycles. The primers used are listed in table 1, which also indicated that the *ACTIN2* gene was used as an internal control. The final data were compiled as the average of three independent experiments, with three technical replicates for each experiment. The statistical significance between control and irradiated groups was analyzed using the Origin 7.5 software (OriginLab Corporation, Northampton, MA, USA).

Statistical analysis

All the results are presented as mean ± standard deviation. The statistical significance of the experiments was determined by performing student's *t* test. A *P*-value of 0.05 or less was considered significant.

Table 1. qRT-PCR primer sequences used for validation of the microarray analysis.

PXMT1-F	5'-CAGCGCTGGAGTTCCTGGTT-3'
PXMT1-R	5'-CCCGTGCACTGCATGTCTTT-3'
AtGSTU9-F	5'-GGTGAACCAACTGTGACGAACG-3'
AtGSTU9-R	5'-TCCACCCGTAGACACCAGGAA-3'
AT2G04050-F	5'-ATGGGGTTGCAAGGGGAAGT-3'
AT2G04050-R	5'-TCCGACCACAACACCACACC-3'
CYP710A1-F	5'-TGTTCGGCGAGGATCACAAA-3'
CYP710A1-R	5'-ACGGACAAGCTGTCGCAGTG-3'
ABR1-F	5'-TTGGCTCGGTACGTTCGACA-3'
ABR1-R	5'-AGCGGTTTGGTGCACAGGTT-3'
LEA7-F	5'-TGTTTGTTGCGTTCGTGAGG-3'
LEA7-R	5'-TGTAATTTCCGTACTAATCACCCG-3'
AT3G21460-F	5'-TTACGAGCAAGGTGTGAGCC-3'
AT3G21460-R	5'-AACAAAAACCGCAGGAACCG-3'
ACTIN2-F	5'-CTAAGCTCTCAAGATCAAAGGC-3'
ACTIN2-R	5'-AACATTGCAAAGAGTTTCAAGG-3'

RESULTS

Microarray-based expression profile

After the microarray experiment, a hierarchical cluster analysis was performed for these genes with ≥ 2-fold expression changes ($P < 0.05$). As shown in figure2, In this map 238 genes were listed for up-regulated expression and 42 genes for down-regulated expression (figure 2). Next, these listed genes were further sorted according to the extent of their fold changes. Fold changes in up-regulated and down -regulated genes were represented by positive and negative numbers, respectively. As shown in figure 3, nearly half of the genes (132 genes) had 2- to 3-fold up-regulated expressions, and expressions of 18 genes increased by more than 7-fold. In contrast, only 42 genes were down-regulated by the root irradiation (≥ 2-fold change), most of which (28 genes) had 2- to 3-fold changes. It is worth noting that the RIBE are typical weak radiation responses, therefore, the genes whose expression changes between 1.5- and 2-fold should also be considered in RIBE. According to this judgment, there were additional 200 up-regulated genes and 183 of down-regulated genes. These results suggest that RIBE might mediate an extensive gene expression changes.

Validation of microarray results by qRT-PCR analysis

To confirm the microarray results, the up-regulated *AtGSTU9, AT2G04050, ABR1, CYP710A1,* and *PXMT1* genes and down-regulated *LEA7* and *AT3G21460* genes were representatively chosen for qRT-PCR analysis. As shown in figure 4, the expression patterns for all tested genes were coincident with the data in microarray analysis although the fold changes were different. The microarray analysis showed that the fold change was 17 for *AtGSTU9*, 13 for *At2G04050*, 6.5 for *ABR1,* 8 for *CYP710A1*, and 5.8 for *PXMT1*, whereas their fold changes in qRT-PCR analysis were 4.3, 3.4, 6.5, 4.1, and 1.6 compared to the controls, respectively. The fold changes in microarray analysis were 8.4 for *LEA7* and 5.3 for *AT3G21460*, whereas their fold changes in qRT-PCR analysis were 1.8 and 9.5 compared to the control groups, respectively. The differences might mainly be due to the different signal extraction and comparative methods between microarray and qRT-PCR analysis [33].

