Investigating Proteome and Transcriptome Defense Response of Apples Induced by *Yarrowia lipolytica*

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A better understanding of the mode of action of postharvest biocontrol agents on fruit surfaces is critical for the advancement of successful implementation of postharvest biocontrol products. This is due to the increasing importance of biological control of postharvest diseases over chemical and other control methods. However, most of the mechanisms involved in biological control remain unknown and need to be explored. *Yarrowia lipolytica* significantly inhibited blue mold decay of apples caused by *Penicillium expansum*. The findings also demonstrated that *Y. lipolytica* stimulated the activities of polyphenoloxidase, peroxidase, chitinase, L-phenylalanine ammonia lyase involved in enhancing defense responses in apple fruit tissue. Proteomic and transcriptomic analysis revealed a total of 35 proteins identified as up- and down-regulated in response to the *Y. lipolytica* inducement. These proteins were related to defense, biotic stimulus, and stress responses, such as pathogenesis-related proteins and dehydrin. The analysis of the transcriptome results proved that the induced resistance was mediated by a crosstalk between salicylic acid (SA) and ethylene/jasmonate (ET/JA) pathways. *Y. lipolytica* treatment activated the expression of isochorismate synthase gene in the SA pathway, which up-regulates the expression of *PR4* in apple. The expression of 1-aminocyclopropane-1-carboxylate oxidase gene and ET-responsive transcription factors 2 and 4, which are involved in the ET pathway, were also activated. In addition, cytochrome oxidase I, which plays an important role in JA signaling for resistance acquisition, was also activated. However, not all of the genes had a positive effect on the SA and ET/JA signal pathways. As transcriptional repressors in JA signaling, *TIFY3B* and *TIFY11B* were triggered by the yeast, but the gene expression levels were relatively low. Taken together, *Y. lipolytica* induced the SA and ET/JA signal mediating the defense pathways by stimulating defense response genes, such as peroxidase, thaumatin-like protein, and chitinase 4-like, which are involved in defense response in apple.

The use of antagonistic yeast for biological control of pathogens has been reported to be a safe and effective method for managing postharvest diseases (Droby et al. 2016). Previous research results showed that competition for nutrients and niche

exclusion are the most important mode of action of the yeast to inhibit the development of pathogens in the fruit wound (Spadaro and Droby 2016). Typically, mechanisms involved in the ability of yeast antagonists to inhibit postharvest pathogens include competition for nutrients and space (Jamalizadeh et al. 2011), induction of host resistance (Tian et al. 2011), oxidative response (Macarasin et al. 2010), and parasitism (Magallon-Andalon et al. 2012). These mechanisms of action have been reviewed by Spadaro and Droby (2016). Among them, host resistance induced by the antagonistic yeast was considered as one of the key components by which the pathogen is inhibited. The plant recognizes the bio-elicitor induction and develops resistance to protect itself from further attacks by the pathogen.

The salicylic acid (SA) signal transduction pathway is an important pathway to acquire induced defense in many plants (Kachroo and Kachroo 2007). SA enhances the biocontrol efficacy of antagonistic yeast by inducing a significant increase in polyphenoloxidase, L-phenylalanine ammonia lyase (PAL), and β -1,3-glucanase activity in cherry fruit (Qin et al. 2003). SA is also involved in the expression of a range of defense pathogenesis-related (PR) proteins, such as chitinase (PR-3), β -1,3-glucanase (PR-2), and peroxidase (Qin et al. 2003). Pathogen infection stimulates plants to synthesize SA, which regulates the expression of PR proteins and it had been reported that PR proteins play an important role in defense against pathogenicity (Umar et al. 2016; van Loon et al. 2006). The antagonistic yeast *Pichia membranaefaciens* induced PR proteins in peach fruit that played an important role in the fruit resistance to pathogens (Chan et al. 2007). Moreover, the antagonistic yeast *Metschnikowia fructicola* up-regulated the PR genes in grapefruit that played an important role in the efficacy of *Metschnikowia fructicola* against *Penicillium digitatum* (Hershkovitz et al. 2012). The generation of PR proteins is one of the important biochemical mechanisms involved in inducing plant resistance against pathogen diseases in postharvest shelf life (Charles et al. 2009; Quaglia et al. 2011; Wang et al. 2009).

Recently, studies revealed the identified PR families mainly comprise 17 groups that most often associated with plant defense response (Hoffmann-Sommergruber 2002, van Loon et al. 2006). In apple stems, expression of apple PR-5 and PR-8 genes were characterized in response to *Candida oleophila* and *Botrytis cinerea* in vitro. The results showed the PR-8 protein has antifungal activity against fungal pathogens (Bonasera et al. 2006). The existence of β -1,3-glucanase (PR-2), chitinase (PR-3), and thaumatin-like protein (PR-5) was observed in *Malus domestica* leaves infected by *V. inaequalis* (Gau et al. 2004). A PR-4 gene identified from *Malus domestica* is involved in defense responses against *Botryosphaeria dothidea* (Bai et al. 2013).

