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感染日日春葉片黃化病植物菌質體之日日春以生長素處理後其病害發展之研究

The Effects of Auxin Treatment on Disease Development in Periwinkle Leaf Yellowing Phytoplasma-Infected Periwinkle Plants

戴肇鋒

Chao-Feng Tai

指導教授：林長平 博士、陳仁治 博士

Advisor: Chan-Pin Lin, Ph.D., Jen-Chih Chen, Ph. D.

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口試委員會審定書

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本論文係 戴肇鋒 君 (R99633014) 在國立台灣大學植物病理與微生物學系、所完成之碩士學位論文，於民國 101 年 7 月 17 日承下列考試委員審查通過口試及格，特此證明

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林長平 博士 林長平

國立台灣大學植物病理與微生物學系教授

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不敢相信的，終於到了要寫致謝的這一天。總是喜歡裝文青寫抒情文的我在此時此刻卻遲遲無法動筆撰寫，一面思考著到底該感謝誰，一面發愣回想這一切。碩士生涯的這段日子裡有開心也有苦悶，回憶起來是如此不真實又珍貴，但如果要我再面對一次，我或許將無心於挑戰。

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我不覺得自己是一個聰明的人，但我希望任何閱讀這個論文的讀者都可以從中學到一些知識或精神，在科學的世界裡一起找到屬於自己的快樂。就算再怎麼害怕改變，我想我還是要勇敢的走入人生的下一個階段吧，在這段旅程結束之後開啟的會是另一個天地，期許自己在往後的人生中可以走出一條不輸給任何人，只屬於自己的道路！

中文摘要

植物菌質體 (phytoplasma) 可感染多種經濟作物，並於感病後常造成枝條增生 (proliferation)、矮化 (dwarfism)、簇葉 (witches' broom) 等病徵，造成農業重大損失。近來已發現植物菌質體之致病蛋白 TENGU 與病徵之誘發相關，且 TENGU 之表現亦使生長素之相關基因如 *Aux/IAAs*、 *SAURs* (small Auxin-up RNAs)、*GH3s* 之表現量下降。又生長素 (auxin) 已證實與植物防禦之相關荷爾蒙如水楊酸 (salicylic acid)、茉莉酸 (jasmonic acid)、乙烯 (ethylene) 互有交互作用，進而調控植物之抗病、感病路徑。另一方面，將感染植物菌質體之日日春枝條，培養於含有高量生長素之培養基中，也可有效使枝條病徵減緩，同時植物體內之菌量也有明顯減少，顯見生長素在植物與植物菌質體交互作用上或在植物之抗性表現上扮演重要角色。本研究以日日春葉片黃化病(periwinkle leaf yellowing, PLY) 植物菌質體為病原、日日春為寄主植物進行研究，試圖了解生長素、植物寄主與植物菌質體三者間交互作用之機制，以期找出對抗植物菌質體病害之新策略。為了釐清前人研究中，生長素訊息傳導相關基因表現量之下降，是否導致植物罹病後對生長素之感受性改變，首先，在本研究中檢查了此類基因於感病後之表現量變化，結果發現生長素訊息傳導相關基因 *IAA9*、*IAA14*、*IAA14-2*、*IAA19* 於罹病後表現下降，而 *IAA8*、*SAUR5* 表現上升，又 *IAA3*、*IAA4*、*IAA12* 之表現量無顯著變化。然而我們亦發現其中僅 *IAA3*、*IAA4*、*IAA12*、*IAA19*、*SAUR5* 受人工合成生長素 NAA (napthaleneacetic acid) 之誘導，而僅有 *SAUR5* 之表現於罹病株中較健株為低，故推論植物菌質體之感染會干擾寄主體內生長素訊息傳導相關基因表現；同時也發現在以生長素處理之罹病株中， *IAAs* 等基因受誘導之表現情形較健株為高，而 *SAUR5* 在罹病株中被誘導之表現則與健株相比稍低，即無法被誘導之情形。本研究進一步觀察植物罹病後，若直接處理生長素是否可造成病徵減緩，故於植物嫁接 PLY 植物菌質體兩周後，以噴灑方式處

理高濃度之 NAA (25 ppm)，定時觀察植株病害發展。結果發現以 NAA 處理後之 PLY 罹病株，可使未顯現病徵之枝條維持健康，而且在處理 NAA 之罹病株上同時也發現出現較多之健康枝條，於其中無法針測到植物菌質體之存在，但在原已顯現病徵之發病部位的花部病徵有加速發展之情形，而且其病枝含有較高之菌量；此外，在觀察以 NAA 處裡後之罹病株病枝中水楊酸、茉莉酸、乙烯相關基因表現量時，亦發現水楊酸相關之 *PR1* 及 *Pr1b* 等系統性抗病相關基因表現受抑制，而於誘導性抗病相關基因之表現中，*LOX2* 在處理 NAA 前後無差別，*AOC* 於處理後表現量未上升，而 *ACO4* 於處理後表現量上升。將 PLY 植物菌質體嫁接至以生長素做前處理之日日春後，發現其較未處理生長素之植株不易罹病，顯見生長素有助於植株健康部位之抗性提升。前人研究顯示胼胝體 (callose) 之累積已被發現與植物限制病原擴散有關，因此本研究亦對處理 NAA 是否造成植株胼胝體沉積之變化進行觀察，結果發現處理 NAA 後，健株有胼胝體之產生，如此或可將處理 NAA 後罹病株含有較多建康枝條之現象，推論乃導因於植物菌質體在移至新枝條之情形受阻。本研究對植物與植物菌質體間之交互作用做進一步之了解，並試圖提出生長素對於植物及植物菌質體間之交互作用影響之可能模式。

關鍵詞：植物菌質體、日日春、生長素、抗病反應。

Abstract

Phytoplasmas, wall-less obligate bacterial pathogens, cause more than one thousand diseases in hundreds of economical crops. Because their disease symptoms are often associated with plant developments, phytohormone imbalance caused by the pathogen was proposed to be the main cause of the symptoms. Recently, a symptom inducing effector, TENGU, was found to suppress expressions of several auxin-related genes. Symptoms caused by phytoplasma can be relieved by treatment of high concentration of auxin in an *in vitro* culture condition. Therefore, in this study, we aimed to realize detail mechanisms of auxin associated defense responses in periwinkles against periwinkle leaf yellowing (PLY) phytoplasma. Consisting with previous finding, expressions of several auxin-related genes were down-regulated after PLY-phytoplasma infection. However, genes with no change and with up-regulated expressions were also observed. Surprisingly, genes examined generally more strongly responded to auxin treatment with *SAUR5* the lone gene showing reduced sensitivity to auxin in diseased plants. After NAA treatment, symptom developments were accelerated in the inoculated shoots while more healthy shoots with no phytoplasma detected were observed. The accelerated symptom developments were associated with early accumulations of phytoplasmas, and suppression of peak

induction on *Pr1* and *Pr1b*. Genes encoding potential JA and ET biosynthesis key enzymes were also analyzed and no conclusive results for involvement of JA biosynthesis in the accelerated symptoms were observed, while a strong induction of *ACO4*, a key ET biosynthesis gene, was only found in diseased shoots treated with NAA. After auxin pre-treatment, periwinkles were more resistant to phytoplasma infection, showing that auxin promote the resistance to phytoplasma in healthy part. Since callose deposition is a basal defense to prevent pathogen spreading, callose deposition were examined and an increased level of callose deposition was observed in healthy plants treated with auxin. The association of reduction in disease rates in callose containing healthy shoots may infer that it may help preventing phytoplasma infection. The study provided clues of interactions of plant-microbe. Scheme of the hypothetic working model among auxin, host plant and phytoplasma was proposed in this study.

Key words:

phytoplasma, periwinkle, auxin, periwinkle leaf yellowing, plant defense

Abbreviations

PAMP	pathogen-associated molecular pattern
PTI	PAMP-triggered immunity
ETI	effector-triggered immunity
ETS	effector-triggered susceptibility
SA	salicylic acid
JA	jasmonic acid
MeJA	methyl jasmonate
ET	ethylene
ACC	1-aminocyclopropane-1-carboxylic acid
IAA	indole-3-acetic acid
NAA	napthaleneacetic acid
SAR	systemic acquired resistance
ISR	induced systemic resistance
PLY	periwinkle leaf yellowing
UBQ	*UBIQUITIN*
amp	*antigenic membrane protein*
Aux/IAA	*AUXIN/INDOLE-3-ACETIC ACID*
SAUR	*SMALL AUXIN UP RNA*
PR	*PATHOGENESIS-RELATED*
LOX	*LIPOXIGENASE*
AOC	*ALLENE OXIDE CYCLASE*
ACO	*AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE*
dpi	day post inoculation

Contents

中文前言

植物菌質體 (phytoplasma) 為一群無細胞壁之革蘭氏陽性細菌，於分類地位上屬於 *Candidatus* Phytoplasma 屬 (IRPCM, 2004)，目前仍無法以人工方式培養於培養基中。於農業上此類植物病原細菌可於上百種經濟作物中造成上千種病害，如翠菊黃萎病 (aster yellows) (O' Mara *et al.*, 1993)、葡萄黃化病 (flavescence dorée) (Margaria *et al.*, 2011)、蕃茄大芽病 (tomato stolbur) (Messiaen *et al.*, 1967)、水稻黃萎病 (rice yellow dwarf) (Jung *et al.*, 2003)、花生簇葉病 (peanut witches'-broom) (Yang, 1985) 等病害，進而造成全球之重大經濟損失 (Agrios, 2005; Doi *et al.*, 1967; Lee *et al.*, 2000)。

植物菌質體之病害防治中，傳統上多以殺蟲劑實行媒介昆蟲之防治，而植物菌質體之媒介昆蟲則包括葉蟬（屬於 Cicadoidea 總科）、木蝨（屬於 Psylloidea 總科）及飛蝨（屬於 Fulgoroidea 總科）等韌皮部取食昆蟲 (Weintraub *et al.*, 2006)。除防治媒介昆蟲之外，清除中間寄主 (intermediate host) 亦為有效之防治策略 (Christensen *et al.*, 2005; Hogenhout *et al.*, 2008) 。於木本植物之植物菌質體病害防治策略上則可以四環黴素做為殺菌之處理，亦有良好之防治效力 (Ishiie *et al.*, 1967) 。然而上述之防治策略可能衍生多種爭議，包括環境汙染問題，或抗生素成本較高，無法普遍施用於所有作物等。近年來學者已開始著手研究植物寄主及植物菌質體間之交互作用，以期可發展出其他良好之防治策略 (Sugio *et al.*, 2011)。

植物於長久之演化歷程中，已發展出多種對抗植物病原菌及食草昆蟲之免疫反應，包含由病原相關分子所引發之免疫反應 (PAMP-triggered immunity, PTI) 及由致病因子所引發之免疫反應 (effector-triggered immunity, ETI) (Pieterse *et al.*, 2009)。 PTI 為植物於辨識病原菌之相關標誌，如鞭毛素 (flagellin)、幾丁質 (chitin)、醣蛋白 (glycoprotein) 等小分子後所引發之免疫反應。病原菌於演化過

程中亦發展出一類致病因子 (effector) ，其可造成致病因子引發之感病反應（effector-triggered susceptibility, ETS）。為了對抗病原菌之致病因子，植物細胞內之 R 蛋白可於辨識致病因子後引發另一免疫反應，即為 ETI。植物觸發上述之免疫反應後，可產生各種防禦相關之植物荷爾蒙並引發下游之各種防禦相關反應，如病程相關蛋白（pathogenesis-related, PR）之表現（Chisholm *et al.*, 2006; De Vos *et al.*, 2005; Jones *et al.*, 2006）。

植物於辨識病原菌後可依其類別產生不同之植物荷爾蒙 (Walters *et al.*, 2007) 。植物病原菌大體上可分為兩類，一為殺生型病原菌 (necrotroph pathogen)，如 *Botrytis cinerea*、 *Pythium spp.*、 及 *Magnaporthe oryzae* 等，可誘導植物產生茉莉酸 (jasmonic acid, JA) (Howe, 2004) 及乙烯 (ethylene, ET) (VonvDahl *et al.*, 2007) 相關之防禦反應。另一類型為活體寄生型病原菌 (biotroph pathogen) ，如 *Xylella fastidiosa*, *Plasmopara viticola*, 及 *Puccinia graminis* ，可誘導植物產生水楊酸 (salicylic acid, SA) 相關之防禦反應 (Loake *et al.*, 2007) 。這些植物荷爾蒙造成之抗病反應亦可分為兩類，分別為由病原菌引發之系統性抗病反應 (systemic acquired resistance, SAR) ，及非病原菌之微生物所引發之誘導性抗病反應 (induced systemic resistance, ISR) (Walters *et al.*, 2007) 。SAR 防禦反應涉及 SA 之相關路徑調控 (Durrant *et al.*, 2004)，而 ISR 防禦反應則涉及 JA 及 ET 等相關路徑調控 (Van Loon *et al.*, 1998; Van Wees *et al.*, 2008) 。

一般於植物之荷爾蒙調節上 SA 與 JA 互為拮抗作用，而 JA 與 ET 則為一加乘效應。於 *Pseudomonas syringae* 之研究中顯示，此類半絕對寄生型病原 (hemibiotriohic pathogen) 可分泌一種 JA 之類似物 coronatine ，其可抑制 SA 之合成，而於阿拉伯芥 (*Arabidopsis thaliana*) 施用 JA 可產生相同之效應 (Feys *et al.*, 1994)。而相對來說 SA 亦可抑制 JA 之生合成。這類由 SA 所誘導產生之 JA 抑制效應主要由一 SA 訊息傳遞 (signaling) 之關鍵基因 *NPR1* 所調控，而另一種 *WRKY* 轉錄因子 *WRKY70* 亦參與其中 (Pieterse *et al.*, 2004; Li *et al.*,

2004; Katagiri, 2004) 。於阿拉伯芥之研究中，JA 及 ET 可共同促進下游防禦相關基因之表現，如 *PDF1.2*、*HEL*、及 *CHIB* 。此外 JA 與 ET 之訊息傳遞路徑經研究發現與抗病基因 *PDF1.2* 之表現相關，其可提升植物對 *Alternaria brassicicola* 之抗病能力 (Kunkel *et al.*, 2002; Norman-Setterblad *et al.*, 2000; Pieterse *et al.*, 2007; Reymond *et al.*, 1998) 。

於植物菌質體病害之研究中，近年來已有報導針對荷爾蒙誘發之免疫反應進行研究。於蕃茄之研究中發現，植株感染植物菌質體後， SA 及 ET 之訊息傳導基因表現量有上升之趨勢 (Ahmed *et al.*, 2011)。另以基因體分析方式觀察受葡萄植物菌質體 (*Ca.* P. solani, Bois noir) 感染之葡萄，亦發現 SA 之訊息傳導相關基因表現量較健康之植株為高 (Hren *et al.*, 2009) 。翠菊黃萎病 (aster yellows – witches'-broom, AY-WB) 植物菌質體中之一致病因子 SAP11，經由功能性分析後發現其可抑制 JA 之生合成，此一機制可能有利於媒介昆蟲於田間之病害傳播 (Sugio *et al.*, 2011)。 另一方面，以 SA 於馬鈴薯及蕃茄做前處理後發現其植株對馬鈴薯紫葉病 (potato purple top, PPT) 之抗性有所助益 (Sánchez-Rojo *et al.*, 2011; Wu *et al.*, 2012) 。此類研究顯示 SA 等荷爾蒙可以某些機制幫助植物抵抗植物菌質體之侵染。

