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Comparison of gene expression profiles in *Bacillus megaterium* treated tobacco leaves using microarray

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The MP agent, prepared from *Bacillus* megaterium isolated from the soil near tobacco fields, can improve metabolic products, and hence the aroma, of tobacco (*Nicotiana tabacum*) leaf. To explore genes regulating metabolic responses in tobacco leaf, we used microarrays to analyze differentially expressed genes in tobacco leaves subjected to various treatments. The expressed genes were classified into six groups based on their expression profile. In total, 753 genes were significantly differentially expressed between microorganism-treated and water-treated samples. Gene ontology (GO) analyses showed that most of these genes were involved in metabolic and cellular processes. Some up-regulated genes were related to the plant defense response, such as *NtMMP1* and *NtACRE231*. Some genes were involved in metabolism responses, such as *NtDXS*. Semi-quantitative reverse transcriptase (RT)-PCR analysis of *NtMMP1* and quantitative RT-PCR analysis of *NtDXS* showed that their expression levels increased after MP agent treatment, confirming the microarray results. We evaluated *NtMMP1* and *NtDXS* in terms of their associations with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Phylogenetic analyses of *NtMMP1*, *NtACRE231,* and *NtDXS* revealed their relationships with homologs in other species. These microarray data increase our understanding of the mechanisms by which MP agent induces a metabolic response in tobacco leaves

Key words: *Nicotiana tabacum*, Microarray, MP agent, plant defense response, aroma.

INTRODUCTION

Tobacco (*Nicotiana tabacum*) is an important economic plant that has been used as a model for plant metabolomics and green bioreactors (Zhang et al., 2011; Poethig and Sussex, 1985; Sang-Wook Park et al., 2007; Tremblay et al., 2010). In plants, secondary metabolites are important for many processes, and are closely related to plant growth and development, and adaptation to the environment. Phenolamides (PAs) are a diverse group of plant secondary metabolites that are found in many dicotyledonous plants, suggesting that they play a role in plant

growth and development (Martin-Tanguy, 1985; Facchini, 2002; Edreva, 2007; Grienenberger and Legrand, 2009). Jasmonates are important plant hormones that mediate plant responses to attack from herbivores and necrotrophic pathogens (Howe and Jander, 2008; Bari and Jones, 2009). In tobacco cell cultures, methyl jasmonate (MeJA) strongly induces the expression of genes related to alkaloid and phenylpropanoid biosynthetic pathways (Goossens et al., 2003). Salicylic acid (SA) is another important signaling compound in plant defense response

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(Verberne et al., 2007). In tobacco, products of second-dary metabolism not only play roles in growth, develop-ment, and defense, but also give a characteristic aroma. The main substances contributing to the aroma are phenolic compounds, terpenoids, and alkaloids, which are formed via the phenylpropanoid metabolic pathway, isoprenoid biosynthetic pathway, and alkaloid biosyn-thetic pathway, respectively (Wang et al., 2008).

In tobacco, treatments with microorganisms have been applied to alter metabolic products, to modify various aspects of plant development and regulation, and to im-prove processing and storage of the tobacco crop. Microorganism treatments were first used by Koller to increase the aroma of tobacco (Koller, 1858). Subse-quently, Tamayo used microorganisms to enhance tobacco aroma and reported that *Bacillus* and *Micrococcus* improved the aroma and decreased the protein content of tobacco leaves (Tamayo and Cancho, 1953). Other studies suggested that pleasant odor components could be produced quickly when tobacco was treated with individual or mixed strains of *Bacillus subtilis* (English et al., 1967). Several bacteria, such as *Pseudomonas convexa* (syn. *Pseudomonas putida*) PC1 (Thacker et al., 1978), *Achromobacter nicotinophagum* (Hylin, 1959) and *Arthrobacter oxydans* (Gherna et al., 1965), can degrade nicotine. In the genus Arthrobacter, the mechanisms of metabolic degradation of nicotine have been charac-terized in detail (Gherna et al., 1965; Schenk et al., 1998; Sachelaru et al., 2005; Ganas et al., 2008). One type of bacillus, *Bacillus megaterium*, has been used to prepare an 'MP agent', which, when sprayed onto tobacco, resul-ted in marked increases in the concentrations of the most important aroma components and decreases in protein content (Wang et al., 2006). However, the mechanism by which these changes occur is still unknown.

Microarray technology has become one of the most important approaches to examining thousands of genes simultaneously. Microarray analyses reveal the global biological functional differences among plants subjected to various treatments, which is the key to deciphering the networks of genes and their regulators that lead to a wide variety of defense responses (Lodha and Basak, 2011). This method has been applied extensively to determine the gene expression patterns in many different orga-nisms, including *Arabidopsis thaliana, Oryza sativa, Zea mays, Glycine max, Solanum lycopersicum* and *Vitis vinifera* (Coughlan et al., 2004; Ma et al., 2005; Lund et al., 2008; Hayes et al., 2010; Mathias et al., 2010; Rohrmann et al., 2011). *Arabidopsis thaliana* is a power-ful model system in the plant kingdom, and the relation-ship between the plant itself and the microorganism has been particularly well studied. Recent microarray studies have showed that the plant immune response is the same among many species at the gene expression level (Wang et al., 2010; Weisman et al., 2010; Van Verk et al., 2011). For example, Arabidopsis BRCA2 and RAD51 proteins are specifically involved in transcription of defense

genes in plant immune responses (Wang et al., 2010). Another two genes, WRKY28 and WRKY46, are both rapidly induced by pathogen elicitors and are related to systemic acquired resistance (Van Verk et al., 2011; Eulgem et al., 2000). In addition, enhanced disease sus-ceptibility1 (EDS1) which interacts with two related pro-teins encoded by Phytoalexin Deficient4 (PAD4) and Senescence Associated Gene101 (SAG101), is an important regulator of plant basal and receptor-triggered immunity (Rietz et al., 2011). It has been reported previously that Arabidopsis CRT2 is another regulator of plant innate immunity that plays a role in regulating plant defense against pathogens (Qiu et al., 2011). Microarray technology has been used widely in studies on tobacco. For example, an Affymetrix tobacco expression micro-array was generated from a set of more than 40 k unigenes and was used to measure gene expression in 19 different tobacco samples to produce the Tobacco Expression Atlas (TobEA) (Edwards et al., 2010). A cDNA microarray prepared from 2831 clones was used to compare gene expression levels in trichome and leaf tissues of tobacco (Cui et al., 2011). In addition, transcript levels in leaves and flowers of transgenic tobacco plants were analyzed using Agilent microarray techniques (Soitamo et al., 2011).

Previous research has suggested that an MP agent prepared from Bacillus megaterium might improve the metabolic products of tobacco leaves (Wang et al., 2006). Therefore, a systematic understanding of genes in *Nicotiana tabacum* and the mechanisms that underlie the improvement of its metabolic products of tobacco leaves is of great interest. In this study, we performed microarray analysis to identify differentially expressed genes bet-ween MP agent-sprayed and water-sprayed tobacco leaves.

MATERIALS AND METHODS

Microorganism cultivation

MP agent was prepared from *Bacillus megaterium* according to the method of Wang et al. (2006). The *Bacillus megaterium* strain was incubated on beef extract peptone medium at 37°C with shaking at 180 rpm until the concentration reached 108 CFU/mL. The super-natant and pellet were harvested after 24 h.

Agilent microarray design

The Agilent "4×44K" microarray chip was designed for *Nicotiana tabacum* cDNA sequences in an effort to cover most tobacco genes with at least one 60-mer oligonucleotide probe. The probes were designed by CapitalBio Corporation (Beijing, China). To investigate differences in gene expression between leaves subjected to various treatments and the control, we sprayed tobacco leaves with four different preparations: supernatant, pellet, and whole bacterial liquid of the MP agent, and water.

Experimental tobacco leaves and RNA isolation

The flue-cured tobacco variety K326 was cultivated in water at a

stable temperature of 25°C. The tobacco leaves were sprayed with supernatant, pellet, whole bacterial liquid of the MP agent, or water at 10 days before maturity. The treated leaves were snap-frozen and stored in liquid nitrogen for RNA extraction. Total RNA was isolated from each sample by TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA templates were quantified by spectrophotometry and subjected to 1.0% formaldehyde denatured agarose gel electrophoresis. The average yield of RNA in each sample was approximately 0.5 µg/mg.

Probes labels and microarray hybridization

The cDNA targets were prepared from 5 µg of total RNA and then labeled with a fluorescent dye (Cy5 and Cy3-dCTP, GE Healthcare Cat. No. PA 55021/ PA 53021). The samples were divided into four groups. In the first group, cDNA from tobacco leaves sprayed with supernatant was labeled with cy5 and cDNA from tobacco leaves sprayed with water (control) was labeled with cy3. In the second group, the cDNA from tobacco leaves sprayed with the pellet and water were labeled with cy5 and cy3, respectively. The labeling cDNA of the third group was fluorescence exchange according to the second group. In the fourth group, the cDNA from tobacco leaves sprayed with bacterial liquid of the MP agent and water were labeled with cy5 and cy3, respectively. The labeled cDNAs were dissolved in 80 µL of hybridization solution containing 3×SSC, 0.2% SDS, 5×Denhardt's solution and 25% formamide, then hybridized at 42°C overnight. After hybridization, slides were washed with washing solution (0.2% SDS, 2×SSC and 2×SSC, respectively) at 42°C for 5 min. Chips were scanned with feature extraction software and images were then analyzed by GeneSpring software (both from Agilent Technologies Co., Ltd.).