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Another response pathway to biotic stresses is mediated by JA/ET signaling, which plants use to deal with the complex environmental effects caused by biotic and abiotic stresses (Santino et al. 2013). In general, JA and ET mediate responses against necrotrophs (Fujita et al. 2006; Glazebrook 2005), but some studies proved that there is crosstalk between SA and JA pathways in response to biotic stress. The crosstalk can be either mutually antagonistic or synergistic (Adie et al. 2007; Beckers and Spoel 2006; Spoel et al. 2007). In *Abrabidopsis*, the invasion of *Botrytis cinerea* induced both the SA and JA signal pathways (Ferrari et al. 2007). *COI1* is a gene that has been proven to play an important role in the JA signal for resistance against fungi (Adie et al. 2007; Chini et al. 2007; Glazebrook 2005). A key step in the activation of induced resistance is mediated by transcription factors (TFs) and TFs control the transcriptional regulation of related defense genes. Several members of the ET-responsive TF (ERF) family have been shown that play a key role in JA-mediated response to pathogens (Berrocal-Lobo et al. 2002; Brown et al. 2003; Lorenzo et al. 2003; McGrath et al. 2005; Moffat et al. 2012). The bHLH transcription factors *MYC2*, *MYC3*, and *MYC4* belong to the TF family; they are all involved in the JA-mediated signal resistance. The *MYC2* is a key point of the JA signaling pathway that regulates the expression of different subsets of JA-responsive genes and also different responsive genes to hormone targets of JAZ repressors (Lorenzo et al. 2004; Shin et al. 2012). Several members of the *WRKY* TF family have been involved in specific aspects of induced responses (Petersen et al. 2000).

Antagonistic yeasts have been reported to act as bio-elicitors of fruit host resistance (Spadaro and Droby 2016). In tomato, the genes involved in metabolism, signal transduction, and stress response were up-regulated by the antagonistic yeast

Cryptococcus laurentii (Sharma et al. 2009). *Rhodosporidium paludigenum* activated gene expression of plant hormones, signaling transduction, stress, and defensive responses in mandarin orange. It has been reported that antifungal metabolites of mandarin orange, indeed, contribute to biocontrol enhancement of yeasts, offer fruit protection, and provide an important basis for understanding the mechanisms of resistance induction in mandarin orange (Lu et al. 2015). In previous studies, *Yarrowia lipolytica* significantly inhibited various fungal diseases via induced disease resistance (Groenewald et al. 2014; Yang et al. 2015). However, the mechanism of the induced disease resistance by *Y. lipolytica* is still not clear.

In the current study, the effects of *Y. lipolytica* on defense-related enzymes such as polyphenoloxidase, peroxidase, chitinase, and PAL were investigated in apple fruit. Additionally, proteome and transcriptome of the apple wound-inoculated tissues were also investigated, to determine the mechanism and the possible pathways involved in the defense induction by the antagonist yeast and the possible signaling elicitors.

RESULTS

Inhibition of *Penicillium expansum* infection by *Y. lipolytica*.

The inhibitory effect on disease incidence was influenced by cell concentrations of *Y. lipolytica* (Fig. 1). The best inhibitory effect was observed using a concentration of 10^9 cells per milliliter. At this concentration, the percentage of disease infection was 24.06%, compared with 84.3% in the control (Fig. 1C). Lower cell yeast concentrations showed very marginal effects on disease incidence. Similarly, the disease lesion diameter was also influenced by the concentration of *Y. lipolytica* (Fig. 1D). The higher the concentration of the yeasts, the less

Fig. 1. Effect of *Yarrowia lipolytica* in mold decay of apples caused by *Penicillium expansum*. **A**, Apples inoculated with *Y. lipolytica* and *P. expansum* and stored for 5 days. **B**, Treatment with *P. expansum*. **C**, The decay incidence caused by *P. expansum*. **D**, The lesion diameter caused by *P. expansum* in apples. Asterisks (*) indicate significant difference.

expansion of the lesion diameter was evident; at a yeast concentration of 10^9 cells per milliliter, the disease lesion diameter was 6.10 mm compared with 21.23 mm in the control (Fig. 1D).

Effect of *Y. lipolytica* treatment on the activity of defense related enzymes.

