

# Analysis of Gene Expressions Associated with Response of Phenolic Metabolism of Allelopathic Rice under Barnyardgrass Stress

ElShamey EAZ<sup>1,2\*</sup>

<sup>1</sup>Rice Research Department, Field Crops Research Institute, Agricultural Research Center, Egypt

<sup>2</sup>School of Plant, Environmental & Soil Sciences, Louisiana State University, LA, USA

## Research Article

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**\*Corresponding author:** ElShamey EAZ, Rice Research Department, Field Crops Research Institute, Agricultural Research Center, Egypt, School of Plant, Environmental & Soil Sciences, Louisiana State University, LA, USA, Tel: +201094194215; Email: [essamelshamey@gmail.com](mailto:essamelshamey@gmail.com)

## Abstract

Allelopathic rice varieties Giza 179 and Sakha 103 were induced by exogenous salicylic acid (SA) to suppress barnyardgrass weed (BYG) and were investigated using suppression subtractive hybridization (SSH) and real-time fluorescence quantitative PCR (qRT-PCR) methods. The results explained that exogenous salicylic acid could induce the allelopathic effect of rice on BYG. The variety Giza 179 showed higher inhibitory effect than Sakha 103 variety on BYG after treated with different concentrations of salicylic acid. Fourteen genes induced by salicylic acid were obtained by SSH analysis from Giza 179 variety. These genes encode ubiquitin carrier proteins, proteins related to phenyl-propanoid metabolism, receptor-kinase proteins, antioxidant related proteins and some growth-mediating proteins. The variations among gene expressions were validated by qRT-PCR in the two rice varieties. Previous studies indicated that cytoplasmic male sterile lines IR69625A, and G46A had general combining ability for panicle weight and grain yield per plant, while the varieties Giza 179 and Giza 178 had general combining ability for grain yield per plant and seed set percentage. In addition, with regard to the variety Giza 179 which have allelopathic potential and restoring fertility genes, so we recommend by using the variety Giza 179 as parental line with the used cytoplasmic male sterile line to produce hybrid rice have highly yield and strong allelopathy.

**Keywords:** Barnyardgrass; Allelopathic Rice

## Introduction

Weeds are the major biological constraint in crop production this problem has traditionally been solved by

hand-weeding. Presently, access to herbicides is helping in decreasing the weed problem. However, increasing farm labour costs and environmental concerns about pesticides usage make it increasingly important to find

alternative and sustainable weed management methods. One ecological strategy of weed control, allelopathic activity, has drawn increased attention. Allelopathy is the inhibition of growth of a plant due to biomolecules released by another [1]. The biomolecules are called allelochemicals and are produced by some plants as secondary metabolites. When the allelochemicals are released into the environment, they inhibit the development of neighboring plants. Allelopathic compounds may be released into the environment from plants by means of root exudation, leaching, volatilization and decomposition of plant residues in the soil.

Allelopathy of rice has been shown to be induced by jasmonic and salicylic acids, which are important signaling molecules regulating inducible defense genes against the pathogen infection. Potential allelochemical isolated from root exudates of the rice cultivar Giza 179 is momilactone B. Momilactone B inhibits the growth of typical rice weeds like barnyardgrass and *Echinochloa colonum* at concentrations greater than  $1\mu\text{mol/L}$  [2]. Rice plants secrete momilactone B from the roots into the rhizosphere over their entire life cycle. The secretion of momilactone B was also confirmed for other rice cultivars. These observations suggest that rice allelopathy may be primarily dependant on the secretion of momilactone B.

In recent years, rice allelopathy has been extensively studied as it is considered as one of the environment-friendly alternative approaches in weed control [3]. Evidences in earlier studies have indicated that rice allelopathy is a quantitative trait, which is mediated by both genetic effects and environmental conditions [4]. The genetic control of allelopathy in rice has been assessed. Studies have shown that rice allelopathy is an inducible trait, which could be mediated by both biotic and a biotic stress conditions such as nutrient starvation and higher accompanying weed densities [5]. Hence, application of plant activators to induce resistance or increase allelopathic potential has become a new strategy for weed control and is expected to be an alternative for effective and sustainable management. However, crucial questions remain unanswered. Investigation of plant activators (e.g., exogenous SA) and their relationship with induced allelopathy would provide valuable information for the understanding of rice plant defense responses and therefore would stimulate discovery of useful resources from allelopathic rice crops and facilitate the development of effective weed control measures. Our work on rice (*Oryza. sativa* L.) showed that salicylic acid

stress enhanced phytotoxic secondary metabolites in both plant tissues and culture solution.