除了 SA 之外，生長素 (Auxin) 及其他荷爾蒙亦有研究指出可協助對抗植物菌質體病害。於被植物菌質體感染之日日春組織培養苗中施用高濃度之生長素後，其病徵有回復之趨勢，而回復之日日春枝條僅能偵測到微量之植物菌質體 (Ćurković-Perica, 2008; Leljak-Levanić *et al.*, 2010)。當菊花接種植物益生菌 *Pseudomonas putida* S1Pf1Rif 後，菊花黃萎病植物菌質體 (chrysanthemum yellows phytoplasma, CYP) 僅能造成微弱之病徵，而植物體內之 IAA 含量有增加之現象，但生長素之含量及病徵減緩間仍缺乏直接之證據 (Gamalero *et al.*, 2010)。於感染葡萄黃萎病之葡萄上施灑高濃度之 IBA，可觀察到病徵減緩之現象，但其回復率與未處理 IBA 之葡萄感病株並無顯著差異。這些研究使生長素

在植物及植物菌質體間扮演之角色趨於困惑 (Kozina *et al.*, 2011)。

於植物菌質體之致病因子研究中，學者已發現強烈之證據證明生長素可能參與植物及植物菌質體之交互作用。利用基因體脂功能性分析，學者從洋蔥黃萎病 (onion yellows, OY) 植物菌質體基因體中發現一致病因子 TENGU (Hoshi *et al.*, 2009)。將此基因已過量表現之方式表現於菸草及阿拉伯芥上皆可觀察到植株之性狀改變為類似植物菌質體感染之病徵。於 TENGU 基因之阿拉伯芥轉殖株中更可發現其生長素相關之基因，如 *Aux/IAA family* 轉錄因子、*SAUR family* 基因、及 *GH3* 基因皆有明顯之抑制現象 (Hoshi *et al.*, 2009)。由於生長素被報導與植物對植物菌質體之抗性有關，以及植物菌質體之致病因子對生長素相關基因有抑制現象，我們進一步推論是否此一現象可能為植物菌質體之致病因子誘發之 ETS，試圖理解生長素如何引發植物對植物菌質體之抗病反應。

生長素向來被歸類於植物器官發育之相關荷爾蒙，但近年來已有許多證據証實其與防禦反應之調控相關 (Kazan *et al.*, 2009)。生長素對於不同之病原菌於防禦反應上扮演不同之角色。大體上絕對寄生型病原菌於侵染過程中可誘發生長素之訊息傳導反應並導致寄主體內之生長素含量提升。 *Pseudomonas syringae* DC3000 可分泌一致病因子 AvrRpt2，於缺乏 R 蛋白之阿拉伯芥 *RSP2* 中可提升生長素之感受性進而使 IAA 之含量增加 (Chen *et al.*, 2007)。菸草嵌紋病毒 (*Tobacco mosaic virus*, TMV) 之複製酶可與菸草之生長素訊息傳遞蛋白 Aux/IAA 產生交互作用，使其無法進入植物之細胞核 (Padmanabhan *et al.*, 2005; Padmanabham *et al.*, 2006) ，而此一交互作用又被證實與 TMV 之侵染有關，複製酶突變之 TMV 無法與寄主之 Aux/IAA 蛋白作用，使其病徵表現大量下降 (Padmanabhan *et al.*, 2009)。由於 Aux/IAA 為生長素訊息傳遞之一抑制子，TMV 的侵染可使其無法進入核中進而促使生長素之下游基因表現，此一現象被認為與 TMV 之病徵發展有關 (Padmanabhan *et al.*, 2008)。 這些絕對寄生型病原之毒性增強被歸因於藉由調控生長素表現，其 SA 之表現量會受抑制所致 (Kazan *et al.*,

2009)。 SA 除了會與 JA 互相拮抗之外，學者也證實其會與生長素互相拮抗。SA 之生合成與 *PR1* 基因之表現會受生長素所抑制，而處理 SA 之植株亦可抑制生長素之生合成 (Wang *et al.*, 2007) 。相較於絕對寄生型病原菌，殺生型病原菌當處於生長素較少之環境時有較佳之致病性。舉例來說，生長素之缺失株對於灰黴病菌 *Botrytis cinerea* 及卵菌 *Pythium irregular* 可造成更強之致病力 (Llorente *et al.*, 2008; Tiryaki *et al.*, 2002)

植物菌質體為一種絕對寄生型之病原菌，然而目前之研究顯示生長素之存在可促進植物對抗植物菌質體之侵染 (Leljak-Levanić *et al.*, 2010)。 但於感染植物菌質體之葡萄植株上施用生長素，顯示較無良好之防治效果，因此，必須要有更多實驗之證據佐證生長素於植物菌質體及寄主交互作用之角色。本實驗將以第一群 (16SrI) 植物菌質體，日日春葉片黃化病 (periwinkle leaf yellowing, PLY) 植物菌質體感染之日日春為研究對象進行研究。我們已於其基因體中發現 *TENGU* 基因之存在 (Fig. S2)。本研究進行罹病之日日春生長素相關基因之表現量觀測，並觀察於罹病株上施用生長素後植株之感並過程是否有減緩之趨勢。然而本研究發現外加之生長素於日日春植株上無法減緩病徵之發展，進而加速病害之發生。本研究亦發現外加生長素後，植物菌質體侵染鄰近部位之速度有下降之現象，而以生長素做前處理之日日春也有減緩病徵發展之趨勢。本研究最後試圖歸納生長素對植物及植物菌質體交互作用之調控機制，以釐清其中確切之相關性。

Introduction

Phytoplasmas, wall-less obligate bacterial pathogens, cause thousands of diseases in hundreds of economical crops, including cruciferous plant (aster yellows) (O' Mara *et al.*, 1993), grape (flavescence dorée) (Margaria *et al.*, 2011), tomato (tomato stolbur) (Messiaen *et al.*, 1967), rice (rice yellow dwarf) (Jung *et al.*, 2003), and peanut (peanut witches'-broom) (Yang, 1985), and result in tremendous yield loss worldwide. (Agrios, 2005; Doi *et al.*, 1967; Lee *et al.*, 2000).

Traditionally, phytoplasmal diseases are controlled by spreading chemical insecticides to manage insect vectors of phytoplasmas since they are transmitted by phloem-sap-feeding insects such as leafhoppers (superfamily Cicadoidea), psyllids (superfamily Psylloidea), and plant hoppers (superfamily Fulgoroidea) (Weintraub *et al.*, 2006), and by eliminating intermediate hosts to limit the sources of the pathogens (Christensen *et al.*, 2005; Hogenhout *et al.*, 2008). For woody crops infected with these pathogens, the tetracycline type of antibiotics was injected into phloem of these crops to kill these bacteria (Ishiie *et al.*, 1967). However, these treatments create issues on environment pollution, are expensive, and are not suitable for all crops. In recent years, scientists have begun researching how plant and phytoplasma interact to each other in order to find new solutions to cope with this notorious pathogen (Sugio

et al., 2011b).

Plants have evolved various immune systems to fight against pathogens and herbivore insects (Pieterse *et al.*, 2009). Two lines of defense mechanisms, PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI), were proposed. PTI features plant recognitions of pathogen signature, pathogen-associated molecular pattern (PAMP), such as flagellin, chitin, and glycoproteins. On the other hand, pathogen may secreted virulence factors, effectors, to counteract plant defense and result in effector-triggered susceptibility (ETS). These effectors can be recognized by plant resistant proteins, R proteins, and trigger ETI. After pathogen recognitions by either PTI or ETI, various of phytohormones are induced to trigger down-stream defense responses including induction of pathogenesis-related (*PR*) genes. (Chisholm *et al.*, 2006; De Vos *et al.*, 2005; Jones *et al.*, 2006).

Plants are able to distinguish different pathogens, and respond with different phytohormone inductions (Walters *et al.*, 2007). In general, necrotroph pathogens, such as *Botrytis cinerea*, *Pythium spp.*, and *Magnaporthe oryzae*, induce jasmonic acid (JA) (Howe, 2004) and ethylene (ET) (Von Dahl *et al.*, 2007)-mediated immunity, and biotroph pathogens, such as *Xylella fastidiosa*, *Plasmopara viticola*, and *Puccinia graminis*, trigger salicylic acid (SA)-mediated immunity (Loake *et al.*, 2007).

Phytohormones also participate in systemic resistance to broad ranges of pathogens. The systemic resistance can be triggered by either pathogens (systemic acquired resistance, SAR) or nonpathogenic microbes (induced systemic resistance, ISR) (Walters *et al.*, 2007). In SAR, SA signaling is required (Durrant *et al.*, 2004). In contrast, ISR goes through a SA independent route, and JA and ET play important roles in this pathway (Van Loon *et al.*, 1998; Van Wees *et al.*, 2008).

Interestingly, the defense in mechanism-mediated by SA and that mediated by JA are mostly antagonistic while JA and ET often show synergistic effects in plant defense. It was found that *Pseudomonas syringae*, a hemibiotrophic pathogen, secreted coronatine, a JA analog to suppress SA production (Feys *et al.*, 1994). Treatment of JA to *Arabidopsis thaliana* also results in the same effect. On the other hand, SA also inhibits JA biosynthesis. This SA-mediated suppression of JA biosynthesis is NPR1, a central mediator in SA signaling, dependent, and goes through actions of a WRKY transcription factor, WRKY70 (Pieterse *et al.*, 2004; Li *et al.*, 2004; Katagiri, 2004). In Arabidopsis, JA and ET synergistically promote expressions of defense genes, including *PDF1.2*, *HEL*, and *CHIB*. In addition, both JA and ET signaling are required for *Alternaria brassicicola* resistance and induction of *PDF1.2* (Kunkel *et al.*, 2002; Norman-Setterblad *et al.*, 2000; Pieterse *et al.*, 2007; Reymond *et al.*, 1998; Xu *et al.*, 1994).

Part of the phytohormone-mediated plant immunities have also been examined for phytoplasma diseases. Marker genes of SA, JA, and ET in plant defense were examined in stolbur phytoplasma-infected tomatos, and it was found that defense genes in SA and ET signaling were induced in there phytoplasma-infected plants (Ahmad e*t al.*, 2011). In agreement with this finding, a transcriptomic study in grapevine using microarray also found that many genes putatively defined as SA signaling genes were up-regulated when grapevine infected with *Ca.* P. solani (Bois noir) (Hren *et al.*, 2009). A phytoplasma effector, SAP11, from AY-WB phytoplasma has been identified through functional genomic approaches to demonstrate its function in JA biosynthesis repression (Sugio *et al.*, 2011a). It was suggested that the repression leads to defeated defense mechanism against sap-feeding insects, and therefore, promotes spreading of the phytoplasma while the role of JA in plant defense against phytoplasma has not been demonstrated. In contrast, exogenous SA pretreatments were found to be effective to induce defense responses and prevent potato purple top (PPT) phytoplasma infection to both potato and tomato (Sánchez-Rojo *et al.*, 2011; Wu *et al.*, 2012). These findings indicate that SA may participate in plant defense against phytoplasma through still undefined pathway.

In addition to SA, auxin was another phytohormone found to be a potential agent for plant defense against phytoplasma. *In vitro* culture of phytoplasma-infected periwinkles in a high auxin concentration medium was able to induce recovery (Ćurković-Perica, 2008; Leljak-Levanić *et al.*, 2010). No or only low concentrations of phytoplasmas were detected in the recovered plants. Chrysanthemums inoculated with a plant-beneficial bacteria, *Pseudomonas putida* S1Pf1Rif, prior chrysanthemum yellows phytoplasma (CYP) infection showed milder symptoms though phytoplasma concentrations were not affected. Since P. *putida* S1Pf1Rif inoculation triggered IAA accumulation, this accumulation was thought to be a reason for the symptom relief though direct evidence is still lacking (Gamalero *et al.*, 2010). Grapevines infected with grapevine yellows phytoplasma were exogenously sprayed with high concentration of IBA, and signs of recovery were observed; however, no significant differences in recovery rate between vines with and without treatment of IBA. This finding made the role of auxin in phytoplasma-plant interaction become puzzling (Kozina *et al.*, 2011).

Another strong evidence supports the involvement of auxin in phytoplasma-plant interaction was from a phytoplasma effector screening. An effector, TENGU, was identified from onion yellows (OY) phytoplasma using a functional genomic approach (Hoshi *et al.*, 2009). Both transient over-expression of this protein in

Nicotiana benthamiana and stable over-expression of that in Arabidopsis resulted in plant phenotypical changes resemble symptoms of onion yellows. A transcriptomic analysis on the transgenic Arabidopsis further revealed that TENGU strongly suppressed expressions of a group of auxin-related genes, including *Aux/IAA* transcription factors, auxin responsive *SAUR* family genes, and *GH3* genes (Hoshi *et al.*, 2009). A similar phenomenon was also found when grapevine infected by Bois noir phytoplasma in which majority of genes in auxin pathways were down-regulated (Hren *et al.*, 2009). The combination of a potential anti-phytoplasma function, and the suppression effects of a phytoplasma effector on auxin prompted us to consider a possibility that phytoplasma may secrete an effector to induce ETS by suppression auxin signaling. Therefore, we aimed to dissect the potential effects of auxin on plant defense against phytoplasmas.

Though auxin has long been considered a primary phytohormone that controls plant development, it has recently emerged as a regulator of plant defense (Kazan *et al.*, 2009). Interestingly, pathogens response to auxin signal differently. In general, biotroph pathogens are thrill under activated auxin signal. Many of them produce auxin or activate auxin production in their host plants. *Pseudomonas syringae* DC3000, secretes an effector, AvrRpt2, to promote accumulation of IAA and enhance auxin sensitivity in Arabidopsis plants lacking a resistant gene, *RSP2*. The

accumulation of auxin in turn makes plant defense against the *P. syringae* vulnerable (Chen *et al.*, 2007). *Tobacco mosaic virus* (TMV) is also able to alter auxin signal through interaction of its replicase with Aux/IAA proteins to prevent the localization of the Aux/IAA proteins to the nucleus (Padmanabhan *et al.*, 2005; Padmanabham *et al.*, 2006). Furthermore, the interaction was found to be associated with symptom development of TMV infection, and loss of the ability for TMV-replicase to interact with Aux/IAA strongly reduced TMV accumulation in its host plants. Since Aux/IAA repressors inhibit auxin signal, the inability for their functions in nucleus to turn on expressions of auxin-responsive genes and to be one of the reasons for TMV caused symptoms (Padmanabhan *et al.*, 2008). One of the reasons for virulence of these pathogens may be due to suppression of SA responses through activation of host auxin responses (Kazan *et al.*, 2009). In addition to antagonistic crosstalk between SA and JA signaling, an antagonistic relationship also occurs between SA and auxin. *PR1* expression and biosynthesis of SA can be inhibited by auxin while SA treatment also suppresses auxin biosynthesis (Wang *et al.*, 2007).

In contrast to biotrophic pathogens, necrotrophic pathogens are often more virulent when attacking plants with defected auxin signaling. For example, auxin-resistant mutants are more susceptible to *B. cinerea*, a necrotrophic fungus, and *Pythium irregular*, an oomycete pathogen (Llorente *et al.*, 2008; Tiryaki *et al.*, 2002).

