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利用代謝體學探討由植物細胞激素 AtCAPE9 調控的免疫反應
Metabolomics Perspective on the Plant Immunity Regulated by
the Cytokine AtCAPE9

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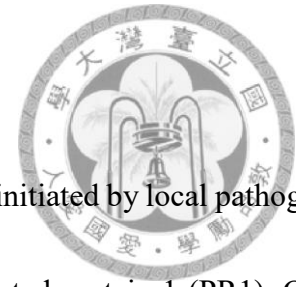
中文摘要



系統性後天抗性 (Systemic acquired resistance, SAR) 是植物在局部病原體感染後啟動的全株防禦反應，主要由水楊酸 (Salicylic acid, SA) 和致病蛋白一號 (Pathogenesis-related protein 1, PR1) 調控。然而，SAR 中系統性信號的傳遞機制仍不明確。在之前的研究中，我們發現 PR1 會被酵素切割產生植物內生性胜肽 AtCAPE9，AtCAPE9 在由細菌鞭毛蛋白衍生胜肽 flg22 誘導的局部和系統免疫反應中發揮了關鍵調節作用。儘管 AtCAPE9 已被證明是 SA 信號途徑中的重要分子，但其在調控 SAR 中的下游機制仍不清楚。為了瞭解這一機制，我們透過液相層析串聯式質譜儀 (LC-MS/MS) 對經 AtCAPE9 處理的阿拉伯芥葉片和韌皮部滲出液進行了代謝體學分析。結果顯示，初級和次級代謝物均發生顯著變化，其中 N-羥基哌啶酸 (N-hydroxypipecolic acid, NHP) 和核黃素 (Riboflavin) 在葉片和韌皮部滲出液中均顯著增加，表明其可能在局部和系統免疫中具有重要作用。進一步的功能研究證實，NHP 在 AtCAPE9 誘導的免疫中是不可或缺的，因為缺乏將哌啶酸 (Pip) 轉化為 NHP 的黃素依賴單加氧酶 1 (Flavin-dependent monooxygenase 1, FMO1) 的阿拉伯芥突變體無法表現 AtCAPE9 誘導的局部和系統免疫。此外，作為 NHP 信號下游的菸鹼醯胺腺嘌呤二核苷酸(磷酸) [NAD(P)]對 AtCAPE9 誘導的氣孔免疫反應至關重要。核黃素同樣在促進局部和系統氣孔免疫反應中發揮了關鍵作用。綜合而言，我們的研究表明，NHP 和 riboflavin 作為 AtCAPE9 的關鍵下游調節因子，調控了支撐 SAR 和氣孔免疫的代謝及免疫信號傳導。

關鍵字：系統性後天抗性、致病蛋白一號、AtCAPE9、液相層析串聯式質譜儀、代謝體學、葉片、韌皮部滲出液、N-羥基哌啶酸、核黃素、菸鹼醯胺腺嘌呤二核苷酸(磷酸)、氣孔免疫反應

Abstract

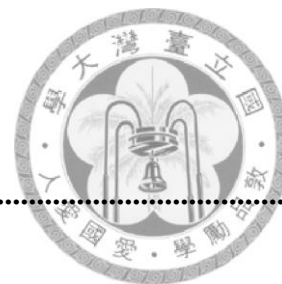


Systemic acquired resistance (SAR) is a plant-wide defense response initiated by local pathogen infection, primarily mediated by salicylic acid (SA) and pathogenesis-related protein 1 (PR1). Our recent study identified AtCAPE9, a cytokine peptide derived from PR1, as a critical regulator of both local and systemic immune responses triggered by the bacterial flagellin-derived peptide flg22. While AtCAPE9 was shown to function as a key mediator in SA signaling, the metabolic pathways involved in its regulation of SAR remained unclear. To elucidate these pathways, we conducted a metabolomic analysis of Arabidopsis leaves and phloem exudates from the plants treated with AtCAPE9 using liquid chromatography-mass spectrometry (LC-MS/MS). This analysis revealed significant alterations in primary and secondary metabolites, with N-hydroxypipecolic acid (NHP) and riboflavin emerging as key metabolites elevated in both leaves and phloem exudates. Functional studies confirmed the essential role of NHP in AtCAPE9-induced immunity, as Arabidopsis mutants deficient in flavin-dependent monooxygenase 1 (FMO1), deficient in NHP biosynthesis, failed to exhibit AtCAPE9-triggered local and systemic immune responses. Furthermore, we found that nicotinamide adenine dinucleotide (phosphate) [NAD(P)], a known downstream component of NHP signaling, is required for AtCAPE9-induced stomatal immunity. Riboflavin also played a pivotal role in promoting both local and systemic stomatal immune responses. These findings demonstrate that NHP and riboflavin acts as an downstream of AtCAPE9, orchestrating metabolic and immune signaling pathways that underpin SAR and stomatal immunity in Arabidopsis.



Keywords: SAR, PR1, AtCAPE9, Arabidopsis, LC-MS/MS, Metabolomics, Leaves, Phloem exudates, NHP, Riboflavin, NAD(P), Stomatal immunity

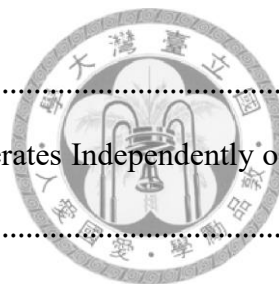
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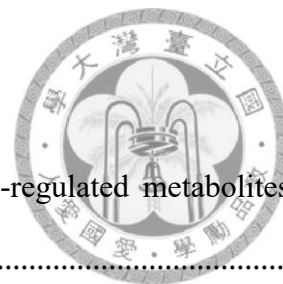


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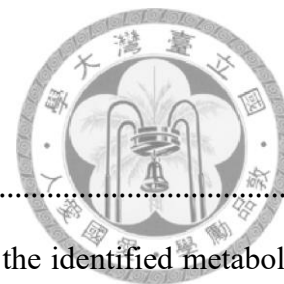


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1. Introduction

1.1 Importance of Immune Systems in Plants

Plants, as sessile organisms, are continuously exposed to a wide array of biotic threats, including bacteria, fungi, viruses, and other pathogens. To survive and grow under such persistent challenges, plants have evolved sophisticated immune systems that enable them to efficiently recognize, respond to, and counteract pathogenic attacks [1]. Unlike animals, which possess specialized immune cells and adaptive immune system, plants rely entirely on their innate immune system for detection and defense [2]. This innate immunity serves as a cornerstone of plant defense, equipping plants with the ability to identify molecular signatures of pathogens and mount rapid responses to neutralize threats [3]. Beyond ensuring survival in pathogen-rich environments, innate immunity is critical for maintaining a delicate balance between growth and defense is also critical in the innate immunity. By fine-tuning their defense mechanisms, plants optimize energy allocation, ensuring robust protection against pathogens while minimizing unnecessary energy expenditure that could hinder growth and reproduction [4]. As such, plant innate immunity plays a pivotal role in safeguarding plants against biotic stressors, contributing to their overall resilience and ecological success [5].

1.2 Overviews of Plant Immune Responses

Plants innate immunity is consisted of two interconnected layers, pattern-triggered immunity (PTI) and effector-triggered immunity (ETI). These systems work synergistically and collaboratively



to recognize and counteract pathogen attacks, ensuring plant survival [1].

PTI is mediated by plasma membrane-localized pattern recognition receptors (PRRs), which detect pathogen- or microbial- associated molecular patterns (PAMPs or MAMPs) and damage-associated molecular patterns (DAMPs) derived from the host tissues. Detection of these molecular patterns activates a series of cellular responses, including calcium influx (Ca^{2+}), reactive oxygen species (ROS) burst, the activation of the mitogen-activated protein kinase (MAPK) cascades, and the transcriptional upregulation of defense-related genes, such as pathogenesis-related (PR) genes (such as PR1, PR2, and PR5) [5, 6].

PRRs primarily consist of receptor-like kinases (RLKs) and receptor-like proteins (RLPs), which are integral membrane proteins critical for plant immunity [1]. RLKs typically are composed of an extracellular domain, a transmembrane domain (TM), and an intracellular kinase domain, also referred to as receptor-like cytoplasmic kinases (RLCKs). This structure enables RLKs to function as signal transducers, recognizing extracellular signals and initiating intracellular signaling cascades. RLPs, on the other hand, lack an intracellular kinase domain and often depend on RLKs to form receptor complexes and propagate immune signaling. These PRRs operate on the plasma membrane, recognizing exogenous PAMPs or endogenous DAMPs, and play a critical role in activating downstream defense pathways [7-9].

PAMPs are highly conserved molecular motifs crucial for microbial survival and interaction with the host. Several well-characterized PAMPs have been identified as central to PTI, including flg22



[10, 11], elongation factor thermo unstable (EF-Tu) [12, 13], certain lipopolysaccharides and oligosaccharides [11, 14, 15], oomycete glutamine aminotransferase and β -glucan, and fungal chitin and glucans [11, 16]. Flg22 is a conserved 22-amino-acid peptide derived from bacterial flagellin [17], and EF-Tu is one of the most abundant bacterial protein, which are specifically recognized by PRRs such as FLAGELLIN SENSING 2 (FLS2) and EF-Tu receptor (EFR) [12, 13], respectively. In addition to PAMPs, DAMPs are endogenous molecules released from damaged or dying plant cells during pathogen infection, activating immune responses to mitigate cellular damage. Furthermore, plants produce immune-modulating peptides known as cytokines, which either directly stimulate immune responses or interact with PRRs to modulate immune responses [18, 19].

Many pathogens have evolved mechanisms to overcome PTI by deploying effector proteins that suppress PRR-mediated immune responses. To counteract this, plants have developed the second layer of immunity mediated by disease resistance (R) protein, primarily nucleotide-binding and leucine-rich-repeat-containing receptors (NLRs). These receptors detect pathogen effectors either directly or indirectly through changes in host target proteins, triggering ETI [20].

There are three types of NLRs: coiled-coil NLRs (CC-NLRs/CNLs), TOLL/INTERLEUKIN-1 RECEPTOR/RESISTANCE NLRs (TIR-NLRs/TNLs), and resistance to powdery mildew 8 (RPW8)-like coiled-coil domain NLRs (RPW8-NLRs/RNLs) [21, 22]. CC-NLRs and TIR-NLRs function as receptor-NLRs, recognizing effectors and triggering immune responses; while RPW8-NLRs act as helper NLRs that transduce downstream signals [23]. Effector recognition by CC-NLRs and TIR-



NLRs often leads to the formation of oligomeric complex called ‘resistosome’, which mediate immune signaling [24]. For example, CC-NLRs oligomerize to form ion channels on the plasma membrane, while TIR-NLRs exhibit NAD⁺-cleaving enzymatic activity to initiate immune responses [24].

ETI provides a more robust and amplified defense response than PTI, often culminating in a hypersensitive response (HR), a localized cell death that restricts pathogen spread. Activation of ETI involves physiological responses such as ROS burst, MAPK activation, transcriptional reprogramming, and biosynthesis of defense hormones such as salicylic acid (SA) and ethylene (ET) [2, 25].

The dual-layered plant immune system was conceptualized as the “zigzag” model by Jones and Dangl in 2006, which remains a foundational framework for understanding plant-pathogen interactions. The zigzag model comprises four phases. First, PRRs recognizes PAMPs or MAMPs, initiating PTI to suppresses pathogen colonization. Second, adapted pathogens deploy effectors into plant cells to suppress PTI, allowing pathogen proliferation. This PTI-compromised state is termed effector-triggered susceptibility (ETS) [26]. Third, plant NLRs recognize pathogen effectors, triggering ETI. This amplified defense response often surpasses the activation threshold required for HR [27]. Fourth, pathogens evade ETI through natural selection, either by losing or altering the recognized effectors or by acquiring additional effectors that inhibit ETI [1].

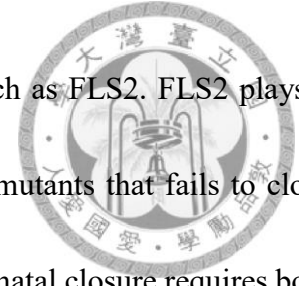
1.3 Stomatal Immunity for Plant Defense



Stomata, microscopic pores primarily located on the underside of plant leaves, are crucial for regulating gas exchange and water loss by modulating the size of their aperture. Each stomata is composed of two guard cells that respond to both abiotic and biotic stimuli. Environmental factors, such as relative humidity, CO₂ concentration, light intensity and temperature, significantly influence the stomatal movements [28, 29]. Beyond their physiological roles in photosynthesis and transpiration, stomata serve as critical entry points for pathogens such as bacteria, fungi, and viruses, thereby making them as integral components of the plant's innate immune defense [30, 31].

Stomatal immunity is a crucial defense mechanism that restricts pathogen entry into the plant. Upon pathogen detection, guard cells initiate stomatal closure within 1-2 hours to limit microbial invasion [32, 33]. This immune response can be triggered by various compounds, including abscisic acid (ABA), SA, ET, methyl jasmonate (MeJA), strigolactones (SLs), and cytokinins. Other elicitors include glucosinolate degradation products like allyl isothiocyanate (AITC), natural polymers such as chitin and its derivative chitosan, fungal elicitor cryptogein, cyclodipeptides, and the Arabidopsis secreted peptide PAMP-induced peptide (PIP1) [30, 34, 35]. Many of these compounds act through ABA or interact with ABA to activate defense responses, highlighting an essential role of ABA in stomatal defense [30].

Under abiotic stress, ABA-induced stomatal closure prevents excessive water loss under drought or low humidity conditions. ABA is synthesized in guard cells in response to these stress conditions



[36, 37]. In biotic stress, PAMPs such as flg22 are detected by PRRs such as FLS2. FLS2 plays a pivotal role in pathogen-induced stomatal closure, as evidenced by *fls2* mutants that fails to close their stomata in response to flg22 treatment [29]. This FLS2-mediated stomatal closure requires both ABA and SA signaling pathways [32, 33]. Mutants such as *eds16/sid2* (SA biosynthesis-deficient) or transgenic plants overexpressing salicylate hydroxylase gene (*nahG*) (SA-deficient) fail to trigger stomatal closure upon pathogen recognition. Similarly, mutants like *aba3* (ABA biosynthesis-deficient) and *ost1* (ABA signaling-deficient) also exhibit impaired stomatal closure in response to flg22 [32]. Experimental evidence indicates that SA acts upstream of ABA in flg22-FLS2 signaling, as exogenous ABA can restore stomatal closure in SA-deficient mutants, but exogenous SA cannot compensate stomatal closure in ABA biosynthesis mutants (e.g., *aba2-1*) [38].

In the absence stress, type 2C protein phosphatases (PP2Cs), specifically ABA-INSENSITIVE 1 (ABI1) and ABI2, inhibit protein kinase OPEN STOMATA 1/SUCROSE NONFERMENTING 1-RELATED PROTEIN KINASE 2.6 (OST1/SnRK2.6) in guard cells, maintaining stomatal opening by suppressing NADPH oxidases and ion channels like Slow Anion Channel-Associated 1 (SLAC1) [39]. During stress, ABA accumulates in leaves and binds to its receptors, PYRABACTIN RESISTANCE 1/PYRABACTIN RESISTANCE 1-LIKE/REGULATORY COMPONENT OF ABA RECEPTOR (PYR/PYL/RCAR), inhibiting PP2Cs activities and activating OST1/SnRK2.6 [40]. This activation phosphorylates and activates SLAC1, aluminum-activated anion channels 12 (ALMT12), and inward-rectifying K⁺-channel KAT1, causing ion efflux from guard cells [41-43].



Additionally, OST1 activate AtrbohF NADPH oxidase for ROS production, further contributing to stomatal closure [44]. Cytosolic Ca^{2+} signaling, also activated by OST1, reinforces SLAC1 activation [45-47]. The flg22-FLS2 pathway also activate MITOGEN-ACTIVATED PROTEIN KINASE 3 and 6 (MPK3 and MPK6), functioning in parallel to ABA signaling to induce stomal closure [48].

In addition to localized responses, stomatal defense can occur systemically. Abiotic stresses such as light, heat, and wounding can trigger rapid systemic stomatal closure within 10-15 minutes in untreated leaves [49, 50]. This systemic response under abiotic stress involves ABA and JA signaling but appears to be independent of SA, as shown by impaired responses in mutants defective in ABA signaling (*ost1*), JA biosynthesis (*aos*), or JA-Ile receptor (*coil*) under light stress. In contrast, *sid2* mutants (SA biosynthesis-deficient) exhibit normal systemic stomatal closure in response to light stress [49]. For pathogen-induced systemic stomatal immunity, inoculation of *Pseudomonas syringae* pv. tomato (*Pst*) DC3000 into Arabidopsis leaves triggers stomatal closure in distant, uninfected leaves [51]. This systemic stomatal defense is dependent on nonexpressor of pathogenesis-related genes 1 (NPR1) and is associated with changes in guard cell proteins, metabolites, and lipids linked to systemic acquired resistance (SAR) [52]. Lipid transfer proteins, such as 1.5.3 DEFECTIVE IN INDUCED RESISTANCE 1 (DIR1) and potato nonspecific transfer protein 10 (StLTP10), has also been implicated in systemic stomatal immunity signaling [53].

Systemic stomatal closure can also be induced by exogenous elicitors, such as chitosan in tomato and SA analog Acibenzolar-S-methyl (ASM) in Japanese radish, which activate ROS-mediated

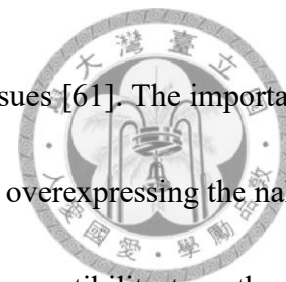


stomatal defenses [54, 55]. These findings suggest similarities between systemic stomatal defense and SAR, though the precise molecular mechanisms and signaling pathways governing systemic stomatal immunity remain poorly understood [56]. Further research is required to elucidate the interplay between local and systemic responses in stomatal defense.

1.4 Systemic Acquired Resistance (SAR)

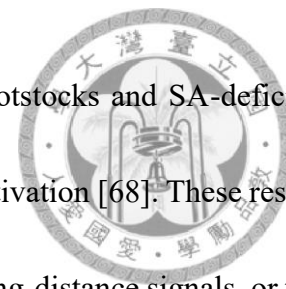
In addition to the immune responses eliciting at the infected sites, plants activate immune mechanisms in distal, uninfected tissues, a phenomenon known as SAR [57]. SAR is a whole-plant defense mechanism triggered by localized exposure to pathogens or pathogen-derived molecular patterns. This defense mechanism equips distal, uninfected tissues with enhanced immunity, enabling the plant to resist subsequent infections by a broad spectrum of pathogens. SAR is characterized by its long-lasting nature and its ability to provide immunity against not only the primary pathogen but also unrelated pathogens [58]. It was first observed in the early 20th century, where plants pre-inoculated with avirulent pathogens exhibited resistance to subsequent infections by virulent pathogens [59].

SAR is initiated in response to localized recognition of pathogen infection, where the two layers of plant innate immunity, PTI and ETI, are activated [1]. The systemic activation of SAR primarily depends on the phytohormone SA, which serve as a key signaling molecule. SA activates the expression of multiple PR genes, mediated by NPR1 [60]. Upon pathogen infection, SA levels



increase at the infected sites and to a lesser content in distal uninfected tissues [61]. The importance of SA in SAR signaling is evident from the studies using transgenic plants overexpressing the *nahG*, which depletes endogenous SA. These plants exhibit increased disease susceptibility to pathogen, demonstrating the pivotal role of SA in SAR activation [58]. PR genes code for a diverse array of low molecular weight proteins, initially identified in tobacco (*Nicotiana tabacum*) leaves infected with tobacco mosaic virus (TMV) [62]. PR proteins, particularly PR1, have been extensively studied as molecular markers for SAR in various plant species, including tobacco, Arabidopsis, and cucumber [58]. Recent studies have highlighted that PR1 possesses antimicrobial activity, obstructing pathogen growth by sequestering essential sterols [63].

A feature of SAR is the generation and systemic movement of mobile signaling molecules from the infected tissue to distal, uninfected parts of the plant [58]. These mobile signals prime distal tissues, enhancing them to mount a more rapid and robust defense upon subsequent pathogen attack. Early studies proposed SA as a potential mobile signal for SAR, as significant levels of SA were detected in phloem exudates from pathogen-infected cucumber and tobacco plants [61, 64, 65]. However, two lines of evidence suggest that SA may not function as the primary long-distance SAR signal. First, in cucumber plants, SAR could still be induced when infected leaves were removed within 6 hours post-inoculation, a time frame before SA accumulation in the phloem [64, 66]. Second, grafting studies in tobacco revealed that SAR could be triggered in WT scions grafted onto SA-deficient rootstocks, indicating that SA accumulation in rootstocks is not essential for SAR induction



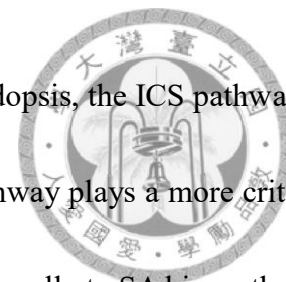
[64, 67]. Conversely, SAR was impaired in chimeric plants with WT rootstocks and SA-deficient scions, suggesting that SA accumulation in scions is necessary for SAR activation [68]. These results suggest that while SA is essential for SAR, it may not act as the primary long-distance signals, or that minimal SA accumulation in infected tissues may be sufficient to trigger systemic responses [64].