The allelopathic potential of rice was closely related to the types and concentrations of phenolic compounds especially under stress conditions, in which the activities of related enzymes involved in the phenyl propanoid metabolic pathway in allelopathic rice was significantly increased [4]. This has been considered to be related to salicylic acid (SA)-mediated signal transduction pathway [6]. Previous studies have indicated that SA is an immanent compound of phenol in plants and is involved in many physiological reactions as well as signal transduction that trigger off cascade reactions functioning in the induction of systemic acquired resistance (SAR). It was also shown that exogenous SA could induce the accumulation of phenylalanine ammonia-lyase (PAL) mRNA and the synthesis of new PAL protein, resulting in increased enzyme PAL activity in crop plants against pest and weed infection [7].

In this communication, we present a study on the effect of exogenous SA on rice varieties and demonstrate the potential of induced allelopathy in suppression of the target weed. The molecular mechanisms underlying the physiological alterations of rice roots in response to SA treatment will exam and analyze using suppression subtractive hybridization (SSH) and real-time fluorescent quantitative polymerase chain reaction (qRT-PCR) methods. The present investigation was undertaken to study the following objectives; (I) investigation of different gene expression patterns in allelopathic and non-allelopathic rice varieties treated with salicylic acid in rice/weed mixtures, (II) avoid increase the weed seed bank in the soil during field screening and increase the selection efficiency for allelopathic activity in rice breeding, (III) increase the yield productivity and improvement the quality of the rice varieties, (IV) decrease both of water usage rate and herbicides during rice season.

## Materials and Methods

### Plant Materials

This experiment was conducted at experimental farm of Rice Research and Training Center (RRTC), Sakha, Egypt during season 2016 and 2017 and School of Plant, Environmental, & Soil Sciences, Louisiana State University, LA, USA, during season 2018. Nineteen genotypes using a line x tester model, which including four CMS lines, three testers and twelve crosses were used to study the effect of salicylic acid as inducer to

allelopathic activity. The experimental design was RCBD in three replicates. Observations were taken on five random plants from each plot. The crop was raised following the package of recommendation practices for summer season at the research farm of RRTC.

### Experiment 1: Rice Allelopathy induced by Exogenous SA

Seeds of Rice varieties Giza 179 high allelopathic potential and non-allelopathic rice Sakha 103 as well as barnyardgrass (*E. crus-galli* L.) will germinate and then sowing on seedling trays, respectively. When the seedlings of rice and barnyardgrass reached 3-leaf and 2-leaf stages, 50 uniform seedlings of each material were selected, transplant into holes spaced at 5 x 6 cm<sup>2</sup> in a Styrofoam plate, and a cotton plug was inserting into each hole to stabilize the seedlings. The Styrofoam plate was float on a pot fill with 10 L Hoagland nutrient solution (normal nutrient condition) according to Kim SY, et al., [8]. Three SA treatments were set at the concentrations of 0.1, 0.2, and 0.3 mM. The rice seedlings were sprayed with 10 ml of each SA solution in triplicate, respectively in the mixture of rice/weed and the control was sprayed with the same volume of distilled water.

After treated for 3 days, the roots of 10 rice seedlings and five accompanying barnyardgrass seedlings were sampled from each treatment and the control for biochemical analysis. The roots were rinsed for 10s in distilled water and then the root physiological activity in terms of the assimilated amounts of methylene blue per unit of time was analyzed following the method described by Xiong J, et al., [4]. At 7d after treatment with SA, the rest of barnyardgrass (five plants) in the mixture of rice/weed were sampled and the harvest tissues were snap-kill in an oven at 105°C for 20min and then dried at 70°C until constant weight. The dry weight (DW) of barnyardgrass was used to determine the inhibition rates (IRs) above control calculated as:  $IRs = (\text{control} - \text{treatment}) / \text{control} \times 100\%$ , which indicates inhibition when  $> 0$  and promotion when  $< 0$  [3]. All data were subjected to analysis of variance using the Statistical Analysis System Program (SPSS). Each value is expressed as the mean of three replicates.

### Experiment 2: Molecular Responses to the Induction of Exogenous SA

The seedlings of allelopathic rice varieties as well as the target weed barnyardgrass were prepared as described in experiment 1. After 7 days of recovery in the Hoagland nutrient solution, 30 rice seedlings and 10 weed

seedlings were transplant into a Styrofoam plate to form a hydroponic rice-weed mixture with the barnyardgrass seedlings as the target plants (receptor plants) in the center, surrounded by the rice seedlings. The root tissues of the rice seedlings were randomly sampled at 72 h after sprayed with 10 ml of 0.2 mM exogenous SA (treatment) or distilled water (control). After harvested, the samples were immediately frozen in liquid nitrogen and stored at -80°C prior to SSH and qRT-PCR analyzes.

