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Little impacts on transcriptome and secondary metabolites of transgenic rice by resveratrol synthase gene

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Abstract

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a stilbenoid that displays a wide range of biological effects, notably as a cardioprotective, antitumor, or neuroprotective agent, as well as an antifungal or antibacterial compound. Obtaining resveratrol safely from the daily diet will contribute to disease prevention and life extension. In this study, the microarray-based transcriptome profiles of two transgenic resveratrol rice lines, Iksan515 (I515) and Iksan526 (I526) were compared with those of the isogenic DJ variety and a non-transgenic common variety Nagdong (ND) as a reference strain, in order to identify the unpredictable effects of gene insertion. Only five genes were differentially expressed in both transgenic lines, suggesting that the impact of insertion of *RS* on the transcriptome was minimal. No gene up- or down-stream from the *RS* insertion showed more than 2-fold change in expression, implying that overexpression of *RS* could occur without causing activation of any unexpected gene networks in secondary metabolic pathways. Three up-stream and one down-stream metabolites from RS, viz., ρ-coumaric acid, vanillin, chlorogenic acid, and biochanin A, demonstrated similar levels between transgenic lines and their counterpart; however, a difference in naringenin level was observed on transgenic line I515 compared to those of I526, DJ, and ND.

Keywords: resveratrol, secondary metabolites, transgenic rice, transcriptome profiles.
Abbreviations: 4CL_4-Coumarate-CoA ligase; C3H_Coumarate 3-hydroxylase; C4H_Cinnamate 4-hydroxylase; CHI_ Chalcone isomerase; CHS_Chalcone synthase; DJ_Dongjin; F5H_Ferulate 5-hydroxylase; FLS_Flavonol synthase; GM_Genetically modified; I515_Iksan515; I526_Iksan526; ND_Nagdong; PAL_Phenylalanine ammonia-lyase; PDT_ Prephenate dehydratase; *RS*_Resveratrol synthase.

Introduction

Resveratrol is a type of natural phenol and possesses antifungal and antibacterial activity as a phytoalexin produced by plants in response to stress, such as wounding or pathogen attack (Langcake and Pryce 1976). Resveratrol is restricted to relatively few species, including grapes, Scots pine, peanuts, and tall fescue, and is biosynthesized through the condensation of one molecule of p-coumaroyl CoA and three molecules of malonyl CoA in a reaction catalyzed by resveratrol synthetase (Rupprich et al.1980). This enzyme is encoded by *RS*, a gene that has been transformed into many types of plants in order to induce phytoalexin production to prevent microbial attack or into medicinal plants to enhance pharmacological activities (Thomzik et al., 1997; Leckband et al., 1998; Zhu et al., 2004). Currently, resveratrol is consumed in the form of numerous biological supplements. It is mostly produced from extracts of the roots of *Polygonum cuspidatum*, a method that is expected to provide an alternative for the use of recombinant microorganisms and plant cell suspensions (Kiselev, 2011). In agriculture, the purpose of increasing resistance against attacks by various pathogens by application of resveratrol is often achieved by means of stilbene synthase or *RS* transformation (Coutos-Thevenot et al., 2001; Delaunois et al., 2009). However, until the development of two resveratrol transgenic rice lines by Baek et al. (2013), no further efforts were made in terms of resveratrol transformation of cereal crops aimed at improving human health through diet. Low expression levels of resveratrol in the transformed plants and the safety of the transgenic foods, particularly of staple crops, were the limiting factors in this field. Due to random insertion of the transgene, unintended traits may be generated through unexpected gene interactions, causing significant effects on plant growth and development. Therefore, biosafety assessment of the transgenic plants is one of the prerequisites for gaining public acceptance of genetically modified crops. The Organization for Economic Co-operation and Development (OECD) put forward guidelines for assessing the safety of genetically modified foodstuffs on the strength of new biotechniques, such as transcriptome, proteome, or metabolome analysis (called “omics”), to identify unintended effects in plant systems (Kogel et al., 2010). To date, such approaches, and particularly transcriptomics, have been used to investigate the unintended effects in a number of transgenic plant systems (Abdeen et al., 2010; Kogel et al., 2010; Coll et al., 2008; 2009; 2010). For example, Coll et al. (2008) compared the transcriptome profiles of the widely commercialized maize MON810 varieties and their non-genetically modified (non-GM) near-isogenic counterparts by means of microarrays. These analyses revealed that the expression profiles of MON810 seedlings are more similar to those of their corresponding near-isogenic varieties than are the profiles of other lines produced by conventional breeding.

Concurrently, they found a small set of differentially expressed sequences that were considered as constituting differences from the genetic background. A subsequent study using the same materials in field trials revealed that most sequences that were differentially expressed in plants cultured in vitro had the same expression values in MON810 and comparable varieties when grown in the field. Moreover, the small set of differentially expressed sequences was not found in two variety pairs grown in the field (Coll et al., 2009). Later, the same GM and non-GM variety pairs were used to identify transcriptomic changes under conventional nitrogen-fertilization and low-nitrogen farming practices (Coll et al., 2010). The results indicated that natural variation explained most of the variability in gene expression among the samples. Relative to the natural differences between varieties and those due to fertilization treatments, the MON810 character had a minor effect on gene expression. When exploring the unpredictable effects occurring in transgenic crops relative to their isogenic plants, most studies concluded that transgenic crops differed little from crops produced by conventional breeding. However, variations in the foreign genes and target organisms, differences in donor plants, transgenic approaches, statistical analyses, duration of observation, and locations require a constantly expanding database on the comparison of GM and non-GM crops. In this study, we performed microarray analysis of two resveratrol transgenic rice lines, I515 and I526, their wild type variety, DJ, and a non-transgenic common variety, ND, grown in the field, in order to evaluate changes in the transcriptome of the transgenic lines, compared to the wild type variety, caused by *RS* insertion. Furthermore, several phenolic metabolites up- and down-stream of the inserted *RS*-encoded enzyme were also analyzed to assess the effects of *RS* insertion on the secondary metabolite pathways.