Interestingly, a piece of evidence has suggested that auxin may induce recovery of disease plants infected with phytoplasma, a biotrophic pathogen (Leljak-Levanić *et al.*, 2010). However, direct auxin treatment on phytoplasma-infected grape did not yield promising results on the recovery induction effect of auxin. Therefore, more evidence will be needed to clarify the role of auxin in plant defense against phytoplasma. We, therefore, tested the effects of auxin on periwinkle leaf yellowing (PLY) phytoplasma (16SrI group)-infected *Catharanthus roseus* (Chen *et al.*, 2011). A *TENGU* gene was also found in PLY phytoplasma genome (Fig. S1); therefore, we tested whether expressions of auxin-responsive genes in disease periwinkles can be affected, and whether auxin treatments can slow down the symptom developments. We were surprised to find that exogenous auxin treatments did not slow down but accelerate the symptom developments in phytoplasma-infected shoots. However, we also discovered that the movement of phytoplasmas into neighboring shoots was slowing down after auxin treatments, and pre-treatment auxin also resulted in retarded phytoplasma infection. The potential mechanisms of auxin-induced responses in PLY phytoplasma-infected periwinkles were also discussed.

Materials and methods

Plant materials and phytoplasma inoculation

Periwinkle plants (*Catharenthus roseus* cv. Pacifica Punch Halo) was used as an experimental host for periwinkle leaf yellowing (PLY) phytoplasma. This phytoplasma was originally isolated from periwinkles collected from Dayuan county, Taoyuan, Taiwan in 2005. The phytoplasma was maintained in periwinkle plants by serial transmissions by side-grafting phytoplasma-infected shoots to 90-day-old plants in a green house condition. For symptom development, "diseased shoot" was defined as a shoot longer than 10 cm showing floral symptoms or proliferation symptoms. Floral symptom severity was recorded according to our previous definition into three stages (Su *et al*., 2011). The three stages are S1 (discoloration of petals), S2 (partial virescence of petals), and S3 (complete floral virescence) (Fig. S2). Proliferaion was defined as a shoot whose number of secondary shoots per number of internodes is larger than two.

Chemical treatments

For time course experiment of napthaleneacetic acid (NAA) treatments on whole plants, 20 ml of 25 ppm (~143μM) NAA was sprayed once per week, after 14 dpi of

side-grafting. In auxin sensitivity analyses, 4 concentrations of NAA were used in which 10 ppm (~57μM) NAA was used for a time course experiment, and 1 ppm (~5.7μM), 0.1 ppm (~0.57μM), and 0.01 ppm (~0.057μM) NAA were used for a dosage-dependent experiment. For these treatments, two newly formed leaves of each shoot on the top were used. For treatments of salicylic acid (SA, 1mM), methyl jasmonate (MeJA, 0.1 mM), and 1-aminocyclopropane-1-carboxylic acid (ACC, 10μM), these chemicals were directly applied on attached leaves for 24 hours. All of the collected samples were divided into two to isolate total DNA and RNA for phytoplasma concentration and gene espression analyses, resepectively.

RNA isolation and cDNA synthesis

Approximately 0.2 g of each leaf sample was used for total RNA extraction using Maestrozol RNA Extraction Reagent (MAESTROGEN, Las vegas, NV, U.S.A.) according to the manufacturer's instruction and treated with TURBO DNase (Applied Biosystems, Foster City, CA, U.S.A.) before cDNA synthesis. Two μg of DNA-free total RNA was used for first-strand cDNA synthesis using oligo d(T) as the primer, and M-MLV reverse transcriptase Kit (Invitrogen Corporation, Carlsbad, CA, U.S.A.) as the synthesis reagents.

Quantitative reverse-transcription PCR

First-strand cDNA was used to be the template and KAPA SYBR FAST qPCR Kit (KAPA Biosystems, Woburn, MA, U.S.A.) was the reagents for quantitative reverse-transcription PCR (q-RT-PCR) in a LightCycler 480 Real-time PCR system (Roche Applied Science, Basel, Switzerland). *UBIQUITIN* was used as the internal control for normalizing the quantity of cDNA. The primer sets used in this study were listed in Table 1. Results were tested in at least two independent experiments, three biological samples, and three technical replications.

Measurement of phytoplasma concentration

Genomic DNA of each sample was first extracted using a method described by Winnepenninckx *et al.* (1993) to be the template for phytoplasma quantification. Approximately 0.2 g homogenized leaf tissues were added with 1 ml 65^{o}C pre-warmed cetyltrimethylammonium bromide (CTAB) DNA extraction buffer (in 100mM Tris-HCl) and 4 4µl RNaseA (20 mg/ml), then incubated at 65 ^{o}C for 40 minutes, and then the extraction mix was centrifuged at 10,000 g for 5 minutes. The supernatant was then gone through phenol-chloroform extraction step to ensure the purity of isolated DNA. Subsequently, genomic DNA in the aqueous phase was precipitated using isopropanol and dissolved in water.

Phytoplasma concentration was determined by an absolute quantification PCR method (Swillens *et al.*, 2004; Liu *et al.*, 2007). In brief, 50 ng genomic DNA was used as the template in this measurement, and a SYBR staining method was used to quantify the amount of PCR product synthesized in LightCycler 480 Real-time PCR system (Roche Applied Science). *UBIQUITIN* was used as internal control to normalize the quantity of plant DNA, and the immunodominant membrane protein gene *amp* was used to calculate PLY phytoplasma concentration. The primer sets used in this study were listed in Table 1. Phytoplasma concentration was calculated to be phytoplasma copy number (Genome unit)/quantity (pg) of periwinkle DNA. Results were tested in at least two independent experiments, three biological samples, and three technical replications.

Callose staining

Callose deposition in leaf tissue was detected by methyl blue staining methods (Eschrich *et al.*, 1964). Newly formed leaves of each shoot on the top were inoculated in acetic acid solution (acetic acid:absolute ethanol, 1:3) for 1.5 hours for cell fixation, and then treated with 1N sodium hydroxide for 6 h to soften tissues. After this step, leaves were washed by potassium phosphate buffer (50mM, pH 7.5) three times for 5 minutes each. Subsequently, leaf samples were soaked in 0.02% methyl blue solution

to stain calloses. Samples were observed using Olympus BX51, and photographed by DP2-BSW Ver. 2.2.

Results

Phytoplasma infection did not reduce overall auxin sensitivity of periwinkles

Previously, auxin-related genes have been shown to be mostly down-regulated after phytoplasma infection (Hoshi *et al.*, 2009; Hren *et al.*, 2009). In order to understand whether the disruption in expressions of auxin-related genes is due to the changes in auxin sensitivity in infected plants, we examined whether the responses of auxin-related genes to auxin have been changed after the infection of PLY phytoplasma. Both of PLY phytoplasma and OY phytoplasma are 16SrI group phytoplasmas and a gene encoding TENGU was also found in PLY phytoplasma genome (Fig. S1); therefore, it is highly possible that PLY phytoplasma suppresses host auxin-related genes after infection. Nine auxin-related genes were identified from expressed tag sequence (EST) database of periwinkle (http://plantta.jcvi.org/), and from a transcriptome database generated by Solexa sequencing (Tseng, unpublished). They are *IAA3*, *IAA4*, *IAA8*, *IAA9*, *IAA12*, *IAA14*, *IAA14-2*, *IAA19*, and *SAUR5* (EG560567, EG558775, EG558303, EG554526, DDScontig51443, TA514_4058, HM165183.1, EG557773, DDScontig51940). Their expressions were examined in both healthy and PLY-infected periwinkles using qRT-PCR. Indeed, four genes, *IAA9*, *IAA14*, *IAA14-2*, and *IAA19*, were down-regulated after the infection. However, *IAA8*

and *SAUR5* were up-regulated after the infection. The expression was no significantly changes in *IAA3*, *IAA4*, and *IAA12* (Fig. 1).

To compare auxin sensitivity of healthy and phytoplasma-infected plants, we treated both groups of plants with different concentrations of NAA (0.01 ppm, 0.1 ppm, and 1 ppm) for 30 minutes or with a higher concentration of NAA (10 ppm) for different time periods (30, 60, 180 minutes). After treatment, samples were collected for RNA isolation and qRT-PCR was used to analyze the expressions of the auxin-related genes. Though most of Aux/IAA transcription factor genes were found to be induced by auxin (Eckardt, 2001), surprisingly, only five of seven genes tested, *SAUR5*, *IAA3*, *IAA4*, *IAA12*, and *IAA19*, were clearly auxin-inducible (Fig. 2). However, *IAA8* and *IAA9* were not induced by NAA treatments. The expression patterns of these auxin-related genes in healthy and PLY phytoplasma-infected periwinkles did not show a consistent tendency (Fig. 2). *IAA3*, *IAA4*, *IAA12* were induced to a higher level at a lower NAA concentration in disease plants, and no differences were observed in the expression patterns of *IAA9* and *IAA19* between healthy and disease plants, while the induction of *SAUR5* by NAA in disease plants was repressed (Fig. 2).

Auxin accelerated symptom developments on PLY phytoplasma-infected shoots

While *in vitro* cultures of phytoplasma-infected periwinkle shoots in a high auxin containing medium have been shown to eliminate phytoplasmas after series of cultures (Ćurković-Perica *et al.*, 2008; Leljak-Levanić *et al.*, 2010), it is still doubtful whether direct spraying of a high concentration of auxin on phytoplasma-infected plants will have a similar effect since no significant enhancement in recovery rate was observed on grapevines with grapevine yellows after exogenous treatments of high concentrations of IBA (0.5, 1.0, 1.5 g/L) (Kozina *et al.*, 2011). We decided to explore the effect of direct auxin spraying on periwinkles, which have been shown to have the effects after *in vitro* cultures in a high auxin containing medium. Periwinkle plants graft-inoculated with PLY phytoplasma were treated with NAA (25 ppm) or with water weekly. Surprisingly, symptom developments of the NAA-treated group were actually accelerated (Fig. 3). Two independent experiments were started in May and September, respectively. The average temperature were 25^{o}C (in May) and 28^{o}C (in September). In the experiment started in May (experiment one), 50% of plants treated with NAA have already exhibited floral symptoms after 22 dpi while the group of plants treated with water reached the mark not until 30 dpi (Fig. 3A). In the other experiment (experiment two, started in September), 50% of plants treated with NAA

or water showed floral symptoms after 18 and 19 dpi, respectively (Fig. 3B). The symptom developments of the experiment one were clearly slower than those of the experiment two (Fig. 3). In addition to floral symptoms, proliferation symptoms of shoots were also more severe after auxin treatment (data not shown).

The phytoplasma concentrations of both groups were also examined, and indeed, in both experiments, PLY phytoplasmas accumulated faster in infected periwinkles treated with auxin (Fig. 4). In agreement with the symptom developments, phytoplasmas also accumulated faster in the experiment two (Fig. 4). In addition to fast accumulation, in the auxin treated group of experiment two, phytoplasmas accumulated to a higher concentration (9.08 phytoplasma GU/ pg plant DNA detected on 42 dpi) than the maximum detected concentration (5.8 phytoplasma GU/ pg plant DNA detected on 70 dpi) in the auxin treated group of experiment one (Fig. 4). In this experiment, healthy periwinkles were also observed (Fig. 4). No phytoplasma were detected in healthy periwinkles.

Differential expressions of phytohormone-regulated genes between disease plants with or without auxin treatments

In order to understand whether other phytohormone-mediated defense mechanisms were involved in the effects caused by auxin treatments, expressions of genes

potentially involved in SA, JA, and ET-mediated defense were examined using relative qRT-PCR (Fig. 5). They were *IAA4*, *IAA12*, and *SAUR5* for auxin-related, *Pr1* and *Pr1b* for SA signaling, two JA biosynthesis genes (*LOX2* and *AOC*) for JA biosynthesis and signaling, and an ET biosynthesis gene (*ACO4*) for ET biosynthesis. These genes were examined to understand whether they are inducible by various phytohormone treatments. It is clear that *LOX2* and *AOC* not only can potentially to be key genes that control JA biosynthesis but also can be induced by JA treatment. However, *ACO4* was not found to be induced by ET but it encodes a key enzyme for ET biosynthesis, and therefore, it was kept in our analysis. On the other hand, *Pr1* and *Pr1b* were able to be induced by SA, JA, and ET (Fig. S3).

In the analysis, both *IAA4* and *IAA12* had no significant change after auxin treatment. However, the expression of *SAUR5* was up-regulated in 56 dpi in the group of untreated PLY phytoplasma-infected periwinkles (Fig. 5A). In *PR*-related genes, after the auxin treatment, induction of both *Pr1* and *Pr1b* found in PLY phytoplasma-infected periwinkles was disappeared. In the expression of JA inducible genes, expression of *LOX2* showed no significant change after auxin treatments, but a peak expression of *AOC* was only detected in PLY phytoplasma-infected plants in 70 dpi (Fig. 5C). Clear induced expressions of *ACO4* were observed after weekly auxin treatment for 8 weeks (Fig. 5D).

Auxin treatment on PLY phytoplasma-infected plants retarded the transmission of phytoplasmas into neighboring shoots

Our results on accelerated symptom developments of a phytoplasma disease after auxin treatment seem in conflict with previous experiments applying *in vitro* culture to eliminate phytoplasma infection in periwinkles. However, we observed a peculiar phenomenon that in general, the disease plants treated with NAA did have a more healthy look after a while of the weekly auxin treatments (Fig. 6A). In order to know whether the transmission of phytoplasmas into neighboring shoots was really retarded after auxin treatments, the percentages of healthy shoots from auxin treated and non-treated plants were carefully recorded monthly. Indeed, over the course of the experiment period, the percentages of healthy shoots were always significantly lower in the non-treated group than those in the auxin treated group (Fig. 6B). The differences were already seen after two months of inoculation in which the percentages of diseased shoots was 26.9% and 53.2% in PLY phytoplasm-infected plants treated with water or NAA, respectively. After three months of inoculation, the percentages in both groups became stable in which in the percentages were 4.1-8.6% and 22-25% in PLY phytoplasm-infected plants treated with water or NAA, respectively (Fig. 6B). No phytoplasma was detected in those healthy shoots from both groups (Fig. 6C).

Auxin pre-treatment enhanced plant defense against PLY phytoplasma infection

Since auxin treatment significantly retarded the transmission of PLY phytoplasma, to further understand whether auxin treatment can promote disease resistance, shoots of plants treated with or without NAA and healthy shoots of plants with PLY phytoplasma infection and NAA treatments were graft-inoculated with PLY phytoplasma after 6 months of treatments, and the symptom developments of inoculated shoots were then recorded. In two sets of independent experiments, symptom developments of healthy plants with no NAA treatment were faster (Fig. 7). In the first test, 50% of shoots from the untreated healthy plants showed S1 floral symptoms while shoots from the other groups remained healthy after 82 dpi. Disease symptoms have not observed until 96 dpi in both NAA treated groups (Fig. 7A). After 124 dpi, the healthy shoots from plants with PLY phytoplasma infection and NAA treatment were least affected by phytoplasma infection with only one out of 9 shoots inoculated showing S1 symptom (Fig 7A). The symptom developments in the second test were clearly progressing faster. In the test, 70% of inoculated-shoots from untreated plants showed various degrees of symptoms while 50% of inoculated-shoots from NAA-treated healthy plants showed symptoms, and the disease symptoms were not yet observed in inoculated-shoots from plants with both PLY phytoplasma

infection and NAA treatments after 82 dpi. There were 80% inoculated-shoots from the untreated group showed S3 floral symptoms, but 60% inoculated-shoots from the NAA pre-treated group showed symptoms and just 50% of them reached S3 floral symptom in 96 dpi (Fig. 7B). Shoots from plants with PLY phytoplasma infection and NAA treatment, again, were least affected by the inoculation with only 20% of shoots infected (Fig. 7B). Healthy shoots from PLY phytoplasma-infected plants but without NAA treatments were also graft-inoculated with the phytoplasma and showed no obvious disease symptoms though since too less shoots were able to be obtained (only three shoots), the group was excluded from the comparison.