Bioinformatics

Microarray analysis

In this research, results were filtered according to the marked flag of feature extraction software. Points marked as "detected" were defined as "active genes". Using the above fluorescent labeling method, we set "sprayed with MP agent": "sprayed with water" (M: W) as the ratio. Then, we averaged the two ratios of the second and third groups, and converted the obtained ratios by a log function to generate data for analyses. When the M:W ratio showed a difference of more than 2-fold, the genes were considered to be differentially expressed (that is, M:W□2, genes were up-regulated; M:W<0.5, genes were down-regulated).

When the analysis data was obtained, whole hierarchical clustering of the average signal intensities was performed using the program Cluster (Cluster 3.0) and the results for all ratios were visualized using the program TreeView. Then, genes were systematically annotated using the following bioinformatics tools: BLASTX was used to search for homologs and Gene Ontology was used for functional classifications (Ashburner et al., 2000). Gene sequences were BLASTXed to the non-redundant (nr) database in GenBank. GO classifications were performed for these species using the BGI-WEGO (Web Gene Ontology Annotation Plotting) web service (http://wego.genomics.org.cn).

Phylogenetic analyses

We used MEGA version 4 software for phylogenetic analyses (Tamura et al., 2007), and ClustalW for multiple alignments of protein sequences (Thompson et al., 1994). All protein sequences used for phylogenetic analyses were downloaded from NCBI.

Pathway analyses

NtMMP1 and NtDXS involved in the plant defense and MEP metabolism pathway were then clarified and graphically displayed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway method (http://www.genome.jp/kegg/).

Semi-quantitative RT-PCR and Quantitative RT-PCR

To investigate the expression level of the *NtMMP1* and *NtDXS* genes, we used RT-PCR and quantitative real-time PCR, respectively. RT-PCR was performed for 25 cycles. The primers designed for NtMMP1 (GenBank accession no. DQ508374.1) was 5'-GAACGGTCTGACGGATAA-3' (sense) and 5'-GCTAAACTCGGGAACATAA-3' (anti-sense).

Quantitative real-time PCR was used to confirm the expression level and microarray data. The primer sequences of NtDXS (GenBank accession no. FN429979.1) was 5'-TATTGGTCCTGTGGATGGT-3' (sense) and 5'-AAACTTGGCTACTCCGTGA-3' (anti-sense). The real-time PCR was carried out using the SYBR Premix Ex Taq kit (TaKaRa, Dalian, China) and each reaction was prepared in 25 µL containing 2 µL complementary DNA, SYBR Premix Ex Taq 12.5 µL, 10 mmol/L primers 0.5 µL (sense and anti-sense). The PCR was set with the following steps: started with 10 s template denaturation at 95°C, followed by 40 cycles of denaturation at 95°C for 5 s, and then combined primer annealing/elongation at 60°C for 31 s according to the manufacturer's instructions. Then the results were read by ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, US). The real-time PCR was performed in duplicate for at least three biological replicates. The amplification of NtGAPDH cDNA sequence was taken as the inner control.

RESULTS

Differences in gene expression among leaf samples from four different treatments

Using the Agilent microarray, we analyzed the gene expression profiles of tobacco leaves subjected to four different spray treatments: supernatant, pellet, and whole bacterial liquid of the MP agent, and water (control). According to "feature extract" analysis software, the points marked as "detected" were defined as "active genes". As a result, we detected expressions of 9,565 genes. Next, we calculated gene-expression ratios (log-ratios) of "sprayed with MP agent":"sprayed with water" (M:W). The genes were clustered into six groups according to their expressions. Group 1 contained all of the up-regulated genes (2867 in total); group 2 contained all of the down-regulated genes (2770 in total); group 3 contained 996 genes that were up-regulated in leaves sprayed with supernatant.

Some of these genes were up-regulated in leaves sprayed with the pellet, and were down-regulated in leaves sprayed with the whole bacterial liquid of the MP agent. Group 4 contained 701 genes that were down-regulated in leaves sprayed with supernatant. Some of these genes were up-regulated in leaves sprayed with the pellet and with whole bacterial liquid. Group 5
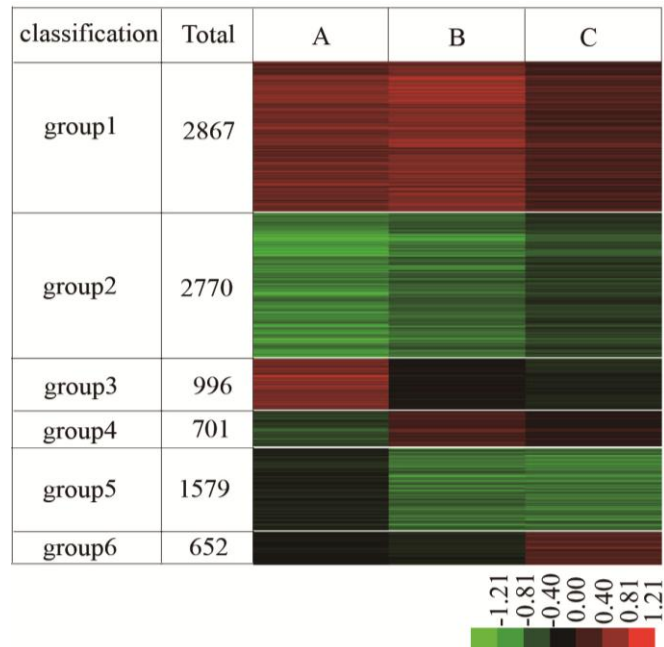
classification	Total	A	B	C
group1	2867			
group2	2770			
group3	996			
group4	701			
group5	1579			
group6	652			

-1.21 -0.81 -0.40 0.00 0.40 0.81 1.21

Figure 1. Hierarchical cluster analysis of genes expressed in response to MP agent- and water-treatments. Gene expression levels are represented by red and green boxes (denoting those with up-regulated and down-regulated expressions, respectively). Horizontal lines represent differential expression of genes of interest. Vertical rows represent different treatments of tobacco leaves (A, B, and C). Ratios represent "Sprayed with microorganism": "sprayed with water" (M: W). A: Sprayed with supernatant of MP agent: sprayed with water; B: sprayed with pellet of MP agent: sprayed with water; C: sprayed with whole bacterial liquid of MP agent: sprayed with water. Genes were classified into six groups based on their expression profile: Groups 1 and 2 included all up-regulated and down-regulated genes, respectively. Group 3 included genes that were up-regulated in leaves sprayed with supernatant of MP agent. Group 4 included genes that were down-regulated in leaves sprayed with supernatant and up-regulated in those sprayed with pellet and bacteria liquid of MP agent. Group 5 included genes that were significantly down-regulated after spraying with pellet and bacteria liquid of MP agent, except for some genes that were up-regulated after spraying with supernatant. Group 6 included genes that were up-regulated only in response to spraying with whole bacterial liquid of MP agent.

contained 1579 genes that were significantly down-regulated; however, some of these were up-regulated after spraying with supernatant. The 652 genes in Group 6 were up-regulated only after spraying with whole bacterial liquid of the MP agent (Figure 1). Among the six groups of genes, 753 genes were identified to differentially express. When expressions were compared between leaves sprayed with MP agent and those sprayed with water, there were 368 up-regulated genes 385 down-regulated genes.