Results

Identification of differentially expressed genes

Microarray analyses were implemented for two transgenic lines, I515 and I526, and their wild type variety, DJ, in order to identify the unintended effects of *RS* overexpression, by comparing the overall gene expression pattern. Furthermore, the transcriptome profile of a common non-transgenic variety, ND, was also analyzed to provide a reference, allowing differentiation of the influence of gene insertion from individual differences or environmental effects. This analysis revealed that 23 and 12 genes were up-regulated in I515 and I526, while 11 and nine genes were down-regulated, respectively, compared to the wild-type DJ, when using a cut-off *P*-value of <0.05 and a fold-change of 2. However, a total of 79 and 101 genes were up- and down-regulated in the non-transgenic ND rice variety, as compared to DJ (Fig 1A). These results indicated that the differences between varieties are larger than those between transgenic rice strains and their wild-type counterparts. By comparing the transcriptional profile of transgenic lines and their wild type counterpart, a total of eight and one genes with enhanced and reduced expression, respectively, were identified for both transgenic lines (Fig 1A). Among these, three up-regulated and one down-regulated gene were also identified for the non-transgenic ND variety, which suggested that these changes arise from environmental factors or individual differences, rather than from *RS* introduction. In addition to the genes with similar variations in the transgenic lines and non-transgenic variety, we identified 15 and three genes with up-regulated expression, and seven and eight genes with down-regulated expression genes in I515 and I526, respectively. In order to validate the results obtained from the microarray analysis, we carried out reverse transcription-polymerase chain reaction (RT-PCR) assays on 13 up-regulated genes using gene-specific primers. Ten of the 13 examined genes exhibited expression patterns that were consistent with the microarray results, confirming the reliability of the microarray data (Fig 1B).

Functional categories of differentially expressed genes

We performed functional categorization of the genes differentially expressed between transgenic and non-transgenic rice varieties by annotation, as shown in Fig 2A (http://www.dna.affrc.go.jp/database/and http://www.arabidopsis.org/tools/bulk/go/index.jsp). Eight of 37 annotated genes belonged to the "cellular composition" category, consisting of four genes encoding plastid proteins, two encoding mitochondrial proteins, one encoding a protein bound to a vesicle membrane, and one encoding an integral membrane protein. Six of 37 differentially expressed genes were categorized under "molecular function," with two genes encoding proteins involved in nucleotide binding, one encoding iron ion-binding proteins, one encoding a protein with nuclease activity, and one encoding a zinc ion-binding protein. Only three of 37 genes were classified under "biological process", comprising one encoding a protein involved in defense responses, one encoding a protein involved in the reproductive process, and one encoding a protein involved in transition metal ion transport. Approximately 15 annotated genes encoded a conserved hypothetical protein or protein domain. In addition, six of 38 genes encoded proteins of unknown function. In general, the genes differentially expressed in ND compared to DJ involved 78 genes categorized as "biological process," 74 as "molecular function," and 80 as "cellular composition" (Fig 2B).

Genes varying by RS insertion

Five differentially expressed genes showed more than 2-fold change in the transcriptome profiles of both transgenic lines as compared to DJ, but there was little difference between varieties (Table 1). Three genes, i.e., Os06g0265100, Os03g0569000, and Os03g0115800, were up-regulated in the ranges of 2.09–5.29, 2.13–2.99, and 3.76–7.00 for the two transgenic lines as compared to DJ; these genes encoded conserved hypothetical proteins. Another two genes, Os02g0161700 and Os09g0359500, encoding a leucine-rich repeat, N-terminal domain-containing protein and a protein kinase-like domain-containing protein, respectively, were up-regulated in ranges of 1.85–2.70 and 1.64–2.70 as compared to DJ. These genes may be involved in signal transduction or other primary metabolic roles.

Genes differentially expressed only in the transcriptome of I515

Fifteen genes with up-regulated expression and seven with down-regulated expression as compared to DJ, using a cut-off *P*-value of 0.05, were uniquely identified in the transcriptome profile of I515 (Fig 1A). Six of the up-regulated genes and one down-regulated gene encoded conserved and/or hypothetical proteins. Four up-regulated and one down-regulated genes had unknown functions. Among them, Os03g0303100, encoding a DUF1645 family protein, may be involved in metabolism and immune response. Ten genes with known functions, consisting of five up- and five down-regulated genes, are described in detail below (Table 2). Two down-regulated genes (Os08g0539700 and Os12g0222800) encode an NB-ARC

Table 1. The same genes of genome-wide transcriptomes in range of 2-fold changes for transgenic rice lines I515 and I526 comparing to their wild type variety DJ but with little difference between varieties (ND to DJ) using a cut-off *P*-value of 0.05.