Callose deposition changed after the treatment of auxin

Callose deposition is a basal defense mechanism that plants may use to limit pathogen transmissions, and has been reported to be activated in many diseases, including phytoplasma diseases and diseases caused by its phylogenic relative, spiroplasma (Koh *et al.*, 2011; Musetti *et al.*, 2010). It was suggested that accumulation of calloses in apple proliferation (AP) phytoplasma-infected apples may plugged sieve pores to prevent phytoplasma spreading inside the host plant (Musetti *et al.*, 2010). AP phytoplasma often loses its ability to re-colonize the upper parts of apple plants when the plants are rejuvenated in spring (Carraro *et al.*, 2004; Seemüller,

1988). This phenomenon was correlated with the occurrence of callose deposition, and it was thought to be the cause (Musetti *et al.*, 2010). Therefore, callose deposition in newly formed leaves was examined. The results showed that auxin treatment on healthy plants was able to enhance callose deposition (Fig. 8). Obvious callose deposition was detected also in healthy shoots of PLY phytoplasma-infected plants regardless auxin treatment or not though much higher densities of calloses were detected in shoots showing disease symptoms (Fig. 8).

Discussion

Plant pathogens often disturb plant developmental processes to benefit themselves. For instance, *Agrobacterium* promotes productions of auxin and cytokinin to induce crown gall formation, and produce required nutrients for its needs (Pitzschke *et al.*, 2010). In general, biotroph and necrotroph pathogens trigger distinct plant responses. Phytoplasma, a biotroph pathogen, however, was reported to down-regulate auxin-related genes (Hoshi *et al.*, 2009; Hren *et al.*, 2009), which often induced by biotroph pathogens through activation of auxin production (Kazan *et al*, 2009).

The responses of auxin-related genes examined to auxin were changed after the infection of phytoplasma, and some of the auxin-related genes were down-regulated after the infection (Fig. 1). The result was consistent with previous studies (Hoshi *et al.*, 2009). However, some genes with enhanced expressions or had no changes after the infection were also observed. An explanation is that phytoplasma does not regulate all auxin-related genes in the same way. The detailed regulation is still unclear.

To compare auxin sensitivity between healthy and phytoplasma-infected plants, different time periods and different concentration were applied to plants. Only five of seven auxin-related genes were clearly auxin-inducible after 30 minutes for high

concentration of NAA (Fig. 2). In both auxin-inducible genes and those not inducible, some genes induced to higher level at lower concentration in diseased plant. However, some genes show no difference (Fig. 2). Overall, the expression patterns of these auxin-related genes in healthy and PLY phytoplasma-infected periwinkles revealed two kinds of tendency. The expression of *IAAs* showed induced much more in PLY phytoplasma-infected periwinkles than healthy, however, the expression of *SAUR5* was not be induced. The *SAURs* represent the function of auxin-responsive genes that might play a role in auxin-mediated cell elongation, but the precise function is still unknown (Knauss, *et al.*, 2003). Those genes were only acquired to express different members in various tissues and in response to light and auxin stimuli in rice (Jain *et al.*, 2006). The result of this experiment showed that after infection of phytoplasma, auxin response of periwinkle was disturbed, and the response was divided to two phase, be induced much more than healthy (*IAAs*) or not be induced (*SAUR5*). The reality of this phenomenon has to be surveyed in the future.

The auxin sensitivity was changed by phytoplasma in periwinkle, observation of the changes to the exogenous treatment of auxin must be useful. We examined the effects of direct auxin spraying on periwinkle and intended to know whether the same effects of symptom relief can be obtained after *in vitro* cultivation of periwinkle shoot in a high auxin containing medium. After direct auxin spraying, the symptoms

speeded up after NAA treatment in diseased plants (Fig. 3). The statement is based on the appearance of floral symptoms or the severity of symptom stages. However, we did not know whether this phenomenon was an effect of auxin in plant development, or its also affected phytoplasma accumulation. In both two experiments, PLY phytoplasma accumulated faster in the plants treated with auxin (Fig. 4). We also observed that symptom developments were processing faster in the second experiment, which was done from September. We suspect that temperature may be one of the reasons causing the progressing of phytoplasma symptoms because the average temperature in green house was 25^{o}C in May, and the average temperature was 28^{o}C in September. In our previous observation, PLY phytoplasma seems to have a better infectious ability in warmer season. Temperature was also found to affect potato zebra chip disease, which is caused by another phloem-limited bacterium *Ca.* Liberibacter solanacearum (Joseph *et al.*, 2011). This bacterium had higher infectious ability in 20-25℃. The symptom slowed down when the temperature was lower than 17℃, and it could not infect host plants when the temperature reaches 33℃ (Joseph *et al.*, 2011).

To understand whether other phytohormone-mediated defense mechanisms were involved in the effects caused by auxin treatments, expressions of genes potentially involved in SA, JA, and ET-mediated defense were examined (Fig. 5). *SAUR5* was

not be induced by auxin in the group of treated PLY phytoplasma-infected periwinkles (Fig. 5A). As that *SAURs* had many unidentified functions, the cause of this phenomenon should be surveyed in the future. Auxin was shown to suppress SA-mediated defense mechanism against infection of biotroph pathogens (Kazan *et al.*, 2009). Our results showed that the PR-related genes, *PR1* and *PR1b*, were not induced by phytoplasma infection when PLY phytoplasma-infected periwinkles received auxin treatments (Fig. 5B). The results may imply that the inhibition of SAR pathway triggered the infection of phytoplasma resulted in the floral symptom development and the accumulation of phytoplasma concentration. The antagonistic croos-talks between auxin and SA have also been demonstrated in Arabidopsis (Kazan *et al.*, 2009). Previous studies also indicated that the amount of JA and ET increased after auxin treatment (Kazan *et al.*, 2009). However, our data did not prove the relationships between auxin and those ISR-related genes in the infection of phytoplasma.

Interestingly, we also found a special phenomenon, that is, the disease plants treated with NAA had a healthier looking after weeks of the auxin treatments (Fig. 6A). To know whether the transmission of phytoplasmas into neighboring shoots was really retarded after auxin treatments, the percentage of healthy shoot had been calculated. An interesting discovery was when periwinkles were given an auxin

treatment, the percentage of healthy shoots was significantly higher than that of periwinkles without treatment (Fig. 6B). Those healthy-looking shoots were convinced that had no phytoplasma been detected in the leaves (Fig. 6C). This finding may suggest that auxin can help plants fight against phytoplasma in healthy branches. In two sets of independent experiments, symptom developments of healthy plants without NAA pre-treatment were progressing faster (Fig. 7). The result demonstrated that pre-treatment of auxin can promote plant immune system against phytoplasma infection to some extend.

Callose deposition was thought to be a mechanism of plant basal defense (Ahmad *et al.*, 2011). In the research of AP phytoplasma, callose was found to occlude in the sieve pores and plug the infection of phytoplasma to the neighboring region (Musetti *et al.*, 2010). Our results showed that auxin treatment on healthy plants was also able to enhance callose deposition (Fig. 8). There were more calloses deposition in PLY phytoplasma-infected periwinkles than in healthy plants (Fig. 8). It has been shown that recovered plants are more difficult to be infected by pathogens (Osler *et al.*, 2000). Therefore, we also like to know that whether there were different between healthy and diseased part in PLY phytoplasma-infected periwinkle because in diseased part, the symptom speeded up, while in healthy part, it has more resistance to phytoplasma. In the result, the diseased part had more callose deposition than healthy

part (Fig. 8), it had the same tendency to the previous study (Leljak-Levanić *et al.*, 2010).

The results of our finding showed that after auxin treatment, the floral symptom was accelerated and was accompanied with the early increase of phytoplasma concentration in diseased part of plant. In previous study of *in vitro* culture, the symptom of diseased shoots recovered after auxin treatment (Ćurković-Perica *et al.*, 2008). The probable explanation of those results is that subculture of plant tissues used newly-formed periwinkle shoots, which may had no phytoplasma after auxin treatment. The growth of phytoplasma in prime tissue is undetected. After times of subculture, the healthy part of periwinkle kept healthy, caused the recovery of symptoms.

In fact, not only auxin-treated PLY phytoplasma-infected periwinkles, but also the untreated plants showed diseased and healthy shoots in the same time (Fig. 6B). The percentage of healthy shoots is lower than auxin-treated plants. There were also no phytoplasma in those healthy shoots. In previous studies in grape, the uneven distribution of phytoplasma was observed, and the sporadic systemic spreading throughout grapevines was reported (Hren *et al.*, 2009). The same phenomenon was proposed in papaya, which infected by *Ca.* P. australiense, causing papaya dieback (Guthrie *et al.*, 2001). It is reasonable to deduce through our results that the different

reactions between healthy and diseased parts may exist naturally, but auxin treatment can magnify this effect.

In our study, we found that after the auxin treatment, plant triggers different reaction in different parts. In Figure 9, we proposed a working model to explain the possible mechanisms involved in the different reactions. In diseased parts, auxin probably suppressed the activation of *PR* genes, and thus may be beneficial to the accumulation of phytoplasma. The reason that expression of auxin-related genes was affected by phytoplasma infection is still puzzling. In healthy parts, auxin may trigger some basal defense, including callose deposition to defend against phytoplasma infection (Fig. 9). There are still many questions for future studies. One of the interesting points is whether plants also apply two different systems in infection site and non-infection regions. Whether callose deposition plays the main role in basal defense after the treatment is another point needed to be clarified. The relationship between *PR* gene expression and phytoplasma infection is still a puzzle. Whether the real content of defense phytohormones in phytoplasma-infected periwinkles parallel to the expression of these hormone-related genes. The detailed mechanism of the aforementioned two-phase working model should be confirmed with more experimental data in the future.

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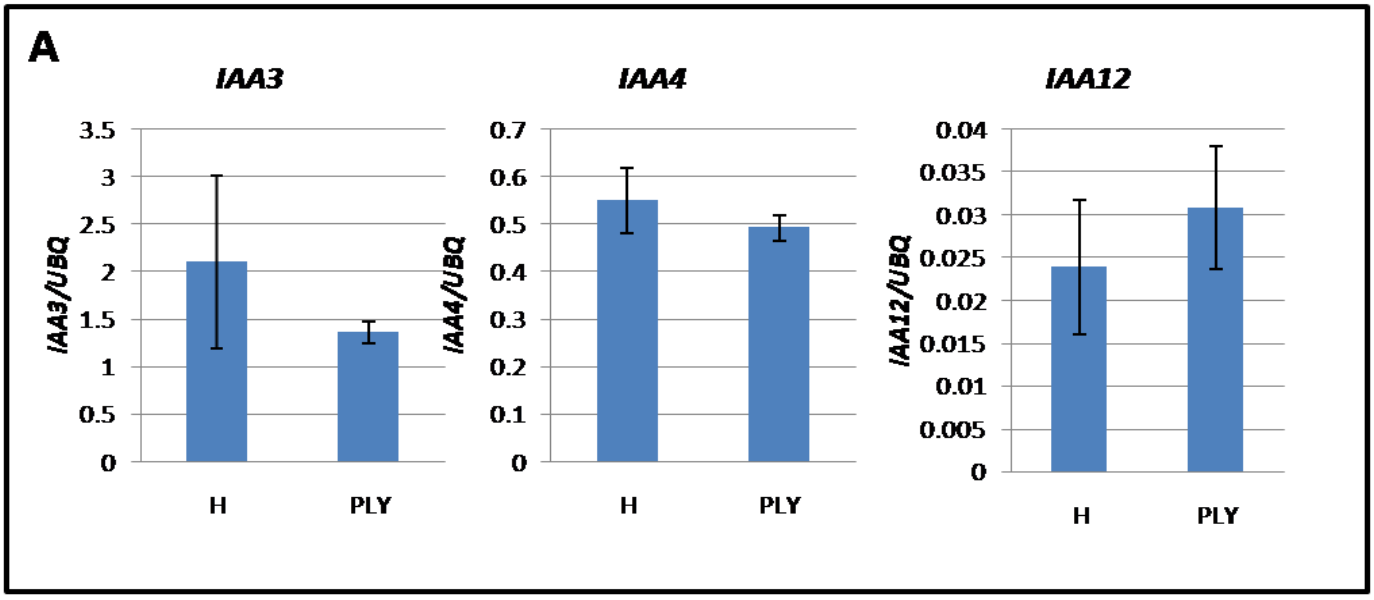
Table and Figures

Table 1. Primers used in this study

Primer	Sequence (5' to 3')	Target	purpose
PLY-amprtF2	TGT AAA AGT AGC GGT TGC TGA TAA TAA	*amp*	detect phytoplasma
PLY-amprtR2	CGC CTA CTA AAC TTA ATA CTT TTG AAC CT	*amp*	detect phytoplasma
UBQ-1252F	GCT GCT CTG GTG ATT GAT GCT	ublquitin	real-time PCR
UBQ-1392R	CCA AAA GGA ACC CGA AAA CA	ubiquitin	real-time PCR
IAA19F	ATG GCC ACC GGT TTG TTC AT	*IAA19*	real-time PCR
IAA19R	TGC CAT AAC AGC CGA AGA GT	*IAA19*	real-time PCR
IAA3F	AGG GAA GGC TAC AAA GGA TCA G	*IAA3*	real-time PCR
IAA3R	ACA ACC CAA GCC TCT TGC TT	*IAA3*	real-time PCR
IAA4F	ATA GCT ACC CTG AGC TCC TCA A	*IAA4*	real-time PCR
IAA4R	AAC ATC TCC TGC CAG CAT CCA A	*IAA4*	real-time PCR
IAA8F	AGG GCA TTG CCA ATC GTG TT	*IAA8*	real-time PCR
IAA8R	AGT GCA CCA GGT CCA GAT TT	*IAA8*	real-time PCR
IAA14F	AGC AGC AGC GTA GCA TTT GT	*IAA14*	real-time PCR
IAA14R	ATT CCC TGT TGG GTC CCA TAG T	*IAA14*	real-time PCR
IAA14-2F	AGC AGC AGC GTA GCA TTT GT	*IAA14-2*	real-time PCR
IAA14-2R	TTC CCT GTT GGG TCC CAT AGT T	*IAA14-2*	real-time PCR
IAA9F	TCT GGC ATA AGT GCT GTG CT	*IAA9*	real-time PCR
IAA9R	AGG TGC ATT GCC ACC GTT AT	*IAA9*	real-time PCR
IAA12F2	TGG TGG CAT GAG CAG GAG TAA AGA	*IAA12*	real-time PCR
IAA12R2	AGT TTG AGC CAA GGT CTC GTA GCA	*IAA12*	real-time PCR
SAUR5F2	GAG GAT TTG TTT GAG GGC TGC TGT	*SAUR5*	real-time PCR
SAUR5R2	CCC TTC TCA TCC TTT CCC TTT ATC CC	*SAUR5*	real-time PCR
Pr1F2	TGT AGG TCC GAT GAG ATG GGA CAA	*Pr1*	real-time PCR
Pr1R2	AGT Cat AGA TCG GCC TAT CGG CAA	*Pr1*	real-time PCR
Pr1bF	TTG CCG AGA GGC GAT TCT ATG ACT	*Pr1b*	real-time PCR
Pr1bR	AAC ACC TAA CCC TAG CAC ACC CAA	*Pr1b*	real-time PCR
LOX2F2	TAG CCA CTG ATG CTT GGC TAT GGA	*LOX2*	real-time PCR
LOX2F2	TTG CAC TAA GTT GTC GAT TGG CCG	*LOX2*	real-time PCR
AOCF	CTG CAG CAG CTC ATC TTT CCG TTT	*AOC*	real-time PCR
AOCR	TCC TCA CAA GCT TTA GCA GCA GGA	*AOC*	real-time PCR
ACO4F	TGA GCA ATT ATC CAC CGT GTC CGA	*ACO4*	real-time PCR
ACO4R	AGT GCC TCA TGG GAG GAA CAT CAA	*ACO4*	real-time PCR
PLY-tenguF	AAA CTG TTA ATA TTT GCT GGC TTT	*PLY-tengu*	detect *PLY-tengu*
PLY-tenguR	TTA GGC ATC TTT CTC GCC CTT T	*PLY-tengu*	detect *PLY-tengu*