Gene ontology annotations and analysis

The 753 differentially expressed genes were subjected to gene ontology (GO) analysis, and GO annotations were obtained for 192 genes. We also analyzed these genes in terms of their participation in biological processes. The results show that most of these genes were involved in metabolic process (118 genes) and cellular process (100 genes), accounting for 61.5 and 52% of total annotated genes, respectively. There was only one gene classified into each of the cellular component organization, multicellular organismal process, and cellular component biogenesis categories (Figure 2). Among the 192 annotated genes, there were many more up-regulated genes than down-regulated genes. The up-regulated genes were involved in processes such as reproduction, reproductive process, multicellular organism process, cellular component biogenesis, and multi-organism process. One
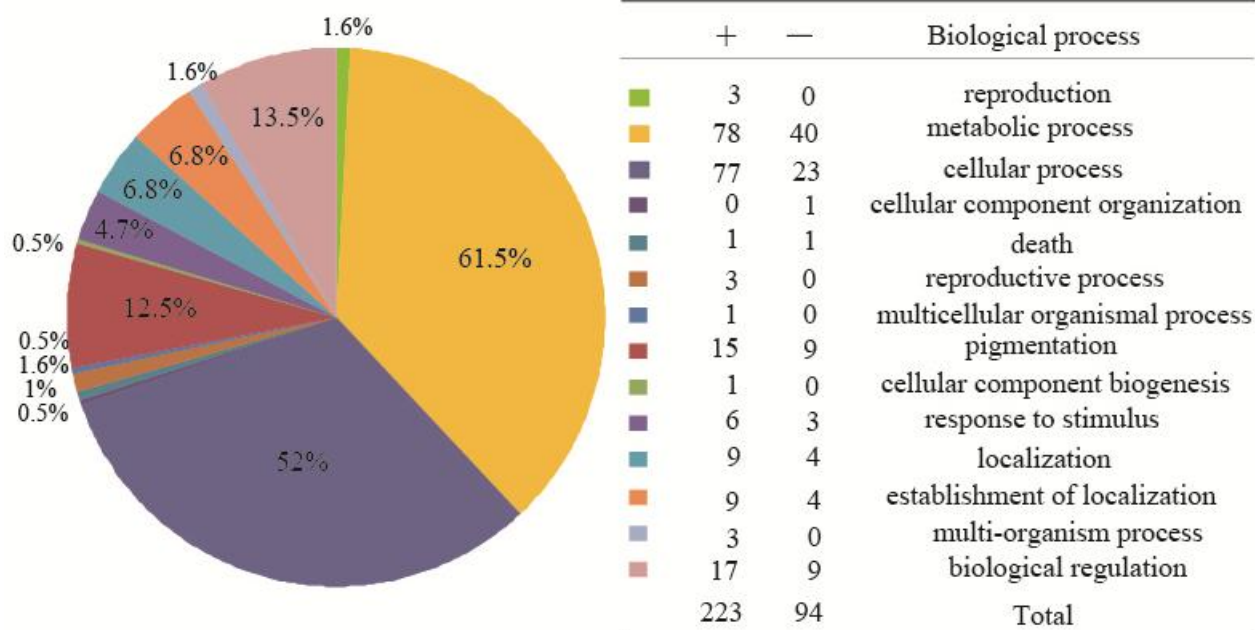
+	−	Biological process
3	0	reproduction
78	40	metabolic process
77	23	cellular process
0	1	cellular component organization
1	1	death
3	0	reproductive process
1	0	multicellular organismal process
15	9	pigmentation
1	0	cellular component biogenesis
6	3	response to stimulus
9	4	localization
9	4	establishment of localization
3	0	multi-organism process
17	9	biological regulation
223	94	Total

Figure 2. Gene ontology categories of the differentially expressed genes. Numbers of up (+) or down.

down-regulated gene was involved in cellular component organization. The analysis suggested that the genes with unknown or other annotated functions may be involved in biological processes.

Differentially expressed genes in response to MP agent treatment

Based on the microarray data, some differentially expressed genes were present in all six groups. According to their functional annotation, some of the up-regulated expression genes were closely related to plant defense response, hormone metabolism, cell cycle regula-tion and enzyme regulation. For instance, expres-sions of NtMMP1, NtACRE231, elicitor inducible LRR protein (EILP), and five WRKY transcription factors, which are involved in the plant defense response, were up-regulated after MP agent treatment (Yamamoto et al., 2004; Schiermeyer et al., 2009; Durrant et al., 2000; Takemoto et al., 2000; Rowland et al., 2005; Park et al., 2006). Some up-regulated genes were closely related to metabolic regulation (example, genes encoding P450 mono-oxygenase (Simon-Mateo et al., 2006) and pyruvate kinase (Grodzinski et al., 1999) and to cell cycle regulation (example, genes encoding cyclin-dependent kinase B1-2 (Sorrell et al., 2001), ribonucleotide reductase (Chaboute et al., 1998), and cyclin A-like protein (Reichheld et al., 1996). At the same time, 385 genes were down-regulated after MP agent treatment. Some down-regulated genes were associated with metabolic regulation, such as genes

encoding an ATP-binding cassette (ABC) transporter (Yazaki, 2005, 2006) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Munoz-Bertomeu et al., 2010). Some down-regulated genes were related to cell cycle regulation, for instance, the gene encoding the ribosomal protein (Turkina et al., 2011), cytochrome C oxidase (Yamashita and Voth, 2011). Other down-regulated genes were involved in plant defense responses (for example, pectinesterase inhibitor, which plays a role in the plant defense mechanism via cell wall fortification (Hong et al., 2010; Jolie et al., 2010), and polyphenol oxidase, a multifunctional enzyme involved in the defense response (Thipyapong et al., 2004; Poiatti et al., 2009). Further analysis of these differentially expressed genes will provide new insights into the molecular mechanisms underlying different responses among tobacco leaves subjected to various treatments.

Analyses of selected genes associated with plant defense responses

There are a number of potential genes related to growth and anti-disease responses in plants. When plants perceive some kind of pathogenic stimulation, expressions of some genes are induced leading to various reactions including resistance or growth stimulation. Previous studies indicated that after spraying with a microorganism MP agent, the levels of some proteins and amino acids decreased in tobacco leaves,

while the main aroma components significantly increased (Wang et al., 2006). According to our microarray results, spraying with a microorganism MP agent resulted in up-regulated expressions of genes involved in the plant defense response such as NtMMP1, NtACRE231, EILP, and WRKY transcription factors, but down-regulated expressions of pectinesterase inhibitor and polyphenol oxidase (Hong et al., 2010; Jolie et al., 2010; Thipyapong et al., 2004; Poiatti et al., 2009; Durrant et al., 2000; Takemoto et al., 2000; Eulgem and Somssich, 2007; Schiermeyer et al., 2009).

Matrix metalloprotease 1

Plant matrix metalloproteinases (MMPs) are conserved proteolytic enzymes that are widely distributed in the plant kingdom. MMPs play crucial roles in many aspects of pathogen defense (Liu et al., 2001), senescence (Delorme et al., 2000) and growth, and development (Golldack et al., 2002). In Nicotiana tabacum, tobacco MMP1 (NtMMP1) participate in the pathogen defense (Schiermeyer et al., 2009). In our research, we analyzed the evolution of MMPs or an unknown protein that contained a characteristic MMP domain in 15 plant species belonging to 10 families. We constructed a phylogenetic tree to examine the relationships of these proteins (Figure 3). The result indicates that NtMMP1 (red, marked with an asterisk) clustered with MMP from *Nicotiana benthamiana*. This group clustered with a zinc metalloproteinase of *A. thaliana* (Cruciferae), a matrixin family protein of *Brassica oleracea* (Cruciferae), a matrix metalloproteinase of *A. thaliana*, and a predicted GPI-anchored protein of A. thaliana. We analyzed whether NtMMP1 exhibited different transcriptional responses after treatment with the MP agent. Semi-quantitative RT-PCR showed that the highest transcript levels of NtMMP1 were observed after spraying with the pellet of the MP agent, followed by the bacterial liquid of the MP agent, then the MP agent supernatant. The lowest transcript levels were observed after spraying with the water control (Figure 4). To determine whether the function of NtMMP1 is similar to that of MMP1, we carried out a pathway analysis. The results indicate that NtMMP1 participates in the Toll-like receptor signaling pathway (Supplementary Figure S1).

Avr9/Cf-9 rapidly elicited protein 231

As mentioned above, plant cells induce an array of defense responses upon perceiving pathogens. Previously, Avr9/Cf-9 rapidly elicited (ACRE) genes from tobacco were identified to encode putative signaling components and may play important roles in the initial development of the defense response (Rowland et al., 2005; Durrant et al., 2000). In our research, we found that the expression level of *Nicotiana tabacum* ACRE231

(NtACRE231) was up-regulated in response to MP agent treatment. Subsequently, we analyzed the evolution of NtACRE231 and its homologs in 16 plant species. We found that NtACRE231 clustered with the glycosyltransferase of *Panax notoginseng* (Supplementary Figure S2).

Analysis of metabolism-related genes in tobacco

Plant secondary metabolites play important roles in plant growth and development, and in adaptation to the environment. As a model plant, tobacco is well characterized in terms of its metabolic responses (Martin-Tanguy, 1985; Facchini, 2002; Edreva, 2007; Goossens et al., 2003; Verberne et al., 2007). In our research, we found that some differentially expressed genes between MP agent-treated and water-treated tobacco were closely related to metabolic responses. These included 1-deoxy-D-xylulose-5-phosphate synthase (DXS) (Walter et al., 2000, 2002; Estevez et al., 2001), and genes encoding P450 monooxygenase (Simon-Mateo et al., 2006), pyruvate kinase (Grodzinski et al., 1999), an ABC transporter (Yazaki, 2005, 2006), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Munoz-Bertomeu et al., 2010).

1-Deoxy-D-xylulose-5-phosphate synthase

Isoprenoids are a large and highly diverse family of natural products involved in primary and secondary metabolism (Buckingham, 1998). The key pathway for production of isoprenoids is the methylerythritol phosphate (MEP) pathway (Walter et al., 2002), in which 1-deoxy-D-xylulose-5-phosphate synthase (DXS) plays a central role (Estevez et al., 2001; Walter et al., 2000, 2002). We found that the expression level of the N. tabacum DXS gene (NtDXS) increased after spraying with MP agent. Considering that DXS is an important player in the MEP pathway, we further examined NtDXS to clarify its role in the regulation of plastidic isoprenoid biosynthesis (Supplementary Figure S3). Quantitative PCR analyses showed that the highest level of NtDXS expression was after spraying with the bacterial pellet of the MP agent, followed by whole bacterial liquid, then the supernatant. The lowest expression was observed after spraying with water (Figure 5). A phylogenetic tree of the gene and its homologs showed that NtDXS clustered with the DXS of Capsicum annuum (CaDXS) (Supplementary Figure S4).