In addition to SA, the searches for SAR-related mobile signals has identified several candidate molecules that act individually or in combination to facilitate long-distance signaling. These include methyl salicylate (MeSA), a conserved three carbon sugar glycerol-3-phosphate (G3P), the lipid transfer protein DIR1, the dicarboxylic acid azelaic acid (AzA), the abietane diterpenoid dehydroabietinal (DA), the free radical nitric oxide (NO) and ROS, pipecolic acid (Pip) and its derivative N-hydroxypipecolic acid (NHP), and extracellular nicotinamide adenine dinucleotide (phosphate) [eNAD(P)] [69-77]. These molecules have been shown to induce systemic resistance when applied exogenously and are required for SAR activation upon pathogen infection.

1.5 Small Molecular Signaling in SAR

1.5.1 Salicylic acid (SA) and Methyl salicylate (MeSA)

SA, a phenolic compound and vital phytohormone, play a central role regulating plant growth, development, and immune responses against biotic and abiotic stresses [34]. SA biosynthesis occurs through two primary pathways: the isochorismate synthase (ICS) pathway and the phenylalanine ammonia-lyase (PAL) pathway, both starting from chorismic acid. The importance of these pathways



in SA biosynthesis varies in different plant species. For instance, in *Arabidopsis*, the ICS pathway is the most predominant contributor to SA accumulation, while the PAL pathway plays a more critical role in rice. In some species, such as soybeans, both pathways contribute equally to SA biosynthesis. In the PAL pathway, SA is synthesized via the conversion of cinnamate catalyzed by the PAL enzymes, as demonstrated in isotopic labeling studies [78]. Silencing PAL genes in tobacco or inhibiting PAL activity with 2-aminoindan-2-phosphonic acid (AIP) in *Arabidopsis*, tomato, cucumber, and potato significantly reduces SA accumulation following pathogen attack [78-82]. In the ICS pathway, chorismic acid is converted into SA through isochorismic acid, a reaction mediated by ICS1 and ICS2 [83]. Among these, ICS1 is the primary contributor to SA biosynthesis during pathogen infection [84, 85]. A deficiency in either pathway can impair or completely abolish SAR, underscoring their essential roles in plant immunity [82, 86].

SA has long been considered as a potential mobile signal for SAR. Radiotracer studies in tobacco have demonstrated that SA produced in pathogen-infected tissues can be transported via the phloem to uninfected, systemic leaves [76, 84, 87]. Furthermore, the enrichment of SA in the phloem exudates of infected leaves supports its mobility within the plant and its role in systemic defense signaling [88]. However, grafting experiments in tobacco have showed that SAR can still be induced in WT scions grafted onto SA-deficient rootstocks. This suggests that while SA plays an essential role in SAR activation, it is unlikely the primary mobile signal for SAR [76, 89].

Instead of SA, MeSA, an inactive, methylated form of SA, has been identified as a mobile signal



for SAR [76]. Grafting experiments in tobacco demonstrated that SA-binding protein (SABP2), an esterase responsible for converting MeSA into its activate form, SA, is required for SAR signal perception in systemic tissues. Mutations that impair the MeSA esterase activity of SABP2 weaken SAR and reduce MeSA levels in infected tissues, phloem exudates, and systemic leaves. Furthermore, inhibiting SA methyltransferase, the enzyme that converts SA into MeSA, prevents SAR induction. However, exogenous application of MeSA can restore SAR in untreated systemic leaves [76], further confirming its role as a mobile signal.

1.5.2 Glycerol-3-phosphate (G3P)

G3P, a universal three-carbon sugar, is an essential element in energy-generating processes like glycolysis and glycerolipid synthesis [90-92]. G3P is produced via dihydroxyacetone phosphate reduction, catalyzed by G3P dehydrogenase (G3PDH), or through glycerol phosphorylation by glycerol kinase (GK) [93, 94]. Mutants deficient in G3P synthesis, such as *gly1* and *gli1*, fail to activate SAR, but this can be restored through exogenous G3P application [70, 95]. Radiotracer studies have demonstrated that the transport of a G3P derivative to distal tissues depends on the lipid transfer protein DIR1, indicating the collaborative role of DIR1 and G3P in SAR [70].

1.5.3 DEFECTIVE IN INDUCED RESISTANCE 1 (DIR1) protein

DIR1 is a non-specific lipid transfer protein that plays a critical role for the generation and/or



translocation of mobile signals required for SAR. The Arabidopsis *dir1* mutant exhibits normal local resistance to pathogens but fails to develop SAR in systemic tissues. Despite exhibiting normal PTI and ETI and SA accumulation in local, infected tissues, the *dir1* mutant exhibits impaired systemic PR genes expressions and SAR-inducing activity in systemic, uninfected leaves. Petiole exudate experiments further highlight the importance of DIR1 in SAR signaling. Exudates collected from pathogen-inoculated *dir1* leaves are incapable of inducing PR1 gene expression in WT plants, while exudates from pathogen-inoculated WT leaves can induce PR1 gene expression in *dir1* plants. These findings suggest that DIR1 is required for the production or translocation of lipid-based SAR signal molecules [96].

1.5.4 Azelaic acid (AzA)

AzA, a nine-carbon dicarboxylic acid, has been identified as a mobile signal for SAR, found in petiole exudates of SAR-induced Arabidopsis leaves [97]. Exogenous application of AzA promotes its accumulation in both petiole exudates and systemic leaves, priming plants for a more robust and faster SA-dependent immune responses against subsequent pathogen infection [97-99]. However, AzA alone does not directly increase SA levels or PR1 expression; instead, it act as a priming factor, enhancing SA-mediated defense upon pathogen challenge [75]. The lipid-transfer protein DIR1 and acid induced 1 (AZI1), an AzA-induced secreted protease-inhibitor/seed-storage/lipid-transfer family protein, are both essential for AzA signaling. This suggests that DIR1 and AZI1 collaborate to mediate

SAR activation and facilitate the translocation of AzA or associated mobile signals [97, 100].



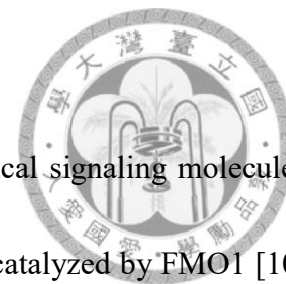
1.5.5 Dehydroabietinal (DA)

DA, an abietane diterpenoids found in the resins of conifer trees, functions as a SAR inducer and is rapidly distributed within plants [71, 98]. Local application of DA induces SA accumulation, PR1 expression, and SAR in both treated and untreated systemic leaves of Arabidopsis, tobacco, and tomato. However, DA-induced SAR is dependent on SA biosynthesis and signaling, as SAR fails in SA-deficient mutants, such as *nahG* plants and *ics1 ics2* double mutants [71, 101].

1.5.6 Pipecolic acid (Pip)

Pipecolic acid (Pip), a lysine-derived metabolite, has emerged as a critical component of SAR. Pip biosynthesis is catalyzed by AGD2-Like Defense Response Protein 1 (ALD1) and SAR-DEFICIENT4 (SARD4) [102]. Pathogen infection trigger the accumulation of Pip in both local and systemic leaves across monocot and dicot plants, including rice, potato, tobacco, and soybean [102-105]. Exogenous application of Pip induces systemic immunity and stomatal closure in Arabidopsis leaves [106]. The *ald1* mutant, impaired in Pip biosynthesis, fails to activate SAR and exhibit reduced Pip levels in petiole exudates [107]. Furthermore, the overaccumulation of Pip in the *fmo1* mutant, which is defective in SAR, highlights the role of flavin-dependent-monooxygenase1 (FMO1) in converting Pip into NHP, another critical SAR signal [75].

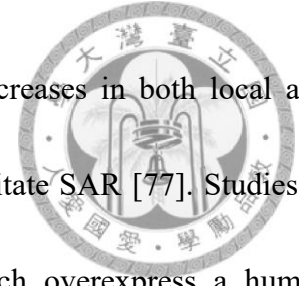
1.5.7 N-Hydroxypipecolic acid (NHP)



NHP, a hydroxylated derivative of Pip, is now recognized as a critical signaling molecule in SAR. NHP is synthesized from Pip through an N-hydroxylation reaction catalyzed by FMO1 [108]. During pathogen infections, high levels of NHP accumulates in infected leaves and systemic, uninfected leaves across diverse plant species [109]. The essential role of NHP in SAR is underscored by the fact that SAR is impaired in *fmo1* mutants despite their high levels of Pip, indicating that NHP, not Pip, is necessary for systemic resistance [110]. Exogenous application of NHP significantly enhance pathogen resistance in the WT plants and restores SAR in *ald1* and *fmo1* mutants, confirming the critical role of NHP in SAR [72, 110]. Recent studies have identified NHP as a mobile SAR signal. In *fmo1* mutants, local application of NHP leads to the detection of NHP metabolite in systemic leaves, demonstrating its transport to distal tissues [72]. Furthermore, NHP application stimulates the expression of ALD1, SARD4, and FMO1 in both local and systemic tissues, suggesting the presence of a positive feedback loop that enhances the production of Pip and NHP during SAR [72]. This self-amplifying mechanism highlights NHP as a pivotal activator of systemic immunity.

1.5.8 Extracellular nicotinamide adenine dinucleotide (phosphate) [eNAD(P)]

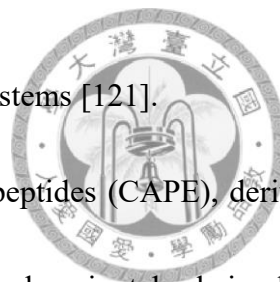
NAD and its phosphorylated form NADP, collectively referred to as NAD(P), are essential pyridine nucleotides that function as electron carriers in metabolic pathways [111]. Beyond their metabolic roles, extracellular NAD(P) [eNAD(P)] have been identified as signaling molecules



involved in plant defense. Upon pathogen infection, eNAD(P) level increases in both local and systemic tissues, acting as a signal to trigger defense responses and facilitate SAR [77]. Studies in transgenic *Arabidopsis* lines deficient in eNAD(P) accumulation, which overexpress a human NAD(P)-hydrolyzing enzyme CD38, or in NAD(P) receptor lectin receptor kinase (LecRK), LecRK-VI.2 or its co-receptor BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 (BAK1), *lecrk-VI.2* or *bak1*, respectively, show compromised SAR, highlighting the critical role of eNAD(P) in systemic signaling. Interestingly, recent studies revealed a crosstalk between NHP and eNAD(P) signaling during SAR. NHP induces the systemic accumulation of eNAD(P) through ROS production mediated by the Respiratory Burst Oxidase Homolog F (RBOHF) [111, 112]. This crosstalk between NHP and eNAD(P) emphasizes a coordinated signaling network that amplifies systemic immunity in plants.

1.6 Cytokines AtCAPE9 Involved in Plant Immune Responses

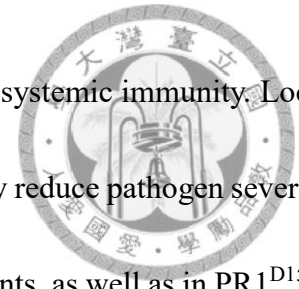
Cytokines are endogenous signaling peptides that play essential roles in plant growth and immune response. The immunomodulate peptide cytokines are released into the apoplast in response to pathogen infections, where they are recognized by their corresponding receptors and co-receptors, triggering downstream signaling pathways [113-117]. Similar to PAMPs, certain cytokines activate canonical PTI responses, including ROS production, Ca^{2+} influx, MAPK activation, and the expression of PTI marker genes [118-120]. By amplifying immune responses and enhancing resistance



against pathogens, cytokines serve as critical regulators of plant defense systems [121].

Among the immunomodulatory peptide cytokine, the CAP-derived peptides (CAPE), derived from PR1 protein, have recently been identified by our group and found to play pivotal role in plant immunity. PR1, a member of the cysteine-rich secretory protein, antigen 5, and pathogenesis-related 1 protein (CAP) superfamily, has long been recognized as a molecular marker for SAR and SA-regulated immunity [122]. However, the function of PR1 remained elusive until 2014, when it was identified as the precursor protein for the highly active cytokine CAP-derived peptide SICAPE1 in tomato [123].

The first CAPE peptide, SICAPE1, was identified in tomato under wounding conditions. SICAPE1 was shown to trigger anti-pathogen and mild anti-herbivore responses, contributing to plant defense without significantly inducing programmed cell death [123]. Building upon this foundational discovery, our subsequent studies identified AtCAPE9 in Arabidopsis and established its pivotal role in enhancing SA-mediated immune responses in Arabidopsis. Biochemical analyses demonstrated that synthetic AtCAPE9 doubles SA concentrations and significantly enhances resistance to *Pst* DC3000, reducing disease severity and pathogen loads in leaves and seedlings [123]. AtCAPE9 operates within a positive feedback loop with SA to amplify immune signaling. SA treatment increases endogenous AtCAPE9 levels approximately six-fold, while AtCAPE9, in turn, enhances SA accumulation and SA-mediated immunity. This reciprocal interaction establishes AtCAPE9 as a crucial mediator of plant defense, linking PR1 processing with SAR activation [124].



In addition to its role in local defense, AtCAPE9 is indispensable for systemic immunity. Local treatment with AtCAPE9 have been shown to trigger SAR and significantly reduce pathogen severity in untreated, systemic leaves. This systemic effect is abolished in *pr1* mutants, as well as in PR1^{D150A} (a mutant with a disrupted cleavage site), PR1^{ΔCAPE9} (AtCAPE9-truncated PR1), and *xcp1* (deficient in the cleavage enzyme responsible for producing AtCAPE9) mutants. These findings underscore the importance of proteolytic release of AtCAPE9 from PR1 and its critical role in systemic signaling. Interestingly, while SAR triggered by the bacterial PAMP flg22 is impaired in mutants deficient in SA biosynthesis (*ics1*) or SA perception (*npr1*) mutants, AtCAPE9 is still able to trigger SAR in these mutants. This suggest that AtCAPE9 can function as a SA downstream or SA-independent signal in systemic immunity [124]. Additionally, unpublished data from our laboratory, provided by Chang, demonstrate that AtCAPE9 can induce both local and systemic stomatal immunity.

1.7 Role of Metabolome in Plant Defense Mechanisms

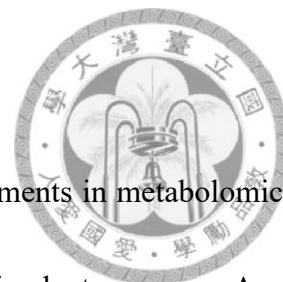
Plant metabolites are fundamental components of plant defense mechanisms, functioning as both signaling molecules and direct defensive agents. Upon recognition of PAMPs or MAMPs, plants initiate molecular signaling cascades that trigger physiological and biochemical changes, leading to resistant, tolerant, or susceptible phenotypes [125]. Similar to transcriptomics and proteomics, metabolomics has emerged as a powerful tool for comprehensive profiling of small molecules involved in plant immune responses [126]. By capturing the metabolic shifts that occur during



pathogen infection, signal transduction, and defense activation, metabolomics provides insights into the dynamic roles of metabolites in plant immunity. Early molecular signaling events in plant defense include the production key signaling molecules such as SA, MeJA, NO and ET [127-129]. These compounds act not only as antimicrobial agents direct against pathogens but also as mediators that prime both local and systemic tissues for enhanced resistance [130]. Furthermore, non-hormonal metabolites such as amino acids [131], lipids [132], and ROS contribute to the intricate signaling that regulate plant defense, often interacting in intricate cross-talk networks to fine-tune immune responses [133, 134].

Plant immune responses depend on the coordinated action of primary and secondary metabolites. Primary metabolites, including carbohydrates, organic acids, amino acids, and lipids, play fundamental roles in structural modifications, energy production, and nitrogen metabolism, all of which are essential for supporting defense responses [135]. For examples, carbohydrates provide the structural components required for cell wall reinforcement, a crucial barrier against pathogen invasion. Similarly, organic acid like malate regulates stomatal closure, restricting pathogen entry [136]. Secondary metabolites, such as phytoalexins, terpenoids, phenolics, alkaloids, and glucosinolates, serve specialized roles in defense, directly targeting pathogen toxicity or signaling for intra- and inter-plant communication [137]. The dynamic interplay between primary and secondary metabolites underscores the complexity of plant defense mechanisms, with metabolomics emerging as a powerful tool for elucidating these processes by capturing both signaling molecules and defense-related

metabolites [126].



The study of plant metabolomes has been revolutionized by advancements in metabolomics, a field that enables the comprehensive profiling of small molecules involved in plant processes. Among the primary analytical techniques used in metabolomics, mass spectrometry (MS) and nuclear magnetic resonance (NMR) are the major analytical techniques used for metabolomics analysis. MS is particularly valuable for plant metabolomics due to its high sensitivity for detecting low-abundance molecules and its ability to analyze a wide range of chemical classes. NMR, on the other hand, provides structural insights without the need for extensive sample preparation, though it is less sensitive compared to MS. To further enhance the performance of MS in metabolomics analysis, it is frequently coupled with, creating robust liquid chromatography (LC)-MS platforms. LC plays a critical role in separating complex mixtures of metabolites prior to their detection by MS, significantly improving resolution and detection accuracy. However, a single LC-MS platform cannot comprehensively profile the entire metabolome due to variations in ionization and separation efficiencies that depend on the physicochemical properties of metabolites. As a result, multiple LC-MS platforms, using different chromatographic separation techniques (e.g., reverse-phase liquid chromatography [RPLC] and hydrophilic interaction liquid chromatography [HILIC]) and ionization modes (positive and negative), are often employed to maximize metabolome coverage. By integrating complementary LC-MS platforms, researchers can achieve a more comprehensive and accurate characterization of the diverse metabolites involved in plant biological and defense processes [126].

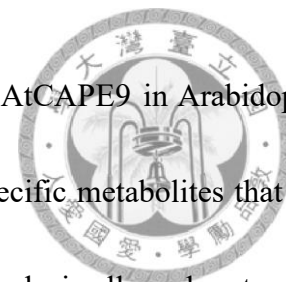
1.8 Propose of This Study



Plants have evolved complex defense mechanisms to counteract pathogen attacks and ensure survival. Among these, cytokines play a crucial role in modulating various immune responses in plants. Our recent discoveries have highlighted the significance of immune peptide AtCAPE9 in modulating plant immunity. In *Arabidopsis*, AtCAPE9, a peptide derived from the PR1 precursor, has been implicated in modulating metabolic pathways that produce immune-related compounds. By inducing specific metabolites, peptides like AtCAPE9 not only strengthen local immune responses but also facilitate long-distance signaling, contributing to the establishment of SAR. Understanding AtCAPE9's orchestration of these metabolic changes is critical to elucidating the mechanisms by which plants orchestrate complex immune responses and maintain resistance across different tissues.

Metabolomic changes are central to plant defense, as metabolites act as either defensive compounds or as signaling molecules to mediate immune responses. In tissues where immune signaling peptides like AtCAPE9 are active, these metabolites may either directly enhance pathogen resistance or function as signaling molecules for downstream events. Investigating the local metabolic changes induced by AtCAPE9 can reveal key metabolites that play pivotal roles in stress responses, immune signaling, and systemic defense induced by AtCAPE9.

The purpose of this study is to elucidate the metabolic signals induced by AtCAPE9 that promote local and systemic immunity in *Arabidopsis*. The research is organized around three primary research objectives focusing on the role of AtCAPE9-induced metabolites in *Arabidopsis* immunity:

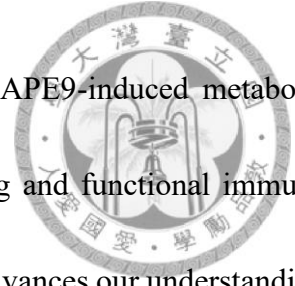


First, we aim to identify the downstream metabolites regulated by AtCAPE9 in *Arabidopsis* leaves. By employing a metabolomics approach, we characterized the specific metabolites that are upregulated or downregulated in response to AtCAPE9 treatment. This analysis allowed us to map the biochemical pathways influenced by AtCAPE9 and to identify compounds that play critical roles in AtCAPE9-induced plant immunity.

Second, we aim to identify the mobile signaling molecules responsible for AtCAPE9-induced SAR. AtCAPE9 has been shown to induce SAR in distal, systemic leaves, highlighting the role of mobile signals in facilitating long-distance immune communication. A mobile signal for SAR is expected to be upregulated in the local, treated tissue and transported through the phloem to systemic, uninfected tissues. To identify such signals, we analyzed the metabolite profile of phloem exudates from AtCAPE9-treated plants and compared them to those of treated leaves. Metabolites that were upregulated in both the leaves and the phloem exudates were considered as candidates for mobile signaling molecules associated with AtCAPE9-induced SAR. Their identities were further verified using the authentic standards. This approach provided critical insights into the systemic signaling mechanisms induced by AtCAPE9.

Third, we evaluated the functional relevance of these candidate metabolites in triggering immune responses. Specifically, we tested their effects on local and systemic stomatal immunity, a key component of the plant's defense against pathogens. By examining how these metabolites influence stomatal behavior, we can directly assess their capacity to induce immune functions in *Arabidopsis*.