A
IAA3
IAA3/UBQ
3.5
3
2.5
2
1.5
1
0.5
0
H
PLY
IAA4
IAA4/UBQ
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
H
PLY
IAA12
IAA12/UBQ
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
H
PLY

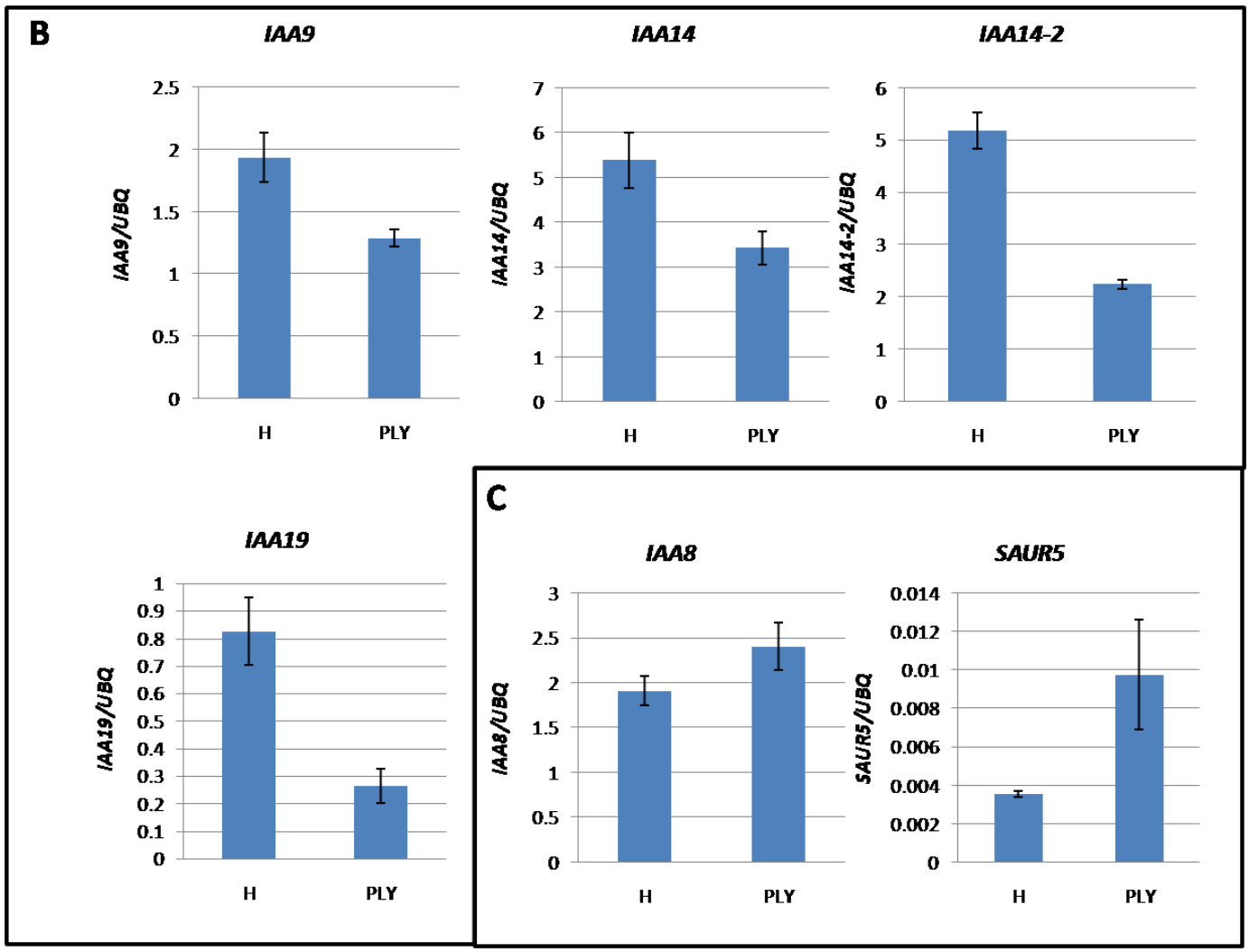
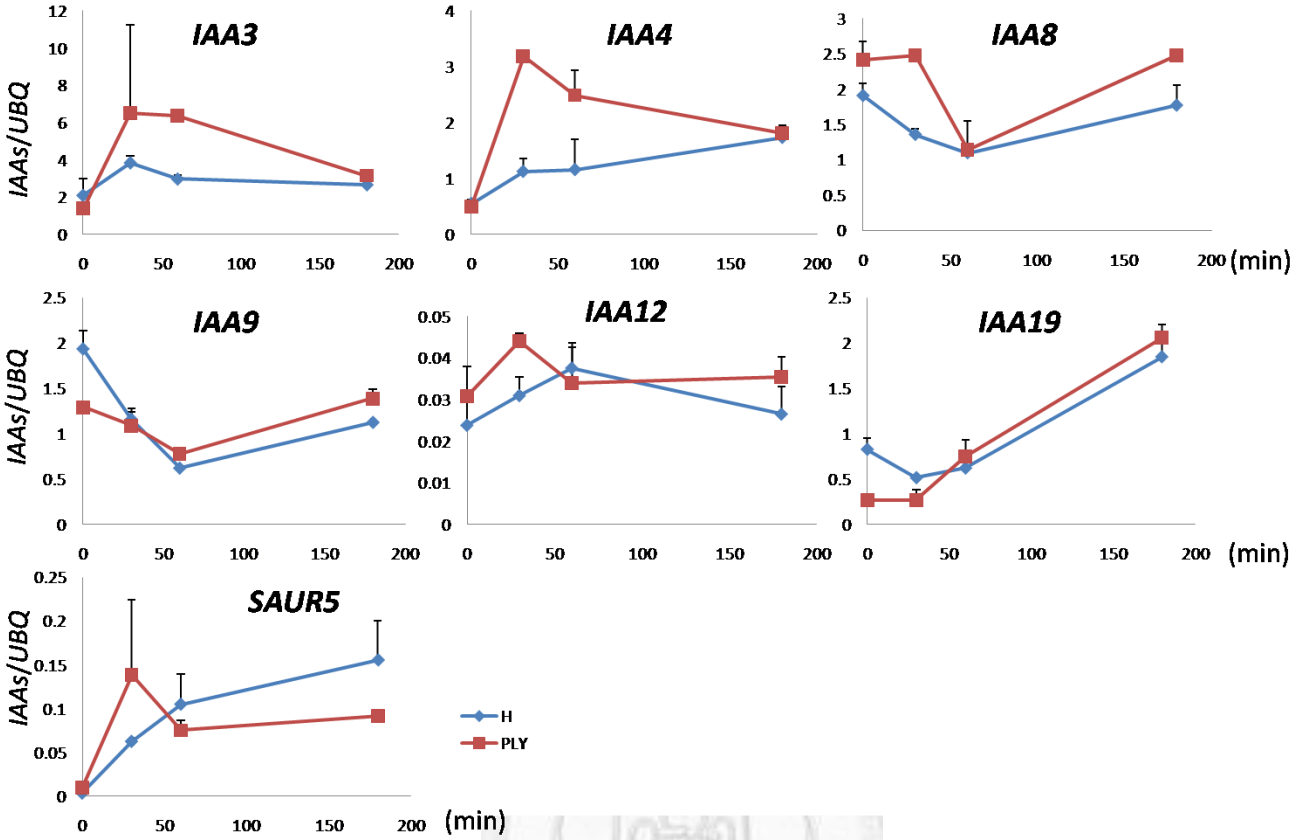

B
IAA9
IAA9/UBQ
2.5
2
1.5
1
0.5
0
H
PLY
IAA14
IAA14/UBQ
7
6
5
4
3
2
1
0
H
PLY
IAA14-2
IAA14-2/UBQ
6
5
4
3
2
1
0
H
PLY
IAA19
IAA19/UBQ
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
H
PLY
C
IAA8
IAA8/UBQ
3
2.5
2
1.5
1
0.5
0
H
PLY
SAUR5
SAUR5/UBQ
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
H
PLY

Fig. 1. The changes of expression of nine auxin-related genes in the periwinkles after PLY phytoplasma infection. In this study, nine auxin-related genes were chosen for our survey. The expression pattern can divided into three groups, (A), (B), and (C). (A) Three genes including *IAA3*, *IAA4*, and *IAA12*, the expression had no significantly changes after infection of phytoplasma. (B) Four genes including *IAA9*, *IAA14*, *IAA14-2*, and *IAA19*, were down-regulated after the infection of phytoplasma. (C) Two genes including *IAA8* and *SAUR5*, were up-regulated after the infection. The pattern of those changes mostly coincident to the previous study (Hoshi *et al.*, 2009). Y axis represent the relative expression of target gene/*Ubiquitin*, and multiply 100 times to be the final number.

A


IAA3
IAA4
IAA8
IAA9
IAA12
IAA19
SAUR5
IAAs/UBQ
(min)
H
PLY

B

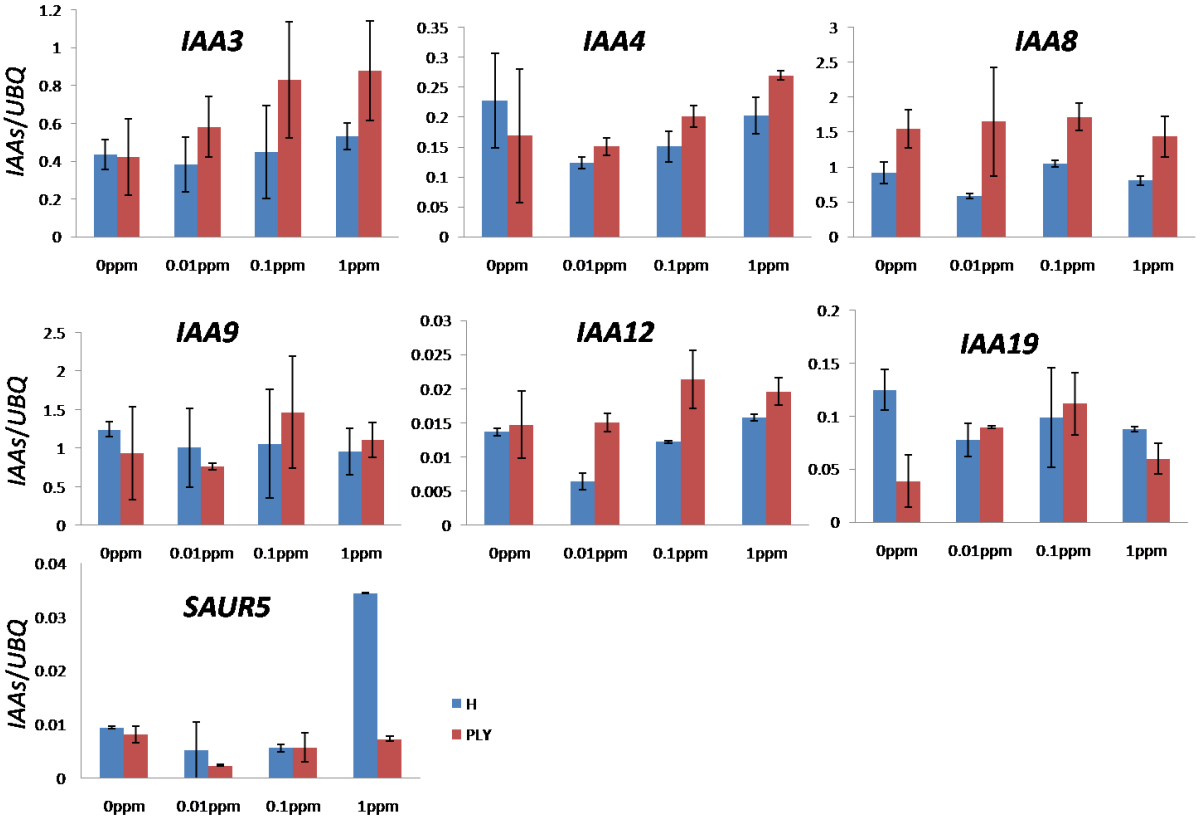

IAA3
IAA4
IAA8
IAA9
IAA12
IAA19
SAUR5
IAAs/UBQ
0ppm
0.01ppm
0.1ppm
1ppm
H
PLY

Fig. 2. Time course experiment and dosage-dependent experiment for auxin sensitivity test. In these experiments seven of nine genes in Fig. 1. were chosen. (A) In time course experiment, 10 ppm (~57μM) NAA was used for auxin treatment, and analyzed the expression after 30, 60, and 180 minutes. Five of seven genes, *SAUR5*, *IAA3*, *IAA4*, *IAA12*, and *IAA19* were induced after auxin treatment, but *IAA8* and *IAA9* were not. After PLY phytoplasma infection, some genes were also induced by auxin, but the pattern between healthy and infected plant were not consist in those seven genes. (B) In dosage dependent experiment, different concentration of NAA, 1 ppm (~5.7μM), 0.1 ppm (~0.57μM), and 0.01 ppm (~0.057μM) were used for the treatment. Samples were collected 30 minutes after, and observed the expression. The expression of *IAAs* were induced much more in PLY phytoplasma-infected periwinkle than healthy. However, the expression of *SAUR5* was not induced. The blue line in (A) and blue bar in (B) represent the healthy periwinkles, and the red line or the red bar represent PLY phytoplasma-infected periwinkles. The Y axis in both (A) and (B) is the relative expression of target gene/*Ubiquitin* and multiply 100 times in the end.