DISCUSSION

Tobacco is a dicot, and is a member of the Solanaceae. Secondary metabolic substances produced by tobacco
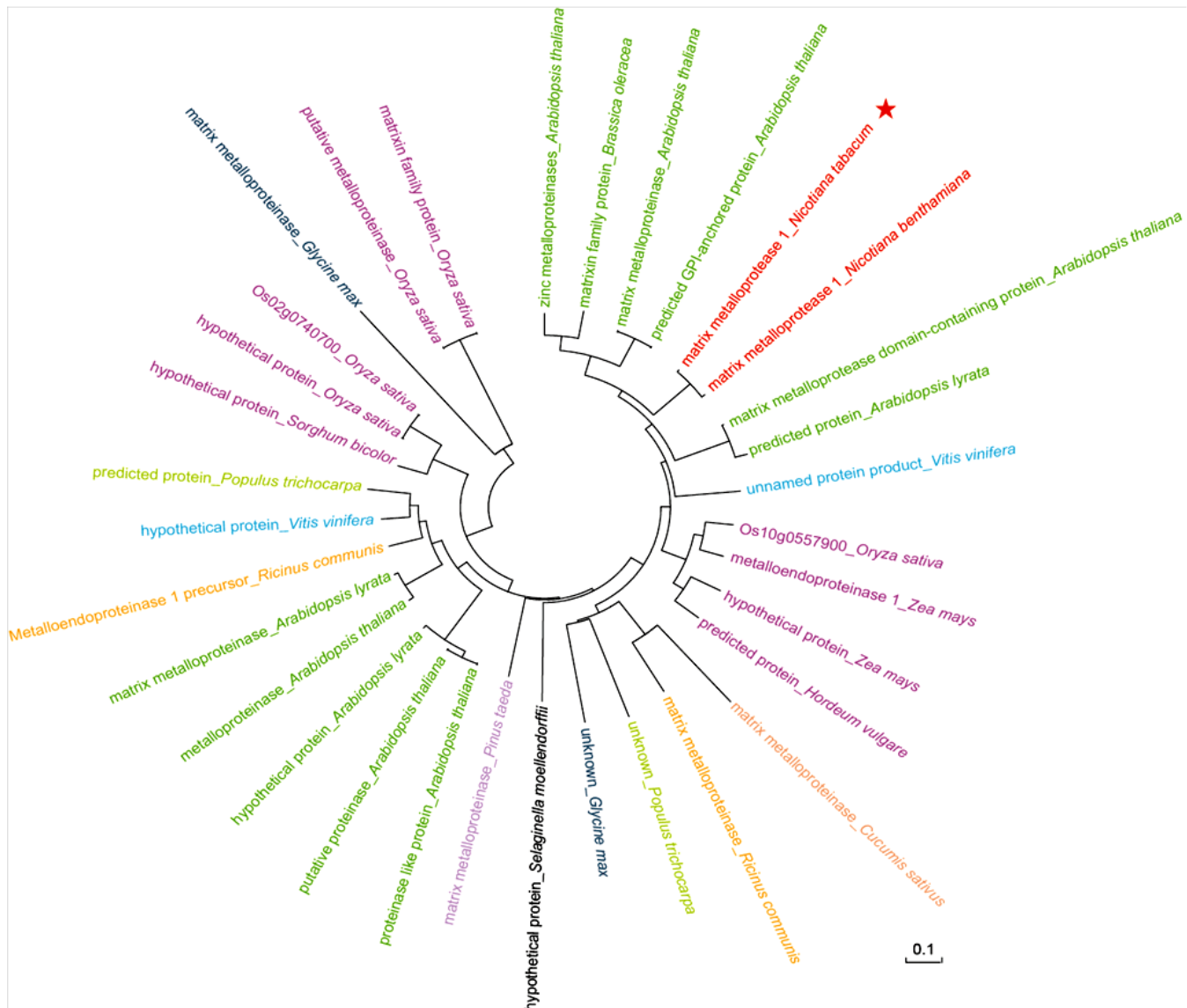
Figure 3. Molecular phylogeny of matrix metalloproteinases (*MMP*). Phylogram was constructed by the Neighbor-Joining method using MEGA4 software and reflects the relationship between *MMP* and its homologs in 15 plant species. Different colors show different plant families: Solanaeae shown in red (tobacco); Brassicaceae shown in green (*Arabidopsis thaliana*, *Brassica oleracea*, and *Arabidopsis lyrata*); Poaceae shown in purple (*Oryza sativa*, *Zea mays*, *Sorghum bicolor,* and *Hordeum vulgare*); Salicaceae shown in light green (*Populus trichocarpa*); Vitaceae shown in brilliant blue (*Vitis vinifera*); Euphorbiaceae shown in orange (*Ricinus communis*); Pinaceae shown in light purple (*Pinus taeda*); Selaginellaceae shown in black (*Selaginella moellendorffii*); Leguminaceae shown in blue (*Glycine max*); and Cucurbitaceae shown in pink (*Cucumis sativus*). Asterisk represents *matrix metalloprotease 1* of *Nicotiana tabacum*. See Materials and Methods for details of the phylogenetic analysis.

play roles in growth, development, defense responses, and in producing an aroma (Onkokesung et al., 2011; Verberne et al., 2007; Lackman et al., 2011; Liu and Thornburg, 2011; Naoumkina et al., 2008). Previously, it was reported that an MP agent produced by Bacillus megaterium, which was screened from soil in tobacco fields, was useful for increasing the aroma of tobacco. biosynthesis (Supplementary Figure S3). Quantitative PCR analyses showed that the highest level of NtDXS

The MP agent was applied to upper leaves in fields, while water was applied to controls, and then the normal processing and maturing steps were conducted. The leaves treated with MP agent showed increased contents of aroma components and decreased total amino acids and protein contents (Wang et al., 2006). In this study, we developed and validated a new 4×44k Agilent microarray, and used it to analyze gene expression after treatment with MP agent. Our results reveal that many
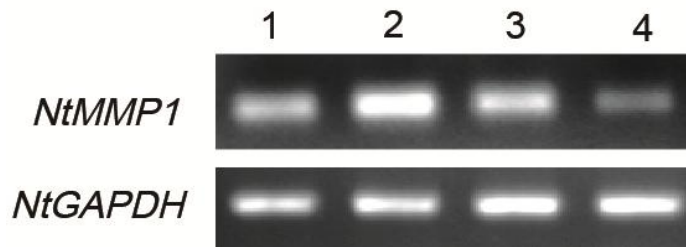
Figure 4. Expression profile of *NtMMP1* in tobacco leaves subjected to different treatments. Lane 1, tobacco leaves treated with supernatant of MP agent; lane 2, tobacco leaves treated with pellet of MP agent; lane 3, tobacco leaves treated with whole bacterial liquid of MP agent; lane 4, tobacco leaves treated with water. *NtGAPDH* mRNA was the internal control.
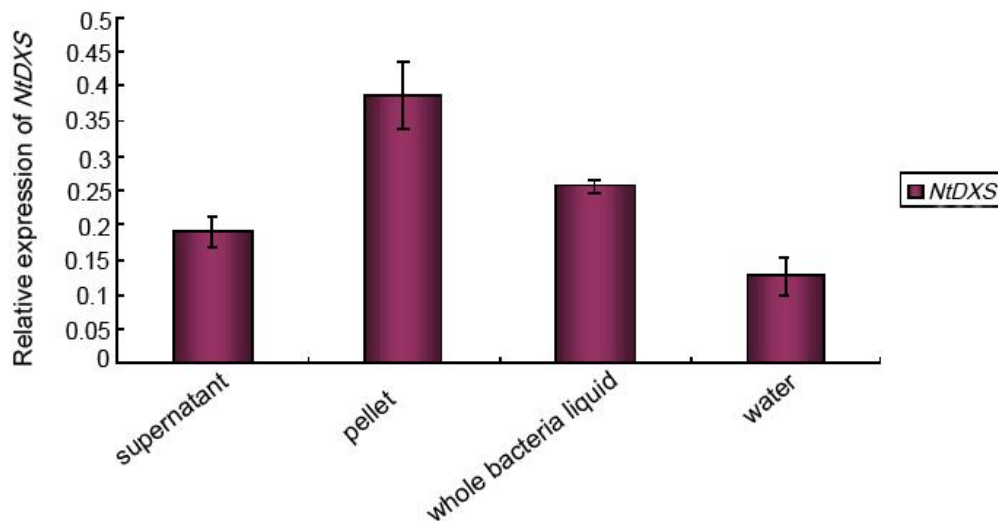

Figure 5. *NtDXS* expression was detected using real-time RT-PCR analysis after treatment with MP agent and water.

genes are differentially expressed between MP agent-treated and water-treated hydroponic seedlings of tobacco. These genes can be classed into six main groups (Figure 1). Our study reveals 753 differentially expressed genes, of which 368 were up-regulated and 385 were down-regulated. Functional analyses using Gene Ontology demonstrated that they are mainly involved in metabolic and cellular processes (Figure 2). We annotated these genes, and found that several genes have been reported previously to participate in plant defense responses. These include genes such as MMP1 (Schiermeyer, Hartenstein et al., 2009), ACRE231 (Durrant et al., 2000; Rowland et al., 2005), EILP (Takemoto et al., 2000), and those encoding WRKY transcription factors (Yamamoto et al., 2004; Park et al., 2006), a pectinesterase inhibitor (Hong et al., 2010; Jolie et al., 2010) and polyphenol oxidase (Thipyapong et al., 2004; Poiatti et al., 2009).