Polyphenoloxidase (PPO), peroxidase (POD), chitinase (CHI), and PAL are the four defense-related enzymes tested in apple wound tissue in response to application of *Y. lipolytica*. As shown in Figure 2A, *Y. lipolytica* caused significant increased PPO activity on days 1 and 2 (20 U per gram of fresh weight), then decreased to 12 U per gram of fresh weight on days 3 and 4, and then, increased to 18 U per gram of fresh weight on day 5. The PPO activity of the control was lower than that in the *Y. lipolytica* treatment at all tested time points except day 3. The results for POD activity (Fig. 2B) indicated that POD activity of apple wound tissue significantly increased ($P < 0.05$) at all the tested time points, with the exception of day 3. The results for activities of CHI (Fig. 2C) and PAL (Fig. 2D) also revealed *Y. lipolytica* increased enzyme activities compared with the control, even through days 5 and 6. The trends of both these two enzymes were the same in apple wounds of *Y. lipolytica* treatment and the control.

Two-dimensional gel electrophoresis (2-DE) analysis of proteins induced by *Y. lipolytica* in apples.

Protein expression levels in apple wounds treated with *Y. lipolytica* was observed to be different than the protein expression levels of the control; there were 42 proteins differentially expressed in the yeast-treated apple tissue at day 3 after inoculation (Fig. 3A and B). Using tandem mass spectrometry (MS/MS), 35 proteins that demonstrated statistically significant changes ($P < 0.05$) relative to protein abundances in treated apples were identified. Among these, 12 proteins were up-regulated and 23 proteins were down-regulated. These proteins were searched with the MS/MS ion searching program MASCOT and were further assessed with MS-BLAST (Table 1). According to the method of functional annotation classification (Bevan et al. 1998), these induced proteins were mainly involved in defense reactions and stress responses (Fig. 3C).

The expression level of PR-10 (spot 5), thaumatin-like protein (PR-5) (spots 6, 7, 12, 14, and 16), and dehydrin (spots 19, 20, and 34) were increased as a result of *Y. lipolytica* treatment (Table 1).

Transcription and functional analysis of genes induced by *Y. lipolytica*.

The transcriptomes of apple fruit wound tissue treated with *Y. lipolytica* and nontreated fruit tissue (control) were analyzed using RNA-Seq technology. A total of 607 DEGs (differentially expressed genes) were detected, in which 461 genes were up-regulated and 146 genes were down-regulated in the *Y. lipolytica*-treated apple tissue. Among these, 229 genes were significantly increased and 20 genes were significantly down-regulated ($\log_2$ fold change >2). These DEGs were clustered by gene function with Gene Ontology (GO) (Fig. 4). The biological processes contained 14 subgroups and the molecular function contained 16 subgroups. The most dynamic GO categories were those in response to biotic stimulus and defense response. The abiotic stress genes under the biological process category were the important genes involved in induced resistance caused by *Y. lipolytica*.

Expression of TFs in SA and JA/ET signal pathways induced by *Y. lipolytica*.

The TFs involved in SA and JA/ET signal pathways were selected and their expression levels were determined by real time-quantitative polymerase chain reaction (RT-qPCR). LOC103407103 and LOC103431866 were respectively annotated to 1-aminocyclopropane-1-carboxylate oxidase (ACO) and isochorismate synthase (SID). These two enzymes were induced by *Y. lipolytica* and belonged to the ET and SA pathways. The expression level of ACO in the yeast treatment group was 32-fold more than that of the control (Fig. 5A). The expression level of SID was also increased by 3.62-fold due to the yeast treatment compared with the control (Fig. 5B). The results indicated that the antagonistic yeast *Y. lipolytica* induced disease resistance of the apples by the SA signal pathway.

The novel 00376 and LOC103437431 were annotated, respectively, to F-box protein (COI) and TF MYC3. COI (Fig. 5C)

Fig. 2. Effect of *Yarrowia lipolytica* on the activities of **A**, polyphenoloxidase (PPO), **B**, peroxidase (POD), **C**, chitinase (CHI), and **D**, L-phenylalanine ammonia lyase (PAL) in apple wounds.

and *MYC3* (Fig. 5D) expression levels were each increased 28.25- and 9.49-fold when induced by *Y. lipolytica* compared with the control. LOC103439587 and LOC103422389, which were both stimulated by *Y. lipolytica*, were annotated as *ERF2* and *ERF4*, respectively. The expression levels of *ERF2* (Fig. 5E) and *ERF4* (Fig. 5F) in the yeast treatment group were increased 8.28- and 3.43-fold compared with that of the control. These genes were all involved in the JA signal pathway and were induced by the yeast in apple fruits. The results indicated that the antagonist yeast *Y. lipolytica* induced the resistance of apple fruit through the JA/ET signal pathway. The expression levels of some other TFs were also analyzed. The WRKY family TFs were also found to be activated by *Y. lipolytica* (Fig. 5G). In addition, TIFY3B and TIFY11B, which are involved in JA signaling, were activated by the yeast, but the gene expression level was relatively low.

