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# Expression of salicylic acid-related genes in *Brassica oleracea* var. *capitata* during *Plasmodiophora brassicae* infection

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**Abstract**

*Brassica oleracea* var. *capitata* (cabbage) is an important vegetable crop in Asian countries such as Korea, China and Japan. Cabbage production is severely affected by clubroot disease caused by the soil-borne plant pathogen *Plasmodiophora brassicae*. During clubroot development, methyl salicylate (MeSA) is biosynthesized from salicylic acid (SA) by methyltransferase. In addition, methyl salicylate esterase (MES) plays a major role in the conversion of MeSA back into free SA. The interrelationship between MES and methytransferases during clubroot development has not been fully explored. To begin to examine these relationships, we investigated the expression of *MES* genes in disease- susceptible and -resistant plants during clubroot development. We identified three MES-encoding genes potentially involved in the defense against pathogen attack. We found that *SS1* was upregulated in both the leaves and roots of *B. oleracea* during *P. brassicae* infection. These results support the conclusion that SA biosynthesis is suppressed during pathogen infection in resistant plants. We also characterized the expression of a *B. oleracea BSMT* gene, which appears to be involved in glycosylation rather than MeSA biosynthesis. Our results provide insight into the functions and interactions of genes for MES and methyltransferase during infection. Taken together, our findings indicate that *MES* genes are important candidates for use to control clubroot diseases.



**Keywords**: Methyl salicylate esterase, Clubroot, Salicylic acid, Methyltransferase

**Introduction**

The *Brassica* genus includes six agriculturally important species that are grown in many countries as vegetable crops. *Brassica* vegetables such as broccoli, cabbage, Chinese cabbage, turnip greens and leaf rape are consumed throughout the world. *Brassica oleracea*, one of the major crops in the genus *Brassica*, is a cruciferous vegetable that is native to coastal southern and western Europe. The yield of some cruciferous vegetables including *B. oleracea* and oil crops is affected by clubroot disease caused by soil-borne *Plasmodiophora brassicae,* which is a major pathogen in temperate regions. Clubroot disease was first reported in Korea in the 1920s and has become a problem in cabbage production. *P. brassicae* spores can persist in the soil for at least 20 years (Dixon 2009), and *P. brassicae* infection is believed to occur in two phases, with a primary phase in root hairs and a secondary phase in the root cortex inducing massive gall structures (Braselton 1995). During infection, plants generally activate centralized defense mechanisms to control the destructive effects of the pathogen. Upon frequent infection, plants develop tolerance through local responses as well as systemic acquired resistance (SAR) via various signaling cascades. Salicylic acid (SA) is key signaling molecule that activates both local and systemic responses of defense mechanisms in plants (Zhao et al. 2009). The exogenous application of SA and jasmonic acid (JA) significantly decreases root gall formation during *P. brassicae* infection in *B. oleracea* (Lovelock et al. 2013). SA can be conjugated to glucose to form SA glucoside (SAG) (Song et al. 2008) and methylated to form methylsalicylate (MeSA) (Seskar 1998), both of which appear to be biologically inactive in plants and lead to increased susceptibility to the pathogens. These inactive SA derivatives can be converted back to free active SA by MeSA esterase which aids in disease resistance (Forouhar et al. 2005). MeSA esterase is significantly expressed in root and leaf of *Populus trichocarpa* during biotic and abiotic stresses (Zhao et al. 2009). Among MeSA esterases, SABP2 (salicylic acid-binding protein 2) esterase activity is involved in the conversion of MeSA into free SA, which is required for the SAR signal in tobacco infected tissues (Park et al. 2009). In tobacco, silencing of *SABP2* suppress the defense response, demonstrating a role for SABP2 in plant immunity (Kumar & Klessig 2003). *SABP2* contains an α/β hydrolase functional domain that is responsible for its esterase activity and plays an important role in conversion of MeSA into SA (Forouhar et al. 2005). The demethylation of acibenzolar-S-methyl, a chemical inducer required for development of SAR in tobacco plants is catalyzed by *SABP2*(Tripathi et al. 2010).

S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase (SAMT) belongs to the SABATH protein family and is involved in MeSA biosynthesis by catalyzing SA methylation. SABATH family methyltransferases utilize a range of substrates similar to SA including benzoic acid (BAMT), SA and BA (BSMT) (Negre et al. 2003; Underwood et al. 2005), jasmonic acid (JMT) (Seo et al. 2001), indole-3-acetic acid (IAMT) (Qin et al. 2005) and gibberellic acid (GAMT) (Kapteyn et al. 2007). MeSA is more hydrophobic than free SA, and can cross membranes and move easily between cells; accordingly plants are required to biosynthesizes MeSA via BSMT when SAR is triggered by pathogen attack (Groux et al. 2014; Vlot et al. 2008) (Vlot et al. 2008) (Groux et al. 2014). In *Arabidopsis thaliana*, 24 SABATH family members are involved in defense responses during biotic and abiotic stresses. For example, AtBSMT in *A. thaliana* utilizes BA and SA as methyl acceptors and shows significant expression during abiotic and biotic stresses (Chen et al. 2003). Recently, a novel methyltransferase *PbBSMT1* was

identified from the pathogen *P. brassicae* and interestingly, was found to show high structural homology to *BSMT1* of the host plant *A. thaliana* (Ludwig-Müller et al. 2015). In addition, Ludwig-Müller et al. (2015) reported that *PbBSMT1* catalyzes MeSA formation by methylation of SA, benzoic and anthranilic acids in *A. thaliana* during clubroot infection. Although, PbBSMT1 has the potential for SA methylation activity during infection, it is unknown whether this protein interacts with plant methyltransferase to act in the host cell or/and is involved in development of SAR during pathogen attack.

The disaccharide sugar trehalose is widely found in bacteria, fungi and accumulates in *P. brassicae*-infected root galls (Elbein 1974). This accumulation of sugars in the root galls can lead to increased starch in the plants during infection; however, accumulation of starch in the root galls themselves has not been clearly demonstrated (Brodmann et al. 2002). By contrast, the amount of starch in *A. thaliana* leaves is reduced during *P. brassicae* infection. This decrease could be due to a lower rate of photosynthesis or to rapid metabolism leading to translocation of carbon from the leaves to infected root galls (Mitchell & Rice 1979). Either way, these results imply a greater demand for carbohydrates and starch in the infected leaves compared to control leaves. In accordance with these terms, carbohydrate demand in the infected tissues could also be due to decrease of SA level in plants. This notion is supported by previous reports that SA increases polysaccharide levels in plants (Jeyakumar et al. 2008; Khodary et al. 2004) and regulates metabolic pathways (Amin A. A. 2007). Spraying leaves with SA affects enzymes activities related to starch biosynthesis and sucrose degradation in yam tuber (JiaShu, Zhou ShengMao, Wang LingPing, Ban MeiLing, Shang XiaoHong, Guo YuanYuan, Huang Hao, Wen JunLi, Liang RenFan, Huang RuKui 2014). Moreover, SA could play an important role in regulating starch levels during biotic and abiotic stress conditions (Evans J 1995; Pandurangam et al. 2014). Consistent with these reports, starch biosynthetic (e.g., starch synthase) genes are upregulated in *Arabidopsis* root galls during *P. brassicae* infection (Siemens, Johannes et al. 2006). These findings suggest the importance of SA accumulation in infected tissues.