Together, this research provided a comprehensive view of the AtCAPE9-induced metabolic response, linking local metabolite changes to systemic immune signaling and functional immune responses. By characterizing these metabolites and their roles, this study advances our understanding the molecular basis of AtCAPE9-induced systemic stomatal immunity.



2. Material and Method



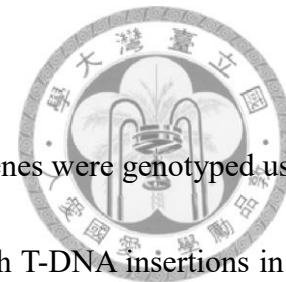
2.1 Chemicals and Reagents

LC-MS grade methanol (MeOH), chloroform, and acetonitrile (ACN) were purchased from J.T. Baker (Gliwice, Poland). Water was produced by a Milli-Q Reagents Water System (Millipore; Burlington, MA, USA). Potassium chloride (KCl), potassium hydroxide (KOH), sucrose, ethylenediaminetetraacetic acid (EDTA), ammonium formate (AmF), ammonium acetate, and formic acid (FA) were purchased from Sigma Aldrich (St. Louis, MO, USA). AtCAPE9 were purchased from Biotools (New Taipei City, Taiwan). Metabolite standards N-hydroxypipecolic acid (NHP) and riboflavin were purchased from MedChemExpress (Monmouth Junction, NJ, USA) and Sigma Aldrich, respectively. 2-(N-morpholino)ethanesulfonic acid (MES) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Half-strength Murashige and Skoog ($\frac{1}{2}$ MS) agar purchased from Duchefa Biochemie (Haarlem, Netherlands).

2.2 Plant Material and Growth Conditions

The wild-type (WT) seeds of *Arabidopsis thaliana* Columbia (Col-0, CS70000), and all T-DNA insertion mutant lines (*pr1*, *fmo1*, *fin4*, and *lecrk-VI.2*) were obtained from the Arabidopsis Biological Resource Center (ABRC). Arabidopsis plants grown in soil were cultivated in a growth chamber with a 10/14 h light/dark cycle (for vegetative growth and delayed flowering) or a 16/8 h light/dark cycle (for seed collection) at 22°C during the light period and 20°C during the dark period.

2.3 Generation and Genotyping of T-DNA Insertion Lines



Homozygous T-DNA insertion lines for the *FIN4* and *LecRK-VI.2* genes were genotyped using primer sequences listed in **Table 1**. WT *Arabidopsis* and mutant lines with T-DNA insertions in the *FIN4* and *LecRK-VI.2* genes were cultured on ½ MS basal medium, supplemented with 10 g/L sucrose, 0.5 g/L MES, and 8 g/L agar. The pH of the medium was adjusted to 5.7 using KOH. To ensure uniform germination, plates were kept in the dark at 4°C for 2 days before being transferred to a climate room. Seedlings were harvested for genomic DNA (gDNA) isolation after 13 days of growth.

For the PCR assays, a Phire Plant Direct PCR Master Mix (Thermo Fisher Scientific) was utilized. Briefly, approximately 2 mm diameter of leaf tissue was harvest from 1 single rosette leaf of 13-day-old plants and ground in a 0.2 mL PCR tube, followed by the addition of 20 µL dilution buffer. The leaf tissue was then crushed, and the plant material was spun down to separate supernatant. A 0.5 µL aliquot of the supernatant was used as the template in a 10 µL PCR reaction. The PCR reaction mixture consisted of 5 µL 2X Phire Plant Direct PCR Master Mix, 0.5 µL of each of forward primer (FP), reverse primer (RP), and base primer (BP), and was adjusted to a total volume of 10 µL with double-distilled water (ddH₂O). This mixture was amplified using a PCR machine (ProFlex PCR System, Biosystems). The PCR products were subsequently analyzed by electrophoresis in a 1% (w/v) agarose gel with 1 x TAE buffer at 200 V for 25 min.

2.4 Metabolomics Analysis of Leaves and Phloem Exudates

2.4.1 Sample Preparation of Arabidopsis Leaves



For untargeted metabolomics of leaves, 12 rosette leaves were detached from three 5- to 6-week-old Arabidopsis plants. The detached leaves were pre-incubated in ddH₂O (molecular biology grade, autoclaved) under light conditions for 2 hours. Following pre-incubation, the leaves were treated with either ddH₂O or ddH₂O supplemented with 500 nM AtCAPE9. Petri dishes containing the treated leaves were covered with lids and incubated under light conditions for 4 hours.

To extract leaf metabolites, fresh leaves tissues were ground into a fine powder under liquid nitrogen. Approximately 0.3 g of the powdered tissue was transferred into a 2 mL Eppendorf tube. A total of 1.125 mL of chilled chloroform-MeOH mixture (2:1; v/v) was added to the frozen powder, followed by gentle agitation for 15 minutes. To facilitate phase separation, 375 μ L of cold ddH₂O was added, and the mixture was agitated for an additional 15 minutes. The samples were centrifuged at $13,000 \times g$ at 4 °C for 10 minutes, resulting in the formation of two distinct layers. The upper hydrophilic layer was carefully transferred to a fresh 2 mL Eppendorf tube and evaporated to dryness using a vacuum centrifugal concentrator.

The dried residue was reconstituted in 100 μ L of 50% MeOH, thoroughly mixed, and centrifugated at $13,000 \times g$ at 4 °C for 15 minutes. The supernatant was carefully transferred to a sample vial for untargeted metabolomics analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques.

2.4.2 Sample Preparation of Arabidopsis Phloem Exudates



For untargeted metabolomics of phloem exudates, 16 rosette leaves from three individual 5- to 6-week-old *Arabidopsis* plants were excised at the petiole near the center of the rosette. The leaves were pre-incubated in ddH₂O under light conditions for 2 hours. Following pre-incubation, the leaves were treated with either ddH₂O (control) or ddH₂O supplemented with 100 nM AtCAPE9. Petri dishes containing the treated leaves were covered with lids and incubate under light conditions for 1 hour.

Phloem exudates was collected using an EDTA-facilitated exudation method, modified from Tetyuk et al., 2013 [138]. To measure the exudation rate per mg of fresh weight (FW), the tissue FW were recorded at the start of the experiment. After the peptide treatment, the base of the petioles (approximately 1 mm) was recut, and the leaves were immediately transferred into reaction tubes containing 20 mM EDTA solution (adjusted to pH 7.5 by NaOH). The leaves were incubated in a dark, humid chamber for 1 hour to prevent the plant's self-sealing mechanism by chelating Ca²⁺ ions [139, 140]. To minimize potential damaging effects and reduce interference from EDTA in the subsequent analysis, the leaves were then transferred to ddH₂O and placed in a dark, humid chamber for an additional 3 hours to collect the phloem exudates [138, 141]. After the collection period, the leaves were removed and discarded, and the exudate were dried using a vacuum centrifugal concentrator. The dried residue was reconstituted in 50 μ L of 50% MeOH, thoroughly mixed, and centrifugated at 13,000 $\times$ g at 4 $^{\circ}$ C for 15 minutes. The supernatant was carefully transferred to a sample vial for untargeted metabolomics analysis using LC-MS/MS techniques.



For both leaves and phloem exudates, quality control (QC) samples were prepared by pooling and mixing equal volumes from all respective samples to enhance metabolite identification and monitor the stability of both the instrument and samples during analysis. Blank samples, consisting of 50% MeOH used for reconstitution, were included to assess potential carryover, background noise, and contamination.

2.4.3 LC-MS/MS Analysis

For untargeted metabolomics profiling, a tribrid mass spectrometer (Orbitrap™ IQ-X™, Thermo Fisher Scientific) was coupled with an online UHPLC system (Vanquish UHPLC, Thermo Fisher Scientific). Two types of columns were employed to achieve differential selectivity of polar metabolites: the Acquity HSS T3 C18 reverse-phase column (1.8 μm , 100 mm $\times$ 2.1 mm; Waters Corporation, Milford, MA, USA) and the Acquity BEH HILIC column (2.7 μm , 100 mm $\times$ 2.1 mm; Waters Corporation).

For the HSS T3 columns, the flow rate was set at 0.4 mL/min, with a mobile phase consisting of 0.1% formic acid in 2% ACN as solvent A and 0.1% formic acid in 100% ACN as solvent B. The elution gradient was set as follows: 0 – 1 minute at 1% B, followed by a linear increase to 99% B over 7.5 minutes, maintained for 1.2 minutes. The column was equilibrated at 1% B for 1.3 minutes before the next run. The injection volume for this column was 2 μL .

For the BEH HILIC column, the mobile phases varied based on the ionization mode, with the

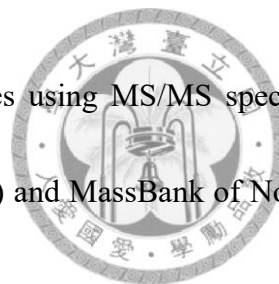


flow rate set at 0.4 mL/min for both modes. In positive ionization mode, the mobile phases consisted of 20 mM ammonium formate in 50% ACN at pH 3 (solvent A) and 20 mM ammonium formate in 90% ACN at pH 3 (solvent B). For negative ionization mode, the mobile phases consisted of 20 mM ammonium acetate in 50% ACN at pH 9 (solvent A) and 20 mM ammonium acetate in 90% ACN at pH 9 (solvent B). Both modes used the same elution gradient: 0 minutes at 99.9% B, a linear decrease to 65% B over 1 minutes, then to 55% B over 5.5 minutes, and finally to 0.1% B over 7.5 minutes, maintained at 0.1% B for 2 minutes. The column was equilibrated at 99.9% B for 2 minutes before the next run. The injection volume for this column was 1 μ L.

The MS utilized a data-dependent acquisition (DDA) method consisting of one MS1 scan and multiple MS2 scans at a fixed cycle time of 0.4 seconds. The MS1 scan was performed across an m/z range of 70 to 1,000 at a resolution of 60,000, while the MS2 scan was conducted at a resolution of 30,000. The intensity threshold for triggering MS2 acquisition was set to 25,000. To enhance the identification of metabolites, four different collision methods were employed for MS2 acquisition: higher energy collision (HCD) at 25 and 45 normalized collision energy (NCE) with 15% stepped energy, and collision-induced dissociation (CID) at 30 and 35 NCE.

2.4.4 MS Data Process and Statistical Analysis

The MS raw data were visualized and processed using MS-DIAL (v4.90) software [142], which facilitate the feature detection, metabolite annotation, and peak alignment between samples.



Metabolite identification was conducted through spectral library searches using MS/MS spectral databases from the National Institute of Standards and Technology (NIST) and MassBank of North America (MoNA).

The principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were conducted using MetaboAnalyst 6.0 [143] with the raw peak area values for all features. PCA was utilized to visualize the unsupervised metabolic variations between AtCAPE9- and mock-treated samples, highlighting global differences in metabolite composition. To further explore treatment-specific metabolic changes, a supervised discriminatory method, OPLS-DA, was applied for pairwise comparisons between the treatment groups. In each OPLS-DA model, metabolites were ranked based on their variable importance in projection (VIP) scores, with features exhibiting VIP values greater than 1 considered key discriminators for distinguishing between treatments.

To identify metabolite features regulated by AtCAPE9, a paired Student's *t*-test (one sample, null hypothesis: no change, mean $\mu = 0$) was applied to the $\log_2$ ratios of metabolite peak areas from AtCAPE9- and mock- treated samples across three biological replicates. Features with a $\log_2$ fold-change greater than 0.58 or less than -0.58 and a *p*-value < 0.05 were deemed significantly up- or down-regulated. For the significantly regulated metabolites, pathway enrichment analysis was performed using MetaboAnalyst 6.0, which integrates comprehensive metabolite pathway databases, including KEGG and the Human Metabolome Database (HMDB). This analysis identified the most

significantly altered metabolic pathways, providing insights into the biochemical pathways influenced by AtCAPE9 treatment.

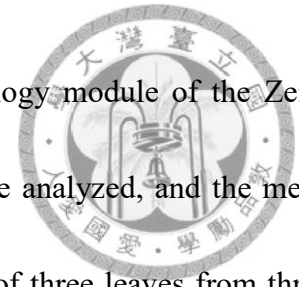


2.5 Local and Systemic Stomatal Aperture Assay

For the local stomatal aperture assay, three rosette leaves were detached from three 5- to 6-week-old *Arabidopsis* plants. To stabilize stomatal opening, the leaves were pre-incubated in 0.5 mL of stomatal buffer (10 mM MES and 30 mM KCl, pH 6.15) for 2 hours under light conditions. After pre-incubation, the leaves were treated with either stomatal buffer (control) or stomatal buffer supplemented with 100 nM AtCAPE9, 0.4 mM riboflavin, 1 mM NHP, 0.2 mM NAD, or 0.4 mM NADP. The petri dishes containing the leaves were covered with lids and incubated under light conditions for an additional 1 hour. For the time course experiment, stomatal responses were monitored at 15, 30, 45, and 60 minutes. Stomatal apertures were subsequently measured to assess the effect of each treatment over time.

For the systemic stomatal aperture assay, three rosette leaves from three 5- to 6-week-old *Arabidopsis* plants were selected (Leaves #7-12) and subjected to pressure infiltration with either stomatal buffer (control) or stomal buffer supplemented with 100 nM AtCAPE9, 0.4 mM riboflavin, 1 mM NHP, 0.2 mM NAD, or 0.4 mM NADP. Following infiltration, the entire rosette was incubated for 1 hour under light conditions. After incubation, systemic (non-infiltrated) leaves (Leaves #13-18) were excised, and stomatal apertures were examined using a light microscope

Stomatal aperture measurements were conducted using the physiology module of the Zeiss AxioVision Rel.4.8 or ZEN 3.4 software. For each leaf, 30 stomata were analyzed, and the mean stomatal aperture width was calculated. Each treatment group consisted of three leaves from three individual plants. Statistical analysis was performed using one-way ANOVA followed by post-hoc Tukey HSD tests to determine significant differences between treatment groups. A *p*-value of less than 0.05 were considered statistically significant. Data were plotted as mean with 95% confidence interval and statistically significant differences between groups were indicated by different letters.



3. Result



3.1 Comprehensive Profiling of Metabolome Changes Induced by AtCAPE9 in Arabidopsis Leaves

The workflow for the comprehensive profiling of metabolome changes induced by AtCAPE9 in Arabidopsis leaves is illustrated in **Figure 1**. To investigate metabolism changes induced by AtCAPE9 in Arabidopsis leaves, a data-dependent acquisition (DDA) UHPLC-MS/MS analysis was employed to analyze the leaves from Arabidopsis WT plants treated with AtCAPE9 or ddH₂O (Mock) for 4 hours post-treatment. To achieve comprehensive metabolites coverage, both reversed phase liquid chromatography (RPLC) and hydrophilic interaction liquid chromatography (HILIC) were utilized, with analyses performed in both positive (POS) and negative (NEG) ionization modes [144, 145]. RPLC, employing an Acquity HSS T3 C18 column (T3), is optimized for detecting less polar to middle polar compounds, such as lipids, organic acids, hormones, and amines. In contrast, HILIC, using a silica-based column, is better suited for highly hydrophilic compounds, including amino acids, biogenic amines, sugars, and nucleotides.

After MS acquisition and data processing, the numbers of detected metabolic features across the four methods are shown in **Figure 2**. A total of 11,627 features were detected in T3 with positive mode (T3_POS), 6,418 in T3 with negative mode (T3_NEG), 7,770 in HILIC with positive mode (HILIC_POS), and 6,566 in HILIC with negative mode (HILIC_NEG). Our DDA analysis successfully acquired MS/MS spectra for a substantial portion of these detected features, with 3,506



(30%) for T3_POS, 2,641 (41%) for T3_NEG, 3,860 (50%) for HILIC_POS, and 2,752 (42%) for HILIC_NEG. Metabolite identification were performed through spectral matching against a combined spectral library consisting of spectra from the National Institute of Standards and Technology (NIST) and MassBank of North America (MoNa). This approach enabled us to obtain the putative metabolite identification for a total of 1,131 (10%) features in T3_POS, 514 (8%) in T3_NEG, 966 (13%) in HILIC_POS, and 478 (7%) in HILIC_NEG.

These identified metabolites were further categorized into ClassyFire Superclass categories [146], revealing a diverse assay of chemical entities detected across the four analytical methods (**Figure 3**). These included lipids and lipid-like molecules, organoheterocyclic compounds, organic acids and derivatives, benzenoids, phenylpropanoids and polyketides, organic oxygen compounds, organosulfur compounds, nucleosides, nucleotides, and analogues, organic nitrogen compound, alkaloids and derivatives, lignans, neolignans and related compounds, hydrocarbon derivatives, organoheterocyclic, organohalogen compounds, and homogenous non-metal compounds. To visualize both unique and overlapping metabolites across the four methods, a Venn diagram of the identified metabolites was generated, revealing a total of 1,764 non-redundant metabolites identified in our metabolomics analysis (**Figure 4A**). Among these, only 18 (1.02%) were consistently identified across all four datasets, while 1,412 (80.05%) were unique to specific datasets. Specifically, 519 and 208 metabolites were uniquely identified using the T3 column in positive and negative ionization modes, respectively, whereas 504 and 162 were unique to the HILIC column in positive



and negative ionization modes, respectively. The classification of metabolites uniquely identified by the T3 column (combining both positive and negative modes) and the HILIC column (combining both positive and negative modes) is depicted in **Figure 4B**, highlighting the distinct chemical classes captured by each column technology and emphasizing their complementary capabilities in metabolite profiling.

To further elucidate the metabolic pathways coverage of our metabolomics analysis, we conducted an in-depth investigation of 1,764 identified metabolites using MetaboAnalyst 6.0 (**Figure 5**). This analysis revealed the involvement of 66 distinct metabolic pathways among these identified metabolites, encompassing a wide range of biochemical categories, including metabolisms of amino acids, carbohydrates, nucleotides, cofactors and vitamins, secondary metabolite, terpenoid and polyketide (**Figure 5**). These pathways play crucial roles in various aspects of plant growth, development, and defense mechanisms (**Table 2**).

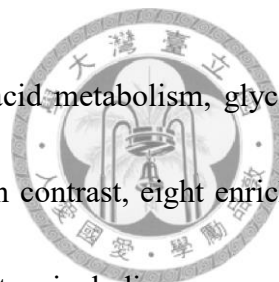
Collectively, these results emphasize the importance of employing multiple analytic methods in metabolomics analysis. Specifically, combining two chromatographic techniques, RPLC and HILIC, with both positive and negative ionization modes enables comprehensive metabolite profiling. This integrative approach facilitates the detection of a broad spectrum of chemical classes, ranging from hydrophilic to hydrophobic compounds, thereby providing a more complete and detailed view of AtCPAE9-regulated metabolome.

3.2 Identification of Metabolites and Key Metabolic Pathways Regulated by AtCAPE9

To visualize the overall metabolic profile changes induced by AtCAPE9 in Arabidopsis leaves, data from all four analytical methods were integrated and subjected to principal component analysis (PCA). The PCA score plot (**Figure 6A**) revealed a moderate separation between AtCAPE9- and mock-treated samples, with PC1 and PC2 accounting for 43.9% and 24.4% of the total variance, respectively.

To further identify the specific metabolites regulated by AtCAPE9, a volcano plot was generated to compare metabolite abundances between AtCAPE9- and mock-treated samples. In the volcano plot, each data point represents a unique metabolite feature, color-coded according to its regulatory status: red for up-regulated features ($p\text{-value} < 0.05$ and fold change > 1.5), blue for down-regulated features ($p\text{-value} < 0.05$ and fold change < 0.67), and gray for unchanged metabolites (**Figure 6B**). This analysis identified 553 up- and 603 down-regulated features in AtCAPE9-treated Arabidopsis leaves, indicating a substantial metabolic change induced by AtCAPE9. Among these, 35 up-regulated and 36 down-regulated were successfully annotated with metabolite identifications through spectral matching and selected as candidate metabolites for subsequent analyses. Detailed information on these candidate metabolites is provided in **Tables 3** and **4**.

To elucidate the metabolic pathways regulated by AtCAPE9, we conducted a pathway enrichment analysis of the regulated metabolites using MetaboAnalyst. This analysis revealed six enriched metabolic pathways associated with the 35 up-regulated metabolites, including purine



metabolism, riboflavin metabolism, zeatin biosynthesis, alpha-linolenic acid metabolism, glycine, serine and threonine metabolism, and glycerophospholipid metabolism. In contrast, eight enriched metabolic pathways were identified with the 36 down-regulated metabolites, including propanoate metabolism, ascorbate and aldarate metabolism, starch and sucrose metabolism, glutathione metabolism, biosynthesis of various plant secondary metabolites, cysteine and methionine metabolism, amino sugar and nucleotide sugar metabolism, and glucosinolate biosynthesis. These results revealed that AtCAPE9 regulates a diverse range of metabolic pathways, several of which are intricately linked to plant immunity, growth, and abiotic stress tolerance, highlighting its central role as a cytokine in modulating complex physiological processes in Arabidopsis. The detailed results of the pathway enrichment analysis, including significance levels and impact values, are summarized in **Table 5** and **6**.