Expression of defense response genes induced by the SA and JA/ET signal pathways.

Expression of several defense response genes downstream of SA and JA/ET signal pathways was determined by RT-qPCR. The peroxidase (LOC103403867) (Fig. 6A), thaumatin-like protein 1b (Fig. 6B), and chitinase 4-like (Fig. 6C) were increased 3.18-, 1.58-, and 3.56-fold, respectively, compared with the control. The most expressed gene was thaumatin-like protein 1a (LOC103426173), whose expression level was increased more than 35-fold compared with the control (Fig. 6D). LOC103434169 and LOC103402179, which were annotated to PR-4 (Fig. 6E) and PR protein-like (Fig. 6F) increased 25.57- and 15.75-fold, respectively, by the yeast compared with the control. In addition, some defense response-related genes were also identified and are shown in Figure 6G. The expression level of PAL, acidic endochitinase-like, glutathione *S*-transferase, and polyphenol oxidase were increased more than twofold

in apple treated with *Y. lipolytica* compared with the control.

DISCUSSION

The aim of this study was to determine the mechanism by which the yeast antagonist *Y. lipolytica* elicits disease resistance in apples. *Y. lipolytica* was proved to be an effective biocontrol agent against *P. expansum* in apples. The activities of disease resistance-related enzymes were activated in apple fruits when the yeast was inoculated into apple surface wounds. The proteome and transcriptome of both the yeast-treated apples and the control were analyzed. A total of 151 differentially expressed proteins were found, in which 77 were up-regulated and 74 were down-regulated. Among these proteins, we selected 35 high-content proteins for analysis. GO and functional categories of the protein enrichment analysis showed that the main category among three was biological processes, and 13 functional proteins were associated with resistance response. A total of 607 DEGs were found, in which 229 genes were significantly up-regulated and 20 genes were significantly down-regulated by *Y. lipolytica* treatment. Functional categories of the DEGs by GO term enrichment analysis showed that defense and response-related genes were strongly induced by the yeast. *Y. lipolytica* is an oleaginous species of yeast that has numerous biotechnological applications in feed and food production (Groenewald et al. 2014; Morin et al. 2011). It also has been shown to have the activity of degrading ochratoxin A to nontoxic products (Yang et al. 2015). *Y. lipolytica* inhibited the growth of *P. expansum* in apple fruits, similar to results by Spadaro et al. (2013), who used *Metschnikowia fructicola* to control *P. expansum* and patulin accumulation in four cultivars of apples.

A successful biocontrol agent is generally equipped with several attributes that often work in concert and may be crucial

Fig. 3. Two-dimensional gels of apple proteins treated with *Yarrowia lipolytica* and the control at 3 days after different treatments. **A**, The control (nonyeast) and **B**, the group treatment with *Y. lipolytica* (yeast) showed the protein spots. **C**, Proteins clustered by function.

for controlling disease development (Droby et al. 2009). The ability to induce resistance through the activation of defense-related enzymes was one important mechanism of action exhibited by the antagonist yeast. POD and CHI were induced by the antagonist *Aureobasidium pullulans* during apple storage (Ippolito et al. 2000). In addition, PAL, POD, CHI, and β -1,3-glucanase activities were found to be involved in the action of plant resistance induced by the yeast *Cryptococcus laurentii* (Yao and Tian 2005). In comparison with our study, the activities of PPO, POD, CHI, and PAL were also induced by *Y. lipolytica* in apples and these four enzymes were actively involved.

The ability to activate an SA-independent induced systemic resistance (ISR) pathway is common for beneficial microbes and occurs in a broad range of plant species (van Loon et al. 1998). In current work, *Y. lipolytica* treatment induced SID gene expression level, which was involved in the SA production pathway. The increased level of SA induced the expression of defense-related genes. Along with SA, plant hormones JA and ET are also important regulators of the plant immune system, *COI* has been described to have a central role in JA signaling for disease resistance against oomycetes and fungi. *COI* is a positive factor of the JA pathway (Chini et al. 2007; Glazebrook 2005). Gene expression of *COI* was increased when induced by *Y. lipolytica* in a robust manner. *ACO* catalyzed the reaction from *S*-adenosyl methionine to ethylene in the ethylene biosynthetic pathway. *ERF2* has a clear impact over *PDF1.2* gene expression by integrating JA/ET signals and *ERF4* repressed *PDF1.2* but induced *VSP2* (Memelink 2009; Moffat et al. 2012). The expression level of *ERF4* was increased, but the increased volume was much less than *COI* and *ERF2* in the

present research. The TF *MYC3* is a key point of abscisic acid and JA signaling and regulates different response genes. *MYC3* act additively with *MYC2* in the activation of JA responses (Fernández-Calvo et al. 2011). *TIF3B* and *TIF11B* are two JAZ (JA-zim domain) family proteins that act as JA coreceptors and transcriptional repressors in JA signaling (Chini et al. 2007). In this study, LOC103442657 and LOC103443669 were annotated to JAZ proteins *TIFY3B* and *TIFY11B* (Fig. 6E). They were triggered by the yeast but gene expression level was relatively low. The above results indicate that *Y. lipolytica* induced disease resistance of the apples by stimulating SA and JA/ET pathways, which mediated the defense response.