Loc_No.	M[a]						Gene feature
	I515/DJ	*P* value	I526/DJ	*P* value	ND/DJ	*P* value	
Os06g0265100[b]	2.14	2.6×10^{-2}	5.28	2.9×10^{-3}	0.02	4.5×10^{-1}	Conserved hypothetical protein.
Os03g0569000	3.04	3.5×10^{-2}	2.13	2.4×10^{-2}	-0.28	2.3×10^{-1}	Conserved hypothetical protein.
Os03g0115800	3.81	2.6×10^{-2}	6.99	2.3×10^{-3}	0.82	2.5×10^{-3}	Conserved hypothetical protein.
Os02g0161700	2.16	2.0×10^{-2}	1.85	2.0×10^{-2}	-1.13	1.7×10^{-1}	Leucine rich repeat, N-terminal domain containing protein.
Os09g0359500	2.70	2.0×10^{-2}	1.64	4.0×10^{-2}	1.90	0.7×10^{-1}	Protein kinase-like domain containing protein
Bar	*6.17*	*3.9* $\times 10^{-3}$	*6.05*	*3.0* $\times 10^{-5}$	*0.33*	*7.9* $\times 10^{-2}$	Selection marker

[a] M indicates $\log_2$ changes in gene expression for transgenic lines and non-genetically modified variety relative to DJ, and is shown in bold font, here and in the next table.
[b] Loc No. with bond font indicate that microarray analysis data were consistent with that of RT-PCR analysis.

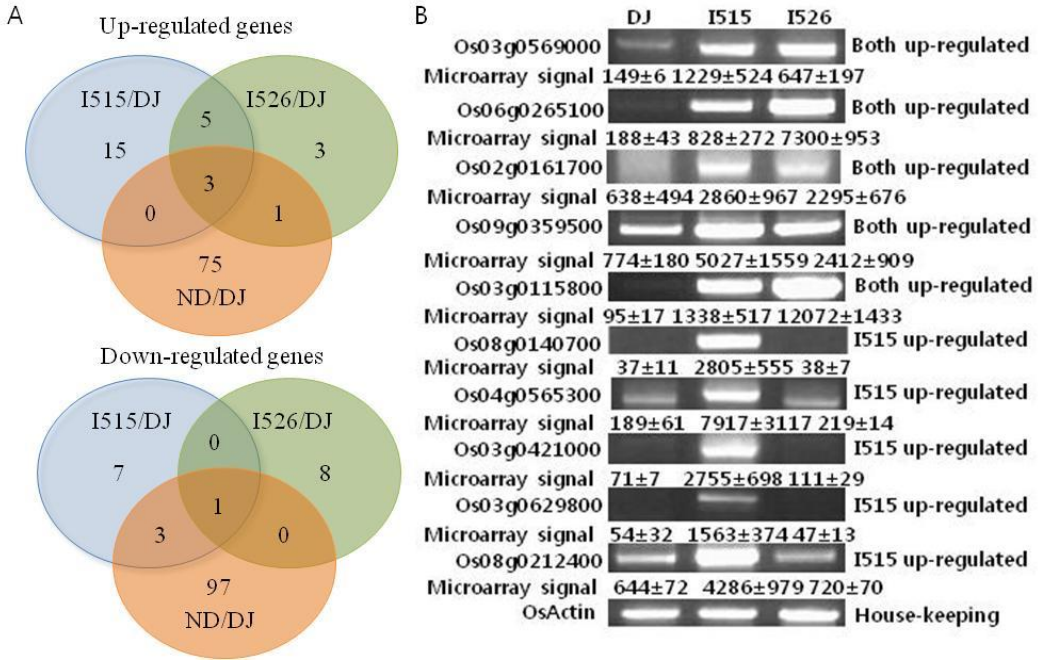

Fig 1. Genes up- and down-regulated by ≥2-fold in transgenic rice lines I515 and I526 ($P < 0.05$) relative to wild type variety DJ and non-transgenic variety ND. A. Differentially expressed gene numbers (up-regulated and down-regulated) in the transcriptome of the two transgenic lines and common variety ND compared to variety DJ. Numbers of genes differentially expressed in I515 compared to DJ are shown in blue, those in I526 in green, those in ND in orange, and the overlapping areas indicate the common changes. B. Microarray data of ten up-regulated genes were consistent with RT-PCR data. Microarray signals of both transgenic lines I515 and I526 and their isogenic variety DJ are separately shown below.

domain-containing protein and a leucine-rich repeat, N-terminal domain-containing protein, respectively. Two genes encoding ethylene-forming enzymes 1-aminocyclopropane-1-carboxylate (ACC) oxidase (X85747 and Os09g0451400), and a transcription factor encoding the MADS 14 protein were down-regulated. Os06g0560000 and Os10g0567900, encoding ferroportin 1 family protein and HAT dimerization domain-containing protein, respectively, were up-regulated in comparison to the wild-type control DJ. Os03g0421000, encoding a zinc finger, FYVE/PHD-type domain-containing protein, was up-regulated approximately 32-fold. The up-regulated Os07g0523600 gene is similar to that encoding the glucose-6-phosphate/phosphate (Glc6P) translocator precursor. Os07g0212400, encoding a transposase from the Ptta/En/Spm family, was also up-regulated.

Genes differentially expressed only in the transcriptome of I526

In transgenic line I526, three genes were uniquely up-regulated, and eight were uniquely down-regulated in comparison to DJ (Fig. 1A); these included five genes with known functions, five genes encoding conserved and/or hypothetical proteins, and one gene with unknown function. One down-regulated gene, Os01g0369900, encodes an enzyme similar to oxo-phytodienoic acid reductase in the jasmonic acid biosynthetic pathway (Table 3). Another down-regulated gene, Os01g0544200, encodes a protein from the cytochrome P450 family. This defense-related gene is often involved in the biosynthesis of secondary metabolites or natural products. Another down-regulated gene, Os02g0202200, encodes SPX, a protein putatively functioning in ubiquitin-protein ligase activity and zinc ion-binding. BAC19867.1, which is homologous to ORF324 of sugar beet, was down-regulated by more than 4-fold compared to the parental control. Os07g0248900, encoding MuDR transposase domain-containing protein, showed an 8-fold down-regulated expression. Os05g0270500, encoding a protein from the ribonuclease P-related protein family, was up-regulated.