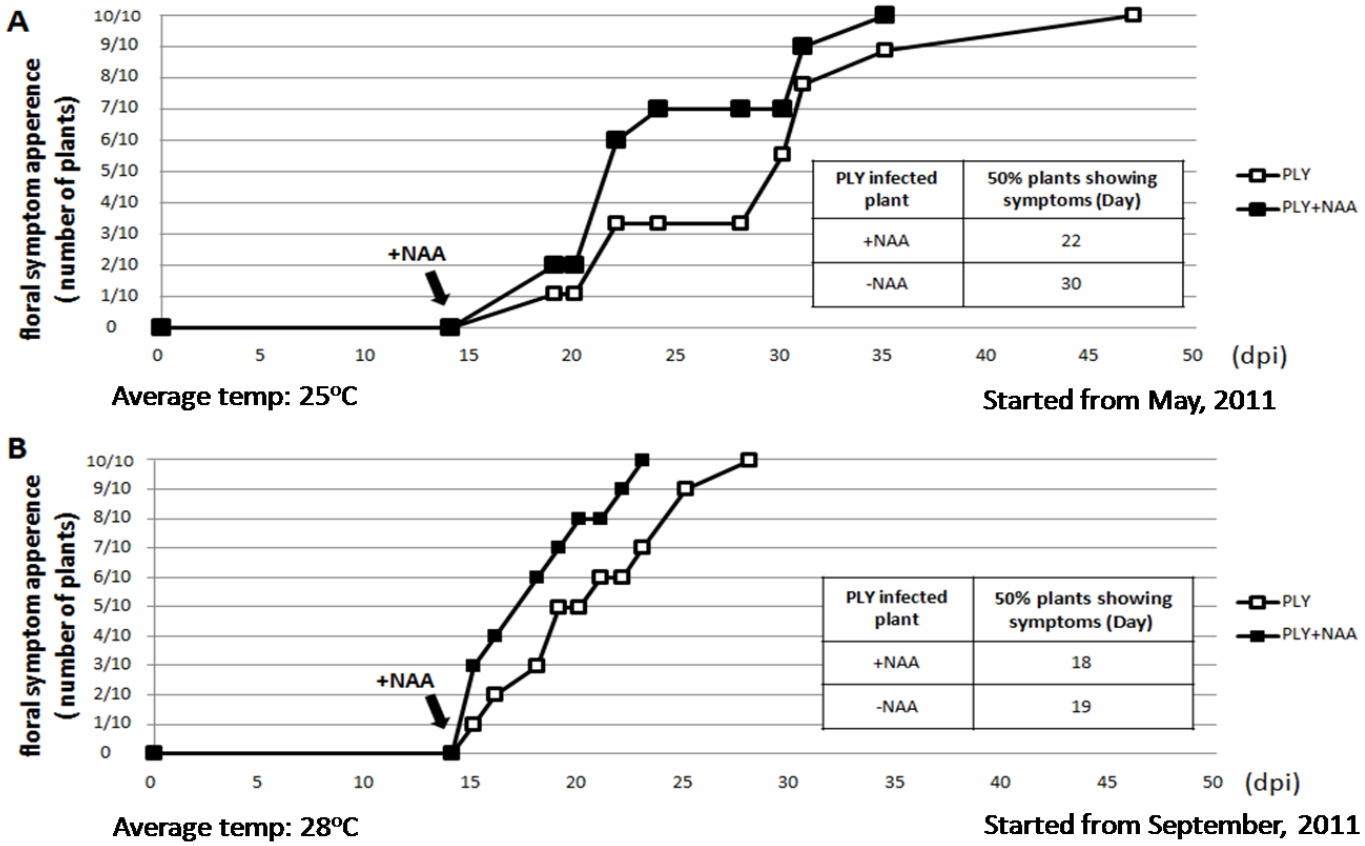


Fig. 3. The percentages of floral symptom showing in the tested plants after inoculation. (A) and (B) are two independent experiments started in May and September, respectively. The average temperature were 25^{o}C (in May) and 28 ^{o}C (in September). Ten periwinkles were used in each experiment. We sprayed 25 ppm (~143µM) NAA was sprayed per plant after 14 days post inoculation (dpi), once a week. The "diseased plant" were calculated when it appeared S1 or other stages of floral symptoms in the first time sight on plants. (A) The day of half plants became diseased was 22 and 30 dpi after treatment or not treatment of NAA, individually. (B) The day of half plants became diseased was 18 and 19 dpi after treatment or not treatment of NAA, individually. Line with black block is PLY phytoplasma-infected periwinkles treated with NAA, and line with white block is PLY phytoplasma-infected periwinkles without treatment. Both experiments showed that plants treated with NAA accelerated the floral symptom development.

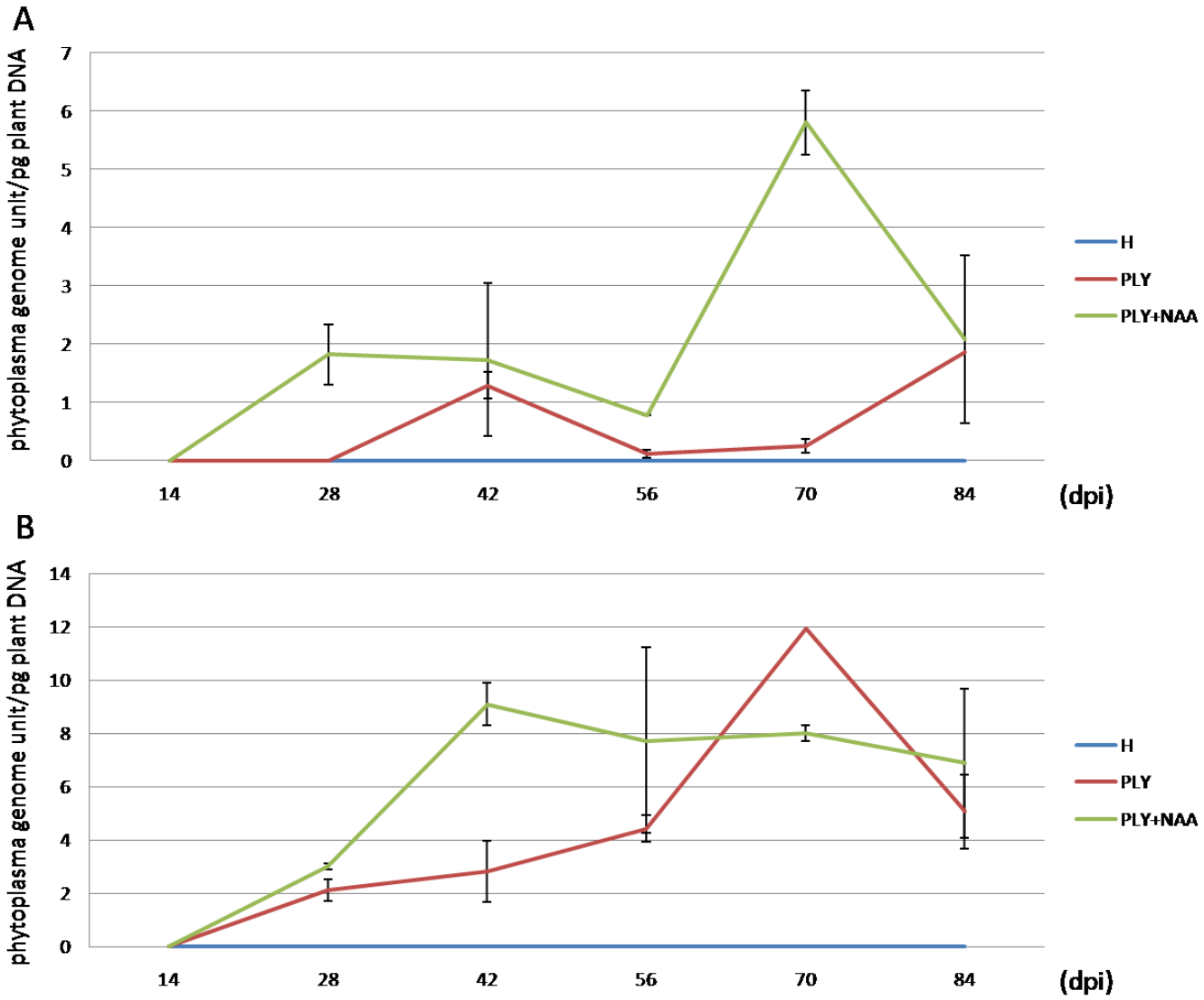


Fig. 4. Measurement of phytoplasma concentration. (A) and (B) are two independent experiments. Both experiments indicated that after treatment of NAA, the concentration of PLY phytoplasma accumulated faster than untreated one. The maximum concentration of PLY phytoplasma in auxin treatment plants were 5.8 and 9.08 phytoplasma genome unit/ per picogram of plant DNA. The blue line represent healthy periwinkles. The red line is PLY phytoplasma-infected periwinkle without treated with NAA, and the green line is PLY phytoplasma-infected periwinkle with treatment. The Y axis is phytoplasma genome unit/ per picogram of plant DNA.

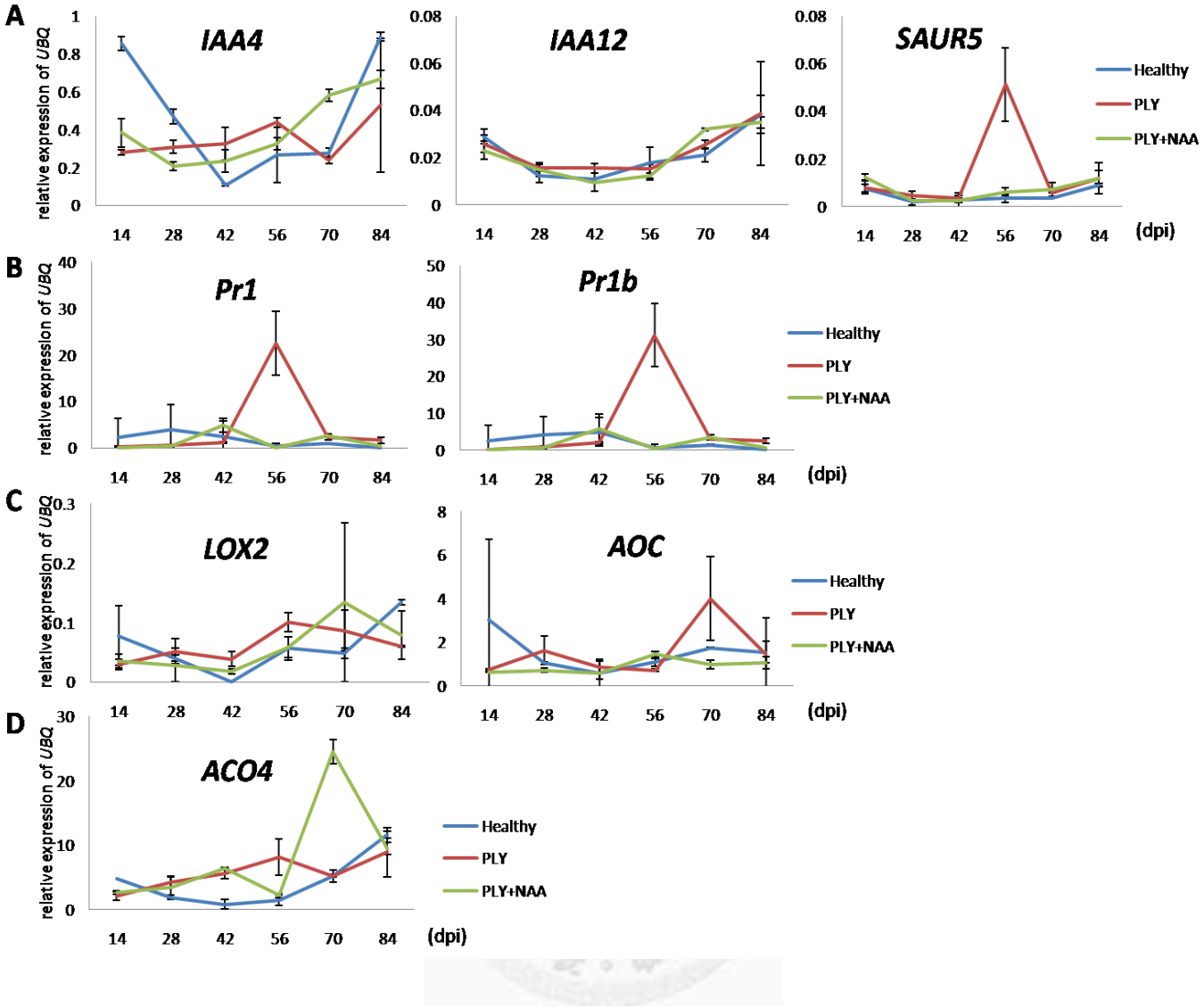
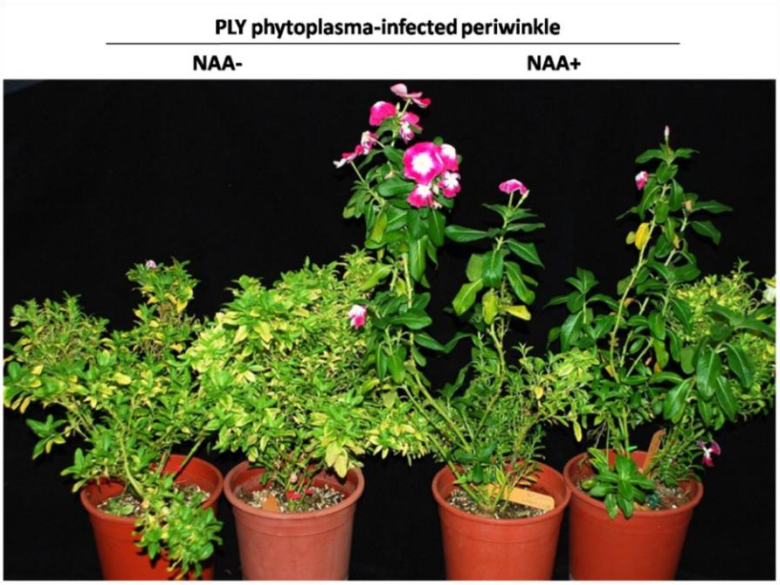
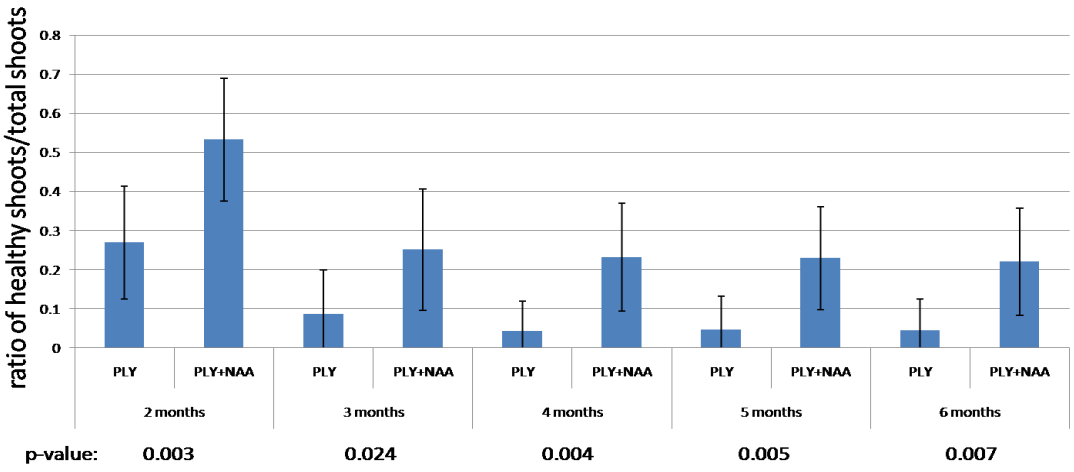

A
IAA4
IAA12
SAUR5
relative expression of UBQ
Healthy
PLY
PLY+NAA
(dpi)
B
Pr1
Pr1b
C
LOX2
AOC
D
ACO4

Fig. 5. Expression of hormone-related genes. The relative expression of auxin-related genes and other defense hormone-related genes were analyzed after auxin treatment. (A) The result showed that the expression of auxin-related genes did not change after the treatment, but *SAUR5* was the only one, that untreated PLY phytoplasma-infected plant had higher expression than other group in 56 dpi. (B) In *PR*-related genes, both *Pr1* and *Pr1b* were evoked the expression in 56 dpi in the untreated PLY phytoplasma-infected plants. (C) In JA related genes, *LOX2* had no difference between treated and untreated plants, but *AOC* was up-regulated in the untreated PLY phytoplasma-infected plants, 70 dpi. (D) In ET related genes, *ACO4* had higher expression in treated PLY phytoplasma-infected plants in 70 dpi. The blue line represent healthy periwinkles. The red line is PLY phytoplasma-infected periwinkle without treated with NAA, and the green line is PLY phytoplasma-infected periwinkle with treatment. The Y axis is the relative expression of target gene/*Ubiquitin*, and multiply 100 times to be the final number.