### Construction of Subtractive Library

Suppression subtractive hybridization (SSH) according to was employ to construct a subtractive library. Total RNA was extract from the roots of SA-treated rice and its control using the Trizol method (Invitrogen) [9]. Genomic DNA was degrading using DNase I (TaKaRa). cDNA was synthesize using SMART PCR cDNA Synthesis Kit (Clontech). SSH was perform using the PCR-Select cDNA Subtraction Kit (Clontech). After two hybridizations, a nested PCR was used to selectively amplify differentially expressed fragments. PCR products were purifying with E.Z.N.A. Cycle-Pure Kit (OMEGA) and insert into pMD18-T Vector (Takara) and then transform into *E. Coli DH5α* cells. The transformed cells were plate on 60μg ml<sup>-1</sup> ampicillin containing LB agar plates, which were overlaid with 10 mM X-Gal and 50 mg ml<sup>-1</sup> IPTG. After incubation overnight at 37 C, white colonies (putative positive clones) were pick and transfer into 1.5 ml tubes containing 500 μl LB liquid medium (AMP<sup>+</sup>).

### Screening and Identification of Clones

Every clone was amplified by PCR (primed with primer 1 and primer 2R in the Clontech PCR-select cDNA subtraction Kit). The clones larger than 300 bp will blot onto Hybond-N<sup>+</sup> nylon membrane (Amersham). Tester cDNA and control cDNA was used as forward and reverse probes, respectively to hybridize with the selected clones. Reverse northern blot will performer using the DIG-High Prime DNA Labeling and Detection Starter Kit (Roche). The hybridization membranes were scanned by ArtixScan 1010 (Microtek).

### Sequence Analysis

The samples which select sent to School of Plant, Environmental, & Soil Sciences, Louisiana State University, LA, USA, for sequencing. DNA sequences were analyzed at NCBI (the National Center for Biotechnology Information, USA, [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) using Blastn and Blastx.

### Real-Time Fluorescent Quantitative Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was used to validate the results from SSH analysis and to determine expressional differences of the related genes rice varieties. cDNAs was synthesized from equal amounts of total RNA isolate from the roots of allelopathic rice and its counterpart as described above using the ExScript RT Kit (Takara), and the reaction mixture was dilute many times for subsequent PCR with gene specific primers. The qRT-PCR was performing according to the method of SYBR Green I. SYBR Premix Ex Taq (Takara) will used.

### Statistic Analysis

All collected data were subjected to statistical analysis using ANOVA as described by ElShamey EAZ, et al., [10]. All statistical analyses were performed using analysis of variance technique by means of "MSTAT" computer software package and "Quantitative genetic analysis".

## Results and Discussions

### Allelopathic Characters

Distinct differences in allelopathic potential were observed among the tested rice genotypes, ranging between 25-84% as shown in Table 1, and Giza 179 showed allelopathic activity 85% on *Echinochloa crus-galli* during 2016 and 2017 season. The parents were crossed with their CMS lines and produced twelve crosses with weed control ranging between 32-87% as shown in Table 2. ElShamey (2016) presented that Rikuto Norin22 and Giza 179 provided that at least 87 and 85% weed control against barnyardgrass in field experiment at RRTC, Egypt. On the other hand, among the crosses, there were some crosses had higher percentage for allelopathic activity as follow; IR69625A × Giza 179, and G46A × Giza 179 and their values were 87 and 86 %, respectively.

For radial area character, it has been found that the parent Giza 179 had higher values among all parents used during 2016 and 2017 seasons as shown in Table 1. This parent was used to be crossed with their CMS lines and produced crosses with values ranging between 11.83–6.33 cm<sup>2</sup>, and the high desirable value which was expressed in IR69625A × Giza 179 (11.83 cm<sup>2</sup>) and G46A × Giza 179 (11.50 cm<sup>2</sup>) during 2015 and 2016 seasons as shown in Table 2.

These results referred to highly genetic effect which controlled these traits since the environmental effect had

no significance. In a similar study, found that the cultivars; Rikuto norine 22, Giza 182 and Vener 1A provided at least 70% weed control in transplanted rice against *E. crus-galli* under Egypt conditions [11].