Gene families, biological processes, and biological pathways involved in RIBE

Fourteen of highly inducible genes included the AT5G55150 (25.34-fold), AT5G62480 (*ATGSTU9*) (17.69), AT2G04050 (13.71), AT2G34500 (*CYP710A1*) (13.55), AT1G13340 (11.64), AT3G22231(*PCC1*) (11.43), AT2G38823 (10.99), AT1G32350(*AOX1D*) (10.26), AT3G49540 (10.22), AT5G01380 (9.09), AT5G24640 (8.51), AT3G54530 (8.12), AT1G66700 (*PXMT1*) (7.93), and AT3G19615 (7.58). Among them, the biological function of the highest induced AT5G55150 gene was not yet characterized, and the second is the gene of Glutathione transferase (GSTs), and the fourth was the gene of C22-sterol desaturase, suggesting an involvement of oxidative stress responses in RIBE. We further classified the genes with ≥2-fold expression changes into different families. As shown in table 2, 12 of the up-regulated genes belong to GST family, accounting for 5.1% of total up-regulated genes identified in the microarray analysis. Other up-regulated genes are the members of the cytochrome P450 enzyme family [5], ethylene response factor (ERF) subfamily [3] and MATE efflux family [3], respectively. Moreover, five down-regulated genes were from the Later Embryogenesis Abundant protein (LEA) family, which account for 11.9% of total down-regulated genes. These results suggest that these gene families might be involved in the RIBE.

We further sorted out these inducible and repressive genes based on their annotated biological processes. These genes were distributed into 10 biological process groups, most of which correlate with oxidative stress responses (table 3). Of these 28 genes were associated with oxidation reduction and 14 with oxidative stress. They were further clustered into six biological pathways, as shown in table 3.

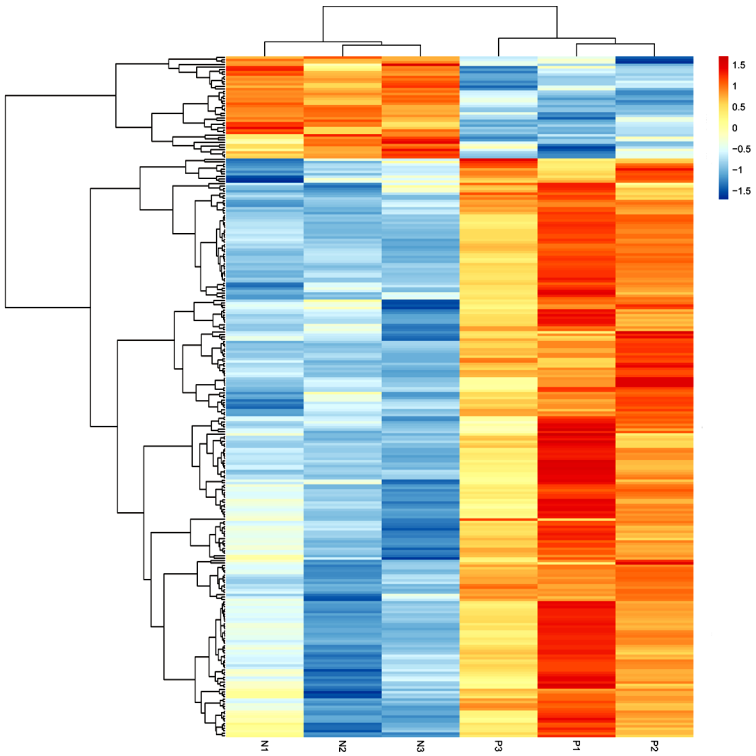

Figure 2. Hierarchical cluster analysis of 280 genes with > 2-fold expression change. Each horizontal line represents the expression data for one gene in aerial plant tissues from control and root-irradiated aerial plants. Colors show the normalized expression level. Induction (or repression) ranges from white to red (or blue) with a fold-change scale bar shown on the right of cluster. N1-N3: control groups, P1-P3: irradiated groups.