Transcript profiles and metabolites change up- and down-stream of the RS insertion

Chalcone synthase (CHS) is a key enzyme catalyzing formation of naringenin chalcone via the condensation of one 4-coumaroyl-CoA and three malonyl-CoA molecules. This condensation reaction represents a branch point in the phenylpropanoid pathway at which CHS channels 4-coumaroyl-CoA molecules towards narigenin chalcone synthesis and the inserted RS towards resveratrol synthesis. Several phenylalanine ammonia-lyase (*PAL*) genes, as well genes encoding cinnamate 4-hydroxylase (*C4H*) and 4-coumarate-CoA ligase (*4CL*) play important roles in the production of resveratrol. To identify the effects of *RS* transformation on transcript profiles, a total of 63 genes expressing proteins that function up- and down-stream of the transgenic *RS* in metabolic pathways were analyzed in I515, I526, the wild-type variety DJ, and the non-transgenic rice variety ND (Fig 3). By using a cut-off *P*-value of <0.05, 43 genes, viz., nine prephenate dehydratase (*PDT*), eight *PAL*, three coumarate 3-hydroxylase (*C3H*), three ferulate 5-hydroxylase (*F5H*), four *C4H*, and 16 *4CL*, functioning up-stream of *RS* showed little change (less than 2-fold) in the two transgenic rice lines as compared to the wild type and non-transgenic common varieties. Changes in 16 *CHS*, one chalcone isomerase (*CHI*), and three flavonol synthase (*FLS*) genes encoding proteins functioning down-stream of *RS* also did not reach 2-fold. In addition, approximately 2.15 ± 0.09 µg/g and 4.34 ± 0.11 µg/g of resveratrol was detected in the brown rice grains of I515 and I526, respectively, implying successful transformation and overexpression of *RS*, but no resveratrol was detected in the DJ and ND varieties, as expected. Six phenolic metabolites from the phenylpropanoid to the flavonoid pathway, occurring up- and down-stream from *RS* were detected in the brown rice grains of both transgenic rice lines and non-transgenic rice varieties. I515 and I526 had similar levels of ρ-coumaric acid, vanillin, and chlorogenic acid, the phenolic metabolites up-stream to *RS*, to those in the DJ and ND varieties (Supplementary Table 2). Down-stream of *RS*, biochanin A levels were similar between transgenic lines and non-transgenic varieties; however, I515 had a 2-fold lower naringenin content than I526, DJ, and ND.

Discussion

Differentially expressed genes and phenotypic traits

In our comparison of the transcriptomes of *RS* transgenic rice lines with non-transgenic varieties, several genes putatively functioning in the control of flowering time, seed setting, plant growth and development, and biotic and abiotic stresses, showed uniquely reduced expression in lines I515 or I526. In I515, a gene (Os08g0539700) encoding a disease-resistance protein of the NB-LRR family, showed nearly 4-fold reduced expression. Expression of a gene encoding the MADS 14 protein was also repressed. Two genes, encoding ethylene-forming enzyme, showed reduced expression in comparison to wild type variety DJ. Moreover, a gene encoding a male sterility-associated cytotoxic protein was down-regulated in line I526. Another gene down-regulated in I526, encoding oxo-phytodienoic acid reductase, is involved in the jasmonic acid biosynthesis pathway. On the other side, agronomic traits and yield components were measured for transgenic rice lines and the DJ variety over 2–3 years (Supplementary Table 3). I515 was significantly different in the 100-grain weight from the wild type variety DJ. Non-significant differences were observed for all agronomic traits of I526. Moreover, increasing susceptibility to bacterial blight, particularly for the K1 and K2 strains, had previously been observed for both transgenic lines as compared to the wild type susceptible variety DJ (Qin et al., 2013b). There is insufficient evidence indicating association between reduced gene expression and a lower seed setting or increased susceptibility of the transgenic lines to bacterial blight. Firstly, the genes with enhanced or reduced expression, shown in Table 2 and Table 3, are uniquely altered in the transcriptome profiles of I515 or I526, but are not seen as common changes in both transgenic lines, indicating that environmental effects, and experimental and statistical error cannot be ruled out. Secondly, many environmental factors, such as location, fertilization treatment, and environmental stresses can affect the transcriptome profiles of field-grown transgenic rice lines. Finally, whether phenotypic differences between I515 and DJ resulted from chromosomal rearrangement during callus culture, or positional effects due to the location of insertion of the foreign gene into the chromosomes, and/or due to other environmental factors, are not clear. Barro et al. (2002) studied the agronomic traits and yield components of transgenic lines and compared these with the non-transgenic parent and a null segregant for 2 years, in the field. Although phenotypic differences for many traits were found, only the heading date and the number of spikelets per spike showed clear genotypic differences: a later heading date and higher number of spikelets per spike were observed in the transgenic plants. However, null segregant lines also exhibited

Table 2. The genes with ≥2-fold enhanced or reduced expression in transgenic line I515 compared to those of the wild type variety, DJ, using a cut-off *P*-value of 0.05.