No.	Parents	Radial area (cm <sup>2</sup> )	Weed control %
1	Giza 179	11.37	85
2	Giza 178	9.33	44
3	Sakha 103	2.3	25
4	IR 69625 A	5.33	39
5	IR 70368 A	6.17	46
6	G46A	5.5	35
7	K17A	6	37

Table 1: Mean performance of radial area and weed control for the parental lines.

No.	Crosses	Radial area (cm <sup>2</sup> )	Weed control %
1	IR69625A × Giza 179	11.83	87
2	IR69625A × Giza 178	8.17	65
3	IR69625A × Sakha 103	6.33	35
4	IR70368A × Giza 179	11.33	83
5	IR70368A × Giza 178	6.33	65
6	IR70368A × Sakha 103	7	30
7	G46A × Giza 179	11.5	86
8	G46A × Giza 178	8	60
9	G46A × Sakha 103	6.5	37
10	K17A × Giza 179	11.33	85
11	K17A × Giza 178	8.5	63
12	K17A × Sakha 103	6.83	37

Table 2: Mean performance of radial area and weed control for the obtained crosses.

### Morphological Characters

A wide range for morphological characters of the parental lines with their crosses was recorded, the data summarized in Table 3 show that the mean values of the three testers and the two CMS lines were (86.71 cm; 93.14 days; 37.11 cm<sup>2</sup> and 30.43 δ) for plant height, days to heading, flag leaf area and flag leaf angle, respectively. But, the mean values for the F<sub>1</sub> hybrids were (97.76 cm; 92.48 days; 36.52 cm<sup>2</sup> and 24.81 δ) for plant height, days to heading, flag leaf area and flag leaf angle, respectively as shown in Table 3.

For plant height, the results in Tables 3 and 4 revealed that the most desirable mean values towards dwarf were found for the parents IR70368A (71.33 cm) and IR69625A (76 cm) in addition to, the crosses and K17A × Sakha103 and K17A × Giza 179 which gave the lowest

values for plant height. The selection of parental lines with appropriate plant height and non-loading characters is important for high yield potential hybrids, particularly interspecific hybrids [12].

No.	Parents	Plant height	Days to heading	Flag leaf area	Flag leaf angle
1	Giza 179	96	90	46.42	32
2	Giza 178	111	98	44	23.33
3	Sakha 103	87.33	93	32.99	35
4	IR 69625 A	76	100	33.33	35
5	IR 70368 A	71.33	99	44.67	26.33
6	G46A	83.33	87	31	28.33
7	K17A	82	85	27.33	33
	Mean	86.71	93.14	37.11	30.43

Table 3: Mean performance of morphological characters for the parental lines.

With respect to days to heading, the most desirable mean values towards the earliness were obtained from the parents; Giza179 (90 days) and Sakha103 (93 days) and the crosses; IR70368A × Giza179, IR69625A × Giza 179 and IR70368A × Giza 178 as shown in Tables 3 and 4, whereas the selection for this trait should be done with the plants which have the lowest days to heading. For flag leaf area, the parents; Giza179 and IR70368A and the

crosses IR69625A × Giza 179 gave the desirable mean values.

Regarding to flag leaf angle, the testers; IR69625A and Sakha 103 and the crosses, IR69625A × Giza 179 and IR58025A×Giza182 gave the desirable mean values, indicating that IR69625A was a good donor for the desirable flag leaf angle.

No.	Crosses	Plant height	Days to heading	Flag leaf area	Flag leaf angle
1	IR69625A × Giza 179	101	93	44.63	35
2	IR69625A × Giza 178	113.33	97	25.73	16.67
3	IR69625A × Sakha 103	97.67	98.67	34.25	26.67
4	IR70368A × Giza 179	101	92	34.28	24.33
5	IR70368A × Giza 178	103.33	93.33	26.51	21.67
6	IR70368A × Sakha 103	94.33	98	31.48	25
7	G46A x Giza 179	97.33	89.5	61.7	25.7
8	G46A x Giza 178	96	91	34.8	25
9	G46A x Sakha 103	93.33	95.87	33.33	24.33
10	K17A x Giza 179	91	79.85	39.51	22.3
11	K17A x Giza 178	94.5	85.2	39.56	25.5
12	K17A x Sakha 103	90.33	96.33	32.5	25.5
	Mean	97.76	92.48	36.52	24.81

Table 4: Mean performance of F1 crosses for some morphological characters.

### Yield and its Component Characters

The mean performance of yield and its component characters i.e., number of panicles per plant, panicle

length, panicle weight, grain yield per plant, number of grains per panicle, 1000-grain weight and seed set (%) for the parental lines are presented in Table 5.