In the course of growth and development, plants face a variety of pathogens, including bacteria, fungi, viruses, and oomycetes. Plants have developed a variety of defense mechanisms against their attackers. However, disease resistance is often governed by a gene-for-gene interact-tions (Dangl and Jones, 2001). Gene-for-gene relation-ships, which are codetermined by a resistance gene (R) and an avirulence gene (Avr), are an important part of plant resistance. The interaction between R and Avr often leads to the hypersensitive response (HR) (Flor, 1971; Keen, 1990). Most R genes have conserved domains, such as leucine rich repeat (LRR), WRKY, and nucleotide-binding site (NBS) domains. Interestingly, we found EILP and five WRKY transcription factors containing LRR and WRKY domains, respectively, showing up-regulated expressions after leaves were sprayed with MP agent. Previous research showed that the product of EILP and WRKY transcription factors may play a central role in

plant immune responses (Bhattarai et al., 2010; Takemoto et al., 2000). Therefore, we can speculate that EILP and WRKY transcription factors play a similar role to that of the R gene after treatment with MP agent; that is, they recognize the complementary pathogen product of Avr, produce a resistance signal to activate a series of signal transduction processes, and activate expressions of defense genes leading to resistance.

The MMP1 gene is involved in a variety of physiological processes including senescence, pathogen defense, and growth and development (Delorme et al., 2000; Liu et al., 2001; Golldack et al., 2002). Our microarray data provide valuable insights into the expression level of NtMMP1 genes after treatment with MP agent and water. Subsequently, semi-quantitative RT-PCR analysis validated that transcript levels of NtMMP1 were significantly increased in response to MP agent treatment, confirming the results from the microarray analysis (Figure 4). Previous research showed that NtMMP1 plays a role in pathogen defense (Schiermeyer et al., 2009). We conducted pathway analysis for this gene and found that it is involved in the Toll-like receptor signaling pathway (Supplementary Figure S1). Plant matrix metalloproteinases (MMP) are collagenases, which are conserved proteolytic enzymes with the ability to degrade proteoglycan and accelerate degradation of the extracellular matrix (ECM) (Nagase and Woessner, 1999). NtMMP1 can degrade the pharmaceutical protein DSPAa1 (Mandal et al., 2010). In our research, we speculate that the increased expression level of NtMMP1 might result in not only enhanced resistance to pathogens, but also increased degradation of some proteins in tobacco leaves. This result reflects the underlying function of NtMMP1 in the metabolic pathway of tobacco.

Most of the aroma components of tobacco are produced via secondary metabolism. Previous studies indicated that a microorganism MP agent could significantly increase main aroma components (Wang et al., 2006). In our research, we found that some of the differentially expressed genes were closely related to metabolic responses. We focused on analyzing the expression level of the *Nicotiana tabacum* DXS gene (NtDXS). Previous research has shown that DXS is a limiting enzyme for plastidic isoprenoid biosynthesis in plants (Estevez et al., 2001; Walter et al., 2002). Isoprenoids participate in a variety of biological functions such as plant defense, photosynthesis, respiretion, growth, cell cycle control, and adaptation to environmental conditions (Estevez et al., 2001). In tobacco, isoprenoids also affect the formation of aroma (Cui et al., 2011). However, DXS, which participates in isoprenoid biosynthesis, is also a candidate gene for the trait of high levels of monoterpenols, which is associated with a distinctive aroma related to the composition of volatiles (Battilana et al., 2011). In our research, we found that the expression levels of NtDXS were increased by MP agent treatment, consistent with our microarray data (Figure 5). DXS is an important enzyme in regulation of the MEP pathway in plants (Estevez et al., 2001; Walter et al., 2000, 2002). Our pathway analysis of NtDXS yielded similar results to those for other DXSs, which are involved in the regulation of plastidic isoprenoid biosynthesis (Supplementary Figure S3). Our results suggest that NtDXS has a similar function to those of NtMMP1, NtACRE231, EILP, and WRKY transcription factors, all of which show increased expression levels after MP agent treatment, leading to increased resistance of tobacco and improved aroma characteristics of tobacco leaves. These results provide valuable information about NtDXS and allow us to clarify its possible function.

In summary, this study summarizes microarray data for tobacco leaves treated with MP agent and water. Analyses of gene expression showed that many genes were differentially expressed, and some of them were involved in plant defense responses. Further analyses of the genes identified in the microarray will increase our understanding of the role of MP agent in the growth and metabolism of *N. tabacum*.

Abbreviations

KEGG, Kyoto Encyclopedia of Genes and Genomes; **rpm,** revolutions per minute; **CFU/mL,** colony-forming units per milliliter; **Cy5 and Cy3,** reactive water-soluble fluorescent dyes of the cyanine dye family; **SSC,** standard saline citrate; **SDS,** sodium dodecyl sulfate; **BLAST,** Basic Local Alignment Search Tool; **BGI-WEGO,** Web Gene Ontology Annotation Plotting; **NCBI,** National Center for Biotechnology Information; **MEP,** methylerythritol phosphate; **RT-PCR,** reverse transcriptase polymerase chain reaction; **GO,** Gene Ontology; **ECM,** extracellular matrix.

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REFERENCES

Bari R, Jones JD (2009). Role of plant hormones in plant defence responses. Plant Mol. Biol. 69:473-488.

Battilana J, Emanuelli F, Gambino G, Gribaudo I, Gasperi F, Boss PK,Grando MS (2011). Functional effect of grapevine 1-deoxy-D-xylulose 5-phosphate synthase substitution K284N on Muscat flavour formation. J. Exp. Bot. 62:5497-5508.

Bhattarai KK, Atamian HS, Kaloshian I,Eulgem T (2010). WRKY72-type transcription factors contribute to basal immunity in tomato and Arabidopsis as well as gene-for-gene resistance mediated by the tomato R gene Mi-1. Plant J. 63:229-240.

Buckingham J (1998). Dictionary of Natural Products on CD-ROM (Version 6.1. London: Chapman & Hall).

Chaboute ME, Combettes B, Clement B, Gigot C, Philipps G (1998).

Molecular characterization of tobacco ribonucleotide reductase RNR1 and RNR2 cDNAs and cell cycle-regulated expression in synchronized plant cells. Plant Mol. Biol. 38:797-806.

Coughlan SJ, Agrawal V,Meyers B (2004). A comparison of global gene expression measurement technologies in Arabidopsis thaliana. Comp. Funct Genomics, 5: 245-252.

Cui H, Zhang ST, Yang HJ, Ji H,Wang XJ (2011). Gene expression profile analysis of tobacco leaf trichomes. BMC Plant Biol. 11:76.

Dangl JL, Jones JD (2001). Plant pathogens and integrated defence responses to infection. Nature 411:826-833.

Delorme VG, McCabe PF, Kim DJ, Leaver CJ (2000). A matrix metalloproteinase gene is expressed at the boundary of senescence and programmed cell death in cucumber. Plant Physiol. 123:917-927.

Durrant WE, Rowland O, Piedras P, Hammond-Kosack KE,Jones JD (2000). cDNA-AFLP reveals a striking overlap in race-specific resistance and wound response gene expression profiles. Plant Cell 12:963-977.

Edreva AM, Velikova VB, Tsonev TD (2007). Phenylamides in plants. Russ J Plant Physiol. 54:287-301.

Edwards KD, Bombarely A, Story GW, Allen F, Mueller LA, Coates SA,Jones L (2010). TobEA: an atlas of tobacco gene expression from seed to senescence. BMC Genomics 11:142.

English CF, Bell EJ, Berger AJ (1967). Isolation of thermophiles from broadleaf tobacco and effect of pure culture inoculation on cigar aroma and mildness. Appl. Microbiol. 15:117-119.

Estevez JM, Cantero A, Reindl A, Reichler S,Leon P (2001). 1-Deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. J. Biol. Chem. 276: 22901-22909.

Eulgem T, Rushton PJ, Robatzek S,Somssich IE (2000). The WRKY superfamily of plant transcription factors. Trends Plant Sci. 5:199-206.

Eulgem T,Somssich IE (2007). Networks of WRKY transcription factors in defense signaling. Curr. Opin. Plant Biol. 10:366-371.

Facchini PJ, Hagel,J., Zulak, K.G. (2002). Hydroxycinnamic acid amide metabolism: physiology and biochemistry. Can J. Bot. 80: 577-589.

Flor HH (1971). Current status of the gene-for-gene concept. Annual Rev. Phytopathol. 9: 275-296.

Ganas P, Sachelaru P, Mihasan M, Igloi GL,Brandsch R (2008). Two closely related pathways of nicotine catabolism in Arthrobacter nicotinovorans and Nocardioides sp. strain JS614. Arch Microbiol. 189: 511-517.

Gherna RL, Richardson SH, Rittenberg SC (1965). The bacterial oxidation of nicotine. VI. The metabolism of 2,6-dihydroxypseudooxynicotine. J. Biol. Chem. 240:3669-3674.

Golldack D, Popova OV, Dietz KJ (2002). Mutation of the matrix metalloproteinase At2-MMP inhibits growth and causes late flowering and early senescence in Arabidopsis. J. Biol. Chem. 277: 5541-5547.