The activities of MeSA esterase (MES), benzoic acid/SA methyltransferase (BSMT) and starch synthase (SS1) are presumed to be involved in the defense response. However, the interrelationships between these genes during clubroot development are yet to be studied in *B. oleracea*. In this context, we focused our study on transcription profiling of salicylic acid-related genes during clubroot infection in *B. oleracea* using two contrasting lines.

## Materials and Methods

### Pathogen preparation

*P. brassicae* (Race 4; Williams differential host (Williams 1966)) infected *B. oleracea* (cabbage) roots containing galls were collected from a nearby field and stored at -20°C in the Department of Horticulture, Sunchon National University, Korea. The mature clubroot galls of cabbage were homogenized and filtered through gauze (25 μm). Suspensions of $10^6$ spores $mL^{-1}$ in water were prepared for inoculation (Agarwal et al. 2011).

**Pathogen inoculation and sample collection**

Seeds of two contrasting *Brassica oleracea* var. *capitata* inbred lines, namely clubroot susceptible CR3 and clubroot resistant CR4, were purchased from Asia Seed Company (Korea). Seeds were grown in a controlled environment (21 °C, 16 h light, 100 µmol photons $s^{-1}m^{-2}$). Fourteen-day-old plants were infected by injecting 2 mL spore suspension ($10^6$ spores $mL^{-1}$) into the soil around the each plant (Siemens, J. et al. 2002). Mock treatments were performed with 2 mL water. Monitoring and assessment of disease was performed until 60 days after inoculation (DAI) with twenty plants within per replicate. The plants were uprooted and observed for macroscopic symptoms of infection in both roots and leaves. Roots and leaves of control and infected plants were harvested at 0, 7, 14, 21, 35, 42, 52 and 60 DAI. The harvested roots were gently washed in water to remove soil particles. Collected samples were immediately frozen in liquid nitrogen and stored at -80 °C for RNA isolation.

**Identification and characterization of sequences**

Annotation-based searches were conducted to identify salicylate esterase- (MES), starch synthase- (SSI) and methyltransferase- (BSMT) encoding genes, in the BRAD database (http://brassicadb.org/brad/) (Cheng et al. 2011), Bolbase (http://www.ocri-genomics.org/bolbase/) and EnsemblPlants database (http://plants.ensembl.org/Brassica_oleracea/Info/Index). The reliability of the identified sequences was confirmed through HMM profiling using the domain sequences of the respective genes. Protein sequences were characterized and their domains were analyzed using the SMART database (http://smart.embl-heidelberg.de/) (Schultz et al. 1998). Phylogenetic trees were constructed based on a condensed alignment in MEGA6.06 using the Neighbor-Joining (NJ) algorithm (Tamura et al. 2013), with parameters modified to 1000 replications for bootstrap values and complete deletion mode, to analyze tree topology and reliability. Primary analysis of the predicted molecular weights, pIs, and stability indexes was done using ProtParam (http://web.expasy.org/protparam/) (Gasteiger et al. 2005). The Gene Structure Display Server (GSDS) web tool (http://gsds.cbi.pku.edu.cn/index.php) (Hu et al. 2014) was used to determine the number of introns and exons, by aligning CDS and genomic sequences of the selected genes. Further, N-glycosylation sites were predicted using the NetNGlyc 1.0 server (Gupta R, Jung E 2004). Subcellular localization prediction of predicted MES proteins was performed using Protcomp 9.0 from Softberry (http://linux1.softberry.com/berry.phtml). The interactions of selected proteins and their association with Arabidopsis proteins, including their functional and physiological interactions were analyzed using STRING software.

**Expression analysis**

Frozen samples were used for total RNA extraction using an RNeasy Mini Kit (Qiagen, USA), followed by RNA cleanup by DNase I treatment (Takara, Japan). Isolated RNA was quantified using an ND-1000 Spectrophotometer and NanoDrop v3.7 software (NanoDrop Technologies, USA). Synthesis of cDNA from RNA extracts was performed with a Superscript III® First-strand Synthesis Supermix kit (Invitrogen, USA) following the manufacturer's instructions. Real-Time quantitative PCR (qRT-PCR) was performed in duplicate for each biological

replicate using 1 µl cDNA in a 20-µl reaction volume with iTaqTM SYBR® Green Super-mix with ROX (California, USA. Thermal-cycler conditions were as follows: 5 min at 95°C, followed by 40 cycles at 95°C for 10 s, 60°C for 10 s, 72°C for 20 s, and then melting at 72°C for 60 s and 97°C for 1 s. The fluorescence was assessed following the last step of each cycle. Product amplification, detection, and data analysis were carried out using a LightCycler96 (Roche, Germany). Relative gene expression levels were calculated using the ΔΔCT method and 0 day time point gene expression was set to 1. *Actin* was used as housekeeping gene.

**Results**

**Sequence identification and characterization of putative genes**

Salicylic acid regulation is essential for plants to acquire SAR. Large amounts of endogenous SA are methylated to form MeSA by benzoic acid/salicylic acid methyltransferase (BSMT) and the reverse reaction is catalyzed methylsalicylate esterase (MES) by to maintain the appropriate SA levels. These SA levels can be modulated by starch synthase (SS1), which regulates the plant defense responses. Hence, we set out to investigate and characterize the corresponding *B. oleracea* genes from the BRAD database (http://brassicadb.org/brad/). In total, 12 genes were collected, including 10 *MES* genes (encoding methylsalicylate esterase), one *BSMT1* (methyltransferase) and one *SS1* (starch synthase). To extend our search for new *BoMES* genes, we used a gene prediction tool (HMMER) and identified other methyl esterase genes (not shown) in the database; however those were not likely to be involved in the activity on MeSA (Huala 2001). In addition, *A. thaliana* MES proteins were used in a BlastN search against *B. oleracea* proteome obtained from the BRAD database. Ten *B. oleracea* (BoMES) proteins homologous to AtMES were obtained (Table S1). Among the 10 MES proteins, seven were found to have α/β hydrolase as their functional domain; the other three genes did not encode this functional domain or not amplified even under different RT-PCR conditions (Table S1). Therefore, the seven *BoMES* genes encoding the α/β hydrolase functional domain were annotated as *BoMES1*, *BoMES2*, *BoMES4* (*BoMES4_1*, *BoMES4_2* and *BoMES4_3*), and *BoMES9* (*BoMES9_1* and *BoMES9_2*) and selected for further analysis. For *BoSS1* and *BoBSMT1*, one gene each was identified and characterized. The ORF sizes and chromosome numbers of the respective genes are presented in Table 1. The α/β hydrolase, methyltransferase_7 and glycogen synthase domains were encoded in *BoMES, BoBSMT1* and *BoSS1* genes, respectively (Table 1). The α/β hydrolase was the only domain encoded in all types of BoMES. Protein sequence alignment to known MES homologs from *A. thaliana* revealed that selected BoMES contained the conserved functional domain (Figure 1). Moreover, a phylogenetic tree was constructed using the domain sequences of the α/β hydrolase and methyltransferase_7 from the respective proteins; *A. thaliana* was used as a reference with *B. oleracea* and *B. rapa* sequences. Genes were conserved in all three species and were classified according to type (Figure S1). Protein characteristics such as protein length, molecular weight, pI, instability index, aliphatic index, grand average hydropathicity and N-Glyc position are tabulated in Table 2. Coding sequences of *BoMES* genes ranged between 218 and 263 bp, while that of *BoBSMT1* was 331 bp and *SS1* was 652 bp. The molecular weight, PI and instability index were predicted as 29-71 kDa, 5.09-5.58 and 26.06-43.40, respectively. Based on their stability index values, BoMES4_1, BoMES9_2 and BoBSMT1 proteins were characterized as unstable and all other proteins

were predicted to be stable. BoMES9_1 and BoBSMT1 were predicted to be located in the cytoplasm, whereas all other BoMES proteins and BoSS1 were predicted to be in the chloroplast (Table 2).