No.	Parents	No. of panicles / plant	Panicle length	Panicle Weight	Grain Yield/plant	No. of grains/ panicle	1000-grain weight	Seed set %
1	Giza 179	15	23	4.83	30.4	167	21.43	92.5
2	Giza 178	22	23.33	4.56	37.67	155	21	96.57
3	Sakha 103	13.33	18	3.74	25.33	126	24.27	81
4	IR 69625 A	16	21	1.53	10	124	22	32
5	IR 70368 A	17	23.17	2.3	17	128	22.67	31
6	G46A	16	21.7	3.1	19	125	26.6	32
7	K17A	18	21.5	3.95	16	123	27.1	29
	Mean	16.76	21.67	3.43	22.2	135.43	23.58	56.3

Table 5: Mean performance of yield and its component characters for the parental lines.

A wide range for yield and its component traits of the parental lines with their crosses was recorded, the data summarized in Table 5 show that the mean values of the three CMS lines and the ten testers were (16.67, 21.67, 3.43, 22.20, 135.43, 23.58 and 56.30) for number of panicles per plant, panicle length, panicle weight, grain yield per plant, number of grains per panicle, 1000-grain weight and seed set (%), respectively. The decrease of the mean values for grain yield per plant referred to the decrease of out-crossing to the CMS lines. But, the mean values performance of F<sub>1</sub> hybrids for these characters were (18.28, 22.14, 4.14, 41.31, 132.25, 25.79 and 79.48), respectively, as shown in Table 6.

From the results summarized in Tables 5 and 6 it could be concluded that for number of panicles per plant character, the highest mean values were obtained by the two parent Giza178 (22.00) and the cross IR70368A×Giza 179 which gave mean values of 26.00 as shown in Table 5.

The desirable mean values for panicle length were recorded by the three parents; Giza 178, IR70368A and Giza 179 with values of (23.33, 23.17 and 23.00 cm), respectively (Table 5) and the crosses; IR69625A × Giza 179 and IR69625A×Giza 178, gave also the desirable mean values (25.00 for first cross and 23.33 for the second cross) as shown in Table 6.

While, for panicle weight, the parents Giza 179 and

Giza178 gave the desirable mean values (4.83 and 4.56), respectively in Table 5, and the crosses; IR70368A × Giza 178 and IR69625A × Giza 178, gave the desirable mean values (4.60 and 4.53), respectively (Table 6).

For grain yield per plant character, the parents Giza 178 and Giza 179 which gave (37.67 and 30.40 gm), respectively and the two crosses IR70368A × Giza179 and IR70368A × Giza 178 which gave (65.00 and 59.40), respectively recorded the highest mean values for this character. While, the tester parents, Giza 179 and Giza 178 with mean values of (167.00 and 155.00), respectively and the two crosses; IR69625A×Giza178 and IR70368A×Giza 178 with mean values of (173.00 and 152.00), respectively recorded the highest mean values for the no. of grains per panicle character.

In addition, with regard to the 1000-grain weight character, the desirable mean values were recorded for the tester lines, Sakha 103 and Giza 179 which gave (24.27 and 21.43 gm), respectively and the crosses; IR70368A × Giza 179 (27.50) and IR69625A × Giza 178.

Also, it could be noticed that the desirable mean values for seed set % character were recorded for the tester Giza 178 which gave (96.57) and the cross IR69625A×Giza178 which gave (98.80). This hybrid may be considered as ideal sources for varieties improvement through pedigree breeding or biparental meeting.

No.	Crosses	No. of panicles / plant	Panicle length	Panicle Weight	Grain Yield/ plant	No. of grains/ panicle	1000-grain weight	Seed set
1	IR69625A × Giza 179	14	25	3.51	45.6	115	26.1	94.87
2	IR69625A × Giza 178	18.33	23.33	4.53	55.67	173	27	98.8
3	IR69625A × Sakha 103	16.67	19.33	2.67	16.33	123	24.33	48.43
4	IR70368A × Giza 179	26	21.5	3.62	59.4	145	27.5	79.27
5	IR70368A × Giza 178	12	23	4.6	65	152	25	98.37
6	IR70368A × Sakha 103	14.33	18.5	2.37	15.33	119	25.33	46.3

7	G46A x Giza 179	21.9	25.6	7.1	64.9	123	26.1	98
8	G46A x Giza 178	20.9	24.8	5.5	48.9	137	24.8	96
9	G46A x Sakha 103	17	18.87	2.87	17.87	119	24.5	52
10	K17A x Giza 179	19.3	23.7	5.3	48	122	28.2	96.8
11	K17A x Giza 178	21	23.1	4.65	42.9	139	25.9	95
12	K17A x Sakha 103	17.87	19	3	15.87	120	24.67	49.87
	Mean	18.28	22.14	4.14	41.31	132.25	25.79	79.48

Table 6: Mean performance of yield and its component characters for the obtained crosses.