PR4 identified from *Malus domestica* was involved in defense responses against *Botryosphaeria dothidea* (Bai et al. 2013). *PR4*, as the response gene of downstream SA and JA signals, could be up-regulated by SA or methyl JA treatment. *PR4* was up-regulated by *Y. lipolytica*, indicating that SA and JA signal pathways were involved in induced resistance in apples. The resistance response was the result of crosstalk between SA and ET/JA pathways. Additionally, according to the proteome and transcriptome results, other defense-related proteins were also found. Thaumatin-like protein belong to the PR proteins 5 family, which has the antifungal ability to permeabilize fungal membranes and destroy cell walls of the pathogen using a series of enzymatic activities (Liu et al. 2010, 2012a and b). Ribonuclease-like PR-10c is homologous with the PR-10 that is an important PR protein in plant defense response (Gao et al. 2005; Liu and Ekramoddoullah 2006; Pulla et al. 2010). The expression level of peroxidase, PR protein, thaumatin-like protein 1a, thaumatin-like protein 1b, and chitinase were higher than the control from the transcriptome data.

Table 1. Differentially expressed proteins identified in apple tissues after treatment

Spot	Protein description	Accession no.	Mass	Organism	PI	Mascot score
↓1	Carbon monoxide dehydrogenase subunit G	gi 739834403	16,169	<i>Sulfobacillus acidophilus</i> DSM 10332	5.21	64
↓2	Dehydrin 9	gi 382948209	31,779	<i>Malus domestica</i>	5.2	233
↓3	Ribonuclease-like PR-10c	gi 15418742	17,694	<i>Malus domestica</i>	5.68	382
↑4	Uncharacterized protein	gi 657975084	49,883	<i>Malus domestica</i>	4.68	637
↑5	Ribonuclease-like PR-10c	gi 15418742	17,694	<i>Malus domestica</i>	5.68	419
↑6	Thaumatin-like protein precursor Mdt11	gi 3643249	26,609	<i>Malus domestica</i>	5.17	145
↑7	Thaumatin-like protein precursor Mdt11	gi 3643249	26,609	<i>Malus domestica</i>	5.17	78
↑8	Aspartic proteinase	gi 21616051	57,088	<i>Malus domestica</i>	4.82	132
↓9	Thaumatin-like protein precursor Mdt11	gi 3643249	26,609	<i>Malus domestica</i>	5.17	77
↓10	Peroxiredoxin-2B-like	gi 657946863	17,550	<i>Malus domestica</i>	5.23	415
↓11	Glutathione S-transferase-like	gi 695055849	41,807	<i>Malus domestica</i>	7.06	267
↑12	Thaumatin-like protein precursor Mdt11	gi 3643249	26,609	<i>Malus domestica</i>	5.17	147
↑13	5'-3' Exoribonuclease 2-like	gi 657979046	22,112	<i>Malus domestica</i>	6.09	318
↑14	Thaumatin-like protein precursor Mdt11	gi 3643249	26,609	<i>Malus domestica</i>	5.17	187
↓15	Predicted protein	gi 168033874	20,676	<i>Physcomitrella patens</i>	5.2	141
↑16	Thaumatin-like protein precursor Mdt11	gi 3643249	26,609	<i>Malus domestica</i>	5.17	299
↓17	Acid phosphatase 1-like	gi 658063964	29,032	<i>Malus domestica</i>	6.10	147
↓18	Thaumatin-like protein precursor Mdt11	gi 3643249	26,831	<i>Malus domestica</i>	4.71	289
↑19	Dehydrin 9	gi 382948209	31,779	<i>Malus domestica</i>	5.20	69
↑20	Dehydrin 9	gi 382948209	31,779	<i>Malus domestica</i>	5.20	113
↓21	Profilin-1	gi 1709779	14,462	<i>Arabidopsis thaliana</i>	4.70	96
↓22	Glutathione S-transferase-like	gi 657955010	23,877	<i>Malus domestica</i>	7.06	75
↓23	Glutamine synthetase cytosolic isozyme-like	gi 525507210	39,423	<i>Malus domestica</i>	5.78	188
↓24	Glutamine synthetase cytosolic isozyme	gi 658036616	39,096	<i>Malus domestica</i>	5.78	115
↓25	L-3-Cyanoalanine synthase 1, mitochondrial	gi 658309724	41,085	<i>Malus domestica</i>	7.61	146
↓26	Apolipoprotein D-like	gi 658003653	21,471	<i>Malus domestica</i>	6.18	192
↓27	Probable aldo-keto reductase 2	gi 657977050	37,340	<i>Malus domestica</i>	5.61	588
↓28	Cytosolic malate dehydrogenase	gi 78216493	35,970	<i>Malus domestica</i>	6.01	299
↓29	Cytosolic malate dehydrogenase	gi 78216493	35,817	<i>Malus domestica</i>	6.01	214
↓30	ATP synthase subunit beta, mitochondrial	gi 658008796	60,115	<i>Malus domestica</i>	6.01	723
↓31	Major allergen Mal d 1	gi 1313966	17,540	<i>Malus domestica</i>	5.72	170
↓32	MLP-like protein 329	gi 658008681	17,823	<i>Malus domestica</i>	5.36	460
↓33	Glycine-rich RNA-binding protein-like	gi 658024705	16,877	<i>Malus domestica</i>	5.60	626
↑34	Dehydrin 9	gi 382948209	31,779	<i>Malus domestica</i>	5.20	477
↓35	Major allergen Mal d 1	gi 2443824	17,514	<i>Malus domestica</i>	5.72	107