**Pathogenesis of *P. brassicae* on *B. oleracea***

*P. brassicae* spores were inoculated to soil surrounding susceptible (S line) and resistant (R line) plants at 14 days old. Pathogenesis of the *P. brassicae* in plants was assessed at eight time points (0, 7, 14, 21, 35, 42, 52 and 60 days after inoculation, DAI) and compared with controls. In the S line, there were no symptoms of clubroot observed on the root until 21 DAI (Figure 2). The first clear symptoms were detected at 35 DAI on the root as well as root hairs. Severe symptoms were observed at 52 DAI as a root gall. Substantial, severe destruction of the root at 60 DAI affected plant growth. In the R line, there was no clear pathogenesis detected throughout the time of pathogen infection. Similarly, no gall was observed on roots of the R line (Figure 3). Overall, differences in susceptibility to *P. brassicae* were characterized based on presence or absence of root galls in the two lines of *B. oleracea*.

**Expression of *MES* and *SS1* genes during *P. brassicae* infection**

In controlled environmental conditions, S (susceptible) and R (resistant) lines of *B. oleracea* were inoculated with *P. brassicae* at the root and hypocotyl parts. To study pathogen-responsive genes, root and leaf samples were collected in seven time courses. Real-time PCR was performed to study the expression of the nine SA-related *B. oleracea* genes we identified above (Figure 1, Figure S1). Additionally, the expression of the *P. brassicae BSMT1* (*PbBSMT1*) gene identified by Ludwig-Müller et al. (2015) was monitored. The primers used in this qPCR analysis are given in Table S2. The inoculated and control plants were examined at the same time courses. Some genes did not fluctuate in expression during infection compared to control, whereas differential expression was observed between two lines. For instance, *BoMES4_1* and *BoMES9_2* genes showed a similar expression pattern in root samples in S line throughout the time course, but infected samples showed higher expression than control samples (Figure S2). In the R line, expression levels of these genes were lower than in the S line. *BoMES1* expression fluctuated in root samples of the S line, whereas upregulation was observed in the R line throughout the time course (Figure S2). The same result was observed in leaf samples, with a difference in expression between the two lines but a similar expression pattern between control and infected samples during the time course (Figure S3). *BoME4_3* expression was higher in infected root and leaf samples of the R line. Interestingly, *BoMES2* and *BoMES4_2* showed increased expression in roots of both lines particularly in the later phase of infection (42 to 60 DAI) during which gall formation occurred (Figure 4a). There was no expression of *BoMES2* until 35 DAI in S line, but a drastic increase after 45 DAI. *BoMES2* transcript accumulation showed an over 20-fold increase at 42 DAI and was substantively reduced to 3- and 2-folds at 52 and 60 DAI, respectively. However, in the R line, *BoMES2* showed a sudden increase in expression from 2- to 3.5-folds after 35 DAI. This could possibly be due to a MeSA level reduction in the R line, whereas *BoMES2* downregulation in the S line might indicate an increase in the level of MeSA during clubroot development. The same pattern was observed for *BoMES4_2*, which showed high expression in the S line at 35 DAI followed by down regulation after 42 DAI (Figure 4b). The maximum fold change was obtained at 42 DAI (15 fold) in the S line,

followed by a substantial reduction over the rest of the time course. In the R line, until 35 DAI there was no expression of *BoMES4_2* detected in uninfected or infected plants. After 42 DAI, there was an up regulation of *BoMES4_2* through 60 DAI in the roots. Specifically, *BoMES2*, *BoMES4_2*, *BoMES9* genes might be involved in the esterase activity to form free SA, supporting their defense activity during fungal infection

Another gene potentially involved in the esterase activity during clubroot development is *BoMES9_1* (Figure 4c). The expression of *BoMES9_1* was increased dramatically in the S line after 35 DAI, with 10, 24 and 23-fold increases at 42, 52 and 60 DAI, respectively. The expression was thus eventually increased through 60 DAI and a similar pattern was obtained in the R line from 42 to 60 DAI but the expression level was less than in the S line. Free SA levels are predicted to be decreased by SS1 activity; to obtain evidence for such activity a playing role in defense responses, we examined *BoSS1* gene expression (Figure 4d). *BoSS1* expression was low until 35 DAI in roots of the S line, whereas it showed a drastic increase in expression after 35 DAI and a substantial 8- to 12-fold increase from 42 to 60 DAI. As expected, *BoSS1* gene activity was higher in the S line than in the R line. Methyltransferase activity of plants would be expected to form MeSA during clubroot formation; however, the expression of *BoBSMT* was not different between the control and infected root samples of both lines (Figure 4e). *BoBSMT* showed moderate expression in the S line than in the R line throughout the time course, which suggests that *BoBSMT* activity is not involved in the galls formation in roots. To examine the activity of *P. brassicae* genes in the root galls, we examined *PbBSMT* expression (Figure 4f). *PbBSMT* showed higher expression in the S line than in the R line, consistent with our assessment of no gall formation in the R line (Figure 4).

Interestingly, most of the potential *MES* genes in *B. oleracea* did not show distinct expression between control and infected leaf samples in the S line (Figure 5). *BoMES2* did show lower expression in infected samples of the S line compared to control samples throughout the time course (Figure 5a). However, there was increased expression in the R line particularly at 52 and 60 DAI. Compared with root samples, *BoMES4_2* and *BoMES9_1* expression was low in leaf samples (Figure 5b, 5c). A similar pattern of expression was observed for these genes in both S and R lines after inoculation. In addition, *BoSS1* showed high expression in infected leaves and was upregulated compared to the control in the S line. The gene regulation was altered in the leaves during the secondary phase infection with *P. brassicae* at 42 to 60 DAI (Figure 5d). However, *BoSS1* showed no alteration in expression in leaves of the R line. Surprisingly, *BoBSMT* expression in leaf samples was significantly higher than in root samples (Figure 5e). There was a drastic increase in *BoBSMT* expression after 35 DAI and a substantial increase from 42 to 60 DAI. *PbBSMT* expression was also lower in leaf than in root samples, as expected (Figure 5f).

**Analysis of protein interaction network**

The interaction network of BoMES proteins and their associations with Arabidopsis proteins were analyzed using STRING software (Figure 6). The protein network showed interaction between MES proteins. The Arabidopsis MES ortholog proteins showed moderate interaction with other proteins. BoMES1 was not predicted to interact with other proteins, whereas BoMES2 was predicted to interact with proline iminopeptidase (PIP), BoMES4 with MES18 and

BoMES9 with MES5 and MES10. In the network, BoSS1 strongly interacted with other carbohydrate biosynthesis proteins including ADP glucose pyrophosphorylase large subunit 1, ADPGLC-PPase large subunit, putative glucose-1-phosphate adenylyltransferase, glucose-1-phosphate adenylyltransferase and ADP glucose pyrophosphorylase 1 (Figure 7a). We found SS1 to be responsive to sugar and starch which strongly associated it with the corresponding Arabidopsis proteins. Similarly, BoBSMT1 showed interaction with UGT74F2 and DIR1, which could play positive regulatory roles in glucosyltransferase and SAR signaling respectively. (Figure 7b).