### Analysis of Variance

The analysis of variance (Table 7) revealed highly significant differences among the 19 genotypes (12 hybrids, 3 testers, and 4 CMS lines) tested for all studied characters. The parental lines and the hybrids showed highly significant differences for all characters. These results indicate that genotypic differences between entries are present. Mean square values of parents and

crosses were found to be highly significant for all studied characters. These results could be used as an indication to average heterosis overall crosses and therefore could be used through hybrid breeding technology to improve such characters. Parents V.S crosses mean square indicated that average heterosis was significant in all crosses for all characters, the variances in all studied traits differed significantly with different combinations, and this is agreement with ElShamey EAZ, et al., [13].

Source of variance	d.f.	Radical area	Weed control	Plant height	Days of heading	Flag leaf area	Flag leaf angle	No. of panicles/plants	panicle length	panicle weight	Grain Yield/Plants	No. of grains/panicle	1000 grain weight	Seed set
Reps	2	0.16 n.s.	0.002 n.s.	0.635 n.s.	1.73 n.s.	0.362 n.s.	2.28 n.s.	0.09 n.s.	1.45 n.s.	0.01 n.s.	0.64 n.s.	4.73 n.s.	0.04 n.s.	0.001 n.s.
Entries	18	27.12**	0.167**	519.91**	49.84**	131.36**	104.26**	43.94**	17.87**	4.48**	1362.35**	1590.88**	10.95**	0.225**
Crosses	11	2.28*	0.018*	14.39*	2.77*	13.28*	9.77*	2.87*	1.87*	0.44*	179.97*	645.17*	87.99**	0.023*
Parents	6	37.19**	0.148**	732.77**	87.9**	33.91**	31.65**	53**	18.07**	5.54**	754.86**	1439.4**	5.81**	0.334**
P*V.C.	1	66.01**	0.880**	2174.06**	110.95**	851.52**	645.09**	191.09**	75.10**	20.11**	682.26**	3128.26**	43.13**	1.072**
Error	18	4.48	0.001	2.073	1.854	1.93	1.75	2.18	1.91	0.04*	2.07	7.85	0.04	0.002

Table 7: Mean square estimates of the ordinary analysis for allelopathic characters, morphological characters, and yield and its component characters.

### Allelopathic Effect in Rice induced by Salicylic Acid

Allelopathic rice varieties Giza 179 showed higher inhibitory effect on the barnyardgrass than Sakha 103 after the two rice varieties were treated with different concentrations of salicylic acid; 0.1, 0.2, and 0.3 mM/L. The inhibitory rates (IRs) was enhanced with the increase of SA concentration and reached a peak at 0.2 mM with 59.81% for suppression of root length, 46.76% for suppression shoot length, and 65.28% for suppression to dry weight of barnyardgrass (Figure 1). However, a slight decreasing tendency of the inhibitory rates was found when the treated concentration of salicylic acid was increased to 0.3 mM. A similar tendency was found for the non allelopathic rice Sakha 103, but its inhibitory effect was always lower than Giza 179 in all of the SA treatments. This result indicated that salicylic acid

treatment with an appropriate dosage could significantly enhance the allelopathic effect of rice on barnyardgrass, especially in allelopathic rice varieties Giza 179.

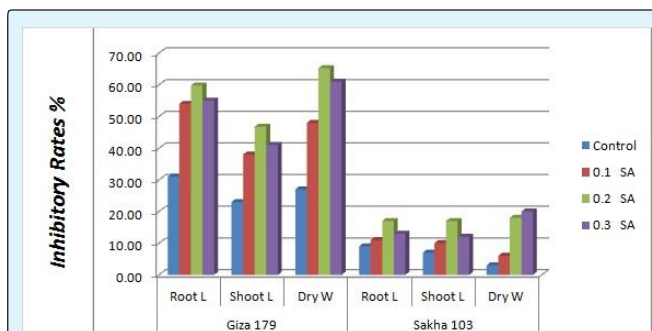


Figure 1: Inhibition rates (IRs) among root length, shoot length and dry weight of barnyardgrass co-cultured with allelopathic Giza 179 and Sakha 103 with different concentrations of salicylic acid (SA).