It had been reported that *LePR5* was up-regulated when post-harvest cherry tomato fruits were treated by biocontrol yeast *Cryptococcus laurentii*, suggesting that *LePR5* plays an important role in the defense system of cherry tomatoes and is involved in the biocontrol mechanism by inducing a resistance protein by yeast against pathogens in postharvest fruit (Guo et al. 2016). They also were consistent with PR-1a and PR-5, which induced elicitor responses to apple pathogen *Erwinia amylovora* (Bonasera et al. 2006). Previous studies have shown that PR-1, PR-3, and PR-4 genes were accumulated in *M. domestica* in response to pathogens (Bai et al. 2013; Gau et al. 2004). In this study, thaumatin-like protein 1a and thaumatin-like protein 1b, which were classified to the PR-5, were increased by *Y. lipolytica*, both in the transcriptome and proteome data. The PR genes were induced by *Y. lipolytica*, suggesting that *Y. lipolytica* could promote PR gene expression and enhance the ability of defense response (Fig. 6G). The expression of defense response genes in apple fruit was mediated by the SA and JA/ET signal pathway (Fig. 7).

Concluding remarks.

Our results demonstrated that *Y. lipolytica* induced the production of SA and ET signal in apple fruit. The SA and ET/JA signal mediated the defense pathway by henceforth stimulating the defense response genes. This led to the PR genes being up-regulated to produce pathogen resistance in the apple fruits. Many genes were up-regulated, but not all of them had a positive effect on the SA and ET/JA signal pathway. Further detailed research on the specific role exhibited by each gene and their resultant interactions with each other is needed.

MATERIALS AND METHODS

Fruit source and yeast.

Apple fruits (*Malus domestica* Borkh 'Red Fuji') were harvested at commercial maturity. The apple fruits were treated

according to the procedure described by Bevan et al. (1998). The antagonist yeast *Y. lipolytica* was obtained from a culture collection maintained by our laboratory. The yeast was cultured following the method described by Morin et al. (2011).

Fruit inoculation and sample preparation.

Three wounds were made, on the equator of each apple fruit, with a sterile cork borer (approximately 5 mm diameter and 3 mm deep). Each wound was inoculated with a 30- μ l suspension of *Y. lipolytica* (1×10^6 to 1×10^9 cells per milliliter), and after 2 h, was inoculated with a 30- μ l suspension of *P. expansum* (1×10^5 spores per milliliter), for analysis of the effect of *Y. lipolytica* in mold decay of apples caused by *P. expansum*. For enzyme activities, proteomic, and transcriptomic analysis, the apple wound was only inoculated with a 30- μ l suspension of *Y. lipolytica* at the concentration of 1×10^8 cells per milliliter and sterile distilled water was used as the control. The fruits were then placed in plastic trays, which were enclosed with polyethylene bags, to keep a high relative humidity (about 95%), and were stored at 25°C. After 3 days, the tissues of the wounds were taken out, were immediately frozen with liquid nitrogen, and were stored at -80°C, before processing. For the proteomic analysis, tissues were removed at 72 h for the quantification of relative mRNA levels.

Analysis of enzyme activities of apple fruits.