**Discussion**

Unlike animals, plants do not possess a specialized immune system to resist pathogen infection. Instead, plants develop SAR by sensing the pathogens and activating signaling via hormones such as SA and JA. These defense-related hormones induce prolonged immunity that is regulated by a complex genetic regulatory mechanism (An & Mou 2011).We retrieved 10 *B. oleracea* genes annotated as *BoMES* from the *Brassica* database (Cheng et al. 2011). Based on the presence of the α/β hydrolase functional domain, seven *BoMES* genes were selected and validated in other databases. The α/β hydrolase domain catalyzes the hydrolysis of various substrates that are important to plant immunity (Forouhar et al. 2005; Ollis et al. 1992). This domain is conserved in all BoMES proteins, which convert MeSA back to SA by their esterase activity (Forouhar et al. 2005) By contrast, BoMSMT1 has a methyltransferase_7 domain, and belongs to the SABATH family, acting on substrates including salicylic acid and jasmonic acid (Ogawa et al. 2001) to biosynthesize MeSA and MeJA (Ludwig-Müller et al. 2015). In Arabidopsis, SABATH family gene *GAMT* plays different biological roles including in leaf and seed development and germination (Varbanova et al. 2007). Other family members, such as *JMT* and *SAMT* are involved in the defense response activities in Arabidopsis (Chen et al. 2003; Seo et al. 2001). Previously, the phylogenetic tree of methyl esterases along with other carboxylesterases and *BSMT1* with other methyltransferases were studied in *Arabidopsis* (Yang et al. 2008; Zhao et al. 2010). To elucidate the evolutionary relationship among *BoMES*, *BoBSMT1* and *BoSS1*, phylogenetic trees were constructed using their respective domain sequences. In our study, *MES*, *BSMT1* and starch synthase (*SS1*) genes of *B. oleracea* and *B. rapa* were found to be well conserved with their respective reference genes from *A. thaliana* (Figure S1). Here, we reported one *BoBSMT* gene, which based on its expression pattern is likely involved in cabbage leaf development but not in the activity related to methylation of SA in the roots during *P. brassicae* infection. This was consistent with the protein interaction prediction that particular gene was showed strong interaction with glucosyltransferase proteins instead of methyltransferase proteins (Figure 7b).

To study the expression of selected genes, two contrasting lines were inoculated with a single spore culture of *P. brassicae* on soil. Resting spores of *P. brassicae* have great capacity to survive in soil and their germination is highly dependent on environmental factors including pH, moisture, temperature and inorganic ions (Kageyama & Asano 2009). Symptoms were observed in the S line from 35 DAI in root hairs, and chronic gall formation was observed at 52 DAI. In various crops, 35 DAI is the favorable period for initial or severe infection of *P. brassicae* (Feng et al. 2014; Siemens, J. et al. 2009). Differences in pathogenesis on host plants strongly depends on degradation of the secondary thickening and cell walls of the xylem (Donald et al. 2008). In plants, JA and SA pathways are predominantly expressed during infection of *P. brassicae* and other important pathogens (Lemarié et al. 2015).

Generally SA is derived from MeSA through methylsalicylate esterase activity in infected roots. Pathogenesis of *P. brassicae* led to gall formation and induced expression of *BSMT1* from the pathogen, which is involved in the biosynthesis of MeSA from SA. Therefore, the plant defense signaling might be suppressed in *B. oleracea* roots during infection, when SA is methylated to form MeSA (Ludwig-Müller et al. 2015). In the S line, some *BoMES* genes were not significantly up-regulated in infected roots, whereas some genes showed dramatic expression at the severe infection period (52 to 60 DAI). Interestingly, *PbBSMT1* expression was detectable in leaf compared to root samples. Although mRNA of *PbBSMT1* was detected in leaf, further pathogen invasion study is needed to confirm whether the pathogen can directly penetrate to leaf or shoot during the secondary phase of infection (Kunkle 1918). Overall, expression of most of the *BoMES* genes and *PbBSMT1* was lower in leaf than in root samples. However, *BoSS1* showed upregulation in the infected leaves of *B. oleracea*, suggesting that starch levels would be lower than in the uninfected leaves. In the S line, *BoSS1* inhibits the accumulation of SA during pathogen infection and leads the plants to be susceptible to the pathogen. Interestingly, *BoBSMT1* was positively regulated in the leaf, where it might be involved in the conversion of MeSA from SA, but its expression pattern did not support a role in infected roots, as proposed by Ludwig-Müller et al. (2015). *BoBSMT1* might be related to the glycosyltransferase activity during pathogen infection in *B. oleracea* roots. This was consistent with the protein interaction prediction that *BoBSMT1* strongly interacted with glucosyltransferase proteins instead of methyltransferase proteins (Figure 7b).

In the R line, except for *BoMES1*, *BoMES4_1*, *BoMES_3*, *BoMES9_2* and *BoSS1*, all remaining *BoMES* genes were substantially upregulated during infection in the root, whereas *BoBSMT1* was downregulated during *P. brassicae* infection. In the case of *PbBSMT1*, even though it showed up-regulation after 42 DAI, there was no high expression in the initial days of infection in the roots. In leaves of the R line, *BoMES* and *BoSS1* were upregulated, whereas *PbBSMT1* was not expressed. *BoMES* was upregulated in the R line and is predicted to lead to hydrolysis of MeSA to biosynthesize SA. By contrast, *BoMES* was downregulated in the S line of infected roots. As reported by Forouhar et al. (2005), MES possesses substrate-specific activity on MeSA and MeJA. However, its highest activity is observed with SA, which acts as a signaling molecule inducing the SAR in plants that inhibits the progress of *P. brassicae* in infected roots of hosts (Lovelock et al. 2013; Zhao et al. 2009). The expression of *BoMES* is positively correlated with clubroot resistance in these two lines of *B. oleracea.* By contrast, *BoMES9_1* showed greater expression in the S line than in the R line, suggesting feedback inhibition particularly in the secondary phase of infection to maintain the level of free SA. Previously, it was reported that the SA signaling during clubroot infection alters the starch synthase activity in infected host plants (Horvath et al. 1998; Ludwig-Müller et al. 2009). *PbBSMT1* shares homology with plant *BSMT1*, however *PbBSMT1* is primarily involved in MeSA biosynthesis and this MeSA can move from root to leaf to be emitted (Ludwig-Müller et al. 2015). The MeSA biosynthesis in susceptible plant roots could be facilitating the development of clubroot infection. In the case of the R line, *BoBSMT1* was not showed increased expression in root or leaf. Even though *PbBSMT1* is upregulated, notable expression was observed only after 52 DAI. Hence, the resistant plants might regulate the pathogenic mechanism of *P. brassicae* through SA signaling molecules that are synthesized by methylsalicylate esterase. Our results were consistent with previous reports that MES in tobacco converts MeSA to SA to induce signal transduction pathways for SAR followed by defense responses (Forouhar et al. 2005; Tripathi et al. 2010).