### Induced Gene Expression in Allelopathic Rice

A library of cDNA was constructed by Suppression Subtractive Hybridization (SSH) for determination of expressed genes in allelopathic rice Giza 179 in response to salicylic acid treatment. A total of 728 clones were obtained from the forward subtractive library, 96 of which were selected for reverse northern blot after PCR (Figures 2A & B). The expressions of 14 clones (genes) were found to be up-regulated. These clones were sequenced, and the cDNA sequences were searched against standard databases ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). According to nucleic acid homologies and encoded protein sequences of the rice (*Oryza sativa L.*) database, clones were successfully assigned. The putative functions of the assigned expressed sequence tags (ESTs) are given in Table 1. Clones 171 and 459 showed the highest homology to a putative receptor-like protein kinase from rice (*Oryza sativa L.*). Clones 163 and 617 encode the same ubiquitin carrier protein, and clones 557, 695 code for a serine/threonine protein kinase-like protein and BAG domain containing protein-like protein, all of which are involved in signal transduction.



Figure 2: Reverse northern blots showing differential screening of partly subtracted library clones in allelopathic rice Giza 179 exposed to SA treatment and barnyardgrass competition. Forward (A) and reverse (B) hybridizations using a tester cDNA probe of Giza 179 under 0.2 SA (A) or control (B) treatments. Arrows indicate positive clones.

Clone 265 encodes a putative glutathione S-transferase functioning in detoxification in rice plants and clones 670, 438, 512, 587 and 316 had the highest homology to a cellulose synthase-4, a putative acetyltransferase, a putative cinnamoyl-CoA reductase, a putative endosperm specific protein SC3, and peptidylprolyl cis-trans isomerase from *Oryza sativa*, respectively, which are related with substance metabolism. Clones 593 and 507 had homology to a putative peroxidase (POD) and phenylalanine ammonia-lyase from *Oryza sativa*, respectively, which are related

with de novo biosynthesis of phenolic allelochemicals involved in plant chemical defence against salicylic acid treatment. These identified genes can be categorized into five groups according to their function; signal transduction, growth-mediated transcription regulation, secondary metabolism, cell defence, protein synthesis and its degradation.

### Validation of Differentially Expressed Genes by qRT-PCR

Differential expression of two genes by qRT-PCR gene expression of both *PAL* and *POD*, which are involved in *de novo* allelochemical synthesis was increased in Giza 179 and Sakha103 when exposed to salicylic acid treatment. The relative expression levels of the two genes in Giza 179, however, were higher than those in Sakha103, showing that the relative transcript abundance of *PAL* in root of Giza 179 increased by 11.38 times, while in Sakha103, they were increased by 1.15 times after three days after treatment with 0.2 SA, respectively. The same was true in the expression of *P450* gene for the two rice varieties as shown in Fig. 3. This finding further confirms the findings of SSH analysis (Table 1).

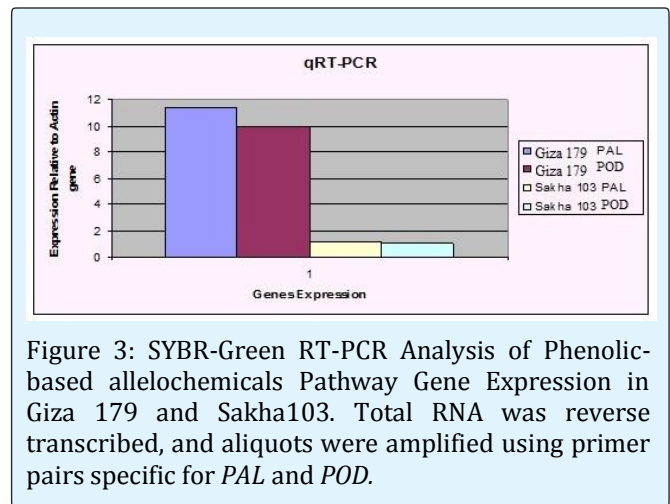


Figure 3: SYBR-Green RT-PCR Analysis of Phenolic-based allelochemicals Pathway Gene Expression in Giza 179 and Sakha103. Total RNA was reverse transcribed, and aliquots were amplified using primer pairs specific for *PAL* and *POD*.