3.3 Role of AtCAPE9-Induced Metabolites in Plant Immune Responses

To further elucidate how AtCAPE9 modulate plant immune responses, we conducted a comprehensive literature analysis of these candidate metabolites (**Table 7**). Among the 35 up-regulated metabolites, N-hydroxy-pipecolic acid (NHP), and 12-oxo-phytodienoic acid (12-OPDA), and riboflavin were identified as central players in pathways related to plant immunity. In contrast, the 36 down-regulated metabolites, including psicose, ascorbic acid, trehalose, and coniferyl aldehyde, were primarily linked to plant immunity. These findings underscore AtCAPE9's



multifaceted role in modulating metabolite profiles to coordinate adaptive immune responses.

Several AtCAPE9-regulated metabolites have been found to play pivotal roles in plant immunity, as summarized in **Table 7**. Among metabolites up-regulated by AtCAPE9, NHP acts as a critical regulator of SAR, which facilitates a ROS-dependent NAD(P) accumulation via RBOH-mediated signaling pathways to amplify SAR. Riboflavin contributes to local pathogen resistance by activating MITOGEN-ACTIVATED PROTEIN KINASE 3 and 6 (MPK3 and MPK6) through ROS-mediated signaling, while also promoting SAR via NPR1-dependent pathways. Additionally, riboflavin exhibits a dual function in modulating ROS dynamics: it stimulates the production of superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2), while also bolstering antioxidant defenses through interactions with reduced glutathione [72]. Moreover, riboflavin mitigates oxidative stress under abiotic stress conditions, such as salinity and heavy metal exposure, thereby enhancing plant tolerance [147, 148].

Dinor-12-oxophytodienoic acid (12-OPDA), another AtCAPE9-induced metabolite, acts as a precursor to jasmonic acid (JA) and its active form, jasmonic acid-isoleucine (JA-Ile). 12-OPDA are integral to both abiotic and biotic stress responses, including drought resilience through abscisic acid (ABA)-mediated stomatal closure and pathogen resistance via downstream signaling pathways. Moreover, 12-OPDA modulates developmental processes such as seed germination and root growth, highlighting its role in balancing growth and stress responses [149].

Conversely, AtCAPE9 treatment downregulates several metabolites, including psicose, ascorbic acid, trehalose, and coniferyl aldehyde, each with unique implications for plant immunity. Psicose, a



rare sugar, has been shown to upregulate plant defense-related PR-protein genes and confer resistance to rice bacterial blight, suggesting its potential as a plant activator for disease resistance [150]. Ascorbic acid is a non-enzymatic antioxidant that neutralize ROS. The reduction in ascorbic acid levels likely alter ROS dynamics by diminishes plant capacity for ROS detoxification. This alteration may indirectly boost pathogen resistance by enhancing ROS-mediated signaling pathways. Trehalose, regulated by TREHALOSE PHOSPHATE SYNTHASE 5 (TPS5), has complex effects on pathogen resistance, enhancing resistance to *Botrytis cinerea* (*B. cinerea*) and while increasing susceptibility to *Pseudomonas syringae* (*P. syringae*) [151, 152]. Coniferyl aldehyde, a phenolic aldehyde, contribute to broad-spectrum resistance to pests and pathogens. These insights underscore AtCAPE9's comprehensive role in modulating plant defense, positioning it as a central regulator in stress adaptation across various signaling pathways [153].

3.4 Identification of Mobile Signals Induced by AtCAPE9 in Arabidopsis

3.4.1 Optimization of Phloem Exudate Collection Using EDTA in Arabidopsis

The workflow for identifying mobile signaling metabolites induced by AtCAPE9 is illustrated in **Figure 8**. Previous study have demonstrated that SAR relies on phloem transport for effective inter-organ communication [140], suggesting that mobile signal molecules induced by AtCAPE9 should be activated not only in local, infected regions but also in the phloem. To identify these AtCAPE9-induced mobile signaling metabolites, the same untargeted metabolomics approach as described in

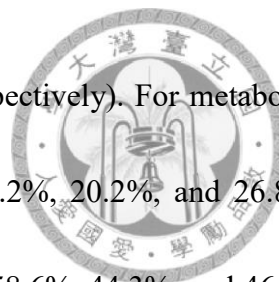


previous sections was employed to analyze phloem exudates collected from WT *Arabidopsis* plants treated with AtCAPE9.

Phloem exudates were collected using the ethylenediaminetetraacetic acid (EDTA)-facilitated exudation method, originally described by King and Zeevert [154]. This method is widely recognized as a reliable and efficient approach for collecting phloem sap while minimizing contamination from surrounding plant tissues, ensuring sample integrity for subsequent metabolomic analyses.

The EDTA-based method involves submerging the cut petiole of the plant into an EDTA buffer, which chelates calcium ions (Ca^{2+}) to inhibit natural self-sealing mechanism in plant. This process allows for the continuous exudation of phloem sap even after the leaves are transferred to water. The exuded sap is collected over several hours, ensuring sufficient sample volume for metabolomic analysis.

For *Arabidopsis* phloem exudate collection, an incubation in water for 1 to 5 hours is generally recommended [140]. To optimize the incubation duration for this study, we evaluated 1, 3, and 5 hours incubation times, followed by LC-MS analysis to assess the number and stability of metabolite recovery. Metabolite features in the phloem exudates were detected and quantified, and the relative standard deviation (RSD%) was calculated across three biological replicates for each incubation time. RSD% values were categorized into four groups: <10%, 10–20%, 20–50%, and >50%, with the proportion of metabolite features within each category analyzed. As shown in **Figure 9**, a 3 hours incubation in ddH₂O resulted in the highest percentage of metabolite features with an RSD% below



10% (6.7%, 16.6%, and 14.6% for the 1, 3, and 5 hours incubations, respectively). For metabolite features with an RSD% between 10 and 20%, the percentages were 21.2%, 20.2%, and 26.8%, respectively. Features with an RSD% between 20 and 50% accounted for 58.6%, 44.3%, and 46.2% of the metabolic features for the 1, 3, and 5 hours incubations, respectively. Lastly, metabolite features with an RSD% greater than 50% were 13.4%, 18.9%, and 12.4%, respectively. Notably, the percentage of metabolite features with an RSD% below 50% was comparable across the three durations, with 86.6%, 81.1%, and 87.6% for the 1, 3, and 5 hours incubations, respectively. These results suggest that while all durations provide reasonable reproducibility, the 3 hours incubation offers a balance of low variability (highest percentage of RSD% <10%) and sufficient metabolite recovery, making it the optimal incubation time for phloem exudate collection in *Arabidopsis* under the conditions of this study.

3.4.2 Metabolomic Profiling of AtCAPE9-Induced Metabolites in Phloem Exudates

For the metabolomics analysis of phloem exudates, WT *Arabidopsis* plants were treated with either ddH₂O (Mock) or AtCAPE9 for 4 hours. Phloem exudates were collected using the ETDA-facilitated method, with a 3 hours incubation in ddH₂O, as determined in previous section to be the optimal duration. The collected exudates were then subjected to the same UHPLC-MS protocol described in Chapter 3.1, leading to the detection of 11,937 metabolic features across the four analytic methods. Among these detected metabolic features, 5,348 features (44.8%) were successfully



obtained MS/MS spectra through DDA analysis. Of these features with DDA spectra, 946 were successfully annotated through spectral matching.

The PCA of the metabolome profiles of phloem exudates from AtCAPE9- and mock-treated samples is depicted in **Figure 10A**, with PC1 and PC2 accounting for 66.5% and 17.6% of total variance, respectively. To further identify the phloem metabolites induced by AtCAPE9, a volcano plot analysis was generated (**Figure 10B**). Red points represent up-regulated metabolite features with a p -value < 0.05 and fold change > 1.5 , blue points indicate down-regulated metabolite features with a p -value < 0.05 and fold change < 0.67 , and gray points correspond to unchanged metabolite features. This analysis revealed 1,010 up-regulated and 34 down-regulated metabolite features in the AtCAPE9-treated samples compared to the mock-treated controls (**Figure 10B**). Of these, 39 up-regulated metabolite features and 1 down-regulated metabolite feature were successfully annotated through MS2 spectral matching. Comprehensive lists of these up- and down-metabolites are provided in **Tables 8** and **9**.

3.5 Verification of NHP and Riboflavin as AtCAPE9-Induced Metabolites in Leaves and Phloem Exudates

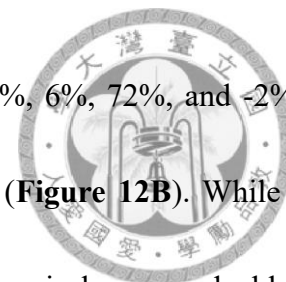
As the mobile signals responsible for AtCAPE9-induced SAR are expected to be activated in both locally infected leaves and in phloem, we analyzed the metabolites that were up-regulated in both datasets (**Figure 11**). This analysis revealed that two metabolites, NHP and riboflavin, were



found to be commonly up-regulated in both datasets (**Table 10**), suggesting their potential roles as mobile signaling molecules in mediating AtCAPE9-induced SAR.

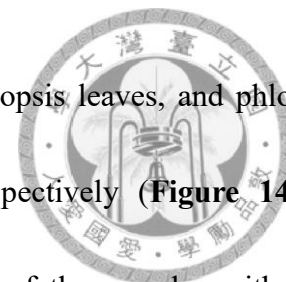
Prior to validating NHP and riboflavin as mobile signaling molecules in AtCAPE9-induced SAR, we confirmed their annotation using authentic standards. Both standards were subjected to the same DDA LC-MS/MS analysis to acquire retention time (RT) and MS/MS spectra (CID at 35 eV and stepped HCD at 45±15%). For NHP, RT values displayed high consistency across the MS1 extracted ion chromatograms (EIC) of the standard, and the metabolic features detected in Arabidopsis leaves and phloem exudates, with RT of 1.08, 1.09, and 1.06 minutes, respectively (**Figure 12A**). We validated the chromatographic consistency by comparing the RT of the samples with those of the authentic standards, calculating the RT error (%) with the formula: $(\text{Sample RT} - \text{Standard RT}) / (\text{Standard RT}) \times 100\%$. The analysis revealed an RT error of 0.9% in the leave samples and -1.9% in the phloem samples, underscoring the similarity in RTs to the standard and confirming the reliability of our identification process.

To further validate NHP identification, we compared the MS/MS spectra fragments from the samples and the NHP standard. Relative intensities for fragments were normalized to the most intense fragment ion, and the differences in relative intensities between the samples and the standard were calculated. In the CID spectra, fragment ions at m/z 129.0362, 128.0699, 101.0414, and 100.0751 exhibited relative intensity differences of -73%, 16%, -45%, and 6% in the leaves, and -39%, 16%, -24%, and 10% in the phloem exudates, respectively. Similarly, in the HCD spectra, fragment ions at



m/z 100.0751, 82.0646, 70.0647, and 55.0539 showed differences of 44%, 6%, 72%, and $\sim$ 2% in leaves, and 16%, 49%, 20%, and 0% in phloem exudates, respectively (**Figure 12B**). While the consistent retention times confirm the identification of NHP in *Arabidopsis* leaves and phloem exudates, the high discrepancies in fragment ion intensities, particularly in leaves, indicate the presence of potential interference from ions with m/z values close to that of NHP.

A detailed survey of our metabolomics data identified a substantial spectral interference at m/z 146.06 during the MS2 acquisition time for NHP in both leaves and phloem exudates, with more pronounced interference in the leaf samples (**Figure 13A**). This interference could compromise the confidence of the compound ID. To mitigate this, we identified and excluded the characteristic fragment ions associated with the interference in the MS2 spectra for further analysis. In CID spectra, the interference signals included fragment ions at m/z 129.0361, 101.0413, 98.0594, and 87.0257, while in HCD spectra, the fragment ions included m/z 128.0699, 110.0594, 100.0751, and 70.0647 (**Figure 13B**). After removing these interference-derived fragments, the refined MS2 spectra demonstrated consistent fragmentation profiles between the NHP standard and *Arabidopsis* leaves. In the CID spectra, discrepancies in relative intensities of fragment ions were calculated as 2%, -3%, and 0% at m/z 128.0698, 110.0594, and 100.0750, respectively. Similarly, in the HCD spectra, discrepancies were -1%, 1%, 1%, and 0% at m/z 128.0699, 110.0594, 100.0751, and 70.0645, respectively (**Figure 13C**). These refined results confirmed the identification of NHP in *Arabidopsis* leaves.



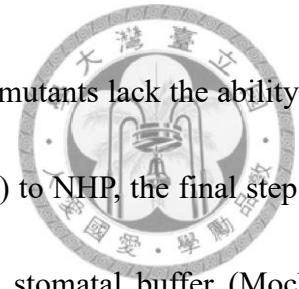
For riboflavin, the RT values in MS1 EIC for the standard, Arabidopsis leaves, and phloem exudates were closely aligned at 3.49, 3.50, and 3.51 minutes, respectively (**Figure 14A**). Consistency in chromatography was confirmed by comparing the RTs of the samples with the authentic standards, yielding RT errors of 0.3% in leaves and 0.6% in phloem. MS/MS spectral concordance was also observed, with CID identifying matching peaks at m/z 359.1346 and 243.0870 in leaves with discrepancies of 1% and 0%, and both showing 0% in phloem (**Figure 14B**). HCD analysis revealed matching peaks at m/z 377.1451, 243.0870, 216.0762, and 172.0864, with differences of -2%, 0%, 0%, and 2% in leaves, and 13%, 0%, -3%, and -4% in phloem, respectively (**Figure 14B**). The consistent RT and MS2 spectral data between the authentic standards and biological samples confirm the accurate identification of NHP and riboflavin.

3.6 Validation of NHP and Riboflavin as AtCAPE9-Induced Metabolites Involved in Local and Systemic Stomatal Immunity

3.6.1 NHP and Its Downstream Signals Essential for AtCAPE9-Mediated Local and Systemic Stomatal Immunity

Our preliminary results have demonstrated that AtCAPE9 treatment would induce local and systemic stomatal closure to reduce pathogen entry. To validate the roles of NHP and riboflavin in AtCAPE9-induced local and systemic immune responses, their effects on stomatal closure in Arabidopsis leaves were assessed (**Figure 15**).

To investigate the roles of NHP in AtCAPE9-induced local and systemic stomatal responses,



WT, *pr1* mutant, and *fmo1* mutant *Arabidopsis* plants were utilized. *fmo1* mutants lack the ability to synthesize NHP, as FMO1 catalyzes the conversion of pipecolic acid (Pip) to NHP, the final step in NHP biosynthesis [155]. Plants and detached leaves were treated with stomatal buffer (Mock), AtCAPE9, NHP, or a combination of AtCAPE9 and NHP for 1 hour. For local stomatal immunity, detached leaves were directly treated and evaluated, while for systemic stomatal immunity, the local leaves (#7-12) were treated, and untreated systemic leaves (#13-18) were assessed. Stomatal apertures were observed using a light microscope, and stomatal pore widths were measured to quantify the immune responses in both local and systemic contexts (**Figure 15**).

For the local stomatal immunity, mock treatment resulted in fully open stomata across all genotypes (**Figure 16A**). AtCAPE9 treatment induced significant stomatal closure in WT and *pr1* mutants but failed to induce stomatal closure in *fmo1* mutants, indicating that NHP is essential for AtCAPE9-induced local immunity. Treatment with NHP alone also triggered stomatal closure in WT and *fmo1* mutants; however, the degree of stomatal closure in *fmo1* mutants was less pronounced compared to AtCAPE9-treated WT plants. Notably, NHP treatment did not induce stomatal closure in *pr1* mutant, suggesting that NHP requires PR1 to activate stomatal immune responses (**Figure 16A**). The combined treatment of AtCAPE9 and NHP resulted in significant stomatal closure across all genotypes, including *fmo1* mutants, further supporting the cooperative role of NHP and PR1 in AtCAPE9-induced signaling (**Figure 16A**).

Systemic stomatal immunity exhibited a similar pattern to the local stomatal immunity (**Figure**



16B). AtCAPE9 treatment induced significant stomatal closure in the systemic leaves of WT and *prl* mutants but not in *fmo1* mutants. Similarly, NHP alone induced stomatal closure in WT and *fmo1* mutants but failed to elicit a response in *prl* mutants. Combined treatment with AtCAPE9 and NHP induced significant stomatal closure in systemic leaves across all genotypes.

These findings collectively demonstrate that NHP is a critical mediator of AtCAPE9-induced local and systemic stomatal immunity. The inability of AtCAPE9 to induce stomatal closure in *fmo1* mutants underscores the essential role of NHP in this response. Additionally, the requirement of PR1 for NHP-induced stomatal closure, as evidenced by the lack of response in *prl* mutants, highlights the dependence of NHP on PR1 to activate stomatal immunity. While NHP has been widely recognized as key regulator in SAR, this study emphasizes its indispensable role in AtCAPE9-induced local and systemic stomatal immunity.

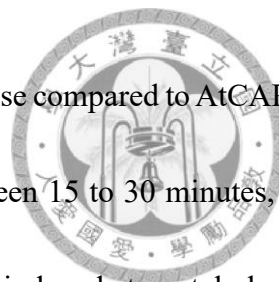
A recent study reported that the NHP triggers SAR through extracellular NAD(P) [156]. As NHP was revealed to be a critical mediator for AtCPAE9-induced stomatal immunity, we hypothesized that NAD(P) may also play roles in AtCAPE9-induced systemic stomatal immunity. To investigate these, we utilized Arabidopsis T-DNA insertion lines deficient in de novo NAD(P) biosynthesis (*fin4*) and NAD(P) receptor function (*lecrk-VI.2*). The mutant lines included SAIL_1145_B10 (*fin4*), SALK_202211C (*lecrk-VI.2* #1), and SAIL_1146_B02 (*lecrk-VI.2* #2). The structures of these mutant loci are illustrated in **Figure 17A** and **17C**. To confirm T-DNA insertions, PCR and sequencing were performed on amplicons spanning the insertion junction, using a combination of three primers: left



primer (LP), and right primer (RP), and base primer (BP). The *fin4* mutant contains a tandem T-DNA insertion. Amplification with LP and RP primers produced the expected 1,145 bp product (without T-DNA insertion), while with LB and RB primers revealed distinct bands below this size, confirming the presence of T-DNA insertion. Homozygous *fin4* plants were identified in lanes 2, 3, 4, and 5, where specific LB and RP products were detected (**Figure 17B**). Similarly, T-DNA insertions in *lecrk-VI.2* #1 and *lecrk-VI.2* #2 mutants were confirmed using LP, RP, and LB primers. The expected band sizes for LP and RP products were 1,101 bp and 1,200 bp, respectively. Amplification with LB and RP primers produced additional bands below the expected size, confirming T-DNA insertions in the mutants. Homozygous plants were identified in lanes 1, 3, 4, 5, and 6 for *lecrk-VI.2* #1 and in lanes 1, 6, and 7 for *lecrk-VI.2* #2 (**Figure 17D**).

To evaluate the roles of NAD(P) in AtCAPE9-induced systemic stomata immunity, local leaves (# 7-12) of WT and *pr1*, *fmo1*, *fin4*, and *lecrk-VI.2* plants were treated with stomatal buffer (Mock), AtCAPE9, NAD, or NADP, and the systemic stomatal responses in untreated, systemic leaves (#13-18) were accessed at 15, 30, 45, and 60 minutes post-treatment (**Figure 18**). In the mock treatment, stomata remained fully open across all genotypes and time points. AtCAPE9 treatment induced significant stomatal closure in WT and *pr1* plants after 60 minutes (**Figure 18A, B**), whereas *fmo1*, *fin4*, and *lecrk-VI.2* mutants exhibited no substantial stomatal closure within the same time period (**Figure 18C-E**), indicating a disruption in the AtCAPE9-induced signaling.

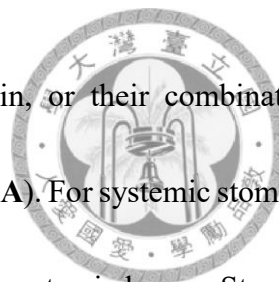
In NAD-treated groups, WT and *pr1* exhibited stomatal closure as early as 15 minutes, sustained



throughout the 60 minutes observation period, demonstrating a faster response compared to AtCAPE9 treatment (**Figure 18A, B**). In *fmo1*, NAD induced stomatal closure between 15 to 30 minutes, but stomata reopened between 45 to 60 minutes (**Figure 18C**). In *fin4*, NAD-induced stomatal closure was consistent throughout the 15 to 60 minutes (**Figure 18D**). In contrast, *lecrk-VI.2* mutants failed to respond to NAD treatment, with stomata remaining open throughout the experiment (**Figure 18E**). Similarly, NADP treatment elicited stomatal closure in WT and *pr1* plants, following a pattern comparable to that observed with NAD (**Figures 18A, B**). In *fmo1*, NADP induced stomatal closure at earlier time points, sustained up to 60 minutes (**Figure 18C**). In *fin4*, NADP treatment also induced sustained stomatal closure (**Figure 18D**), while no response was observed in *lecrk-VI.2* mutants (**Figure 18E**). These results suggest that NAD(P) functions as a crucial downstream signal in AtCAPE9-induced systemic stomatal immunity, operating downstream of NHP.