Loc_No.	M				Gene features
	I515/DJ	*P* value	I526/DJ	ND/DJ	
Genes with known functions					
Os08g0539700	-1.98	1.1×10^{-2}	-0.69	-0.16	NB-ARC domain containing protein
X85747[a]	-1.56	2.0×10^{-2}	-0.76	0.81	ACC oxidase 1) (Ethylene-forming enzyme) (EFE)
Os03g0752800	-1.23	2.1×10^{-3}	-0.31	0.25	Similar to Isoform 2 of MADS-box transcription factor 14
Os12g0222800	-1.21	4.5×10^{-3}	-0.30	-0.32	Leucine rich repeat, N-terminal domain containing protein
Os09g0451400[a]	-1.19	3.9×10^{-2}	-0.52	0.64	ACC oxidase 1) (Ethylene-forming enzyme) (EFE)
Os07g0523600	1.58	4.1×10^{-2}	0.02	0.80	Similar to Glucose-6-phosphate/phosphate-translocator precursor.
Os06g0560000	1.82	5.0×10^{-2}	1.90[c]	0.64	Ferroportin1 family protein
Os07g0212400 [b]	2.68	1.1×10^{-2}	0.17	0.23	Transposase, Ptta/En/Spm, plant domain containing protein
Os10g0567900	2.74	4.3×10^{-2}	0.25	0.23	Similar to F-box protein interaction domain containing protein, expressed
Os03g0421000	5.23	1.0×10^{-2}	0.66	0.24	Zinc finger, FYVE/PHD-type domain containing protein.
Genes encoding conserved and/or hypothetical proteins					
Os07g0641400	-1.27	3.9×10^{-2}	-0.29	0.46	Hypothetical protein.
Os05g0425600	1.17	1.0×10^{-2}	0.09	0.01	Hypothetical protein.
Os04g0120100	1.65	2.9×10^{-2}	0.52	0.66	Conserved hypothetical protein.
Os02g0216200	2.12	7.9×10^{-3}	0.02	0.09	Conserved hypothetical protein.
Os03g0629800	4.81	9.6×10^{-3}	-0.19	0.74	Conserved hypothetical protein.
Os04g0565300	5.34	2.5×10^{-2}	0.23	0.16	Hypothetical protein.
Os08g0140700	6.20	6.5×10^{-3}	0.05	-0.36	Conserved hypothetical protein.
Genes with unknown functions					
Os02g0160900	-1.53	9.3×10^{-4}	-0.29	-0.24	Protein of unknown function wound-induced domain-containing protein
Os02g0265300	1.02	2.0×10^{-2}	0.08	-0.14	Protein of unknown function DUF3778 domain-containing protein
Os03g0303100	1.10	1.0×10^{-2}	0.69	0.29	Protein of unknown function DUF1645 family protein.
Os04g0276600	1.11	3.9×10^{-2}	-0.18	0.04	Protein of unknown function DUF303, acetylesterase putative domain-containing protein
Os04g0587500	1.15	2.7×10^{-3}	0.17	0.29	Domain of unknown function DUF250 domain-containing protein

[*a] Genes involved in biosynthesis of ethylene from methionine

[b] Loc No. with bond font indicate that microarray analysis data were consistent with that from RT-PCR analysis

[c] Non-significant at 0.05 level (P > 0.05)

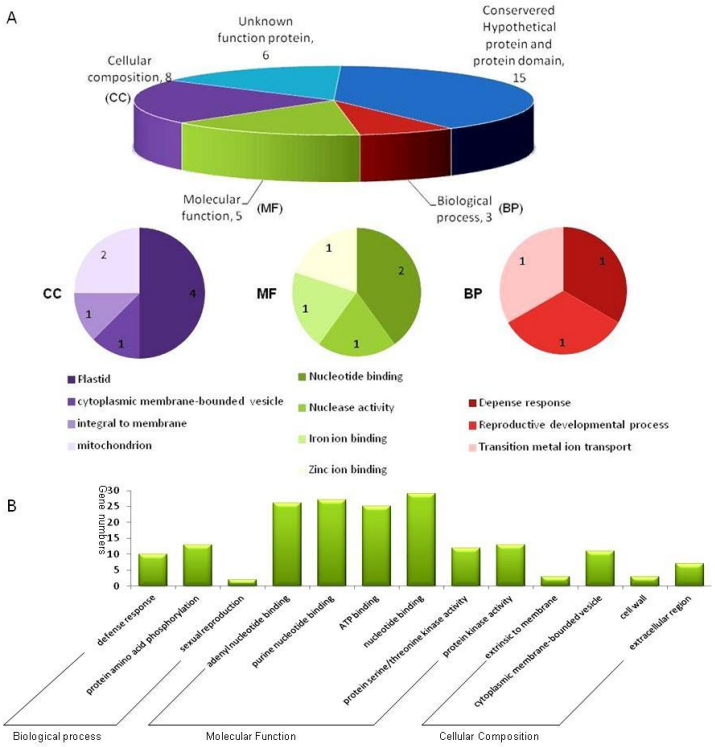

Fig. 2. Functional category classifications of genes differentially expressed between transgenic lines and the common variety ND as compared to the wild type variety DJ. A. Pie charts showing the functional category classifications of genes differentially expressed in transgenic lines compared to those in DJ. Genes involved in cellular composition (CC), molecular functions (MF), and biological processes (BP) were separately shown in purple, green, and red; B. Functional categories of the genes differentially expressed in non-transgenic variety ND from DJ.

the same trends as the transgenic lines, indicating that variations in these traits may be more related to somaclonal variation, induced by in vitro culture, rather than by foreign gene introduction. In order to distinguish between unpredictable metabolic changes resulting from gene insertion and tissue culture, Zhou et al (2012) obtained the descendants of the same transformant from different breeding programs, including both transgenic and null-segregant progeny. Then, variations caused by transgenes were compared between transgenic and their respective null-segregant plants, while null-segregant and wild-type control plants were compared to identify the influences of tissue culture. Likewise, the metabolic profiles suggested that more changes resulted from tissue culture than from foreign gene insertion.