## Conclusion

In this study seven *BoMES*, one *BoSS1* and *BoBSMT1* genes were identified, characterized and analyzed for gene expression. Among seven *BoMES* genes, three genes are potentially involved in the activity to control the clubroot formation in *B. oleracea* crops. Additionally, the *PbBSMT1* gene from *P. brassicae* was used for expression analysis to confirm the infection stability. The expression pattern of *BoMES* genes reveals the mechanism underlying variation in susceptible and resistant lines of *B. oleracea* plants. Here, we showed that during infection *SS1* plays an important role in the *B. oleracea* leaves and roots. In addition, methyl salicylate-related genes are essential for the development of SAR in *B. oleracea* in response to a chronic pathogen, likely by synthesizing SA. Here we reported one *BoBSMT* gene likely involved in the cabbage leaf development but not in the activity to methylate SA in the roots during *P. brassicae* infection. The genes studied in this work might interact each other and could be responsible for susceptibility due to their activity in cabbage. Expression analysis is not sufficient to determine the possible interrelationships of these genes, and further studies are needed to elucidate their potential roles during pathogen infection. Nonetheless, the expression profiling information for these genes will be valuable in molecular breeding to develop plants with biotic resistance.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.



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**Table 1. Domain analysis and position of selected genes**

| Gene name | Locus ID | ORF (bp) | Chromosome | Protein | |
|---|---|---|---|---|---|
| | | | | Domain | Domain position |
| **BoMES1** | Bol031679 | 789 | Scaffold000053 | Abhydrolase | 10-253 |
| **BoMES2** | Bol045801 | 792 | C08 | Abhydrolase | 10-253 |
| **BoMES4_1** | Bol012862 | 774 | Scaffold000192 | Abhydrolase | 8-247 |
| **BoMES4_2** | Bol031680 | 657 | Scaffold000053 | Abhydrolase | 10-209 |
| **BoMES4_3** | Bol031681 | 786 | Scaffold000053 | Abhydrolase | 9-252 |
| **BoMES9_1** | Bol018638 | 774 | C07 | Abhydrolase | 6-248 |
| **BoMES9_2** | Bol028953 | 774 | C01 | Abhydrolase | 5-248 |
| **BoSS1** | Bol022442 | 1,959 | C06 | GT1_Glycogen_synthase | 143-643 |
| **BoBSMT1** | Bol010926 | 996 | C03 | Methyltransferase | 6-331 |

**Table 2.** ***In-silico*** **analysis of Salicylic acid (SA) Related Proteins**

| Gene name | Protein Length (aa) | Molecular weight (Da) | Theoretical pI | Instability index | Aliphatic index | Grand average of hydropathicity | N-Glyc position | Protein stability | Subcellular localization |
|---|---|---|---|---|---|---|---|---|---|
| **BoMES1** | 262 | 29334.8 | 5.58 | 35.16 | 82.60 | -0.043 | - | Stable | Chloroplast |
| **BoMES2** | 263 | 29470.7 | 5.29 | 34.13 | 81.52 | -0.176 | - | Stable | Chloroplast |
| **BoMES4_1** | 257 | 28567.0 | 5.13 | 40.59 | 100.62 | 0.047 | - | Unstable | Chloroplast |
| **BoMES4_2** | 218 | 24500.6 | 5.39 | 26.06 | 97.52 | 0.130 | 48, 149 | Stable | Chloroplast |
| **BoMES4_3** | 261 | 29300.6 | 5.35 | 31.29 | 90.04 | -0.045 | 47, 180 | Stable | Chloroplast |
| **BoMES9_1** | 257 | 28912.4 | 5.48 | 36.67 | 85.29 | -0.114 | 43, 182 | Stable | Cytoplasmic |
| **BoMES9_2** | 257 | 28708.0 | 5.23 | 40.49 | 84.51 | -0.072 | 42, 182 | Unstable | Chloroplast |
| **BoSS1** | 652 | 71821.0 | 5.53 | 36.23 | 79.72 | -0.234 | 19, 93, 187, 194, 627 | Stable | Chloroplast |
| **BoBSMT1** | 331 | 37865.6 | 5.09 | 43.40 | 80.63 | -0.289 | 74, 91, 318 | Unstable | Cytoplasmic |

**Figure captions**

**Figure 1.** Comparison of the deduced aminoacid sequences of BoMES and AtMES. Underline indicates the functional domain. Solid boxes indicate the sequences not taken for expression analysis. Numbers in the right margin indicates the position of amino acid residues. Identical amino acids are indicated by a dark background, while >50% similarities are indicated by a light background.

**Figure 2**. Clubroot development in a susceptible (CR3) line of *B. oleracea* **(a)** inoculated with *P. brassicae* compared to **(b)** uninfected plants at 0 to 60 days after inoculation. Arrows indicate characteristic root gall formation.

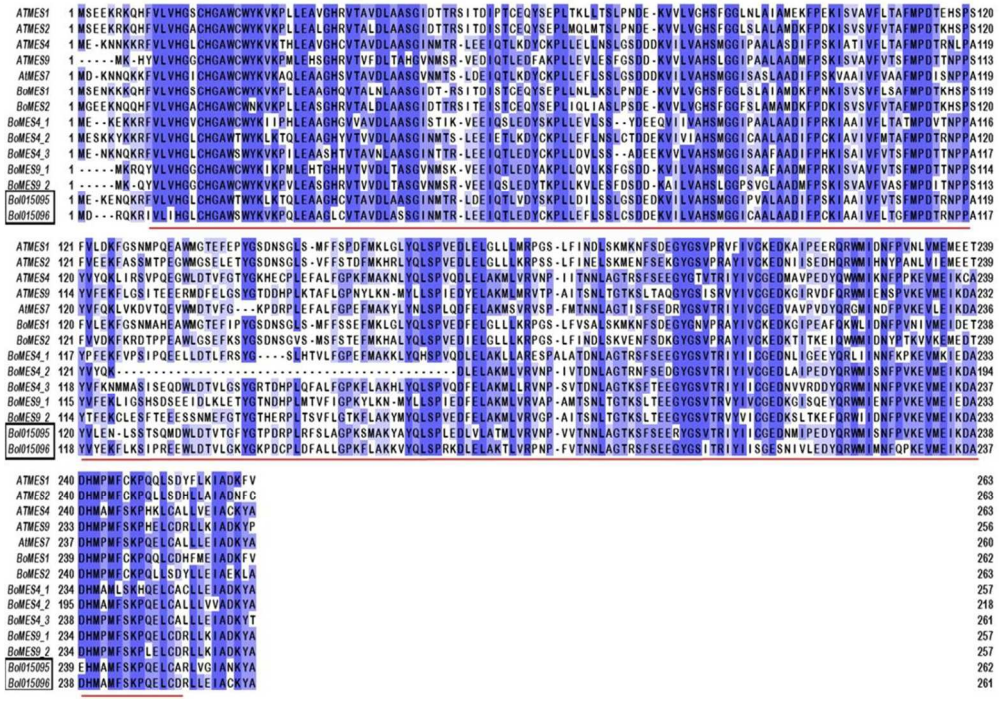
**Figure 3.** The resistant (CR4) line of *B. oleracea* from 0 to 60 days after *P. brassicae* infection **(a)** compared to **(b)** uninfected plants at the same time points.