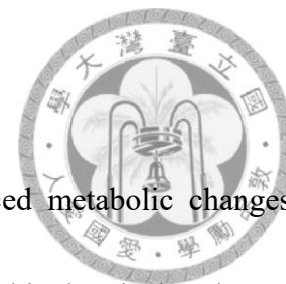
3.6.2 Riboflavin-Induced Local and Systemic Stomatal Immunity Operates Independently of or Downstream to PR1

Riboflavin, a precursor to flavin cofactors, has been recognized as an elicitor of systemic resistance and an activator of plant defense signaling pathways [157]. To investigate its potential role in the defense mechanisms induced by AtCAPE9, the local and systemic stomatal responses were assessed in WT and *pr1* mutant plants treated with either AtCAPE9, riboflavin, or a combination of both, as described in previous section. For local stomatal immunity, mock treatment resulted in fully



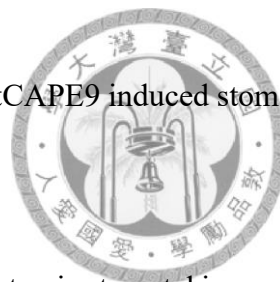
open stomata across all genotypes. Treatment with AtCAPE9, riboflavin, or their combination induced significant stomatal closure in both WT and *pr1* mutants (**Figure 19A**). For systemic stomatal immunity, similar stomatal responses were observed in systemic, untreated systemic leaves. Stomata remained wide open under mock treatment but exhibited significant closure following treatment with AtCAPE9, riboflavin, or their combination in both WT and *pr1* plants (**Figure 19B**). Although riboflavin biosynthesis or receptor mutants were not investigated in this study, our findings suggest that riboflavin can induce both local and systemic stomatal immunity via a pathway that operates either independently of or downstream of PR1. Further studies using riboflavin biosynthesis and receptor mutants are necessary to comprehensively elucidate its signaling function and clarify its role within AtCAPE9-induced immune responses.

4. Discussion



This study provides a comprehensive analysis of AtCAPE9-induced metabolic changes in *Arabidopsis* leaves, highlighting its pivotal role in orchestrating complex biochemical pathways to enhance plant immunity and stress resilience. Using an integrative metabolomics approach combining RPLC and HILIC with dual ionization modes, we identified 1,764 metabolites. This represents a significant improvement in metabolome coverage compared to previous studies relying on single-column approach, highlighting the value of complementary chromatographic techniques for achieving a more comprehensive metabolic profile [162, 163].

With the integration of metabolomics datasets from *Arabidopsis* leaves and phloem exudates, we identify NHP and riboflavin as candidate mobile signaling molecules involved in AtCAPE9-induced SAR, with both metabolites significantly upregulated by AtCAPE9 in treated leaves and phloem exudates. Their identities were rigorously confirmed in the metabolomics datasets through retention time and MS2 spectral matching using authentic standards. Stomatal assays further revealed their critical roles in both local and systemic stomatal immunity induced by AtCAPE9. Interestingly, while NHP induced stomatal closure within 1 hour in WT plants, its effect was less pronounced compared to AtCAPE9, highlighting potential differences in their signaling kinetics. Notably, the precursor of NHP, Pip, requires approximately 2 hours to induce stomatal closure [106], likely due to the enzymatic conversion of Pip to NHP and subsequent activation of downstream signaling molecules such as NAD(P). Regardless, AtCAPE9-induced local and systemic stomatal immunity



was abolished in *fmo1* mutants, confirming NHP is indispensable for the AtCAPE9 induced stomatal immunity.

Our study also highlights NAD(P) as a critical downstream signal in systemic stomatal immunity regulated by AtCAPE9 via NHP, as AtCAPE9-induced systemic stomatal immunity was abolished in *fin4* mutants. Notably, we did not observe significant changes in total NAD and NADP levels in the leaves and phloem exudates after AtCAPE9 treatment. This is consistent with a previous study indicating that during SAR, extracellular NAD(P) [eNAD(P)] accumulates in the apoplast, while the total NAD(P) levels in the leaves remain unchanged [156]. This suggests that eNAD(P) functions specifically within the apoplast to amplify immune responses. Future studies should examine apoplastic washing fluids (AWF) to determine whether AtCAPE9 induces eNAD(P) accumulation, which would provide further insights into its role in AtCAPE9-induced immune signaling.

Additionally, riboflavin was shown to induces stomatal closure in both WT and *pr1* mutants, supporting its role in AtCAPE9-mediated immunity. However, further experiments involving riboflavin-deficient mutants are necessary to confirm its relationship with AtCAPE9 [158]. Additionally, the potential interaction between riboflavin and NHP in SAR and stomatal immunity warrants further investigation. These findings collectively advance our understanding of AtCAPE9-mediated defense strategies and its broader implications in plant resilience.

Future investigations into the temporal dynamics and regulatory interplay between Pip, NHP, and NAD(P) will be critical for understanding the precise mechanisms underlying stomatal immunity

and the broader defense strategies orchestrated by AtCAPE9. These insights could pave the way for targeted applications of these signaling pathways in enhancing plant resilience to environmental stresses.



5. Conclusion



This study provides a comprehensive analysis of AtCAPE9-induced metabolic changes in *Arabidopsis* leaves, revealing its pivotal role in regulating pathways associated with plant defense and stress adaptation. Using a dual-column metabolomics approach integrating RPLC and HILIC with dual ionization modes, we identified 1,764 metabolites, achieving broader metabolome coverage than previously reported studies. Through enrichment analysis and a literature survey of AtCAPE9-regulated metabolites, we uncovered the metabolic pathways and key metabolites associated with AtCAPE9-induced immune responses. By integrating the metabolomics analysis of phloem exudates, NHP and riboflavin were identified as critical regulators of AtCAPE9-induced plant immunity. Both metabolites were upregulated by AtCAPE9 in local leaves and phloem exudates, suggesting their roles as mobile signals in AtCAPE9-induced SAR. Functional assays demonstrated that NHP is essential for both local and systemic stomatal immunity induced by AtCAPE9, operating via PR1-dependent pathways. Furthermore, NAD(P), a downstream signal of NHP, was shown to be essential for AtCAPE9-induced systemic stomatal immunity. Riboflavin induced stomatal closure in both WT and *pr1* mutants, suggesting its role through a PR1-independent or downstream mechanism. These findings collectively position AtCAPE9 as a central modulator of plant immunity, orchestrating a sophisticated network of metabolic and signaling pathways to enhance resilience against biotic and abiotic stresses (**Figure 20**).

Metabolite Extraction from Arabidopsis Leaves with and without AtCAPE9 Treatment



LC-MS/MS Metabolomics Analysis

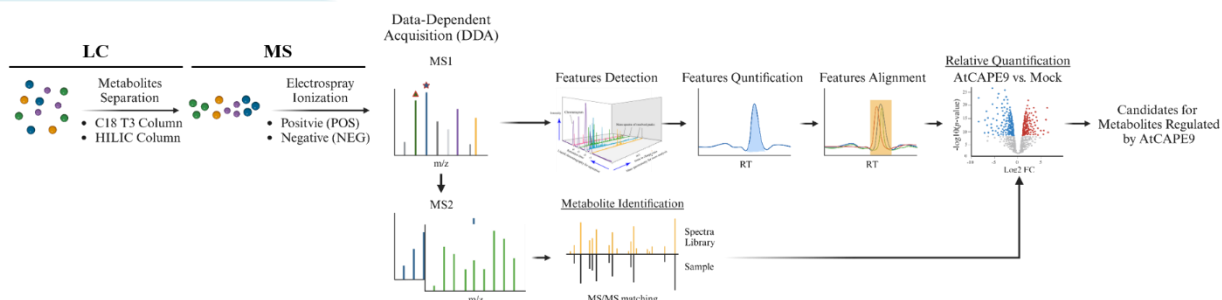


Figure 1. Workflow of metabolomics analysis for identifying AtCAPE9-regulated metabolites in Arabidopsis leaves

WT leaves were treated with AtCAPE9 or ddH₂O (Mock), followed by metabolite extraction and untargeted mass spectrometry analysis. To achieve comprehensive metabolite coverage, reversed-phase liquid chromatography (RPLC) and hydrophilic interaction liquid chromatography (HILIC) were employed for chromatographic separation, along with positive (POS) and negative (NEG) ionization modes. Mass spectrometry operated using Data-dependent acquisition (DDA) mode, collecting MS1 and MS2 spectra for downstream analysis. MS1 spectra underwent extensive processing for feature detection, quantification, and alignment across samples, while MS2 spectra were used to compound identification through spectral matching against reference libraries. Statistical analyses were conducted to identify differentially regulated metabolites between AtCAPE9-treated and mock-treated leaves. These AtCAPE9-regulated metabolites with compound identification were further subjected to pathway integration and literature surveys to elucidate their roles in AtCAPE9-induced plant immunity and defense responses.

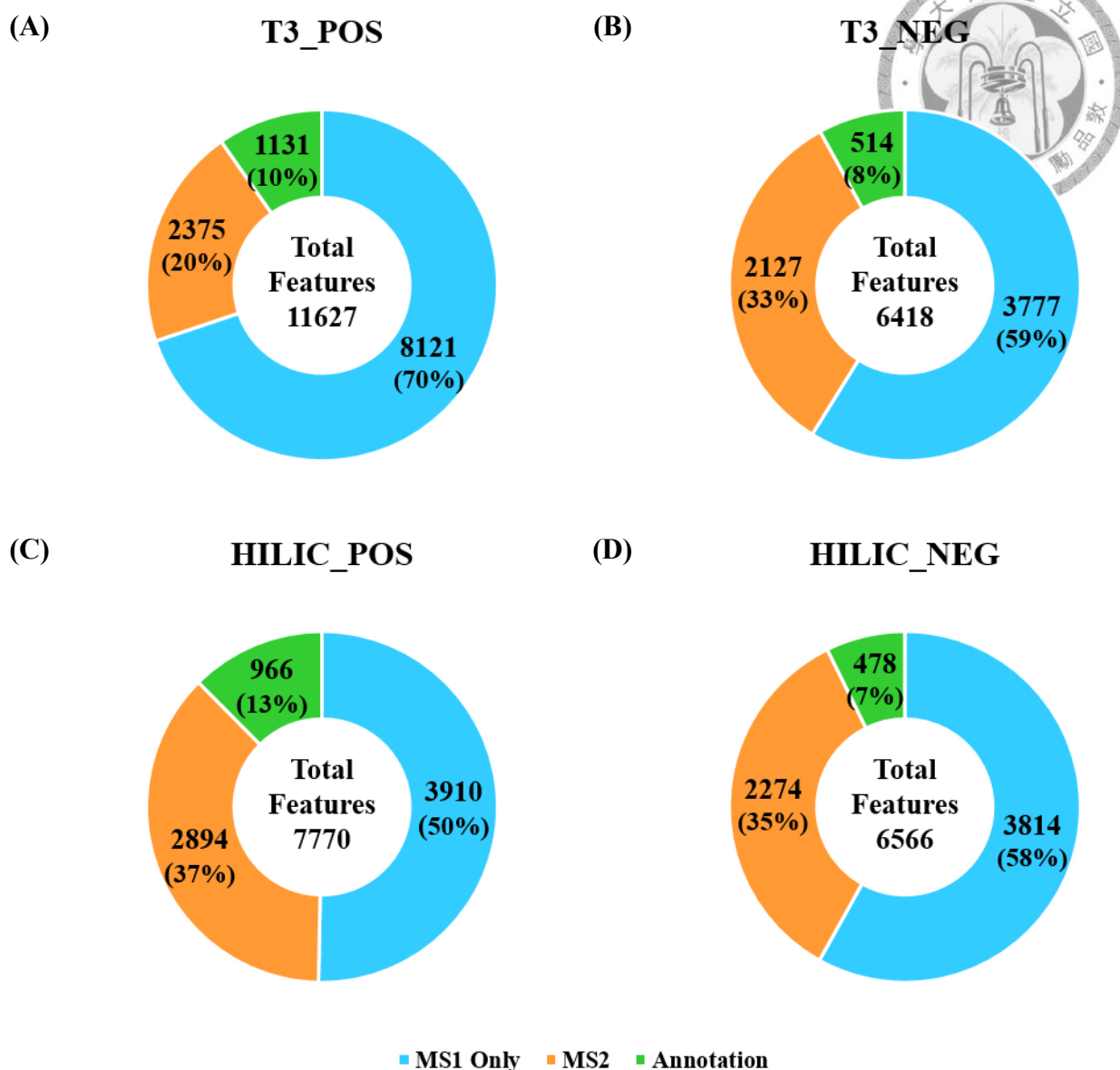


Figure 2. Numbers of metabolic features detected across four mass spectrometry platforms

The figure illustrates the numbers of metabolic features detected in *Arabidopsis* leaves using RPLC in positive (A, T3_POS) and negative (B, T3_NEG) ionization modes, as well as HILIC in positive (C, HILIC_POS) and negative (D, HILIC_NEG) modes. Detected metabolic features are categorized into three groups based on the results of MS data processing: MS1 only (blue), representing features detected with MS1 spectra but without MS2 spectra acquisition; MS2 (orange), representing features with both MS1 and MS2 spectra but without annotation through spectral matching; and Annotation (green), representing features identified with MS1 and MS2 spectra and successfully annotated through spectral matching.

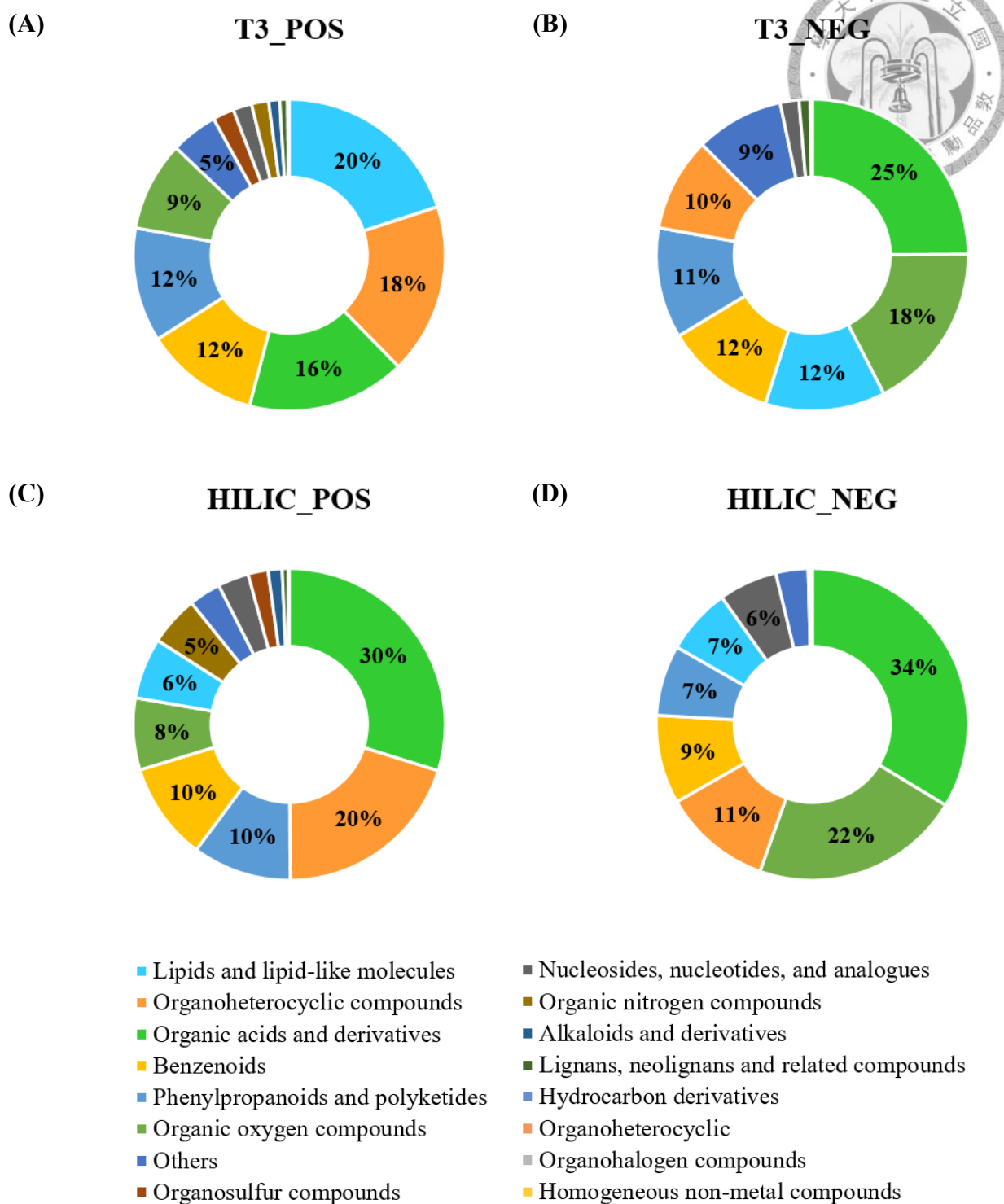


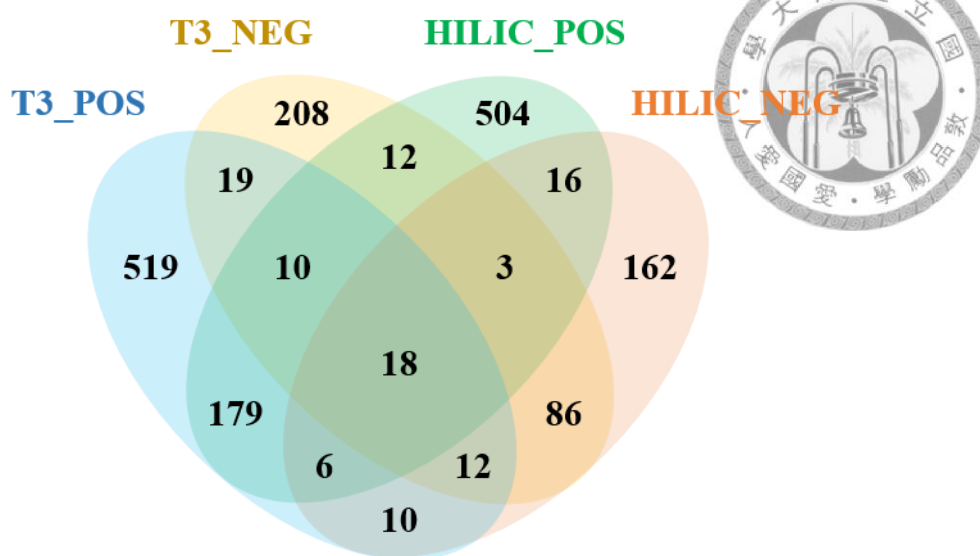
Figure 3. Classification of the metabolites identified in Arabidopsis leaves across four mass spectrometry platforms

Pie charts illustrate the distribution of metabolites identified in Arabidopsis leaves, categorized by ClassyFire Superclass categories. The metabolites were identified using RPLC in positive (A, T3_POS) and negative (B, T3_NEG) ionization modes, as well as HILIC in positive (C, HILIC_POS) and negative (D, HILIC_NEG) modes. Each pie chart represents the percentage

composition of metabolite classes, highlighting the diversity of chemical entities detected across the four mass spectrometry platforms.



(A)



(B)

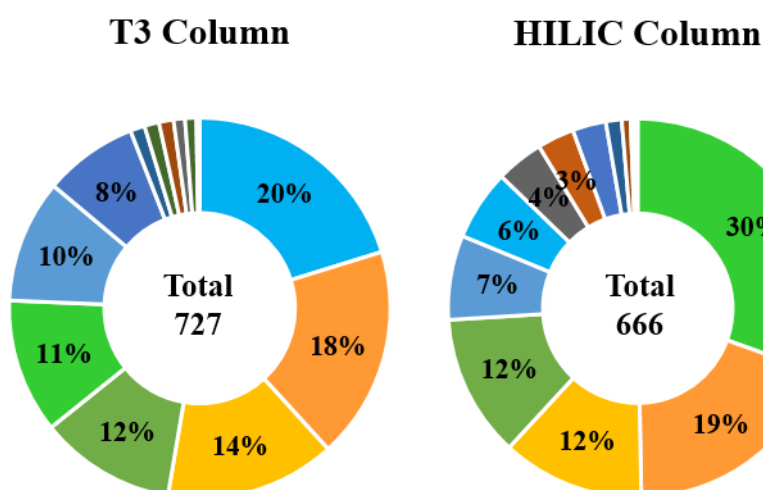


Figure 4. Comparative analysis of metabolites identified across four mass spectrometry platforms

(A) Venn diagram that delineates the unique and overlapping metabolites identified across four mass spectrometry platforms: RPLC in positive (T3_POS) and negative (T3_NEG) ionization modes, as well as HILIC in positive (HILIC_POS) and negative (HILIC_NEG) modes. The numbers within each segment of the diagram represent the metabolites uniquely detected or shared among the four platforms, highlighting their complementary metabolic profiling capabilities. (B) Pie charts illustrate the classification of metabolites exclusively identified by the T3 column (combining both positive and negative modes) and the HILIC column (combining both positive and negative modes). Each pie chart represents the percentage composition of metabolite classes, demonstrating the chemical diversity captured by each column technology and their specialization in profiling distinct chemical classes.