Goossens A, Hakkinen ST, Laakso I, Seppanen-Laakso T, Biondi S, De Sutter V, Lammertyn F, Nuutila AM, Soderlund H, Zabeau M, Inze D,Oksman-Caldentey KM (2003). A functional genomics approach toward the understanding of secondary metabolism in plant cells. Proc Natl. Acad. Sci. U S A, 100:8595-8600.

Grienenberger E, Besseau S, Geoffroy P, Debayle, D., Heintz, D., Lapierre C, Pollet B, Heitz T, Legrand M (2009). A BAHD acyltransferase is expressed in the tapetum of Arabidopsis anthers and is involved in the synthesis of hydroxycinnamoyl spermidines. Plant J. 58 246-259.

Grodzinski B, Jiao J, Knowles VL, Plaxton WC (1999). Photosynthesis and carbon partitioning in transgenic tobacco plants deficient in leaf cytosolic pyruvate kinase. Plant Physiol. 120: 887-896.

Hayes KR, Beatty M, Meng X, Simmons CR, Habben JE,Danilevskaya ON (2010). Maize global transcriptomics reveals pervasive leaf diurnal rhythms but rhythms in developing ears are largely limited to the core oscillator. PLoS One 5: e12887.

Hong MJ, Kim DY, Lee TG, Jeon WB, Seo YW (2010). Functional characterization of pectin methylesterase inhibitor (PMEI) in wheat. Genes Genet. Syst. 85: 97-106.

Howe GA,Jander G (2008). Plant immunity to insect herbivores. Annual Rev. Plant Biol. 59: 41-66.

Hylin JW (1959). The microbial degradation of nicotine. II. The mode of action of Achromobacter nicotinophagum. Arch. Biochem. Biophys.

83: 528-537.

Jolie RP, Duvetter T, Van Loey AM,Hendrickx ME (2010). Pectin methylesterase and its proteinaceous inhibitor: a review. Carbohydr. Res. 345: 2583-2595.

Keen NT (1990). Gene-for-gene complementarity in plant-pathogen interactions. Annual Rev. Genet, 24: 447-463.

Koller JB (1858). Der tabak in naturwissenschaftliche. Augsburg: Landwirt schafilieher and technischerbe zichung.

Lackman P, Gonzalez-Guzman M, Tilleman S, Carqueijeiro I, Perez AC, Moses T, Seo M, Kanno Y, Hakkinen ST, Van Montagu MC, Thevelein JM, Maaheimo H, Oksman-Caldentey KM, Rodriguez PL, Rischer H, Goossens A (2011). Jasmonate signaling involves the abscisic acid receptor PYL4 to regulate metabolic reprogramming in Arabidopsis and tobacco. Proc. Natl. Acad. Sci. U S A 108: 5891-5896.

Liu G,Thornburg RW (2011). Knockdown of MYB305 disrupts nectary starch metabolism and floral nectar production. Plant J.

Liu Y, Dammann C,Bhattacharyya MK (2001). The matrix metalloproteinase gene GmMMP2 is activated in response to pathogenic infections in soybean. Plant Physiol. 127: 1788-1797.

Lodha TD, Basak J (2011). Plant-Pathogen Interactions: What Microarray Tells About It? Mol. Biotechnol.

Lund ST, Peng FY, Nayar T, Reid KE,Schlosser J (2008). Gene expression analyses in individual grape (Vitis vinifera L.) berries during ripening initiation reveal that pigmentation intensity is a valid indicator of developmental staging within the cluster. Plant Mol. Biol. 68: 301-315.

Ma L, Chen C, Liu X, Jiao Y, Su N, Li L, Wang X, Cao M, Sun N, Zhang X, Bao J, Li J, Pedersen S, Bolund L, Zhao H, Yuan L, Wong GK, Wang J, Deng XW (2005). A microarray analysis of the rice transcriptome and its comparison to Arabidopsis. Genome Res. 15: 1274-1283.

Mandal MK, Fischer R, Schillberg S,Schiermeyer A (2010). Biochemical properties of the matrix metalloproteinase NtMMP1 from Nicotiana tabacum cv. BY-2 suspension cells. Planta, 232: 899-910.

Martin-Tanguy J (1985). The occurrence and possible function of hydroxycinnamoyl acid-amides in plants. Plant Growth Regul, 3: 381-399.

Mathias PC, Jones SI, Wu HY, Yang F, Ganesh N, Gonzalez DO, Bollero G, Vodkin LO,Cunningham BT (2010). Improved sensitivity of DNA microarrays using photonic crystal enhanced fluorescence. Anal Chem, 82: 6854-6861.

Munoz-Bertomeu J, Cascales-Minana B, Alaiz M, Segura J,Ros R (2010). A critical role of plastidial glycolytic glyceraldehyde-3-phosphate dehydrogenase in the control of plant metabolism and development. Plant Signal Behav. 5: 67-69.

Nagase H, Woessner JFJr. (1999). Matrix metalloproteinases. J Biol. Chem. 274: 21491-21494.

Naoumkina MA, He X, Dixon RA (2008). Elicitor-induced transcription factors for metabolic reprogramming of secondary metabolism in Medicago truncatula. BMC Plant Biol. 8:132.

Onkokesung N, Gaquerel E, Kotkar H, Kaur H, Baldwin I,Galis I (2011). MYB8 controls inducible phenolamide levels by activating three novel hydroxycinnamoyl-CoA: polyamine transferases in Nicotiana attenuata. Plant Physiol. 158(1):389-407.

Park CJ, Shin YC, Lee BJ, Kim KJ, Kim JK, Paek KH (2006). A hot pepper gene encoding WRKY transcription factor is induced during hypersensitive response to Tobacco mosaic virus and Xanthomonas campestris. Planta 223:168-179.

Poethig RS, Sussex IM (1985). The developmental morphology and growth dynamics of the tobacco leaf. Planta, 165: 158-169.

Poiatti VA, Dalmas FR,Astarita LV (2009). Defense mechanisms of Solanum tuberosum L. in response to attack by plant-pathogenic bacteria. Biol. Res. 42: 205-215.

Qiu Y, Xi J, Du L, Roje S, Poovaiah BW (2011). A dual regulatory role of Arabidopsis calreticulin-2 in plant innate immunity. Plant J. 69(3):489-500.

Reichheld JP, Chaubet N, Shen WH, Renaudin JP,Gigot C (1996). Multiple A-type cyclins express sequentially during the cell cycle in Nicotiana tabacum BY2 cells. Proc Natl Acad Sci U S A, 93: 13819-13824.

Rietz S, Stamm A, Malonek S, Wagner S, Becker D, Medina-Escobar N, Vlot AC, Feys BJ, Niefind K, Parker JE (2011). Different roles of Enhanced Disease Susceptibility1 (EDS1) bound to and dissociated from Phytoalexin Deficient4 (PAD4) in Arabidopsis immunity. New Phytol. 191:107-119.

Rohrmann J, Tohge T, Alba R, Osorio S, Caldana C, McQuinn R, Arvidsson S, van der Merwe MJ, Riano-Pachon DM, Mueller-Roeber B, Fei Z, Nesi AN, Giovannoni JJ,Fernie AR (2011). Combined transcription factor profiling, microarray analysis and metabolite profiling reveals the transcriptional control of metabolic shifts occurring during tomato fruit development. Plant J. 68(6):999-1013.

Rowland O, Ludwig AA, Merrick CJ, Baillieul F, Tracy FE, Durrant WE, Fritz-Laylin L, Nekrasov V, Sjolander K, Yoshioka H,Jones JD (2005). Functional analysis of Avr9/Cf-9 rapidly elicited genes identifies a protein kinase, ACIK1, that is essential for full Cf-9-dependent disease resistance in tomato. Plant Cell 17: 295-310.

Sachelaru P, Schiltz E, Igloi GL, Brandsch R (2005). An alpha/beta-fold C--C bond hydrolase is involved in a central step of nicotine catabolism by Arthrobacter nicotinovorans. J Bacteriol. 187:8516-8519.

Sang-Wook Park, Evans Kaimoyo, Dhirendra Kumar, Stephen Mosher,Klessig DF (2007). methyl salicylate is a critical mobile signal for plant systemic acquired resistance. Science 318:113-116.

Schenk S, Hoelz A, Krauss B, Decker K (1998). Gene structures and properties of enzymes of the plasmid-encoded nicotine catabolism of Arthrobacter nicotinovorans. J. Mol. Biol. 284: 1323-1339.

Schiermeyer A, Hartenstein H, Mandal MK, Otte B, Wahner V,Schillberg S (2009). A membrane-bound matrix-metalloproteinase from Nicotiana tabacum cv. BY-2 is induced by bacterial pathogens. BMC Plant Biol. 9: 83.

Simon-Mateo C, Depuydt S, CL DEOM, Cnudde F, Holsters M, Goethals K, Vereecke D (2006). The phytopathogen Rhodococcus fascians breaks apical dominance and activates axillary meristems by inducing plant genes involved in hormone metabolism. Mol. Plant Pathol. 7: 103-112.

Soitamo AJ, Jada B, Lehto K (2011). HC-Pro silencing suppressor significantly alters the gene expression profile in tobacco leaves and flowers. BMC Plant Biol. 11: 68.