A


PLY phytoplasma-infected periwinkle
NAA-
NAA+

B


ratio of healthy shoots/total shoots
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
PLY
PLY+NAA
2 months
3 months
4 months
5 months
6 months
p-value:
0.003
0.024
0.004
0.005
0.007

C

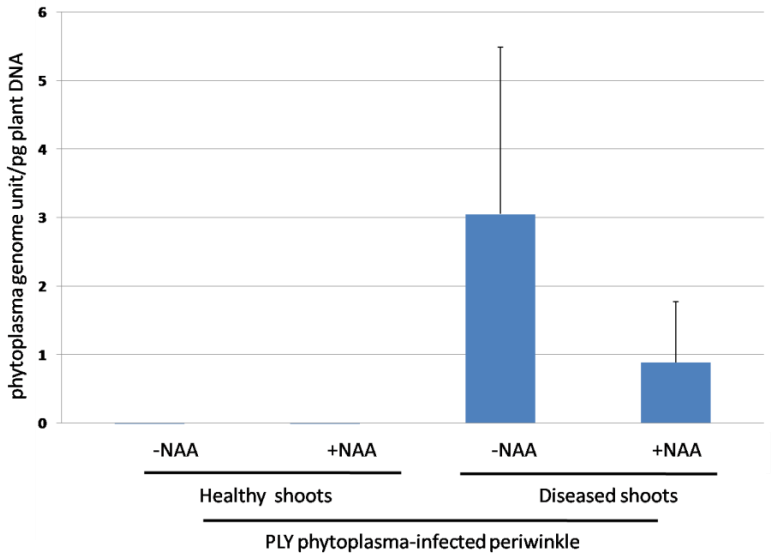

phytoplasma genome unit/pg plant DNA
6
5
4
3
2
1
0
-NAA
+NAA
-NAA
+NAA
Healthy shoots
Diseased shoots
PLY phytoplasma-infected periwinkle

Fig. 6. The symptom expressions in the phytoplasma-infected periwinkles with or without the auxin treatment. (A) With the progress of symptom development, there were quite different phenotype between NAA-treated and untreated PLY phytoplasma-infected periwinkles. The left two plants were PLY phytoplasma-infected periwinkles which untreated NAA, almost all shoots were diseased. However, the right two plants were PLY phytoplasma-infected periwinkles which NAA treatment, producing more healthy shoots. (B) The ratio of healthy shoots from 2 months to 6 months were calculated, and a significantly different was found between those two groups. Untreated PLY phytoplasma-infected plant indeed had more severe symptoms than treated one. (C) The concentration of phytoplasma in diseased and healthy shoots. Both diseased shoots were detected phytoplasma whether treated auxin or not. On the other hand, both healthy shoots were not detect phytoplasma whether treated auxin or not.

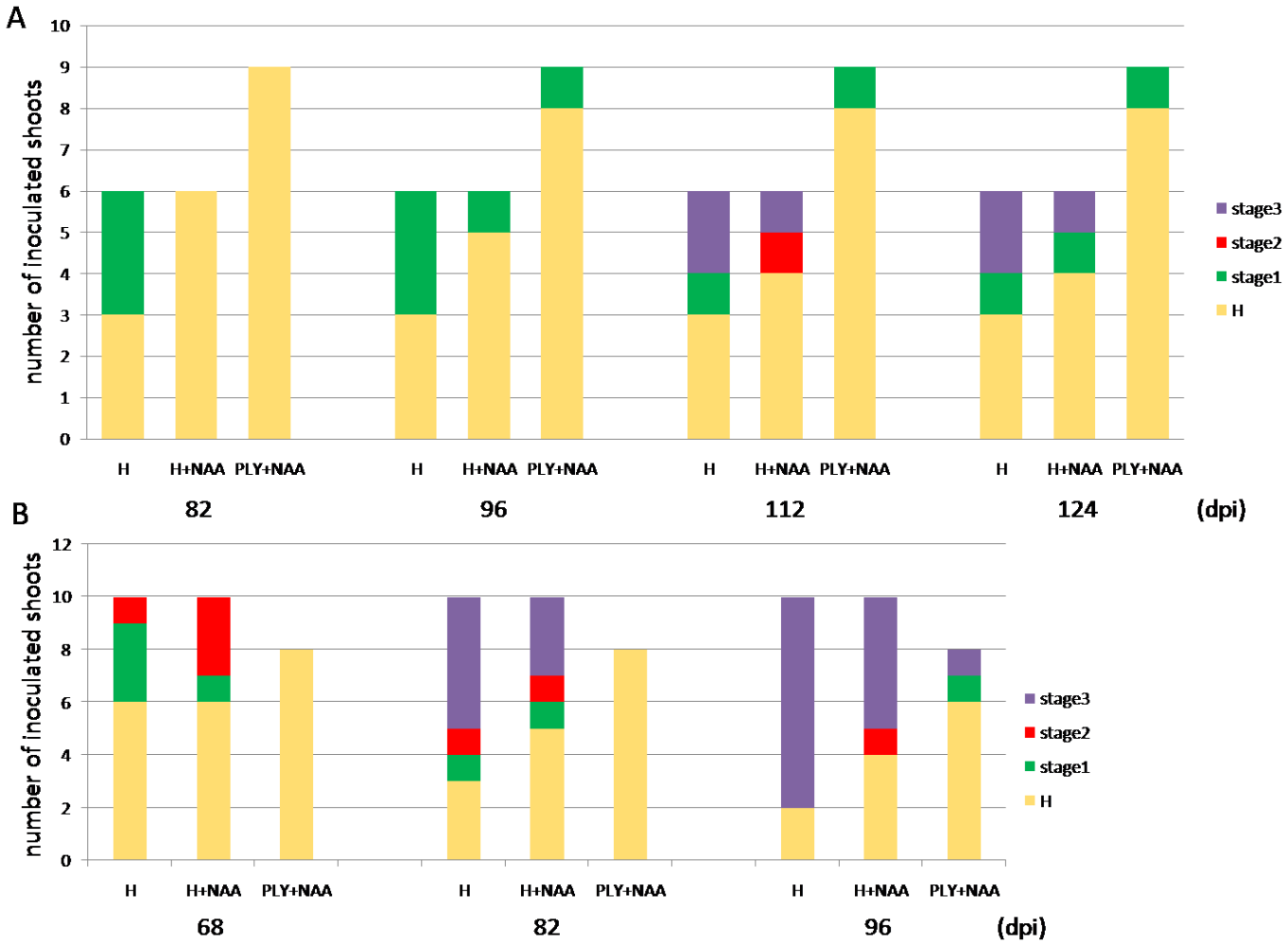


Fig. 7. Reverse inoculation test. After 180 dpi on plants treated or untreated NAA, PLY phytoplasma scions were side-grafted in healthy shoots. The healthy shoots on PLY phytoplasma-infected periwinkles treated with NAA also side-grafted PLY phytoplasma scions. (A) and (B) were two independent experiments. (A) The floral symptom development was calculated in 83, 100, 114, 128 dpi. (B) The floral symptom development was calculated in 68, 82, and 96 dpi. Both of the experiments showed after auxin treatment, the shoots became more resistant to the infection of phytoplasma, and the healthy shoots on PLY phytoplasma-infected periwinkles treated with NAA was the most resistant. The yellow bar represent healthy flower, and the green, red, and purple bar represent S1, S2, and S3 floral symptoms, respectively.

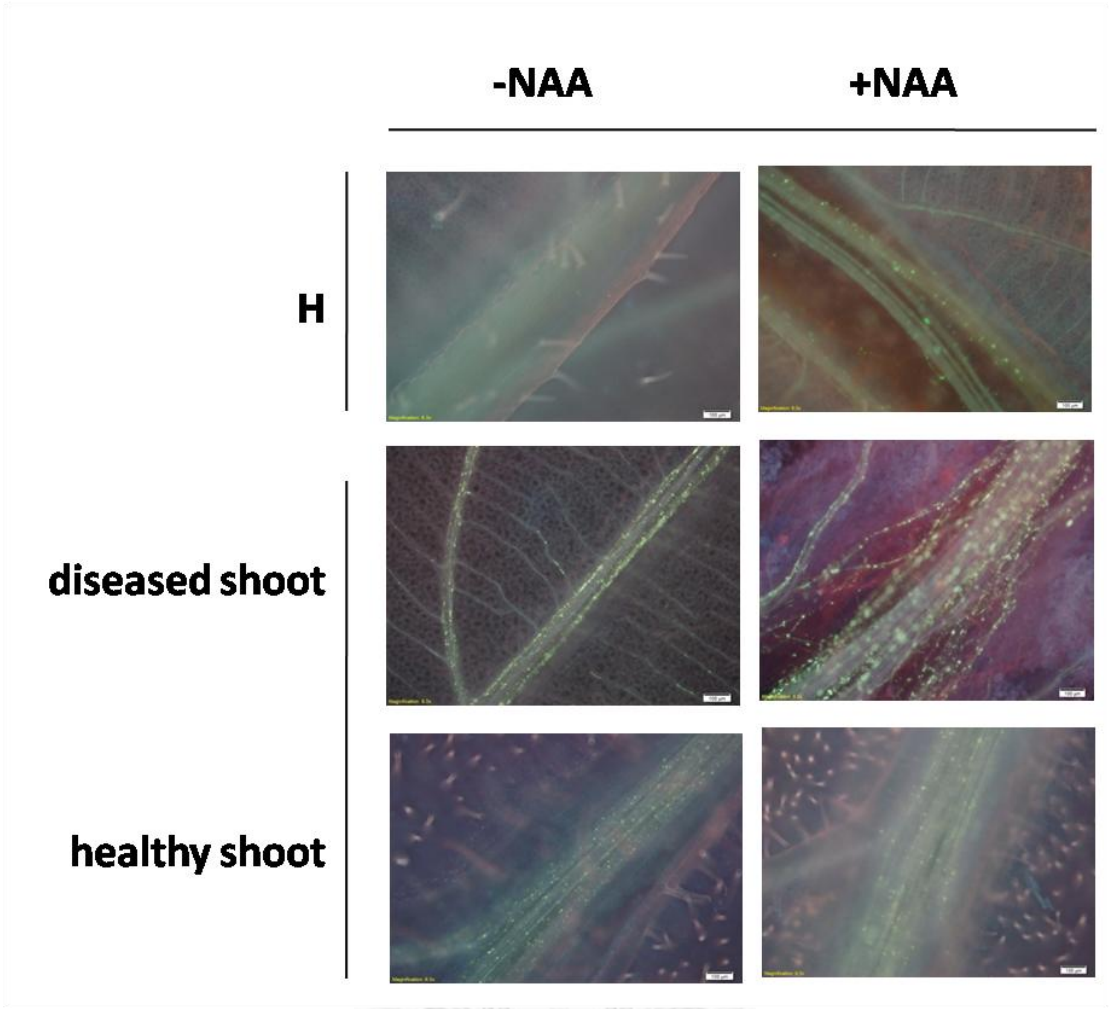


Fig. 8. Callose deposition test. Methyl blue method and florescence microscopy were used to observed the situation of callose deposition. The spot light in the vascular bundle are calloses. In healthy periwinkles, the tissue treated with NAA had more calloses in the field. Comparision of healthy and PLY phytoplasma-infected plant, more callose deposited in the diseased tissue. The diseased tissue had more callose deposition than healthy tissue.

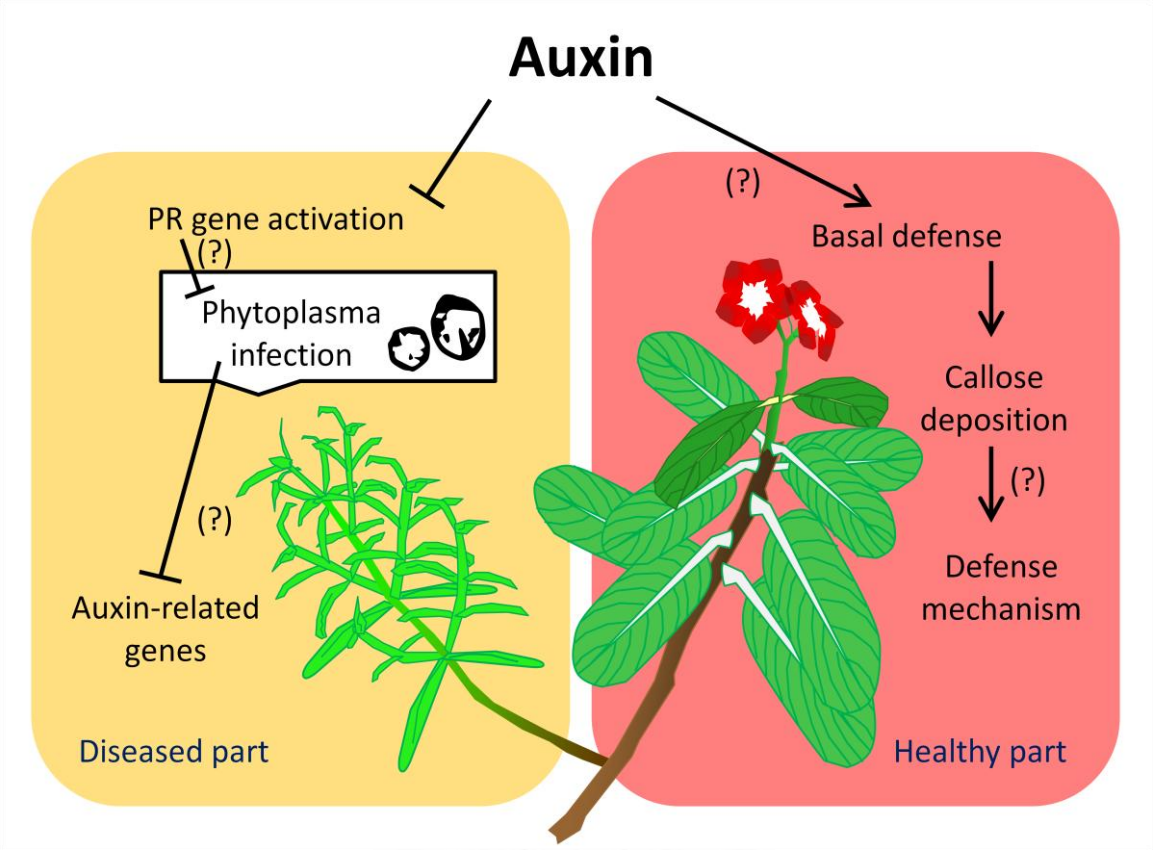
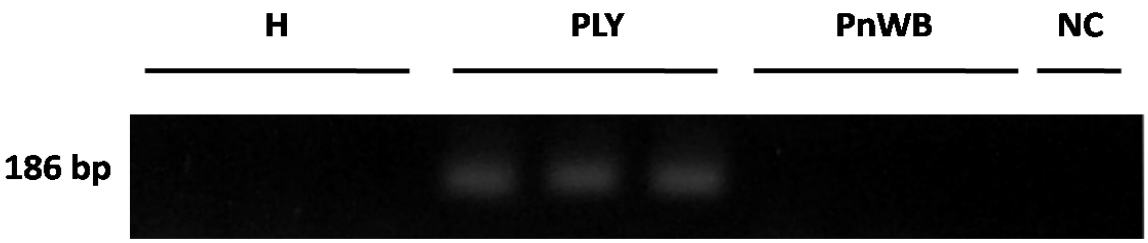


Fig. 9. Scheme of the hypothetic working model in this study. After auxin treatment, plant will trigger two-phase mechanism. In diseased part, auxin probably suppressed the activation of *PR* genes. The suppression of *PR*s was beneficial for the accumulation of phytoplasma. The reason that phytoplasma affect the expression of auxin-related genes is still puzzle. In healthy part, Auxin may trigger some basal defense, including callose deposition. The detailed mechanism should be studies in the future.