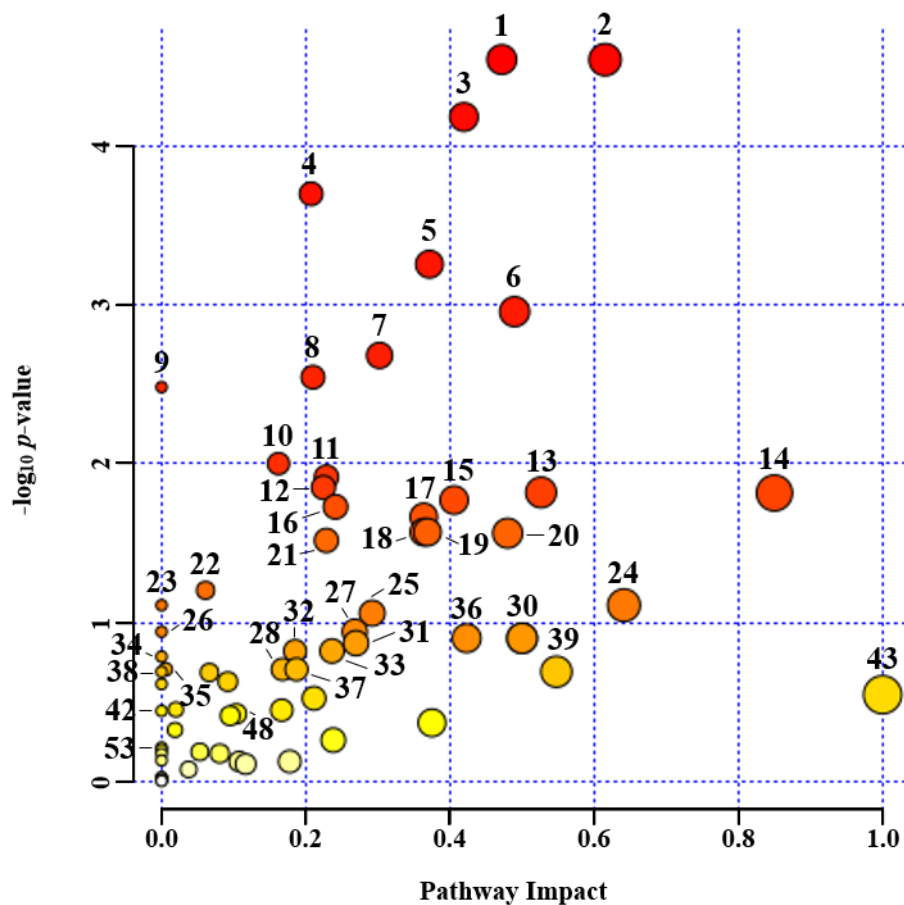
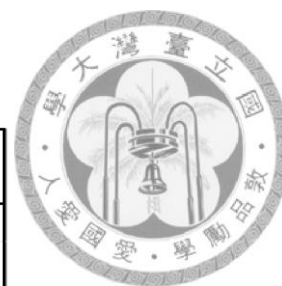
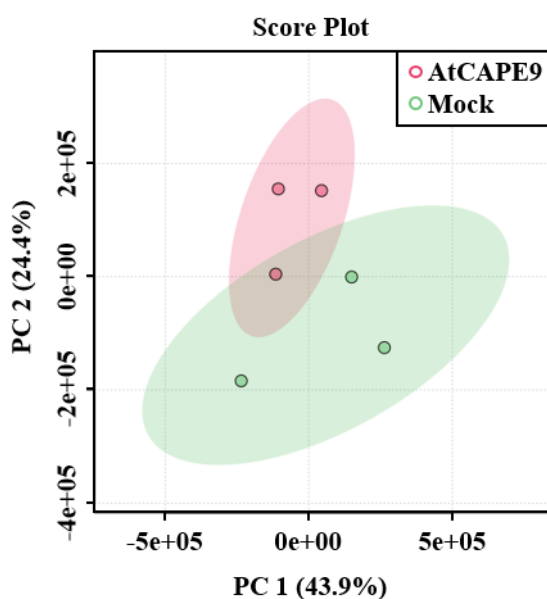


Figure 5. Metabolic pathway analysis of the identified metabolites in Arabidopsis leaves

Metabolomics pathway analysis was conducted using MetaboAnalyst 6.0, based on the metabolites identified in Arabidopsis leaves. Each circle represents a metabolic pathway, with its color indicating the *p*-value (darker colors correspond to more significant pathway enrichments) and its size reflecting the pathway impact score. A detailed summary of the 66 involved metabolic pathways is provided in Table 2.

(A)



(B)

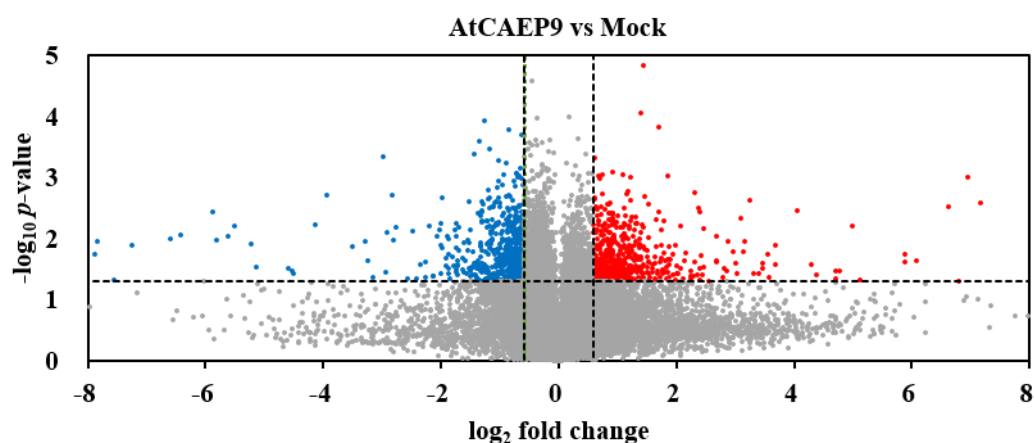


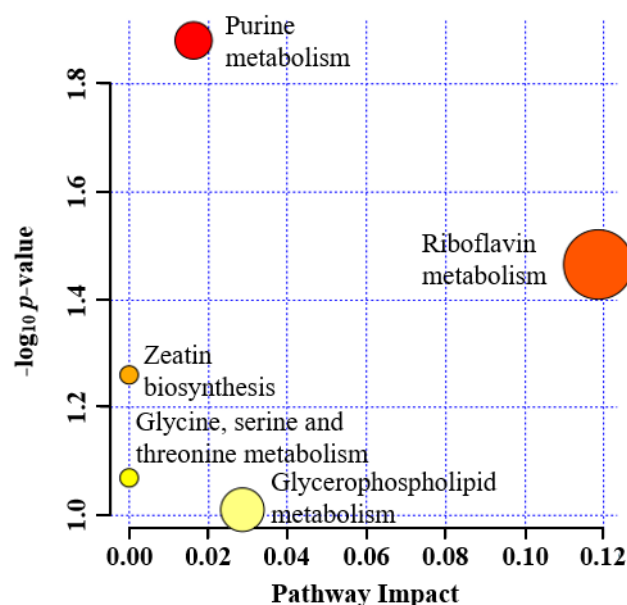
Figure 6. Metabolic profile and metabolite changes induced by AtCAPE9 in Arabidopsis leaves

(A) Principal component analysis (PCA) score plot was used to visualize the overall metabolic profile changed induced by AtCAPE9 in Arabidopsis leaves. The red circles represent the metabolome profiles of AtCAPE9-treated samples, while the green circles represent mock-treated samples. (B) Volcano plot was used to depict the log₂-transformed fold changes and -log₁₀ *p*-values of metabolite abundances between AtCAPE9- and mock-treated samples. Metabolites with *p* < 0.05 and a fold change > 1.5 or < 0.67 were identified as up-regulated (red) or down-regulated (blue) metabolites by AtCAPE9, respectively, while gray indicates metabolite features with no significant difference.



(A)

Pathway analysis for metabolites up-regulated by AtCAPE9



(B)

Pathway analysis for metabolites down-regulated by AtCAPE9

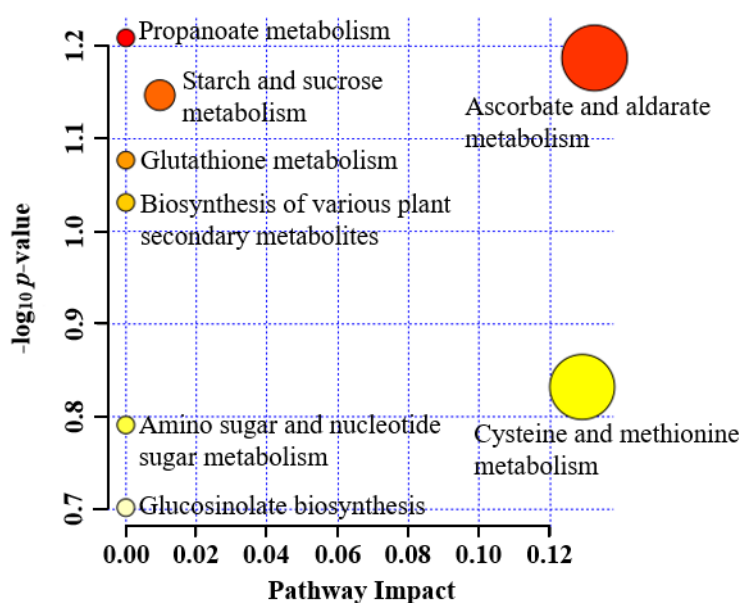


Figure 7. Metabolic pathway analysis of the identified metabolites in Arabidopsis leaves regulated by AtCAPE9

Metabolic pathway analysis, generated by MetaboAnalyst software, for AtCAPE9 (A) up-regulated and (B) down-regulated metabolites is presented. Circles indicate the matched pathways, with the color of each circle reflecting the p -values (darker colors denote more significant changes in metabolites within the pathway), while the size of the circle represents the pathway impact score. Detailed results of pathway analysis are listed in the Table 5 and 6.

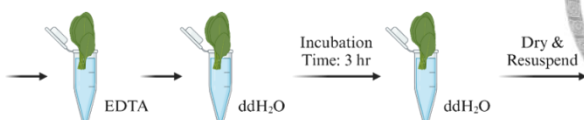
Extraction of metabolites from Arabidopsis phloem exudates with or without AtCAPE9 treatment

Sample Treatment

Arabidopsis Plants: WT

- Mock (n=3)
- AtCAPE9 (n=3)

Phloem Collection Using EDTA-Facilitated Method



LC-MS/MS Metabolomics Analysis

Same with Aim 1

Identification of Potential Mobile Signals Induced by AtCAPE9 in Local Leaves and Phloem Exudates

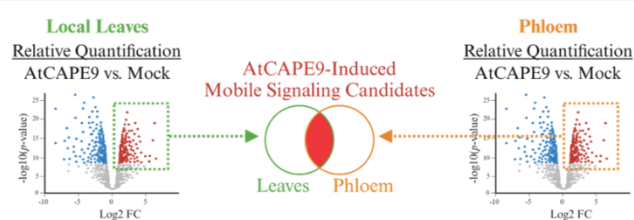


Figure 8. Workflow for identifying AtCAPE9-induced mobile signaling metabolites in Arabidopsis

WT Arabidopsis leaves were treated with AtCAPE9 or ddH₂O (Mock), and phloem exudates were collected using an EDTA-facilitated method. The collected exudates were subjected to metabolomics analysis and data processing as described in previous sections. Metabolites up-regulated in both AtCAPE9-treated leaves and phloem exudates were identified as candidate metabolites for AtCAPE9-induced mobile signals.

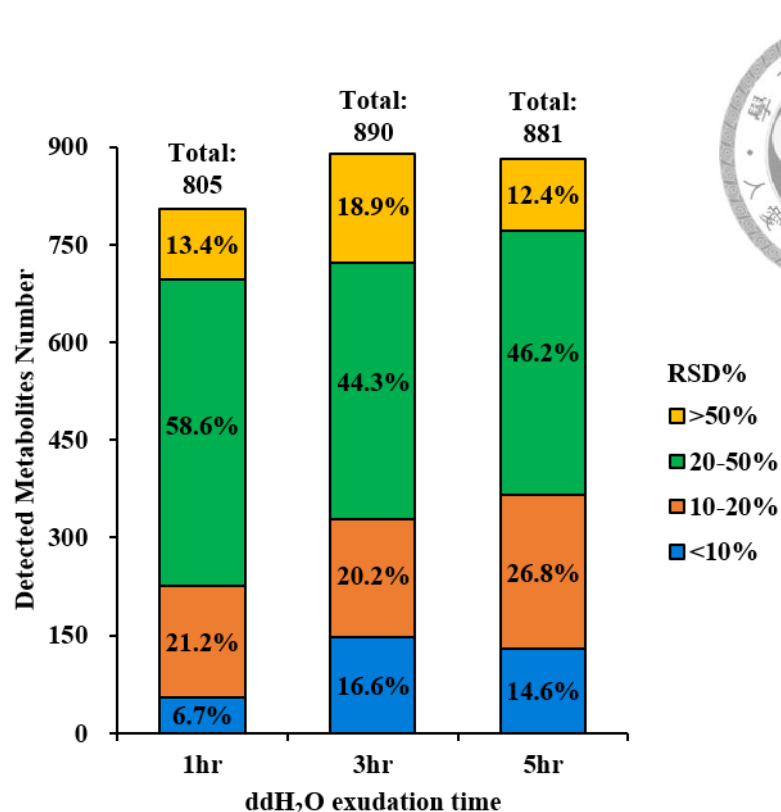
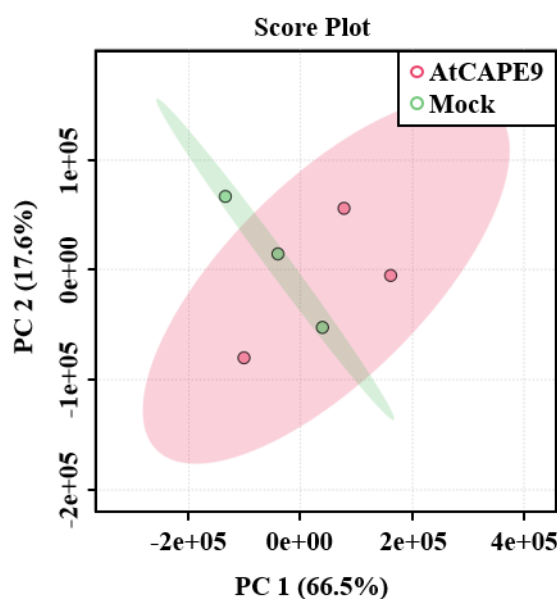


Figure 9. Number and abundance reproducibility of metabolite features detected from phloem exudates collected at different incubation durations

Phloem exudates were collected by first incubating cut petioles in a 20 mM ethylenediaminetetraacetic acid (EDTA) solution for 1 hour, followed by transferring them to ddH₂O for incubation periods of 1, 3, or 5 hours. The exudates were subsequently dried, reconstituted, and analyzed for metabolite features by LC-MS. The stability of metabolite feature abundances was evaluated using the relative standard deviation (RSD%) values from three biological replicates. The RSD values were categorized into four groups: < 10% (blue), 10-20% (orange), 20-50% (green), and > 50% (yellow). The data demonstrate variability in metabolite reproducibility across the different incubation durations, with the 3 hours incubation yielding the highest proportion of metabolite features with RSD% < 10%, indicating optimal reproducibility and reliability for metabolomic analysis.

(A)



(B)

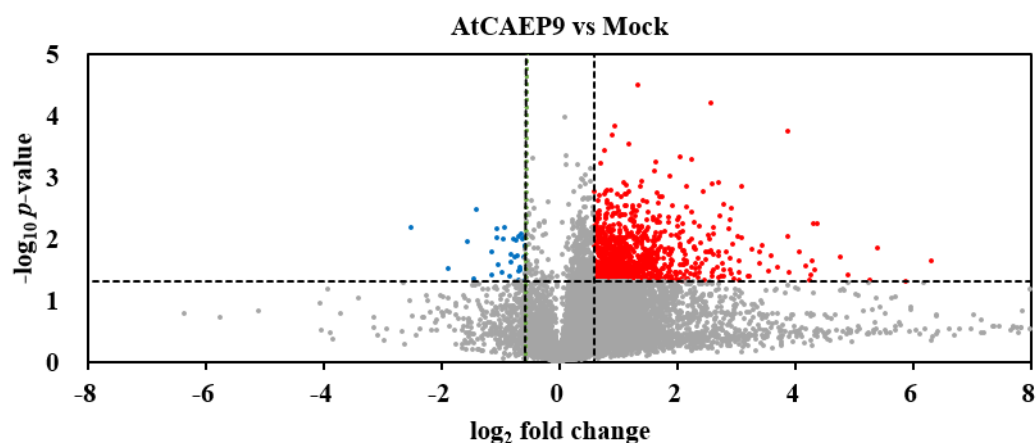
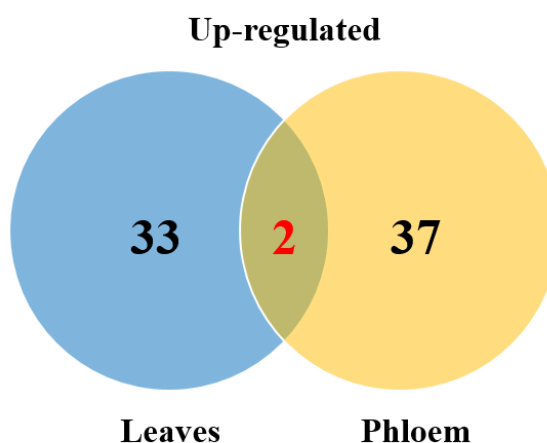


Figure 10. Metabolic profile and metabolite changes induced by AtCAPE9 in Arabidopsis phloem exudates

(A) PCA score plot was used to visualize the overall metabolic profile changed induced by AtCAPE9 in phloem exudates. The red circles represent the metabolome profiles of AtCAPE9-treated samples, while the green circles represent mock-treated samples. (B) Volcano plot was used to depict the $\log_2$ -transformed fold changes and $-\log_{10} p$ -values of metabolite abundances between AtCAPE9- and mock-treated samples. Metabolites with $p < 0.05$ and a fold change > 1.5 or < 0.67 were identified as up-regulated (red) or down-regulated (blue) metabolites by AtCAPE9, respectively, while gray indicates metabolite features with no significant difference.

(A)



(B)

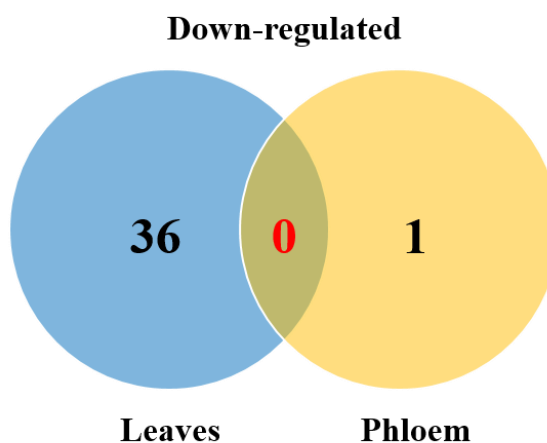
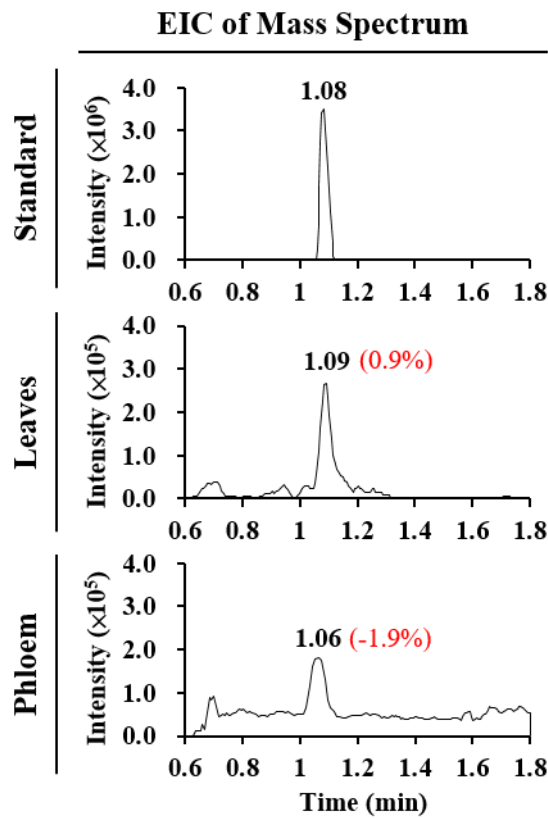


Figure 11. Venn diagrams of AtCAPE9-regulated metabolites in Arabidopsis leaves and phloem exudates

The Venn diagrams illustrate the unique and overlapping metabolites that were significantly (A) up-regulated and (B) down-regulated in Arabidopsis leaves (blue) and phloem exudates (yellow) following AtCAPE9 treatment. The analysis revealed two metabolites that were commonly up-regulated in Arabidopsis leaves and phloem exudates following AtCAPE9 treatment, whereas no metabolites were found to be commonly down-regulated.