**Figure 4**. Real-time PCR expression analysis after infection with *Plasmodiophora brassicae* in *B. oleracea* root samples. Data represent an average ± s.e.m of three biological replicates, with transcripts normalized to *Actin*. S- Susceptible and R- Resistant line

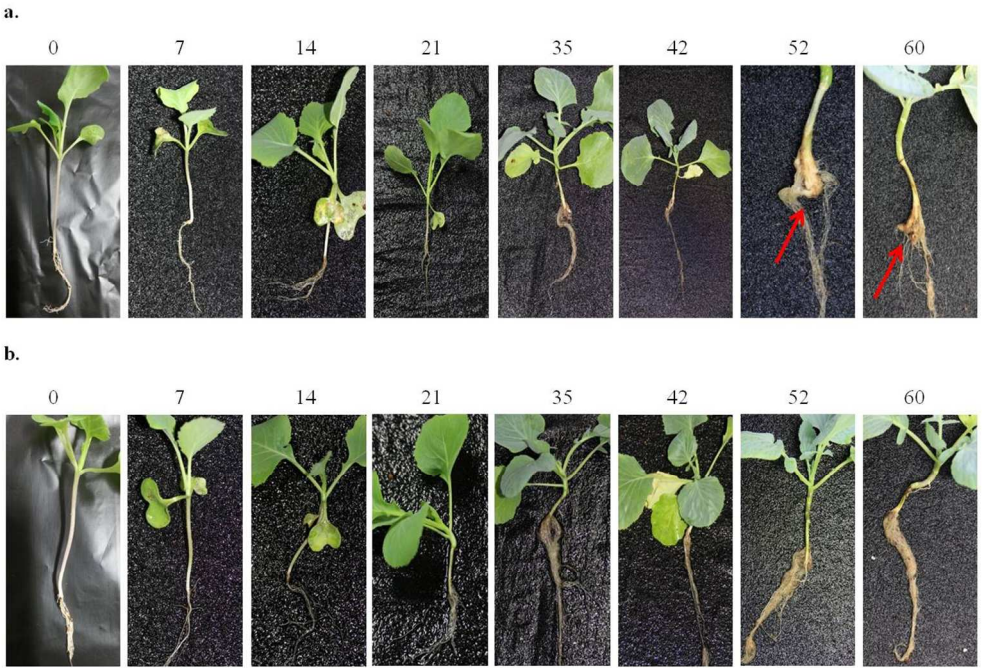
**Figure 5**. Real-time PCR expression analysis after infection with *Plasmodiophora brassicae* in *B. oleracea* leaf samples Data represent an average ± s.e.m of three biological replicates, with transcripts normalized to *Actin*. S- Susceptible and R- Resistant line

**Figure 6**. Interaction network among BoMES and related proteins of *Arabidopsis thaliana*. Stronger associations are represented by thicker lines.

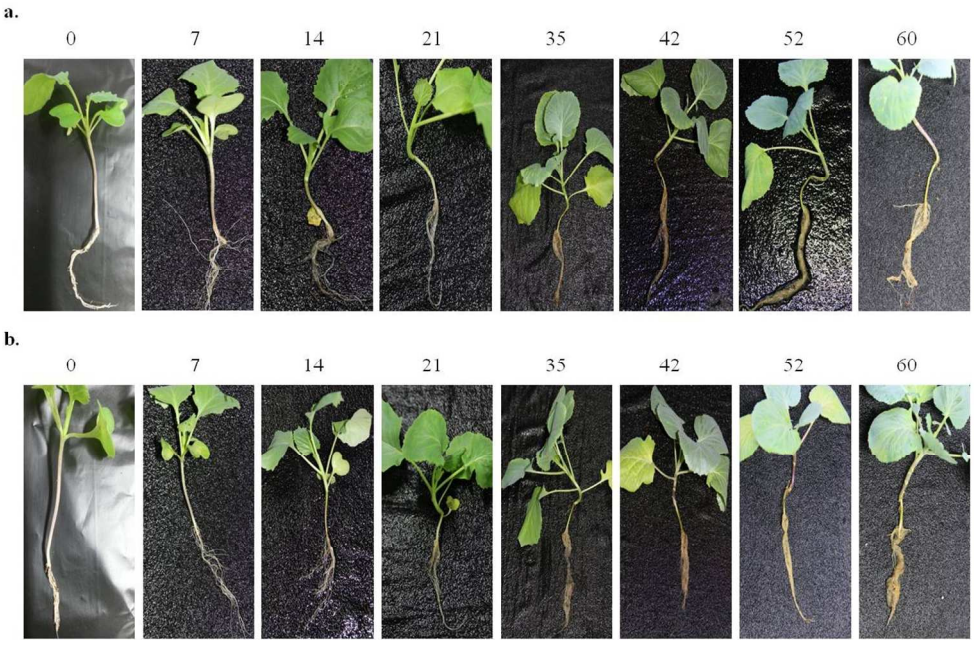
**Figure 7**. Interaction networks for (a) BoSS1 and (b) BoBSMT1 with related proteins of *Arabidopsis thaliana.* Stronger associations are represented by thicker lines.



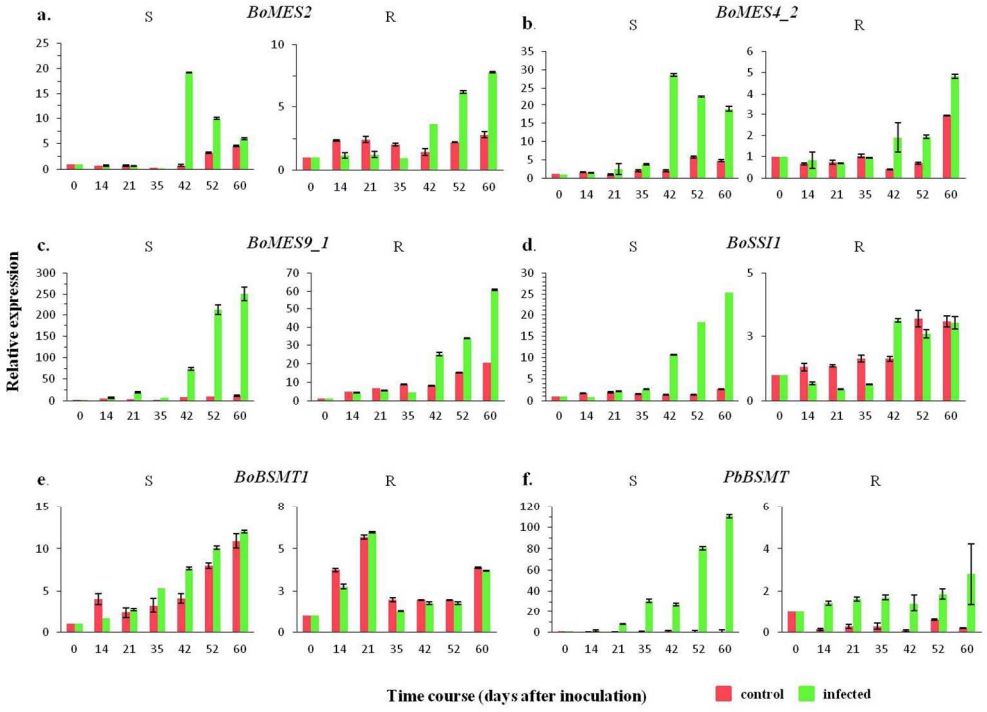
226x161mm (150 x 150 DPI)