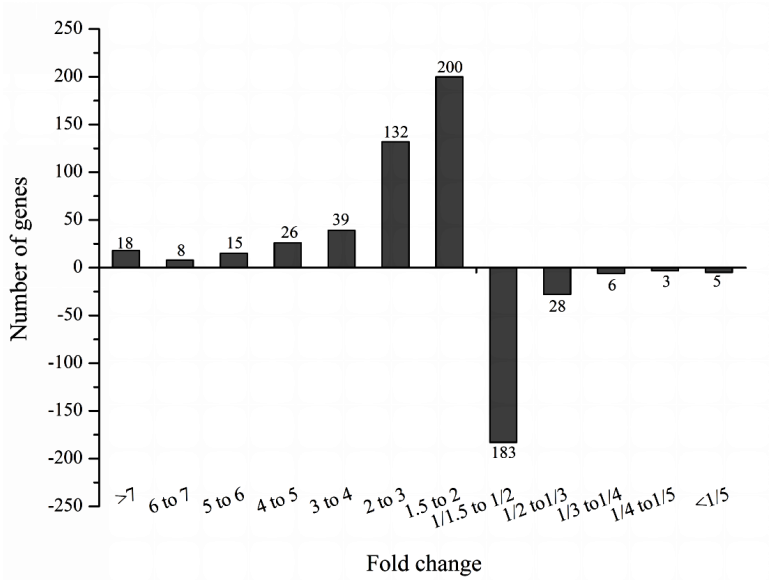

Figure 3. The number of RIBE-mediated genes with different fold changes, and the numbers above the column represent the number of genes with the specific fold changes.

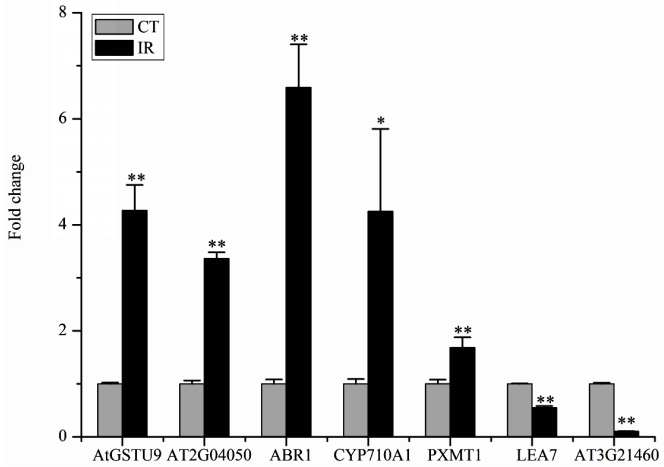

Figure 4. qRT-PCR analysis of 7 representative genes. These genes were selected from the significantly up-regulated or down-regulated gene groups for validation of the microarray analysis. Results are expressed as the means ± SD (n=3, t test * P<0.05 and ** P < 0.01).

Table 2. The high frequency of gene families involved in RIBE.

Gene family	Gene	Fold change	regulation
GSTs	ATGSTU9	17.68889	up
	ATGSTL1	5.962991	up
	ATGSTU25	4.605921	up
	ATGSTU11	4.441361	up
	ATGSTF6	3.820138	up
	ATGSTF2	3.350726	up
	ATGSTU4	3.16003	up
	ATGSTF3	3.107264	up
	ATGSTF7	2.694439	up
	ATGSTF12	2.399328	up
	ATGSTF9	2.327342	up
	ATGSTF11	2.073808	up
Cytochrome P450 enzyme	CYP710A1	13.546226	up
	CYP76C2	5.886769	up
	PAD3	5.855027	up
	CYP79F1	2.956539	up
	CYP83A1	2.118532	up
MATE efflux family protein	AT2G04050	13.71277	up
	AT2G04066	3.94306	up
	AT2G04070	3.086136	up
ERF (ethylene response factor) subfamily	ABR1	6.540907	up
	AT2G47520	4.83663	up
	AT5G13330	2.129644	up
Late embryogenesis abundant protein (LEA) family protein	AT1G52690	8.352396	down
	AT3G17520	4.750286	down
	AT5G06760	3.013772	down
	AT3G02480	2.796134	down
	AT2G41280	2.005858	down

Table 3. Gene ontology analysis of the biological processes and pathways involved in RIBE. p-value is the probability of seeing at least x (count) differential expression gene out of the total genes in the list annotated to a particular term. The q-value is the adjusted p-values found using an optimized FDR approach.

Biological processes (GO Term)	Count	*p*-Value	*q*-Value
GO:0009407 toxin catabolism	13	1.78E-20	3.35E-18
GO:0055114 oxidation reduction	28	6.1E-13	3.82E-11
GO:0006979 response to oxidative stress	14	2.36E-11	1.11E-09
GO:0009098 leucine biosynthesis	5	1.48E-09	5.57E-08
GO:0019761 glucosinolate biosynthesis	5	1.93E-07	5.19E-06
GO:0009414 response to water deprivation	6	8.73E-05	0.001263
GO:0009768 photosynthesis, light harvesting in photosystem I	2	0.000143	0.00192
GO:0007568 aging	4	0.0002	0.002198
GO:0042631 cellular response to water deprivation	2	0.000214	0.002198
GO:0009737 response to abscisic acid stimulus	7	0.000222	0.002198
Biological Pathways			
Metabolism of xenobiotics by cytochrome P450	5	7.04E-13	9.24E-13
Drug metabolism - cytochrome P450	5	9.24E-13	9.24E-13
Glutathione metabolism	5	1.17E-10	7.78E-11
Valine, leucine and isoleucine biosynthesis	3	8.26E-07	4.13E-07
Phenylpropanoid biosynthesis	3	0.000119	4.78E-05
Naphthalene and anthracene degradation	2	0.000677	0.00018