(A)



(B)

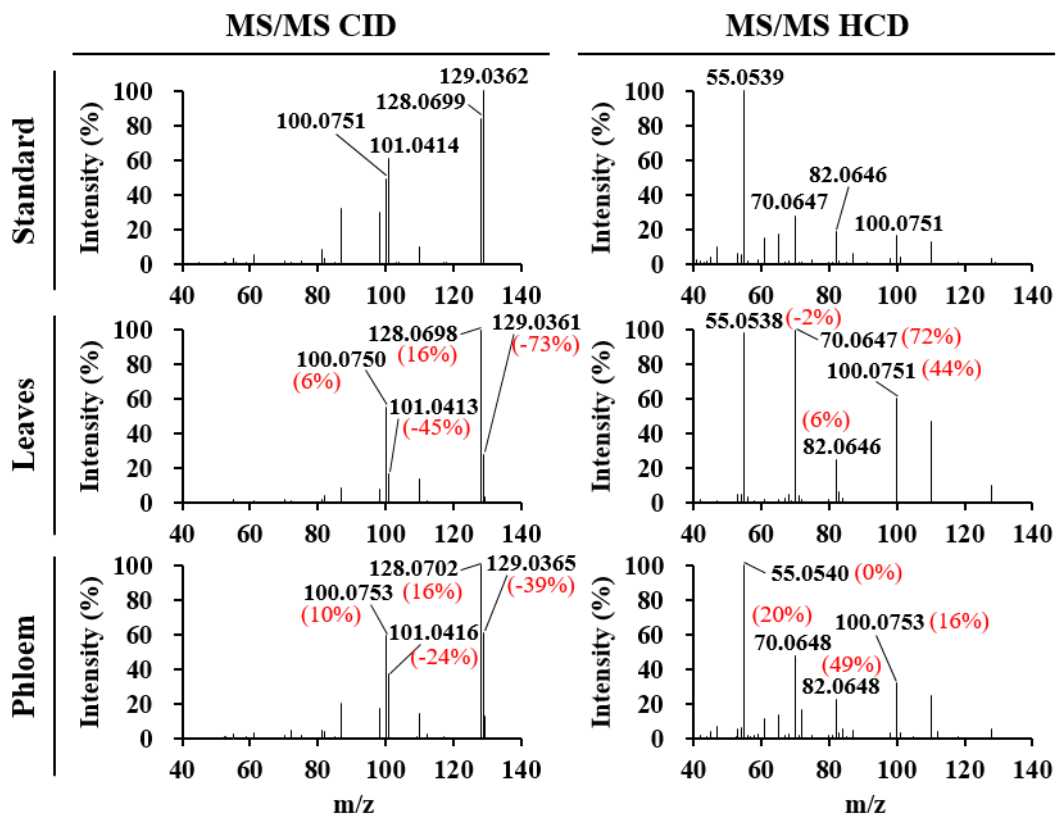
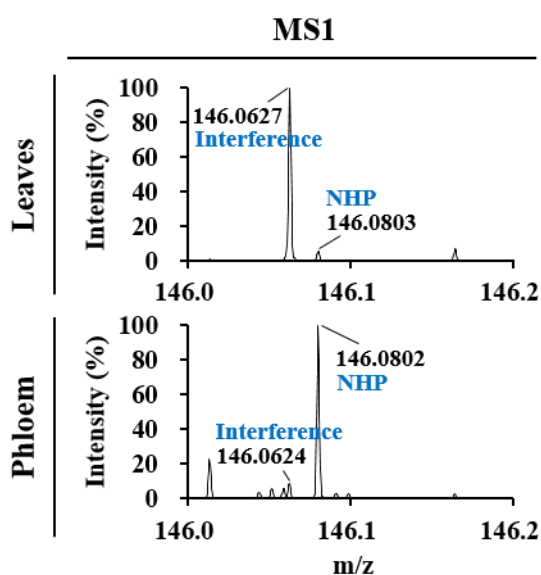


Figure 12. Validation of the identification of N-hydroxypipelicolic Acid (NHP) in Arabidopsis leaves and phloem exudates using authentic standard

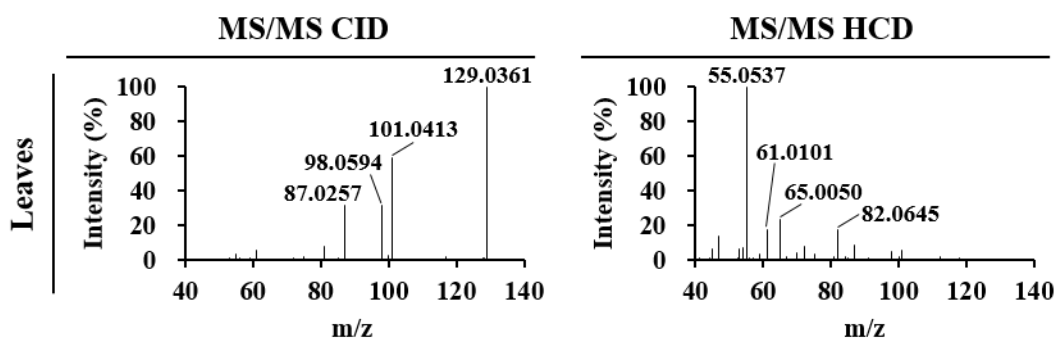
(A) Extracted ion chromatograms (EICs) for m/z 146.0817 are shown for the NHP standard (top), Arabidopsis leaf samples (middle), and phloem exudates (bottom). Discrepancies in retention time (RT) between the samples and the standard were calculated as $(\text{Sample RT} - \text{Standard RT}) / \text{Standard RT} \times 100\%$, with values highlighted in red brackets. (B) MS2 spectra for m/z 146.0817 were obtained using collision-induced dissociation (CID, left) and higher-energy collisional dissociation (HCD, right). Fragmentation patterns of NHP in the standard, Arabidopsis leaves, and phloem exudates are shown, with differences in relative ion intensities calculated as $(\text{Sample relative intensity} - \text{Standard relative intensity})$. While consistent retention times confirm the identification of NHP in Arabidopsis leaves and phloem exudates, high discrepancies in fragment ion intensities suggest potential interference from ions with m/z values close to that of NHP.



(A)



(B)



(C)

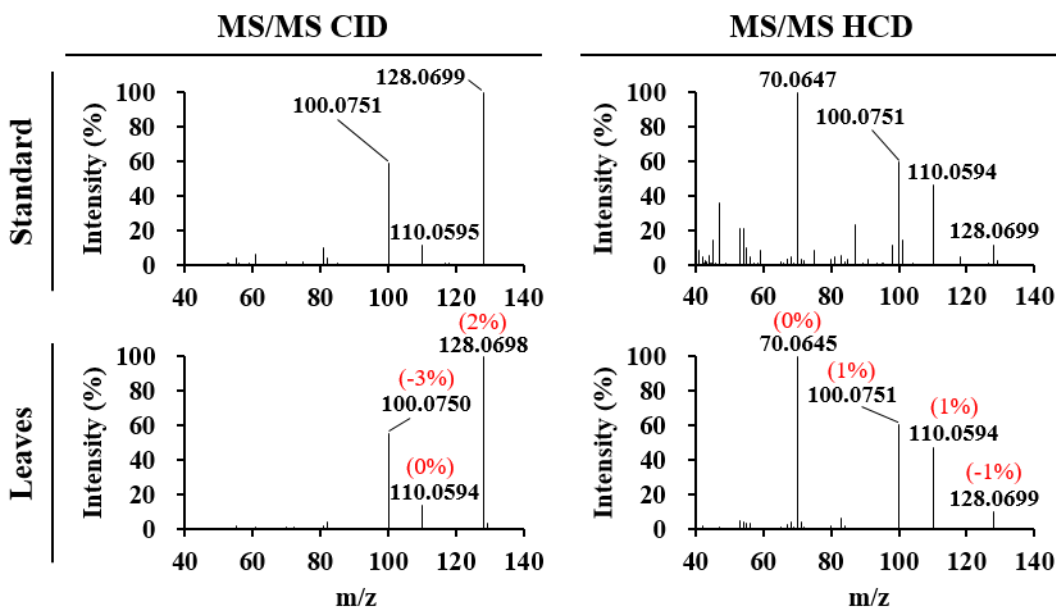
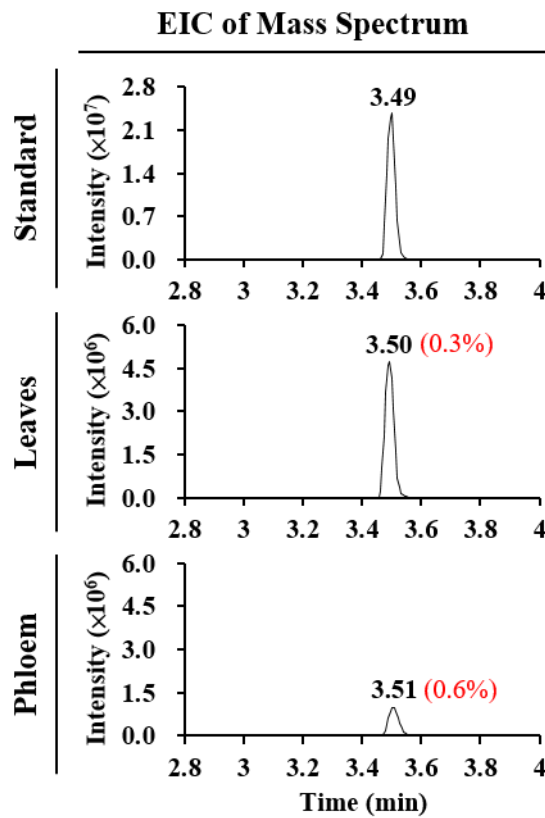


Figure 13. Validation of NHP identification in Arabidopsis leaves using authentic standard after interference removal

(A) MS1 spectra at the retention time of NHP MS2 acquisition in Arabidopsis leaves (top) and phloem exudates (bottom) revealed the presence of an interference ion with an m/z value (146.06) close to that of NHP (m/z , 146.0817). Notably, spectral interference at m/z 146.0627 is prominent in leaf samples but relatively minor in phloem exudates. (B) The CID (left) and HCD (right) MS2 spectra of the interference ion display its characteristic fragment ions. (C) Refined MS2 spectra for m/z 146.0817 from NHP standard and Arabidopsis leaves, post-removal of interference characteristic fragment ions, demonstrate the consistent fragmentation profiles between the NHP standards and Arabidopsis leaves. This confirms the identification of NHP in Arabidopsis leaves. Differences in relative ion intensities between the samples and the standard are calculated as (Sample relative intensity - Standard relative intensity).

(A)



(B)

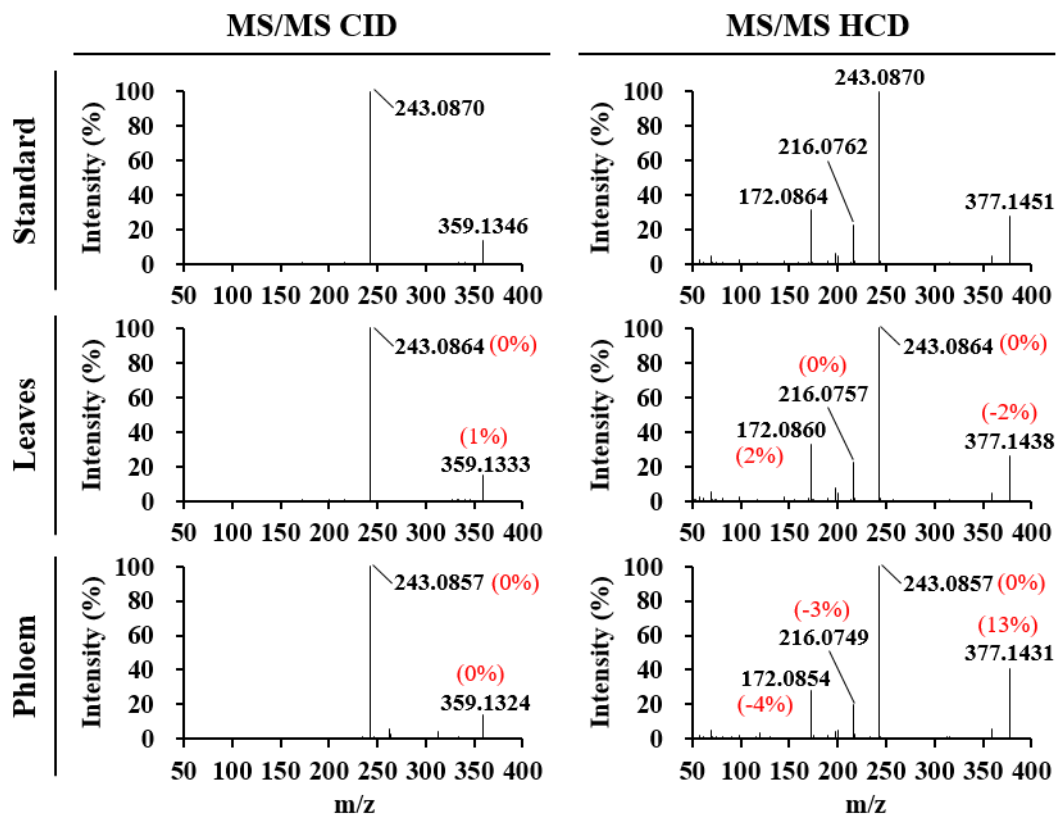


Figure 14. Validation of the identification of riboflavin in Arabidopsis leaves and phloem exudates using authentic standard

(A) EICs for m/z 377.1461 are shown for the riboflavin standard (top), Arabidopsis leaf samples (middle), and phloem exudates (bottom). Discrepancies in RT between the samples and the standard were calculated as $(\text{Sample RT} - \text{Standard RT}) / \text{Standard RT} \times 100\%$, with values highlighted in red brackets. (B) MS2 spectra for m/z 377.1461 were obtained using collision-induced dissociation (CID, left) and higher-energy collisional dissociation (HCD, right). The fragmentation patterns for riboflavin in the standard, Arabidopsis leaves, and phloem exudates are depicted, with differences in relative ion intensities calculated as $(\text{Sample relative intensity} - \text{Standard relative intensity})$. Both the retention time and MS2 spectra showed consistent patterns across standard, leaf, and phloem exudate samples, confirming the identification of riboflavin in Arabidopsis leaves and phloem exudates.

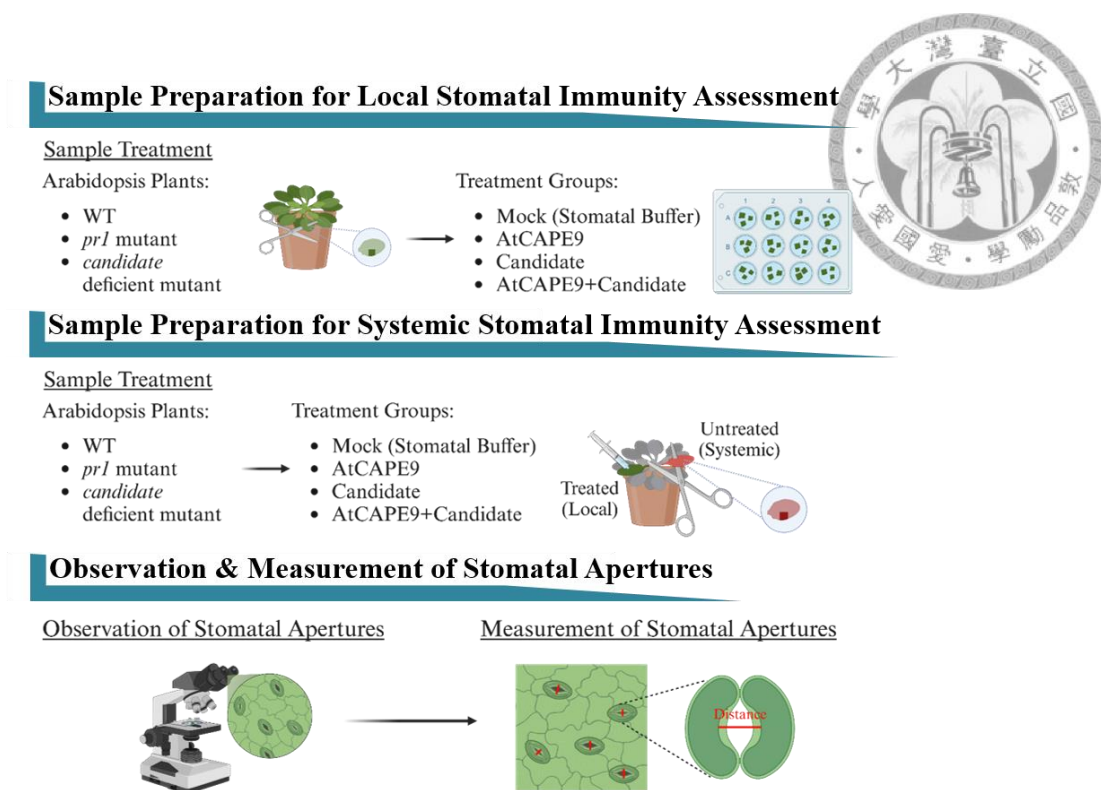
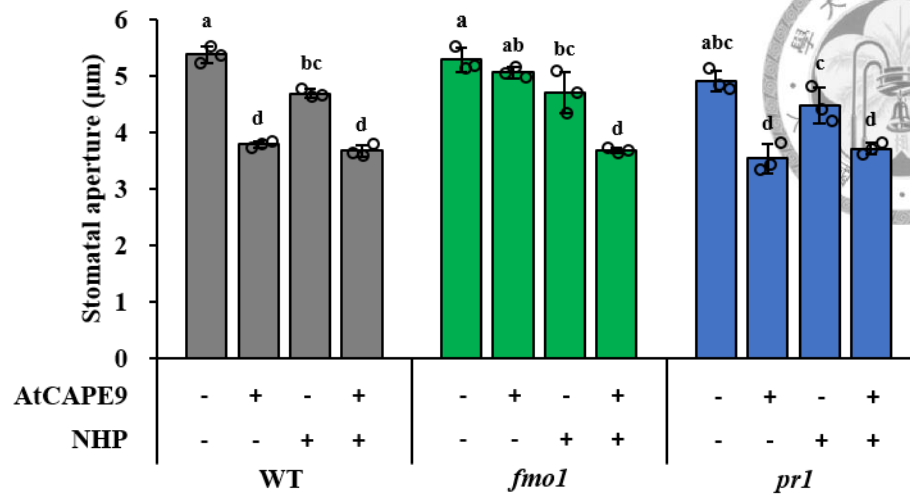


Figure 15. Workflow for accessing the roles of candidate metabolites in AtCAPE9-induced local and systemic stomatal immunity in Arabidopsis

This figure illustrates the workflow for evaluating the roles of candidate metabolites in AtCAPE9-induced local and systemic stomatal immunity. Stomatal aperture widths in local and systemic leaves were measured in Arabidopsis plants, including WT, *pr1* mutants, and candidate-deficient mutants. For local stomatal immunity, detached leaves were incubated with either stomatal buffer (mock), AtCAPE9, candidate metabolites, or a combination of AtCAPE9 and candidate metabolites (top panel). For systemic stomatal immunity, local leaves (# 7-12) were infiltrated with either stomatal buffer (mock), AtCAPE9, candidate metabolites, or a combination of AtCAPE9 and candidate metabolites, and untreated systemic leaves (# 13-18) were analyzed to evaluate the distal effects of these treatments (middle panel). Stomatal apertures were observed using a light microscope, with stomatal pore widths measured to quantify immune responses. Comparative analyses across different treatment groups and genotypes provided insights into the roles of AtCAPE9 and candidate metabolites in modulating local and systemic stomatal immune responses.

(A)



(B)

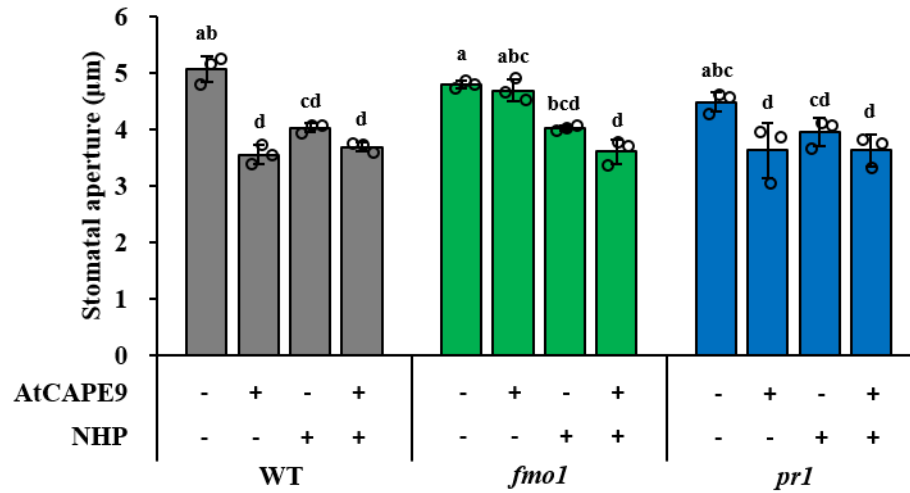


Figure 16. NHP is required for the AtCAPE9-induced local and systemic stomatal immunity
(A) Local and (B) systemic stomatal apertures were measured after 1 hour treatments with stomatal buffer (Mock), 100 nM AtCAPE9, 1 mM NHP, or a combination of AtCAPE9 and NHP in Arabidopsis WT, *fmo1*, and *pr1* mutants. Data are presented as means $\pm$ standard deviation (SD) from three independent plants. Letters above the bars indicate statistically significant differences between treatment groups, as determined by one-way ANOVA ($p < 0.05$).