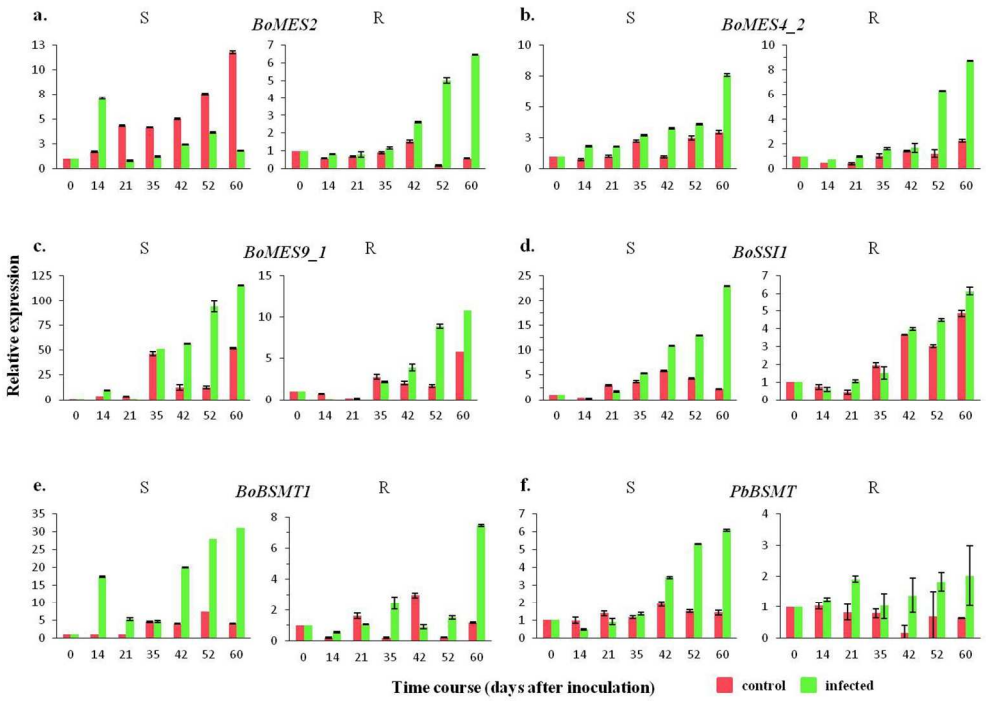

235x159mm (150 x 150 DPI)



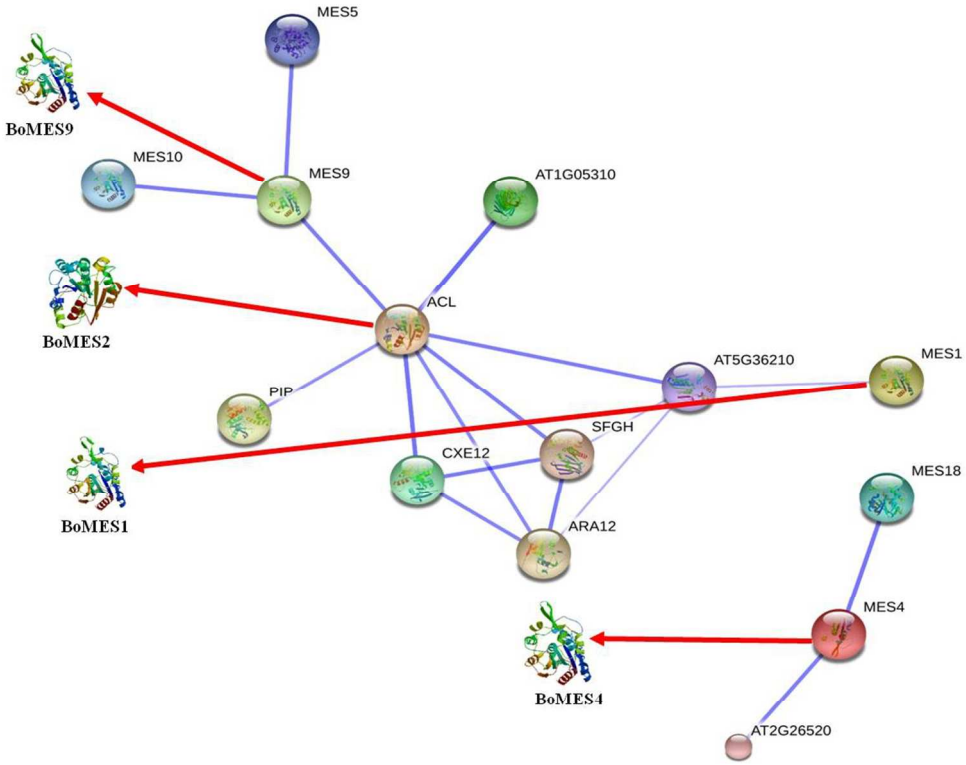

239x154mm (150 x 150 DPI)



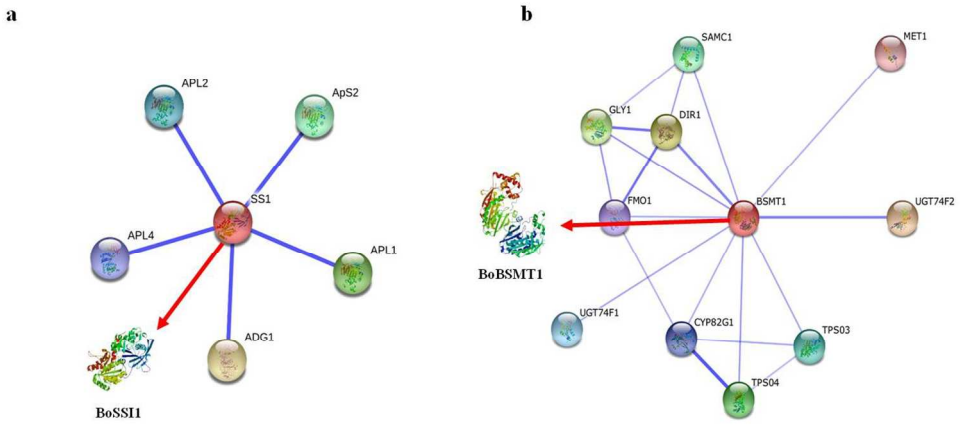

251x179mm (150 x 150 DPI)




247x175mm (150 x 150 DPI)




202x167mm (150 x 150 DPI)




247x107mm (150 x 150 DPI)

**Table S1.** *A. thaliana* protein similarity search against *B. oleracea* proteome

| Sl. | Gene name | *A. thaliana* | *B. oleracea* Identity | |
|---|---|---|---|---|
| **1** | AtMES1 | AT2G23620 | Bol031679* | 83% |
| | | | Bol045801* | 77% |
| **2** | AtMES2 | AT2G23600 | Bol045801 | 76% |
| **3** | AtMES4 | AT2G23580 | Bol015096 | 76% |
| | | | Bol031681* | 72% |
| | | | *Bol031682* | 72% |
| | | | Bol012862* | 67% |
| | | | Bol031680* | 67% |
| **4** | AtMES7 | AT2G23560 | *Bol015095* | 73% |
| | | | *Bol015096* | 70% |
| **5** | AtMES9 | AT4G37150 | Bol018638* | 80% |
| | | | Bol028953* | 76% |

*These proteins are annotated based on their *A. thaliana* high homology (>60%) and taken for this study. Italicized IDs (Bol015095 and Bol015096 are not amplified; Bol031682 does not have α/β hydrolase domain) are not taken for this study.