Extraction procedures were conducted at 4°C. A quantity of 2 g of the fresh tissue around the wound from six apples was homogenized with 10 ml of cold 50 mM sodium phosphate buffer (pH 7.8) containing 1.33 mmol of EDTA and 10 mg per milliliter of polyvinylpyrrolidone. The homogenates were then centrifuged using a TGL-16 M centrifuge (XiangYi) at 12,000 rpm at 4°C for 15 min, and the supernatants were assayed. PPO activity was measured as described by Kiewning et al. (2013). POD activity was measured using the method described by Lurie et al. (1997), with some modifications by our research team

Fig. 4. Gene Ontology (GO) analysis of genes from the transcriptome data.

(Zhao et al. 2013), using guaiacol as a substrate. CHI activity was measured using the method described by El Ghouth et al. (2003), PAL activity was measured as described by Assis et al. (2001).

Protein extraction and solubilization.

Total protein was extracted from pooled samples using a phenol-based protocol (Spadaro et al. 2013). The tissues of apple wounds were ground into a fine powder in liquid nitrogen, using a mortar and pestle. The powder was subsequently suspended in an extraction buffer (0.8 M sucrose, 0.1 M Tris-HCl, pH 7.5, 10 mM EDTA, 0.1 M KCl, 2 mM phenyl-methylsulfonyl fluoride, and 2% β-mercaptoethanol), and an equal volume of Tris-saturated phenol (pH 7.9) was added. The mixture was kept at −20°C for 20 min and was vortexed every

10 min, and then, was centrifuged for 30 min at 10,000 rpm. The upper phenol phase was collected and the aqueous phase was re-extracted. The combined phenol phases were washed twice with extraction buffer. Then five volumes of cold methanol containing 0.1 M ammonium acetate were added and kept at −20°C overnight. After centrifugation at 10,000 rpm for 20 min, the precipitated protein pellets were washed, twice with cold methanol containing 0.1 M ammonium acetate and twice with cold acetone. The pellets were then vacuum-dried and resolution in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% dithiothreitol (DTT), 1% ampholyte, pH 3 to 10). Protein concentration was measured according to the methods of Spadaro et al. (2013) and Yao and Tian (2005), using bovine serum albumin as the standard. Two replicates were conducted

Fig. 5. The relative expression levels of the transcription factors involved in the salicylic acid and ethylene/jasmonate pathways. CK = the control; Y-T = the apple treated with *Yarrowia lipolytica*; log₂ (Fold change) = the result acquired from the transcriptome data. One asterisk (*) indicates significant difference ($P < 0.05$) and two asterisks (**) indicate extremely significantly difference ($P < 0.01$).

for each step. The protein suspension was stored at -80°C until 2-DE analysis.

2-DE analysis.

2-DE and image analysis were performed as described by van Loon et al. (1998), with some modifications. Proteins were first separated by isoelectric focusing (IEF), using a GE EttanIPGphor 3 and ImmobilineTM Drystrip gels (24 cm) (GE Healthcare) with a nonlinear pH gradient (3 to 10), according to the manufacturer's instructions. After IEF, strips were equilibrated for 15 min in 10 ml of equilibration buffer (7 M urea, 2% sodium dodecyl sulfate, 0.075 M Tris-HCl, pH 8.8, 30%

glycerol) containing 1% DTT and, subsequently, for 15 min in 10 ml equilibration buffer containing 2.5% iodoacetamide. The second dimensional electrophoresis was run on a 12% polyacrylamide gel in an Ettan DALT System (GE Healthcare). Protein spots in 2-DE gels were visualized by Coomassie blue staining. Three replicate gels were run for each sample. Image analysis was performed with the IPM7.0 software (GE Healthcare). A quality score of <40 was adopted to define low-quality protein spots, which were eliminated in further analysis (Fernández-Calvo et al. 2011; Memelink 2009). After normalization, only those spots with an average fold change ≥ 2 at least at one point in time after treatment as well as those that

Fig. 6. The relative expression level of the pathogenesis-related (PR) genes. CK = the control; Y-T = the apple treated with *Yarrowia lipolytica*; log₂ (Fold change) = the result acquired from the transcriptome data. One asterisk (*) indicates significant difference ($P < 0.05$) and two asterisks (**) indicate extremely significant difference ($P < 0.01$).

exhibited the same expression pattern among the three replicates were considered as differentially expressed proteins (Liu et al. 2010). Proteins were first separated by IEF using a Pro-tean IEF Cell and ReadyStrip IPG stripgels (24 cm) (GE Healthcare) with a linear pH gradient of 4 to 7, according to the manufacturer's instructions.

In-gel digestion.

Differentially expressed protein spots were excised manually from the gels and were destained with a 50 mM ammonium bicarbonate, 50% acetonitrile solution. The gel pieces were then reduced in 10 mM DTT for 45 min at 55°C and were alkylated in 55 mM iodoacetamide for 45 min at room temperature. After washing with 50% acetonitrile in 0.025 mM NH_4HCO_3 , the dried gels were incubated overnight at 37°C in 10 μl of a 10-ng/ml solution of trypsin (Sigma-Aldrich). The supernatant was collected for MS analysis.