A



B

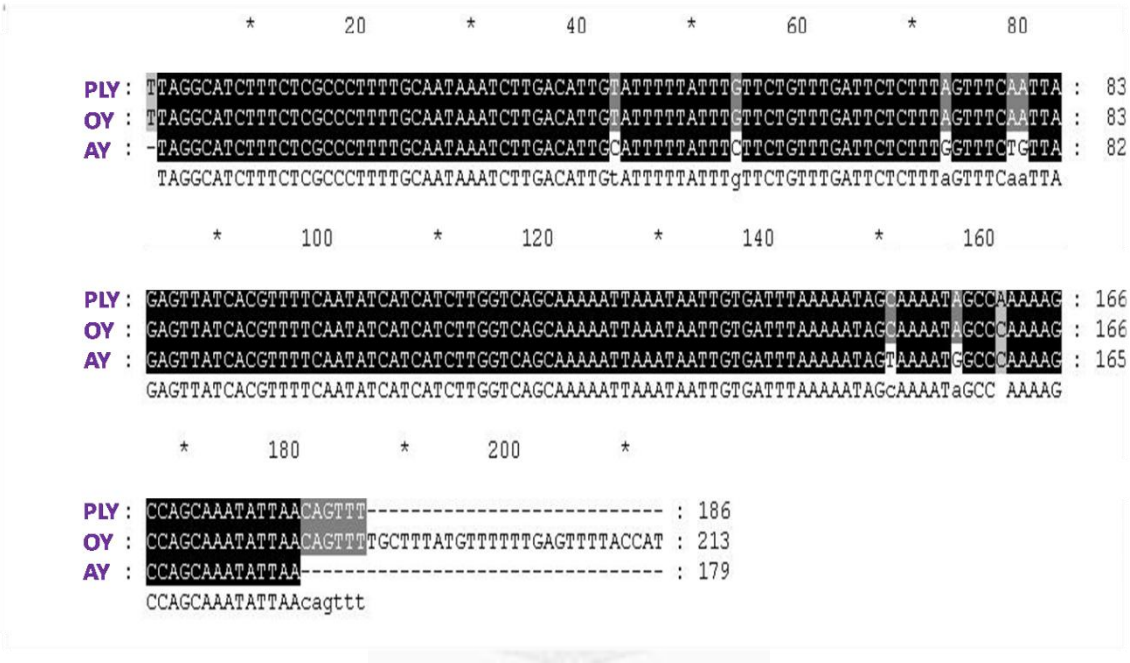


Fig. S1. Search for *PAM765* in different phytoplasma species. The process of searching was to extract total RNA from phytoplasma-infected periwinkle first, preparing of cDNA by using random priming, and subsequently to find the gene using PCR technique. (A) The result of PCR. A 186-bp sequence fragment was cloned from PLY phytoplasma-infected periwinkle. (B) The alignment data with other representative phytoplasma. We named the fragment *PLY-tengu*, and did alignment with OY and AY phytoplsama. *PAM765* seems have a high conservation in 16SrI phytoplsama. H: healthy periwinkle; PLY: PLY phytoplasma-infected periwinkle; PnWB: peanut witches'-broom (PnWB) phytoplasma-infected periwinkle, which belong to 16SrII phytoplasma; NC: negative control.

Fig. S2. Three stages of the symptom development of PLY phytoplasma-infected periwinkle. The progress of floral symptoms were defined by Su *et al.* (2011). (A) Healthy periwinkle flower. (B) Flower of floral symptom stage 1 (S1): discoloration of flower. (C) Flower of floral symptom stage 2 (S2): partial virescence of flower. (D) Flower of floral symptom stage 3 (S3): complete floral virescence. The red arrow shows the condition of smaller flower compare to normal one.

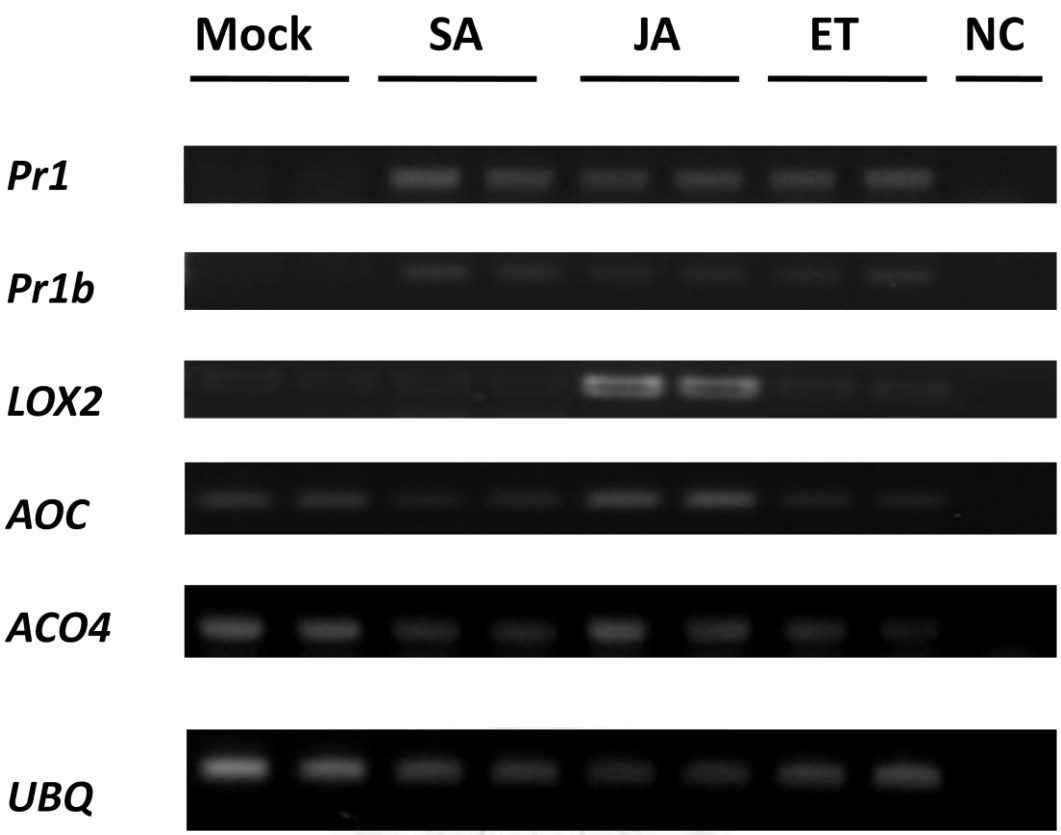
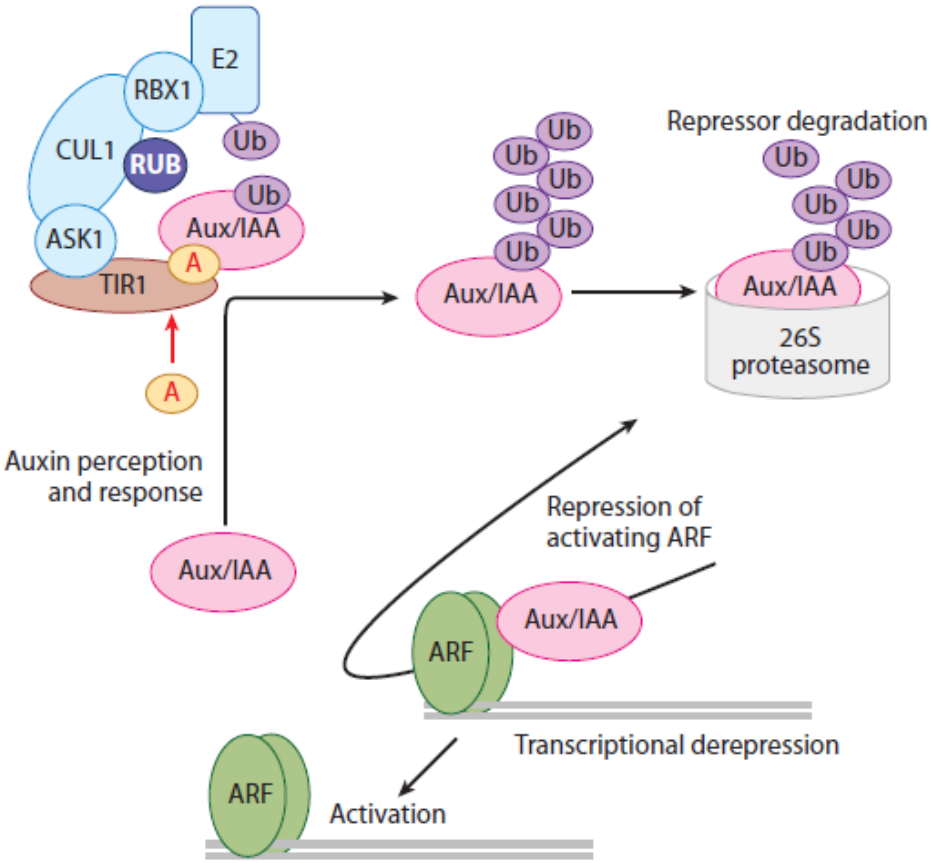


Fig. S3. The induction of defense signaling genes. SA, JA, and ET related genes were surveyed as marker genes in the hormone related test. *Pr1* and *Pr1b* were chosen as PR-related marker genes, *LOX2* and *AOC* as JA marker genes, and *ACO4* as ET marker gene. All of the primers were tested after plant samples treated with SA, JA, and ET for 24 hours, separately. Mock: treated water as control; SA: SA treatment; JA: JA treatment; ET: ET treatment; NC: negative control.

A


E2
RBX1
CUL1
RUB
Ub
ASK1
Aux/IAA
A
TIR1
Repressor degradation
26S proteasome
Auxin perception and response
Repression of activating ARF
ARF
Transcriptional derepression
Activation

B

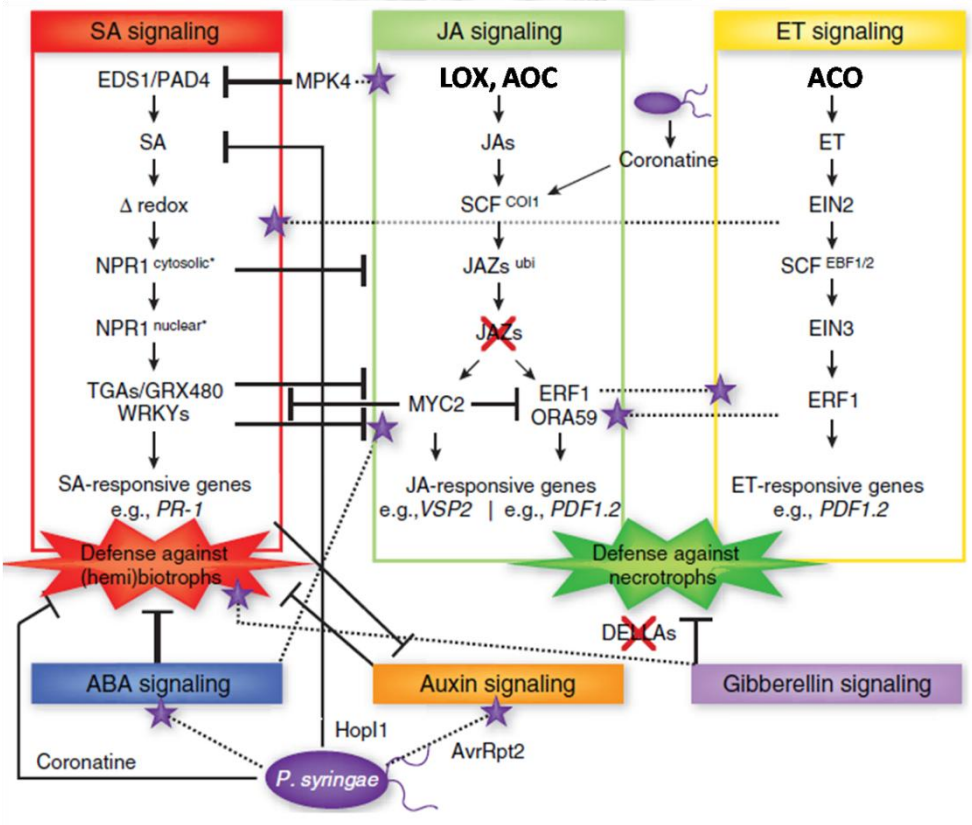

SA signaling
JA signaling
ET signaling
EDS1/PAD4
MPK4
LOX, AOC
ACO
SA
JAs
Coronatine
ET
Δ redox
SCF COI1
EIN2
NPR1 cytosolic*
JAZs ubi
SCF EBF1/2
NPR1 nuclear*
JAZs
EIN3
TGAs/GRX480
WRKYs
MYC2
ERF1
ORA59
ERF1
SA-responsive genes
e.g., PR-1
JA-responsive genes
e.g., VSP2 | e.g., PDF1.2
ET-responsive genes
e.g., PDF1.2
Defense against (hemi)biotrophs
Defense against necrotrophs
DELLAs
ABA signaling
Auxin signaling
Gibberellin signaling
HopI1
AvrRpt2
Coronatine
P. syringae

Fig. S4. Pathways surveyed in this study. (A) Auxin signaling pathway is mediated by degradation of Aux/IAA repressors through ubiquitination. When plant cells perceive high content of auxin (A), F-box protein TIR1 binds to Aux/IAA substrate, and Aux/IAA is degraded by 26S proteasome. After the degradation, auxin-inducible genes can be activated by auxin response factors (ARFs). E2, Ub-conjugating enzyme. (From Mocaitis, K., and Estelle, M. 2008. Auxin receptor and development: a new signaling paradigm. Annu. Rev. Cell. Dev. Biol. 24:55-80.) (B) Cross-talks between plant hormones mediate plant defense efficiently against various pathogens. Pathogens may also secrete effectors, for instance HopI1 and AvrRpt2, to disturb proper hormone communications. Signaling pathways of SA, JA, and ET highlight the main plant defense, and other hormones feed into the complicated network. (from Pieterse, C. M. J. *et al.*, 2009. Net working by small molecule hormones in plant immunity. Nat. Chem. Biol. 5:308-316.)