Transcriptomes and secondary metabolites

Hanhineva et al. (2009) considered that increased susceptibility of transgenic lines to grey mold fungus might be induced by the reduced production of phenolic compounds and flavonoids, caused by competition for the same substrate with *RS*. In the present study, no genes encoding components of the secondary metabolic pathway were found to show more than a 2-fold change, implying that overexpression of *RS* could occur without causing activation of any unexpected gene networks in secondary metabolic pathways. Levels of six phenolic metabolites up- and down-stream of RS were not markedly changed in both transgenic lines compared to the isogenic and common varieties. Even though I515 showed a 2-fold lower naringenin content than DJ, a similar trend was not detected in the other transgenic line, I526, which at least suggested that the *RS* insertion did not influence the secondary metabolic pathway in I526. Remarkably, except for a difference in the insertion site, there was no difference in the molecular characteristics in terms of the inserted T-DNA structure, copy number, and RNA expression between I515 and I526 (Qin et al., 2013a). Likewise, microarray analysis showed that bar expression signals were 34929 ± 5304 in I515 and 32006 ± 526 in I526. The similar average expression signals indicated that the inserted T-DNA expression in I515 was similar to that in I526. However, it remains unclear whether the large standard deviation in the microarray signal implied unstable expression of T-DNAs in line I515. However, the resveratrol content of I515 was 2-fold lower than that of I526. A similar trend was found for naringenin, a downstream metabolite of naringenin chalcone, which is encoded by *CHS* (Fig. 3). Experimental and statistical errors or positional effects derived from the *RS* insertion may explain the differences in resveratrol content between the transgenic lines. The expression levels produced by the two *RS* copies inserted in I515 resulted in only half the levels of metabolites compared to those of I526; further metabolite profiling of both transgenic lines will be required to elucidate the reason for this phenomenon. In the present study, only three of five differentially expressed genes encoding conserved hypothetical proteins could be considered as probably resulting from the gene insertion, but this was not conclusive, because of the complexity of going from transcript level to phenotypic trait. In conclusion, the impact of the *RS* insertion on the transcriptomes was minimal in both transgenic lines. For I526, the secondary metabolites up- and down-stream of RS, and the agronomic traits of the transgenic line were not different compared to the counterpart DJ and the common ND varieties. For I515, the lower grain weight and greater susceptibility to bacterial blight were caused by tissue culturing, a common

Table 3. The genes with ≥2-fold enhanced or reduced expression in transgenic line I526 compared to those of the wild type variety, DJ, using a cut-off *P*-value of 0.05.

Loc_No.	M				Gene feature
	I515/DJ	I526/DJ	*P* value	ND/DJ	
Genes with known functions					
Os07g0248900	-0.13	-3.79	4.0×10^{-4}	0.14	Transposase, MuDR, plant domain-containing protein.
Os01g0369900[a]	-0.41	-1.12	1.2×10^{-3}	-0.28	Similar to Oxo-phytodienoic acid reductase.
Os02g0202200	-0.10	-1.08	1.9×10^{-4}	-0.16	SPX, N-terminal domain-containing protein.
Os01g0544200	0.18	-1.33	4.7×10^{-3}	0.09	Cytochrome P450 family protein.
Os05g0270500	0.89	1.28	1.6×10^{-4}	0.13	Ribonuclease P-related protein family protein.
Genes encoding conserved and/or hypothetical protein.					
BAC19867.1	0.09	-2.71	4.0×10^{-2}	0.50	Hypothetical protein
Os07g0153100	0.31	-1.89	3.2×10^{-2}	0.31	Hypothetical protein.
Os11g0636200	0.50	-1.51	3.8×10^{-2}	0.31	Conserved hypothetical protein.
Os08g0153550	-0.55	-1.17	2.1×10^{-3}	-0.03	Hypothetical protein.
Os04g0531800	0.48	1.20	4.4×10^{-2}	0.03	Conserved hypothetical protein.
Genes with unknown functions					
Os01g0537250	0.87	1.13	1.6×10^{-2}	0.29	Protein of unknown function DUF3778 domain-containing protein

[a] Genes involved in jasmonic acid biosynthesis

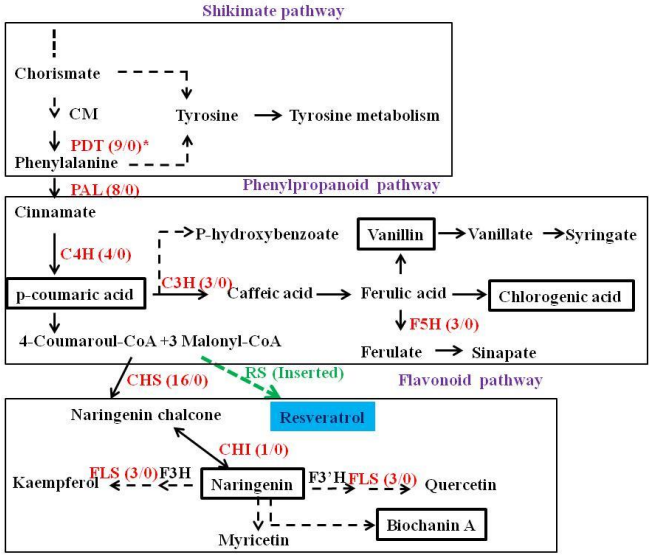

Fig 3. The inserted RS was involved in secondary metabolite pathways, from the phenylpropanoid pathway to the flavonoid pathway. The gene expression of enzymes involved up- and down-stream of the inserted RS are marked in red letters; *(A/B) indicates the number of genes involved/number of genes with a ca. 2-fold change in the transgenic lines as compared to the wild type variety DJ. The green arrow indicates the inserted resveratrol synthase gene, and resveratrol production is shown in blue. The box labeled metabolites indicates the metabolites analyzed in brown rice grains of transgenic lines, their non-transgenic counterpart, and a common variety.

property of all transgenic plants, or by changes in the down-stream metabolite levels due to *RS* insertion, which need a further profiling of metabolites from the phenylpropanoid to the flavonoid pathway and comparative analysis between I515 and I526 in future.