Sorrell DA, Menges M, Healy JM, Deveaux Y, Amano C, Su Y, Nakagami H, Shinmyo A, Doonan JH, Sekine M, Murray JA (2001). Cell cycle regulation of cyclin-dependent kinases in tobacco cultivar Bright Yellow-2 cells. Plant Physiol. 126: 1214-1223.

Takemoto D, Hayashi M, Doke N, Mishimura M,Kawakita K (2000). Isolation of the gene for EILP, an elicitor-inducible LRR receptor-like protein, from tobacco by differential display. Plant Cell Physiol. 41:458-464.

Tamayo AI,Cancho FG (1953). microbiology of the fermentation of Spanish tobacco. International Congress of Microbiology: 48-50.

Tamura K, Dudley J, Nei M,Kumar S (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24:1596-1599.

Thacker R, Rorvig O, Kahlon P, Gunsalus IC (1978). NIC, a conjugative nicotine-nicotinate degradative plasmid in Pseudomonas convexa. J. Bacteriol. 135:289-290.

Thipyapong P, Hunt MD,Steffens JC (2004). Antisense downregulation of polyphenol oxidase results in enhanced disease susceptibility. Planta 220:105-117.

Thompson JD, Higgins DG, Gibson TJ (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673-4680.

Tremblay R, Wang D, Jevnikar AM,Ma S (2010). Tobacco, a highly efficient green bioreactor for production of therapeutic proteins. Biotechnol. Adv. 28: 214-221.

Turkina MV, Klang Arstrand H, Vener AV (2011). Differential Phosphorylation of Ribosomal Proteins in Arabidopsis thaliana Plants during Day and Night. PLoS One 6: e29307.

van Verk MC, Bol JF, Linthorst HJ (2011). WRKY transcription factors involved in activation of SA biosynthesis genes. BMC Plant Biol. 11: 89.

Verberne MC, Sansuk K, Bol JF, Linthorst HJ, Verpoorte R (2007).

Vitamin K1 accumulation in tobacco plants overexpressing bacterial genes involved in the biosynthesis of salicylic acid. J. Biotechnol. 128: 72-79.

Walter MH, Fester T,Strack D (2000). Arbuscular mycorrhizal fungi induce the non-mevalonate methylerythritol phosphate pathway of isoprenoid biosynthesis correlated with accumulation of the 'yellow pigment' and other apocarotenoids. Plant J. 21: 571-578.

Walter MH, Hans J, Strack D (2002). Two distantly related genes encoding 1-deoxy-d-xylulose 5-phosphate synthases: differential regulation in shoots and apocarotenoid-accumulating mycorrhizal roots. Plant J. 31: 243-254.

Wang C, Dai Y, Zhu L, Tan X, Zhang D, Feng S (2006). Experiments on improving leaf quality by foliar spraying of microbic agent before harvesting. Tobacco sci. 31-34.

Wang S, Durrant WE, Song J, Spivey NW, Dong X (2010). Arabidopsis BRCA2 and RAD51 proteins are specifically involved in defense gene transcription during plant immune responses. Proc Natl Acad Sci U S A, 107: 22716-22721.

Wang X, Yang T, Yin Q, Zhai Z, Yang Z,Shi Z (2008). Research Advances in Tobacco Flavor--related Main Secondary Metabolic Pathways. China Tobacco Sci. 29: 47-50.

Weisman D, Alkio M,Colon-Carmona A (2010). Transcriptional responses to polycyclic aromatic hydrocarbon-induced stress in Arabidopsis thaliana reveal the involvement of hormone and defense signaling pathways. BMC Plant Biol. 10: 59.

Yamamoto S, Nakano T, Suzuki K, Shinshi H (2004). Elicitor-induced activation of transcription via W box-related cis-acting elements from a basic chitinase gene by WRKY transcription factors in tobacco. Biochem. Biophys. Acta. 1679:279-287.

Yamashita T, Voth GA (2011). Insights into the Mechanism of Proton Transport in Cytochrome *c* Oxidase. J. Am. Chem. Soc. 134(2):1147-1152.

Yazaki K (2005). Transporters of secondary metabolites. Curr Opin. Plant Biol. 8: 301-307.

Yazaki K (2006). ABC transporters involved in the transport of plant secondary metabolites. FEBS Lett. 580:1183-1191.