MS analysis and database query.

A sample of each peptide solution was mixed with an equal volume of a matrix solution (70% acetonitrile, 0.1% trifluoroacetic acid, 10 mg of α -cyano-4 hydroxycinnamic acid per milliliter). The mixture was immediately spotted onto the target plate and was dried at room temperature. MS data were obtained using an ultraflexmatrix-assisted laser desorption-ionization time-of-flight mass spectrometer. The resulting peptide mass fingerprinting (PMF) were queried against the National Center for Biotechnology Information non-redundant database, using MASCOT software with the following search parameters: viridiplantae (green plant), trypsin, up to one missed cleavage, carbamidomethylation of cysteine, peptide tolerance ± 0.9 Dal, mass value MH⁺ and monoisotopic.

Total RNA isolation, integrity, and quantification.

RNA was extracted according to the procedure of Liu et al. (2012a and b) with some modification. Diethyl pyrocarbonate (DEPC)-treated water was used for all solutions. The extraction buffer without β -mercaptoethanol (2% CTAB, 2% polyvinylpyrrolidone K-30 [soluble], 100 mM Tris HCl [pH 8.0], 25 mM EDTA, and 2.0 M NaCl) was autoclaved and stored at room temperature, and 2% β -mercaptoethanol was added before use. The protocol used was as follows. Frozen fresh apple tissue (3 g) was ground in liquid nitrogen, and then, the ground tissues

were transferred into 50-ml polypropylene tubes with a chilled autoclaved spatula and were kept in liquid nitrogen until ready. Afterward, 10 ml of preheated (65°C water bath) extraction buffer was added to each tube, and samples were vortexed and stored at 65°C for 10 min. Samples were vortexed every 2 min during the period. RNase were used to spray gloves occasionally. An equal volume of chloroform and isoamyl alcohol (Chl:Iaa) (24:1) was mixed and was immediately vortexed for 2 min. Samples were transferred to 50-ml tubes that were cleaned and prelabeled and were then vortexed. The mixture was homogenized with a vortex in an ice bath for 10 min and, then, was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was then transferred into a new tube. The aqueous phase was carefully pipetted and transferred into another tube, making sure that the white phase was not included. The mixture was re-extracted with an equal volume of Chl:Iaa after centrifugation at 10,000 rpm for 10 min at 4°C. Thereafter, the supernatant was transferred again into a new 2-ml centrifuge tube (RNase-free nonstick) by carefully pipetting all supernatant excluding the white layer. Steps 4 and 5 were repeated. Subsequently, the supernatant was transferred into a new 2-ml centrifuge tube and an equal volume of isoamylalcohol was added and the solution was vortexed. The supernatant was kept at room temperature for 10 min and was later centrifuged at 12,000 rpm for 15 min at 4°C, and the supernatant was discarded. Pellets were washed with 1 ml of 75% ethanol-vortexed oscillator for about 5 s and, then, was centrifuged at 7,500 rpm for 1 min. The remaining liquid was carefully pipetted and the pellets were air-dried for 5 to 10 min and were dissolved with an appropriate volume of DEPC-treated water. RNA was stored at -80°C. RNA degradation and contamination was monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer spectrophotometer (Implen) at wavelengths of 230, 260, and 280 nm. RNA concentration was measured using the Qubit RNA assay kit in a Qubit 2.0 flurometer (Life Technologies). RNA integrity was assessed using the RNA Nano 6000 assay kit of the Bioanalyzer 2100 system (Agilent Technologies).

Real-time-qPCR.

RT-qPCR analyses were performed with independently generated samples from yeast treatments and control at the time. Primers for RT-qPCR were designed using the Primer 5 software and were synthesized by Sangon Biotech. cDNAs were reverse transcribed from 2 mg of total RNA, using the PrimeScript RT reagent kit (Takara), and RT-qPCR analyses were performed on an ABI Prism 7500 real-time PCR system (Applied Biosystems). The apple ACTIN gene was used as an internal control to normalize the expression data (Liu and Ekramoddoullah 2006). Each RT-qPCR experiment was repeated three times. The relative expression of genes was calculated using the $2^{-\Delta\Delta C_t}$ method (Gao et al. 2005), and standard deviation was calculated between three biological replicates. The gene-specific primers are listed in Supplementary Table S1.

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Fig. 7. Salicylic acid (SA) and ethylene/jasmonate (ET/JA) signal pathways induced by *Yarrowia lipolytica* in apple fruit. Red words represent the genes that were up-regulated by the *Y. lipolytica*.

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