Materials and Methods

Plant materials

Resveratrol transgenic rice lines I515 and I526, their wild type variety DJ, and the non-transgenic common variety ND were planted in a Suwon experimental field. The I515 and I526 lines were transformed with the pCAMBIA 3300 vector encoding the resveratrol synthase *AhSTS1* (*RS3*) gene from *Arachis*, under the control of the maize ubiquitin promoter, and the bar as a selectable marker, under the control of the CaMV 35S promoter. Flanking sequence analysis indicated that two copies of T-DNA with inverted repeats were inserted in intergenic regions, nucleotides 928,858,739–28,858,786 on chromosome 4 of I515 and nucleotides 330,872–330,907 on chromosome 12 of I526, respectively. The stable *RS* transcript was previously detected in both transgenic lines (Qin et al., 2013a).

RNA isolation and RT-PCR

Total RNA was isolated from the leaves of field-grown transgenic rice lines I515 and I526, as well as DJ and ND varieties, in three replicates. Specifically, frozen samples were homogenized with a mortar and pestle in liquid nitrogen. Then, TRIZOL and chloroform were added to the ground powders and vortexed well; thereafter, samples were centrifuged at 13,000 × g for 15 min at 4°C using a MX-301 centrifuge (Tomy Seiko). The same volume of isopropanol as the supernatant of each tube was added and samples then incubated for 10 min at room temperature. Samples were then again centrifuged at 13,000 × g at 4°C. The pelleted RNA samples were further purified using the RNeasy mini plant kit (Qiagen, Valencia, CA, USA) and DNA was removed from the samples by using TURBO DNA-Free DNase (Ambion, Austin, TX, DNA). Total RNA was quantitated by measuring absorbance at 260 nm and 280 nm, using a Nanodrop ND 1000-spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, NC, USA). Total RNA (5 μg) from each sample was used to synthesize cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). For PCR amplification, 1 μl of the resulting cDNA reaction was used as a template. PCR reactions were carried out in a volume of 20 μl using 10 pmol of each primer pair (Supplementary Table 1). Cycling involved pre-denaturation for 3 min at 94°C, 25–28 cycles of denaturation at 94°C for 30 s, annealing at 50–65°C for 30 s, and extension for 1 min at 72°C, followed by a final extension by 5 min at 72°C. RT-PCR was performed in triplicates. Rice actin (*OsActin1)* mRNA was used to monitor equal loading.

Microarray experiment and data analysis

Three biological replicates of DNase-treated RNA from the leaves of transgenic rice lines I515, I526, as well as the DJ and ND varieties, grown in the field, were prepared for microarray analysis. An *Oryza sativa* 135K microarray containing 31589 probes was employed, using DJ as a control, by GGBIO Tech Inc. (Myongji University, Yongin, Gyeonggi-do, Korea). Differential gene expression was analyzed using a moderated *t*-test with empirical Bayes smoothing, and a change of 2-fold of the Log2 ratio was used to identify differentially expressed genes after primary data processing. RT-PCR of 13 up-regulated genes was carried out to confirm the accuracy of microarray analysis.

Phenolic compound analysis

Six phenolic compounds, viz., vanillin, ferulic acid, chlorogenic acid, ρ-coumaric acid, naringenin, and biochanin A, were extracted from 100 mg brown rice grain powders of the resveratrol transgenic rice lines and non-transgenic varieties DJ and ND. Extractions were analyzed by gas chromatography time-of-flight (TOF) mass spectrometry using a model 7890A gas chromatograph (Agilent, Atlanta, GA, USA) coupled with a Pegasus HT TOF mass spectrometer (LECO, St. Joseph, MI, USA) as described by Kim et al. (2013). Resveratrol was extracted and analyzed by high-performance liquid chromatography as described by Baek et al. (2013). All transgenic and non-transgenic samples were naturally dried over 2 months after harvesting and three independent repeats were sampled at different plots of the same field in 2012.

Agronomic and yield trait evaluation and statistical analysis

Nine agronomic and yield traits consisting of culm length, panicle length, panicle number, 100-grain weight, weight per plant, unfilled grain number, filled grain number, grain number per panicle, and seed set were measured for 100 samples of both transgenic lines, and the DJ variety, from 2011 to 2013, at the Suwon GMO experimental field. Mean values and standard deviations were calculated, and *t*-tests were performed using Microsoft Excel.

Conclusion

Microarray based transcriptome profiling was carried out on two transgenic lines, I515 and I526, their wild type variety DJ, and a non-GM variety, ND, to find unintended effects on gene networks especially in the secondary metabolic pathway activated by *RS* insertion. A total of 34 and 21 genes showed differential expression on I515 and I526 comparing to the wild type variety DJ, respectively. Among these, only five genes with similar changes were observed in the transcriptomes of both transgenic lines, eliminating the influences of environmental or variety-based variations. Comparing to 180 genes differentially expressed in ND compared to DJ, transcriptome variations caused by *RS* insertion were far less than those due to cultivar differences. Changes in genes encoding proteins functioning up- and down-stream of the inserted RS did not reach 2-fold in both transgenic rice lines, which indicated that the *RS* insertion did not cause activation of any unexpected gene networks in the secondary metabolic pathway. No significant differences in ρ-coumaric acid, vanillin, and chlorogenic acid levels, metabolites up-stream of RS, were detected; however, naringenin, a metabolite found down-stream of RS, showed a 2-fold lower content in I515, compared to I526, the DJ counterpart, and a common variety. In conclusion, the inserted *RS* gene had little impact on the transcriptome of both transgenic lines, but differences in the contents of secondary metabolites accompanied by resveratrol production suggested that global metabolite profiling and comparative analysis of both transgenic lines should be carried out in future.