Zhang J, Zhang Y, Du Y, Chen S,Tang H (2011). Dynamic metabonomic responses of tobacco (Nicotiana tabacum) plants to salt stress. J. Proteome Res. 10: 1904-1914.
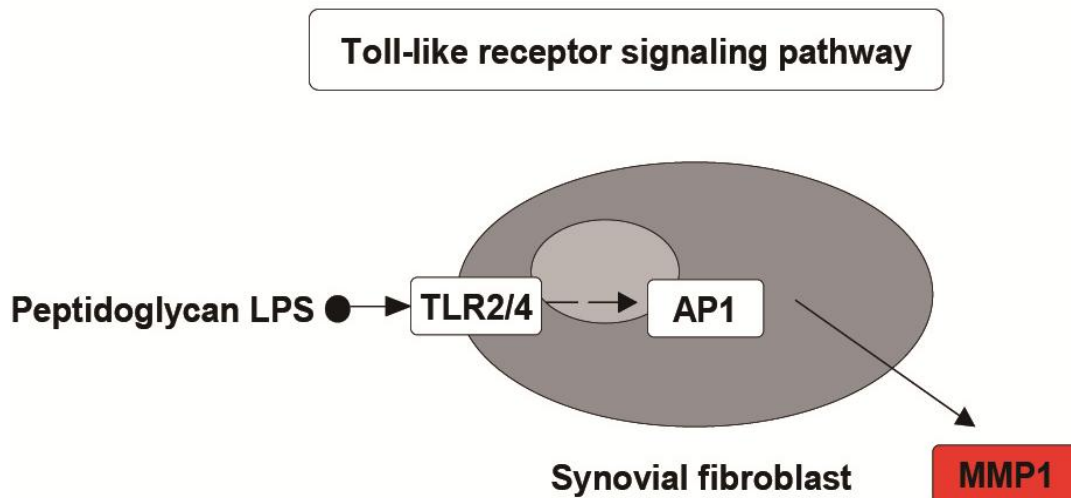
Supplementary Figure S1. Toll-like receptor signaling pathway which *MMP1* participate in. TLR2/4, Toll-like receptor 2/4; AP1, activator protein 1.
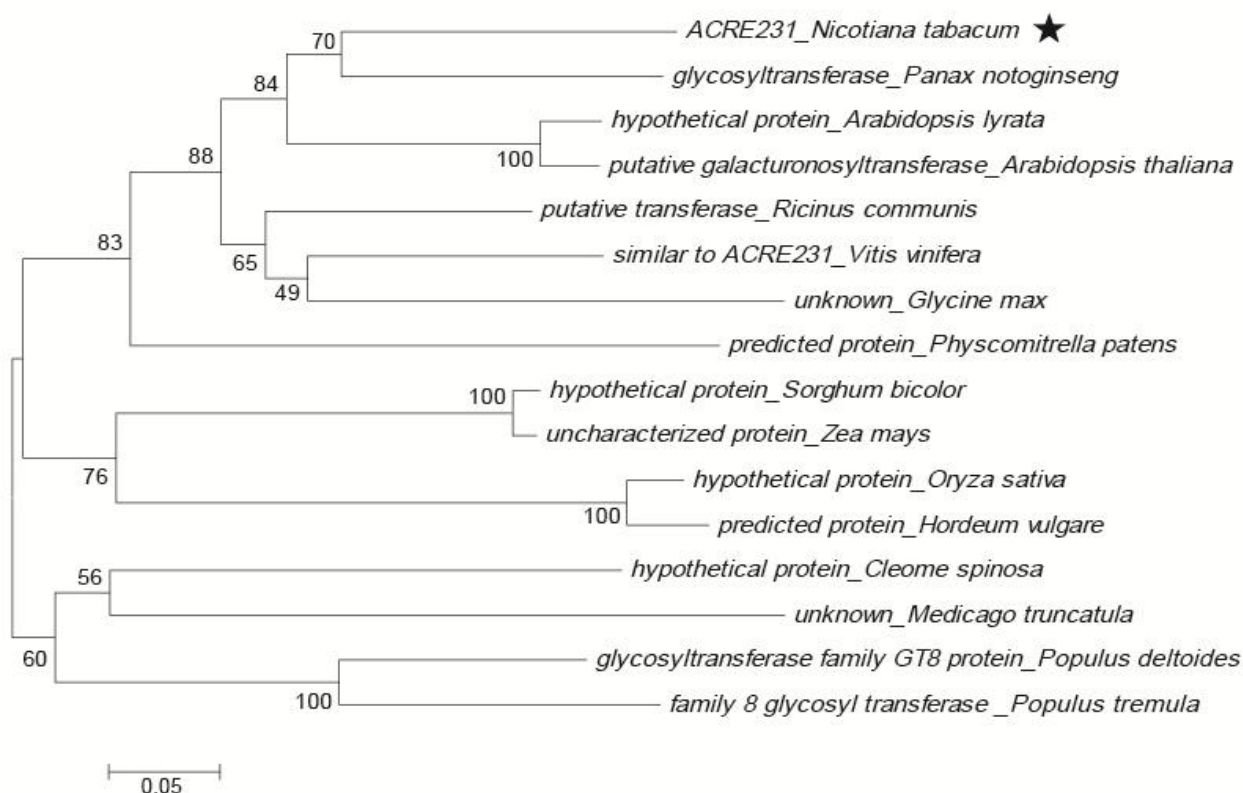
Supplementary Figure S2. Phylogenetic tree of the *Nicotiana tabacum* ACRE231 and homologous proteins. Phylogram was constructed by the Neighbor-joining method using MEGA4 software and shows relationships among 16 amino acid sequences of ACRE231-like proteins. Support for each branch, as determined from 1,000 bootstrap samples, is indicated by value at each node (in percent). Only bootstrap values above 50% are shown. Scale bar indicates evolutionary distance estimated by amino acid substitutions per position.
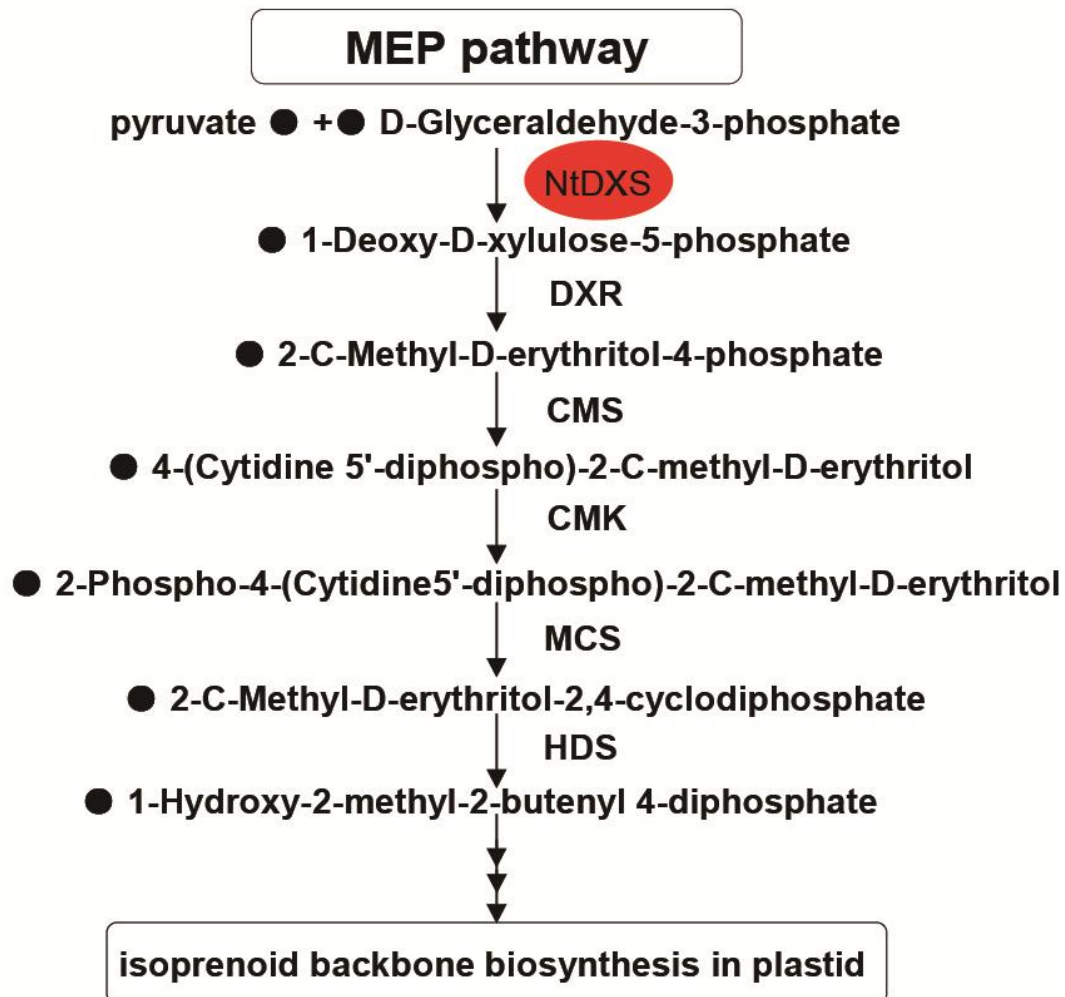
Supplementary Figure S3. Isoprenoid biosynthetic pathways in *Nicotiana tabacum*. Diagramatic representation of the plastidic MEP pathways is shown. NtDXS, 1-deoxy-d-xylulose 5-phosphate reductoisomerase in *Nicotiana tabacum*. DXR, 1-deoxy-d-xylulose 5-phosphate reductoisomerase; CMS, 4-diphosphocytidyl-2C-methyl-D-erythritol synthase; CMK, 4-diphosphocytidyl-2C- methyl-D-erythritol kinase; MCS, 2C-methyl-D-erythritol-2,4-cyclodiphosphate synthase;   HDS, hydroxymethylbutenyl diphosphate synthase.
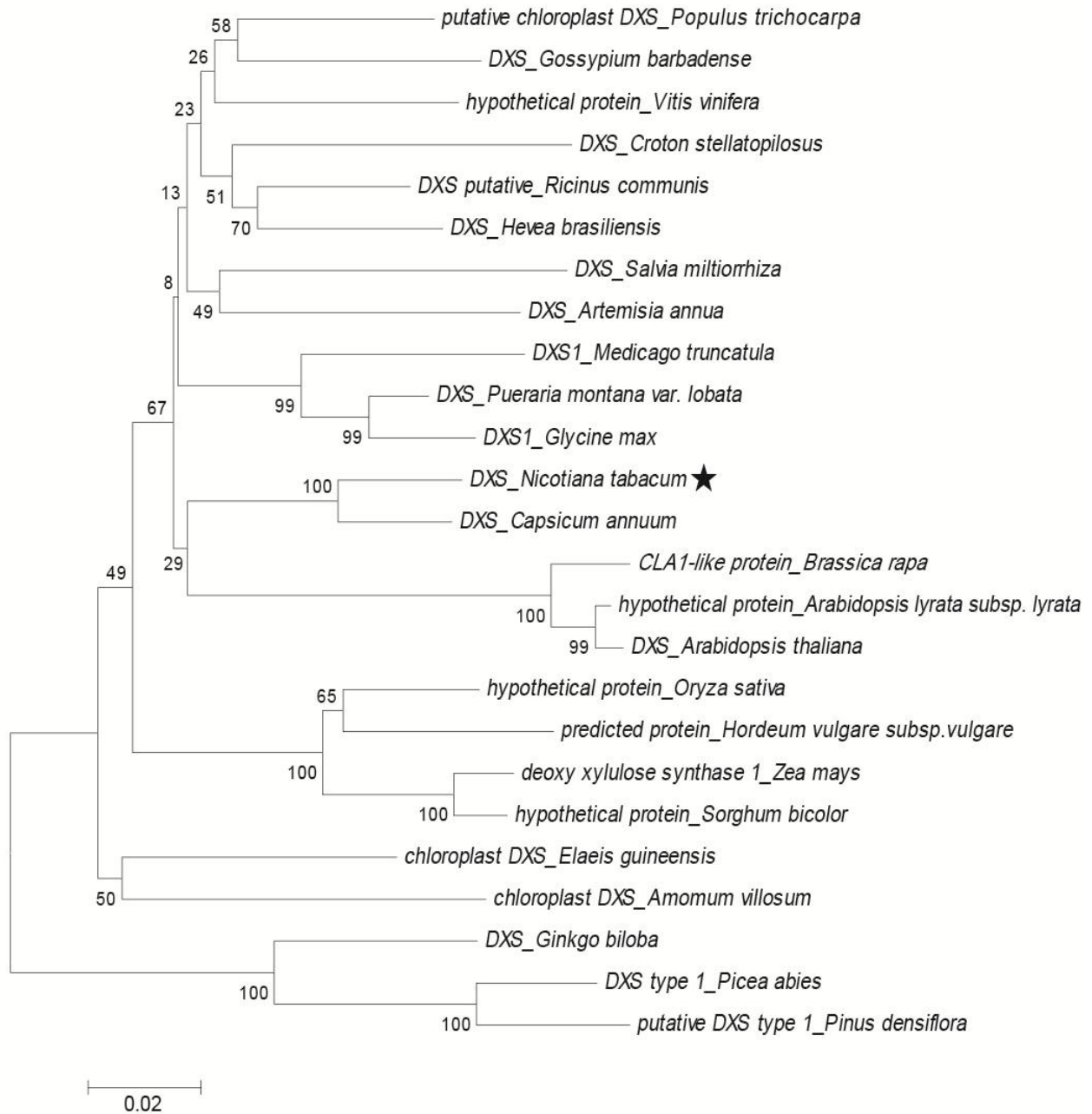
Supplementary Figure S4. Phylogenetic tree of *Nicotiana tabacum DXS* and its homologs. Phylogram was constructed using the Neighbor-joining method with MEGA4 software and reflects relationships among 25 amino acid sequences of *DXS*-like proteins. Support for each branch, as determined from 1,000 bootstrap samples, is indicated by value at each node (in percent). Only bootstrap values above 50% are shown. Scale bar indicates evolutionary distance estimated by amino acid substitutions per position.