The present results confirmed that SA could serve as an elicitor to induce higher allelopathic potential of donor rice to suppress the target weed plants. This inducible effect is responsive to the dose of SA treatment. The allelopathic inhibitory effect of Giza 179 on barnyardgrass was significantly increased after the rice varieties were treated with SA. The activities of protective enzymes *PAL* and *POD* as well as root physiological capability were all enhanced in Giza 179, implying that the plant chemical defence in rice has taken effect in the suppression of the



target weed. Here, we present an update on the effects of SA treatment on the expression of genes involved in rice plant chemical defence response using the combined

approach of SSH and qRT-PCR. Our study showed that many genes in the rice varieties can be significantly up-regulated by exogenous SA treatment.

Clone	Length (bp)	Accession no.	Best homologue database	score	E-value/Sequence identify (%)	Source
171	324	AC131374	Putative receptor-like protein kinase	278	2e-73/100	<i>Oryza sativa</i>
187	382	AA134132	Putative acetyltransferase	229	8e-05/92	<i>Oryza sativa</i>
245	195	ABR25322	Phenylalanine ammonia-lyase	176	2e-73/100	<i>Oryza sativa</i>
311	221	AP003734	Putative cinnamoyl-CoA reductase	231	3e-34/100	<i>Oryza sativa</i>
418	219	AY224431	Serine/threonine protein kinase-like protein	356	2e-45/97	<i>Oryza sativa</i>
434	297	AP005578	Putative endosperm specific protein SC3	143	2e-64/100	<i>Oryza sativa</i>
519	313	AP003252	BAG domain containing protein-like	143	4e-105/100	<i>Oryza sativa</i>
543	349	CM000126	Ubiquitin carrier protein	76	0/97	<i>Oryza sativa</i>
567	245	AP000263	Putative peroxidase	45	4e-127/98	<i>Oryza sativa</i>
590	314	CM000126	Ubiquitin carrier protein	77	5e-92/98	<i>Oryza sativa</i>
598	221	AC131374	Putative receptor-like protein kinase	111	3e-90/100	<i>Oryza sativa</i>
601	248	AP000528	Cellulose synthase-4	196	2e-54/100	<i>Oryza sativa</i>
623	291	AC092236	Putative glutathione S-transferase	111	8e-125/99	<i>Oryza sativa</i>
667	288	CM000126	Ubiquitin carrier protein	166	5e-154/98	<i>Oryza sativa</i>

Table 8: Sequence similarity comparison display fragments genomic library of the root in allelopathic rice Giza 179 under 0.2 SA treatments.

Both defense-related proteins and the key enzymes such as PAL, CCR, GST, and POD, etc., which are associated with phenylpropanoid metabolism and plant defense, showed significantly positive expression in allelopathic rice Giza 179. It has been reported that phenylalanine ammonia-lyase (PAL), which can be induced by biotic and a biotic factors, is a key enzyme of plant metabolism catalyzing the first reaction in the phenylpropanoid biosynthesis from L-phenylalanine to a wide variety of natural products based on the phenylpropane skeleton [14,15]. More recently, work by has suggested that under conditions of decreased nitrogen availability, plant phenolic content and PAL activity might increase because of decreased demand for proteins involved in growth, and ammonium ions released by PAL could be assimilated to increase nitrogen cycling [16]. The result also indicated that the gene coding for CCR, which is the entry-point enzyme of the lignin specific biosynthetic pathway, was up-regulated in this SA-mediated process. Previous studies showed that the product lignin released from the donor plants functioned as specific inhibitors of the detoxifying enzymes (e.g., P450s) present in the receiver plant (e.g., barnyardgrass) to block the ability of modifying toxic agents [17]. As a result, chemical defence could take effect at a lower dosage of phytotoxin. The present result suggested that the SA mediated cascade reaction in rice to

defend against barnyardgrass attack might be similar to the responses to nutrient deficiency through the regulation of carbon-nutrient balance. This in turn results in the accumulation of phenolics and more phenolic allelochemicals being released into external environment (culture solution) from the roots to depress the target weed growth including the inhibition of protective enzyme activity and root uptake ability, which was consistent with the previous studies [18]. This raises the question as to why the auto-toxicity does not occur during this process.

Our present work elucidated that the donor plants possess a detoxifying system and that the up-regulation of the enzyme might play an important role in this process. It has been reported that this enzyme, which could be activated by SA, was associated with xenobiotic detoxification, limiting oxidative damage, and other stress responses in plants [19]. The present work suggested that the variety Giza179 is strong allelopathy, and from obvious studies we found the variety Giza179 as restorer line [15]. So, we recommend by using variety Giza 179 as parental line to produce hybrid rice IR69625A x Giza179 and IR70368A x Giza 179 which have highly yield and strong allelopathy.

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