**Table S2.** Primers used for Realtime-PCR analysis

| Gene name | Locus ID | Primers | |
|---|---|---|---|
| | | **Forward** | **Reverse** |
| **BoMES1** | Bol031679 | GCCACCAAGTAACCGCCTTA | AAGCTCGAGGAACATTGCCA |
| **BoMES2** | Bol045801 | AGACCTAGCTGCTTCGGGTA | GAGGCTTGCAGAACATTGGC |
| **BoMES4_1** | Bol012862 | GTGGTTGCAGTGGATCTTGC | ACGCTGGTACTCCTCACCTA |
| **BoMES4_2** | Bol031680 | GAGGCTGCAGGTCACTATGT | ACCACCAAGAGAAGAGCACAG |
| **BoMES4_3** | Bol031681 | AGAAGCGGTTTGTGCTTGTC | AGGCTTGGAGAACATTGGCA |
| **BoMES9_1** | Bol018638 | CGTCTGGTGTGAACATGAGC | AAAGTTCTTGCGGCTTGGAG |
| **BoMES9_2** | Bol028953 | TACAAGGTCAAACCGGTGCT | CTTTTGGTCCCCGTCAGGTT |
| **BoSS1** | Bol022442 | ACCGTCAAAGCCGAAGTGAT | TGTTCTCGCCCATGTTGGAA |
| **BoBSMT1** | Bol010926 | ACCAGGCTCCTTCTACGCTA | TCCAAAGTGAGCAACGAGCA |
| **PbBSMT** | N106050 | CATATCGGTGCTGAAATCGG | GAATAGTCATGCCAGGTGAAC |
| **Actin** | KF218591 | TCTCACACTGTGCCAAT | CCACGTCACACTTCAT |

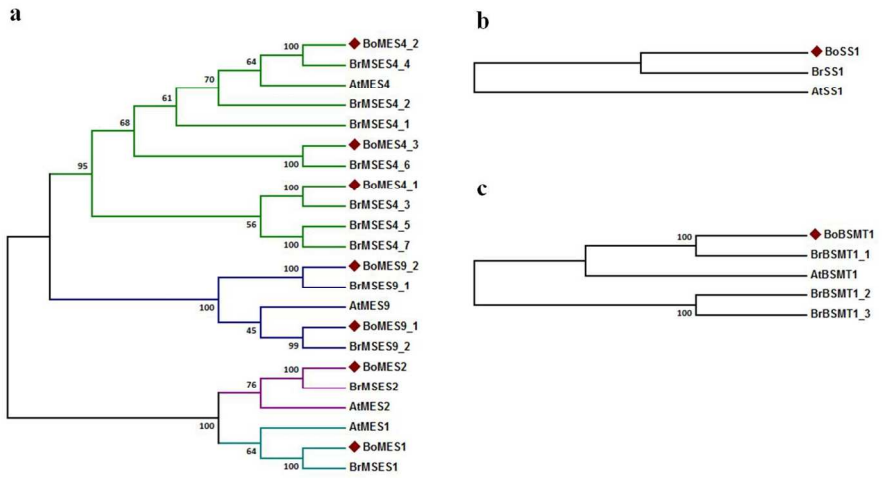



**Figure S1.** Phylogenetic analysis of SA-related genes from *A. thaliana* (At), *B. oleracea* (Bo), *B. rapa* (Br). Sequences were aligned using ClustalW, and the tree was constructed using the Maximum likelihood (ML) method employing the JTT matrix along with 1000 bootstrap replications. The bootstrap value at the tree nodes represents robustness of the tree. **(a)** MES genes, **(b)** SS1 genes and **(C)** BSMT genes.

254x152mm (150 x 150 DPI)

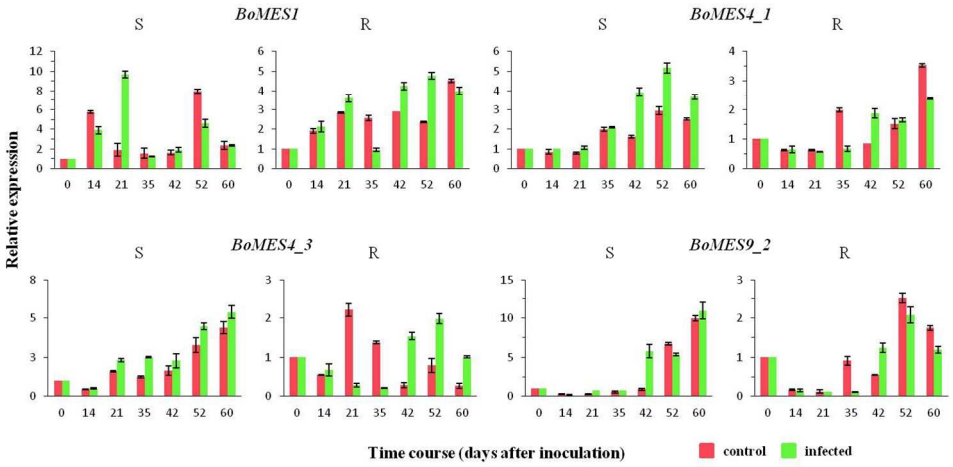



**Figure S2**. Real–time PCR expression analysis after infection with *Plasmodiophora brassicae* in *B. oleracea* root samples. a. *BoMES1*, b. *BoMES4_1*, c. *BoMES4_3*, d. *BoMES9_2*. Data represent an average ± s.e.m of three biological replicates, with transcripts normalized to *Actin*. S- Susceptible and R- Resistant line.

254x146mm (150 x 150 DPI)

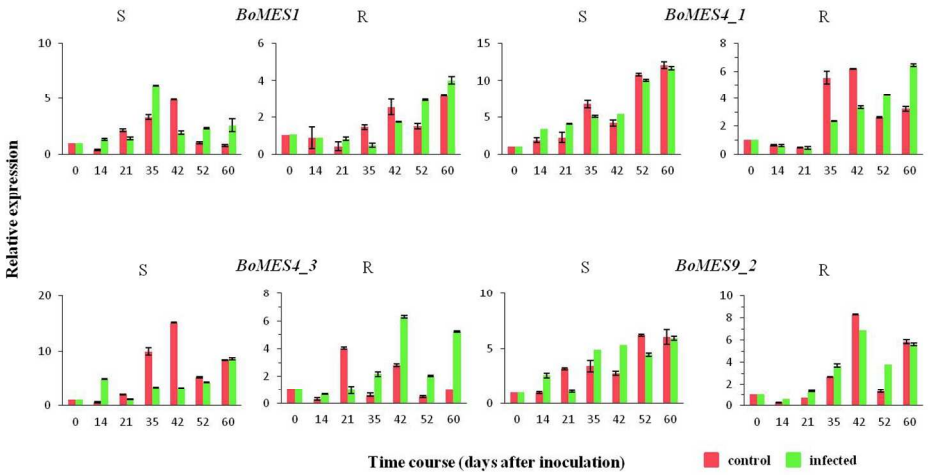



**Figure S3**. Real–time PCR expression analysis after infection with *Plasmodiophora brassicae* in *B. oleracea* leaf samples. a. *BoMES1*, b. *BoMES4_1*, c. *BoMES4_3*, d. *BoMES9_2*. Data represent an average ± s.e.m of three biological replicates, with transcripts normalized to *Actin*. S- Susceptible and R- Resistant line.

254x149mm (150 x 150 DPI)