DISCUSSION

In this study, we analyzed the genome-wide responses of *A. thaliana* to RIBE based on the microarray-based transcriptomic profile. It is well known that unlike irradiation-targeted effects, RIBE consist of production of bystander signals in the hit tissues (cells) and irradiation responses in non-hit bystander tissues (cells). In the experiments of the root-localized irradiation, due to the low-penetration potential of α-particles, the root cells directly traversed by α-particles cannot be accurately defined and separated from the naïve root cells. Therefore, in this study we only investigated the transcriptomic responses in bystander aerial tissues. Interestingly, a γ-induced transcriptomic profiles in aerial leaf tissues of *A. thaliana* has been reported [33]. Comparing to the transcriptomic profiles in directly irradiated tissues in the work by Kim et al, we found that gene expressions in two irradiation patterns were completely different, and the direct irradiation leads to more than 3000 differentially expressed genes over 280 genes by the RIBE. This definitely indicates that the RIBE are weak radiobiological response relative to the direct radiation effects. However, in these two experiments, the differences in radiation quality (α-particles versus γ-rays) and radiation doses (10 Gy versus 200 Gy) also attenuated the reasonability of the comparison. Therefore, further experiments for comparing the transcriptomic responses in directly irradiated and bystander plant tissues should be carried out under the same irradiation conditions.

It has been reported that ROS play a pivotal role in mediating RIBE in plants [22, 24, 28], in accordance with the up-regulated gene expressions of P450 family in the microarray analysis, in which five family members and two pathways were activated (tables 2, 3). Considering the role of P450 in oxidation of various substrates [34], they might mainly contribute the oxidative stress in bystander aerial tissues. The cells can accordingly initiate antioxidant mechanisms to eliminate the oxidative stress. It has been reported that GST family takes part in this process [35], and the microarray analysis also showed that 12 genes of this family were highly inducible (table 2). In *A. thaliana*, the GST family contains 97 members that are organized into seven classes: *phi* (*GSTF*), *tau* (*GSTU*), *zeta* (*GSTZ*), *theta* (*GSTT*), *lambda* (*GSTL*), *dehydroascorbate reductase* (*DHAR*) and *tetrachloro-p- hydroquinone dehydrogenase-related* (*TCHQD*) [36-38]. In the present study, four *tau* genes and seven *phi* genes were found among the activated GST genes, indicating that the GSTU and GSTF were two important GST sub-families in the RIBE.

The microarray analysis also provided some new clues about the irradiation responses in bystander aerial tissues, such as the up-regulated expressions of *ERF* genes and the down-regulated expressions of the *Late Embryogenesis Abundant protein* (*LEA*) family. It is well accepted that the plant hormone ethylene is a regulator of a variety of developmental and stress responses in plants [39]. However, there is no yet evidence about its involvement in RIBE. Moreover, it has been reported that the acquisition of desiccation tolerance during the late stages of seed development is correlated with the induction of LEA proteins [40]. The *LEA* genes usually function in the late stages of seed development and their down-regulation in RIBE is hard to explain. We speculate that the RIBE might disturb plant development, leading to repressed expressions of *LEA* genes as a secondary reaction to plant development arrest.

In addition to these genes with > 2-fold expression change, the genes with 1.5- to 2-fold expression change should also be included in the RIBE, mainly considering that the RIBE are weak radiobiological responses relative to the radiation-targeted effects. In our previous studies, the DNA repair genes and TGS-silenced genes were definitely up-regulated in the bystander aerial tissues [22, 28]. Their expression changes were between 1.5- and 2-fold in the microarray analysis. With the judgment of 1.5–2 -fold change, there were 200 up-regulated and 183 down-regulated genes. The deeper exploration to these genes might provide more insights into the mechanisms underlying the RIBE.

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***Conflicts of interest*:** *Declared none.*

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