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References

Abdeen A, Schnell J, Miki B (2010) Transcriptome analysis reveals absence of unintended effects in drought-tolerant transgenic plants overexpressing the transcription factor ABF3. BMC Genomics. 11:69-90

Baek SH, Shin WC, Ryu HS, Lee DW, Moon EJ, Seo CS, Hwang E, Lee HS, Ahn MH, Jeon YJ, Kang HJ, Lee SW, Kim SY, DSouza R, Kim HJ, Hong ST, Jeon JS (2013) Creation of resveratrol-enriched rice for the treatment of metabolic syndrome and related diseases. PLoS ONE. 8:e57930

Barro F, Barcelo P, Lazzeri P, Shewry P, Martin A, Ballesteros J (2002). Field evaluation and agronomic performance of transgenic wheat. Theor Appl Genet.105:980-984

Coll A, Nadal A, Palaudelmas M, Messeguer J, Mele E, Puigdome'nech P, Pla M (2008) Lack of repeatable differential expression patterns between MON810 and comparable commercial varieties of maize. Plant Mol Biol. 68:105-117

Coll A, Nadal A, Collado R, Capellades G, Messeguer J, Mele E, Palaudelmás P, Pla M (2009) Gene expression profiles of MON810 and comparable non-GM maize varieties cultured in the field are more similar than are those of conventional lines. Transgenic Res. 18:801-808

Coll A, Nadal A, Collado R, Capellades G, Kubista M, Messeguer J, Pla M (2010) Natural variation explains most transcriptomic changes among maize plants of MON810 and comparable non-GM varieties subjected to two N-fertilization farming practices. Plant Mol Biol. 73:349-362

Coutos-Thevenot P, Poinssot B, Bonomelli A, Yean H, Breda C, Buffard D, Esnault R, Hain R, Boulay M (2001) In vitro tolerance to *Botrytis cinerea* of grapevine 41B rootstock in transgenic plants expressing the stilbene synthase Vst1 gene under the control of a pathogen-inducible PR 10 promoter. J Exp Bot. 52:901-910

Delaunois B, Cordelier S, Conreux A, Clement C, Jeandet P (2009) Molecular engineering of resveratrol in plants. Plant Biotech J. 7:2-12

Hanhineva K, Kokko H, Siljanen H, Rogachev I, Aharoni A, Kärenlamp SO (2009) Stilbene synthase gene transfer caused alterations in the phenylpropanoid metabolism of transgenic strawberry (Fragaria × ananassa). J Exp Bot. 60:2093-2106

Kim JK, Park SY, Lee SM, Lim SH, Kim HJ, Oh SD, Yeo YS, Cho HS, Ha SH (2013) Unintended polar metabolite profiling of carotenoid-biofortified transgenic rice reveals substantial equivalence to its non-transgenic counterpart. Plant Biotechnol Rep. 7:121-128

Kogel KH, Voll LM, Schäfera P, Jansen C, Wu YC, Langen G, Imani J, Hofmann J, Schmiedl A, Sonnewald S, Wettsteina DV, Cook RJ, Sonnewal U (2010) Transcriptome and metabolome profiling of fieldgrown transgenic barley lack induced differences but show cultivar-specific variances. Proc Natl Acad Sci USA. 107:1-6

Kiselev KV (2011) Perspectives for production and application of resveratrol. Appl Microbiol Biotechnol. 90:417-425

Langcake P, Pryce CA (1976) The production of resveratrol by *Vitis vinifera* and other members of Vitaceae as a response to infection or injury. Physiol Plant Pathol. 9:77-85

Leckband G, Lörz H (1998) Transformation and expression of a stilbene synthase gene of *Vitis vinifera L*. in barley and wheat for increased fungal resistance. Theor Appl Genet. 96:1004-1012

Qin Y, Ahn HI, Kweon SJ, Baek SH, Shin KS, Woo HJ, Cho HS, Lee JH, Lim MH (2013a) Molecular characterization of transgenic rice producing resveratrol. Plant Breed Biotech. 1:406-415

Qin Y, Kim SM, Ahn HI, Lee JH, Baek SH, Shin KS, Woo HJ, Cho HS, Kweon SJ, Lim MH (2013b) Bioassay for the response of resveratrol transgenic rice lines to bacterial and fungal diseases. Plant Breed Biotech. 1:251-263

Rupprich N, Hildebrand H, Kindl H (1980) Substrate specificity in vivo and in vitro in the formation of stilbenes biosynthesis of rhaponticin. Arch Biochem Biophys. 200:72-78

Thomzik JE, Stenzel K, Stöcker R, Schreier PH, Hain R, Stahl DJ (1997) Synthesis of a grapevine phytoalexin in transgenic tomatoes *(Lycopersicon esculentum Mill*.) conditions resistance against *Phytophthora infestans.* Physiol Mol Plant Pathol. 51:265-278

Zhou J, Zhang L, Li X, Chang YW, Gu Q, Lu X, Zhu Z, Xu G (2012) Metabolic profiling of transgenic rice progeny using gas chromatography–mass spectrometry: the effects of gene insertion, tissue culture and breeding. Metabolomics. 8:529-539

Zhu YJ, Agbayani R, Jackson MC, Tang CS, Moore PH (2004) Expression of the grapevine stilbene synthase gene *VST1*, in papaya provides increased resistance against diseases caused by *Phytophthora palmivora*. Planta. 12:807-812