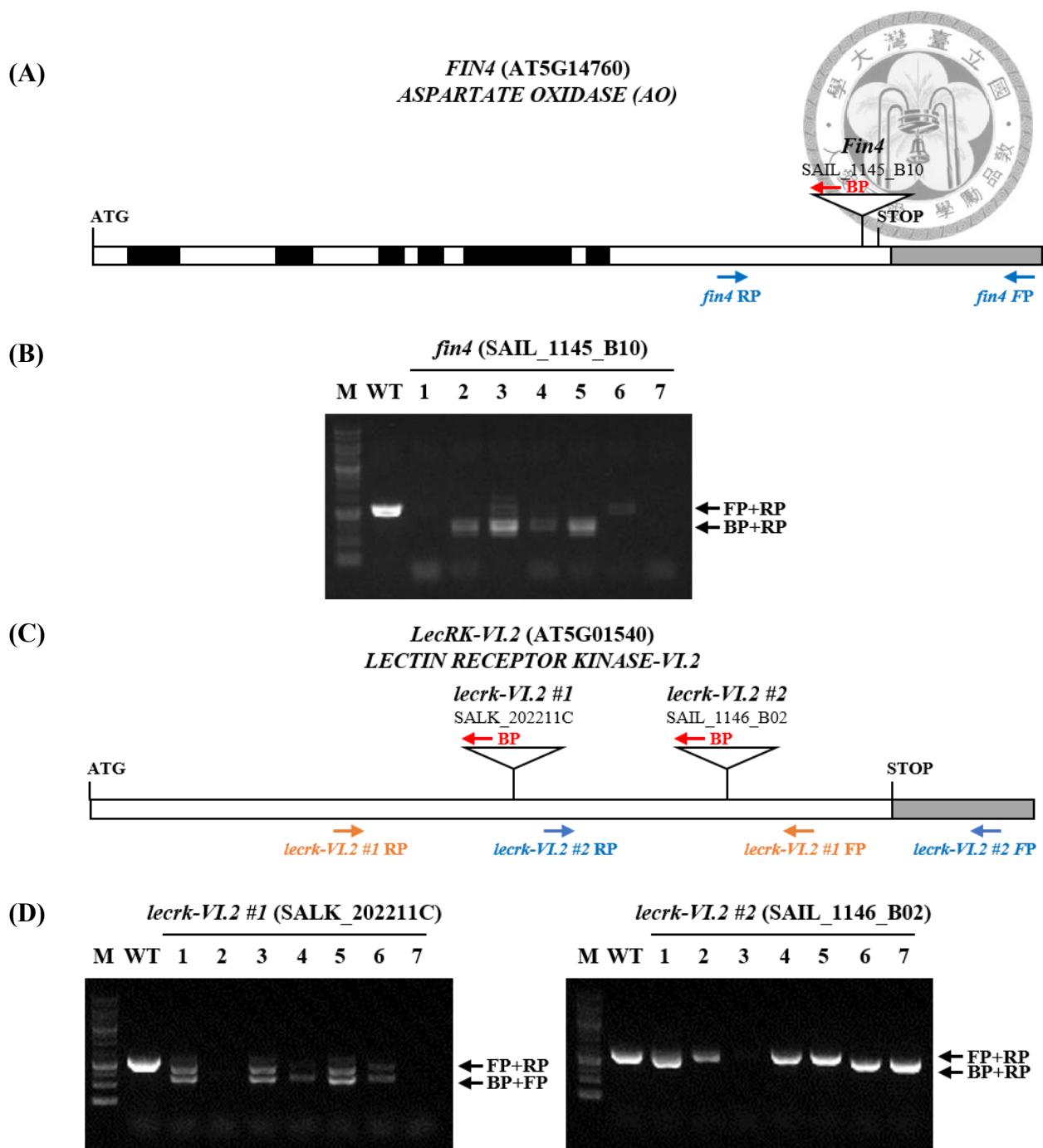


Figure 17. Schematic representation and PCR verification of T-DNA insertion lines of the *Arabidopsis fin4* and *lecrk-VI.2* mutants

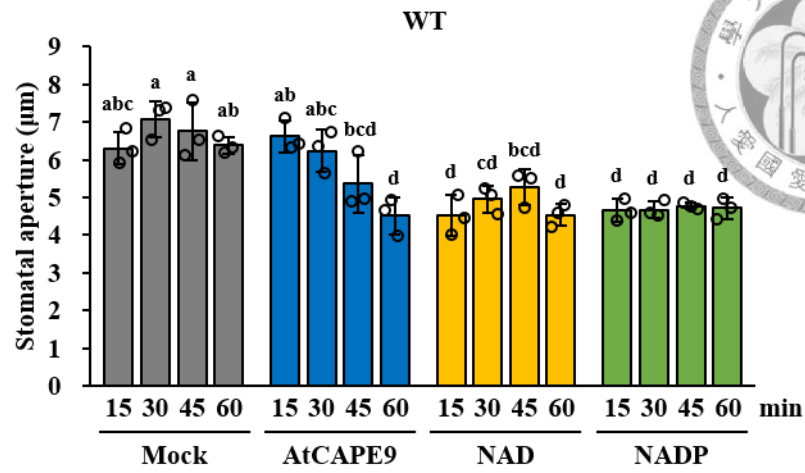
Schematic diagrams depict the gene structures of (A) *FIN4* (AT5G14760) and (C) *LecRK-VI.2* (AT5G01540), showing T-DNA insertion sites and primer location in the *fin4* (SAIL_1145_B10) and *lecrk-VI.2* #1 (SALK_202211C) and *lecrk-VI.2* #2 (SAIL_1146_B02) mutants. PCR analysis was conducted to confirm the presence of T-DNA insertions and zygosity of (B) *fin4* and (D) *lecrk-VI.2* mutants. Key genomic features include: ATG (start codon), STOP (stop codon), BP (base primers), FP (forward primers), and RP (reverse primers). Black boxes represent introns, while white boxes represent exons. Gel electrophoresis shows sample order (from left to right): M, size

marker; WT, Col-0 wild-type; and individual sibling lines from the T-DNA insertion mutants, denoted by numerical labels.

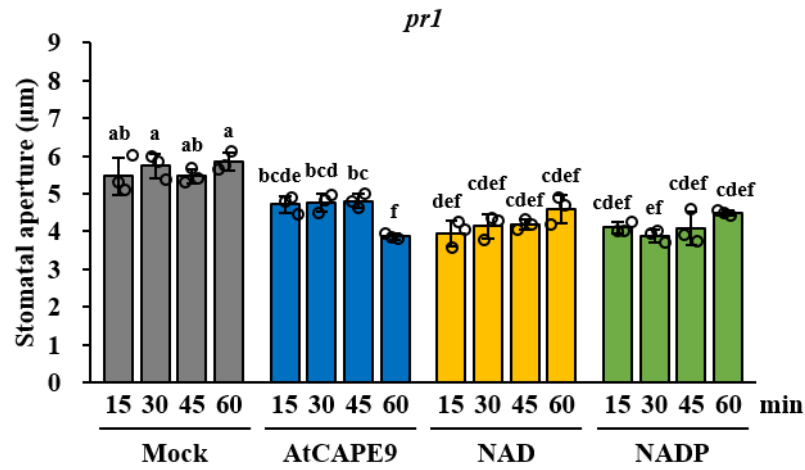




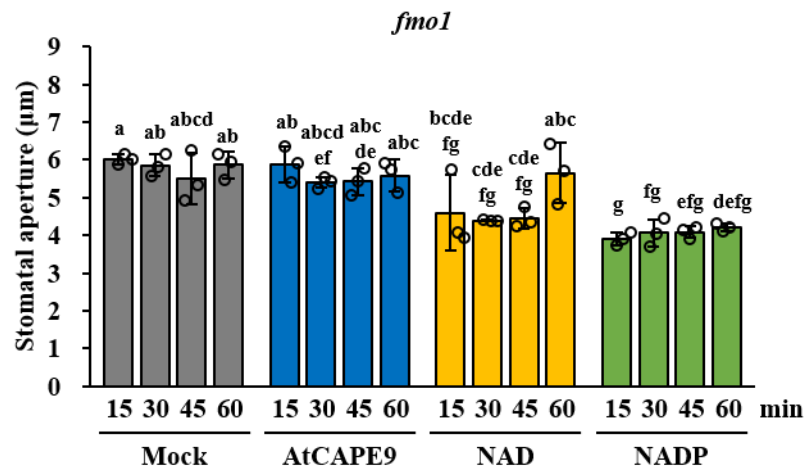
(A)



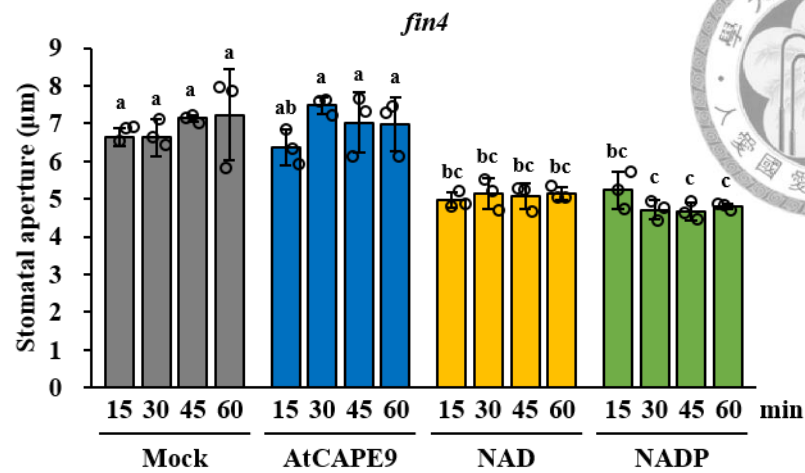
(B)



(C)



(D)



(E)

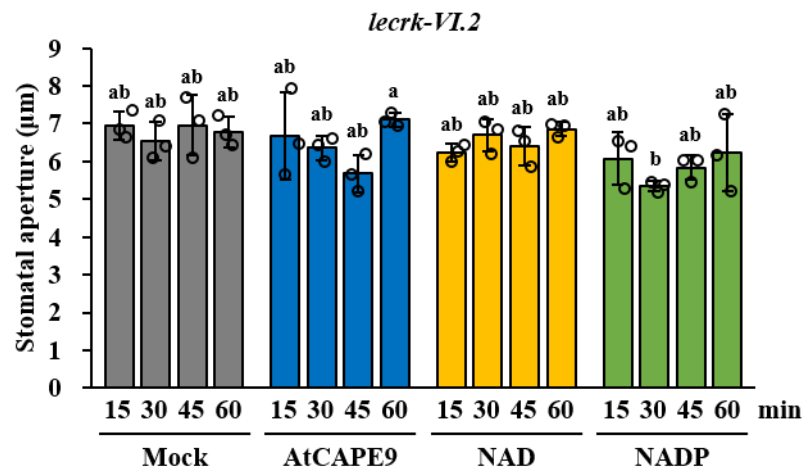
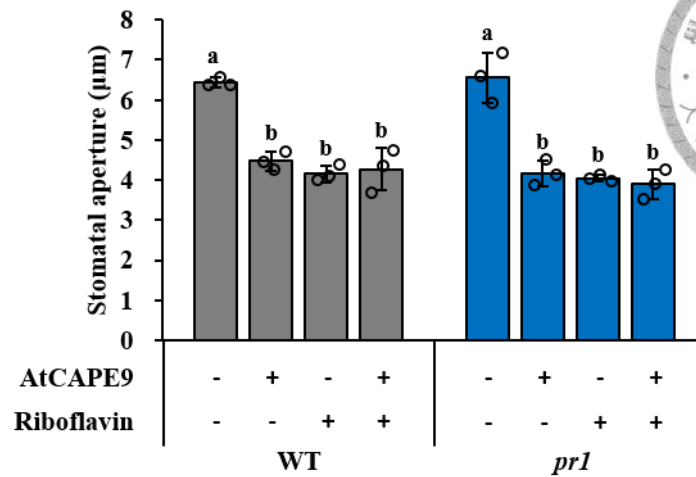


Figure 18. NAD(P) functions as a key downstream signal enhancing NHP-AtCAPE9-mediated stomatal closure in systemic leaves in Arabidopsis

Systemic stomatal apertures were measured after 15, 30, 45, and 60 minutes of treatment with stomatal buffer (Mock), 100 nM AtCAPE9, 0.4 mM NAD, or 0.4 mM NADP in Arabidopsis (A) WT, (B) *pr1*, (C) *fmo1*, (D) *fin4*, and (E) *lecrk-VI.2* mutants. Data are presented as means $\pm$ standard deviation (SD) from three independent plants. Letters above the bars indicate statistically significant differences between treatment groups, as determined by one-way ANOVA ($p < 0.05$).



(A)



(B)

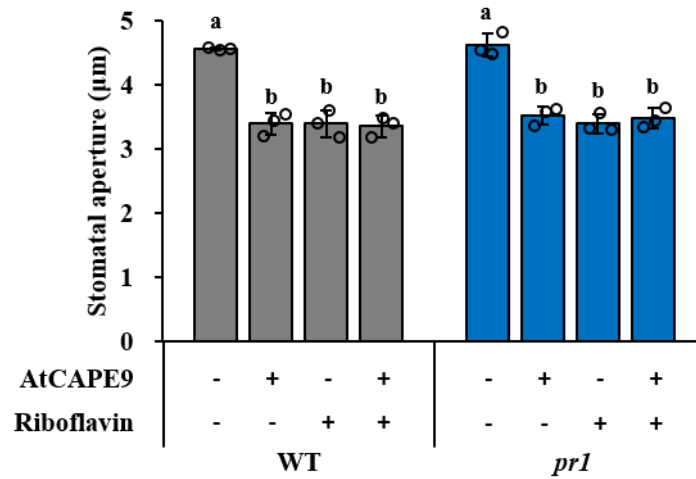


Figure 19. Riboflavin-induced stomatal closure in local and systemic leaves in WT and *pr1* Arabidopsis plants

(A) Local and (B) systemic stomatal apertures were measured after 1 hour treatment with stomatal buffer (Mock), 100 nM AtCAPE9, 0.4 mM riboflavin, or a combination of AtCAPE9 and riboflavin in Arabidopsis WT and *pr1* mutants. Data are expressed as means $\pm$ SD from three independent plants. Letters above the bars represent statistically significant differences among treatment groups, as determined by one-way ANOVA ($p < 0.05$).

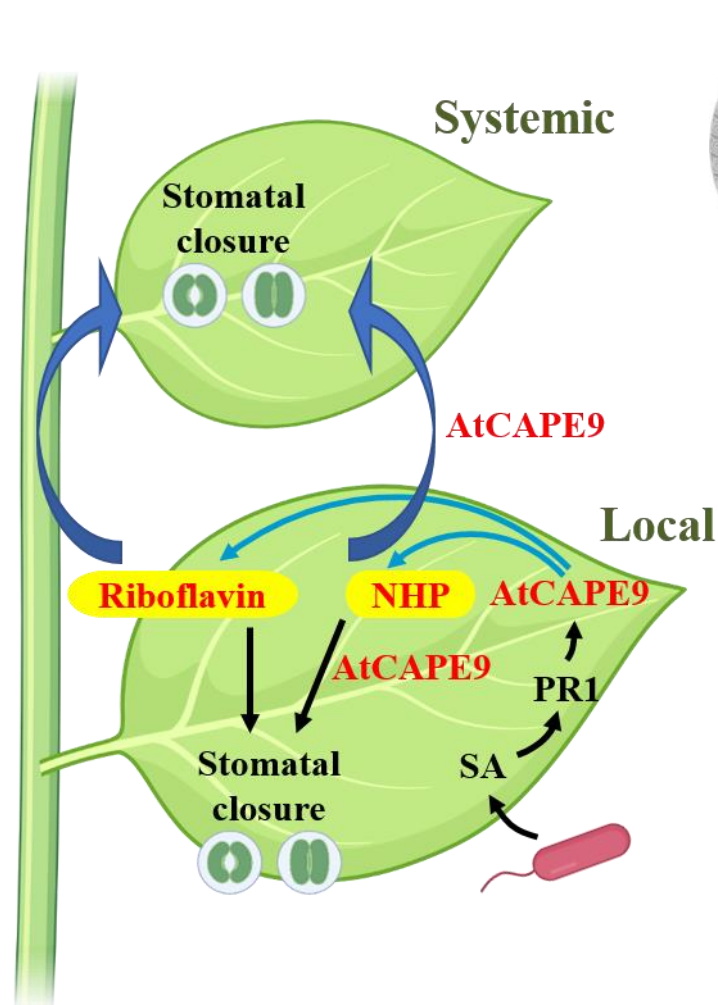


Figure 20. Proposed model for AtCAPE9-induced stomatal immunity featuring NHP and riboflavin

The schematic illustrates the proposed model for AtCAPE9-induced stomatal immunity in Arabidopsis, highlighting the role of NHP and riboflavin. AtCAPE9 stimulate the accumulation of NHP and riboflavin in local leaves as well as in phloem exudates, supporting their roles as mobile signals for AtCAPE9-induced systemic acquired resistance (SAR). NHP is required for AtCAPE9-induced stomatal immunity; however, the NHP-induced stomatal immunity partially dependent on AtCAPE9. Riboflavin is suggested to induce stomatal closure through a PR1-independent mechanism or downstream of the PR1 signaling pathway.

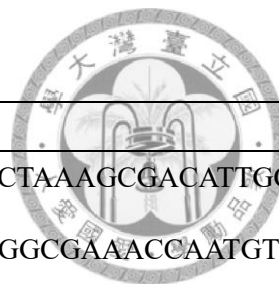


Table 1. Sequence of the primers used in this study

Gene Name	AGI No.	Name	Primer	Sequence
Flagellin-insensitive 4 (Fin4)	AT5G14760	SAIL_1145_B10	Forward	5'-GCTGTTCTAAAGCGACATTGC
			Reverse	5'-TTACAAGGCGAAACCAATGTC
Lectin Receptor Kinase-VI.2 (LecRK-VI.2)	AT5G01540	SAIL_1146_B02	Forward	5'-TGGTTCAACGATCAAAAATGG
			Reverse	5'-GTTCGAGAATTTGTCGCAGAG
		SALK_202211C	Forward	5'-GCACTGAGAATCTCACCGTTC
			Reverse	5'-CGCGAGATTAGGGTTTAAACC

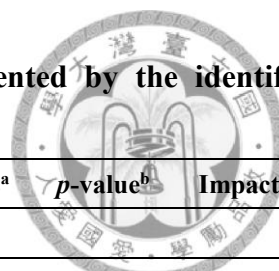
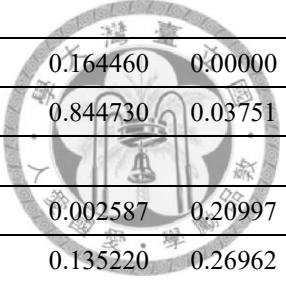


Table 2. Summary of the 66 involved metabolic pathways represented by the identified metabolites

No.	Pathway Name	Match Status ^a	<i>p</i> -value ^b	Impact ^c
Amino acid metabolisms				
1	Arginine biosynthesis	9/18	0.000028	0.47185
2	Alanine, aspartate and glutamate metabolism	10/22	0.000029	0.61511
3	Glycine, serine and threonine metabolism	12/33	0.000066	0.41930
4	Valine, leucine and isoleucine biosynthesis	9/22	0.000200	0.20738
5	Arginine and proline metabolism	10/32	0.001104	0.48959
6	Lysine biosynthesis	4/9	0.010005	0.16216
7	Cysteine and methionine metabolism	10/47	0.021641	0.36359
8	Tyrosine metabolism	5/17	0.027033	0.36872
9	Tryptophan metabolism	6/29	0.078007	0.64123
10	Phenylalanine metabolism	3/12	0.125900	0.42308
11	Phenylalanine, tyrosine and tryptophan biosynthesis	4/22	0.197820	0.16767
12	Histidine metabolism	3/16	0.236160	0.09184
13	Valine, leucine and isoleucine degradation	5/37	0.354360	0.01982
14	Lysine degradation	3/20	0.357390	0.16667
Carbohydrate metabolism				
15	Citrate cycle (TCA cycle)	8/20	0.000554	0.37151
16	Glyoxylate and dicarboxylate metabolism	9/29	0.002083	0.30233
17	Ascorbate and aldarate metabolism	6/20	0.014145	0.22449
18	Amino sugar and nucleotide sugar metabolism	11/52	0.016976	0.40575
19	Galactose metabolism	7/27	0.018726	0.24121
20	Butanoate metabolism	5/17	0.027033	0.36363
21	Pyruvate metabolism	5/23	0.087585	0.29205
22	Fructose and mannose metabolism	4/18	0.114490	0.26789
23	C5-Branched dibasic acid metabolism	2/6	0.125950	0.50000
24	Starch and sucrose metabolism	4/22	0.197820	0.18716
25	Pentose and glucuronate interconversions	2/17	0.552380	0.23810
26	Propanoate metabolism	2/19	0.614680	0.00000
27	Glycolysis / Gluconeogenesis	2/26	0.780910	0.11665
Biosynthesis of other secondary metabolites				
28	Flavone and flavonol biosynthesis	4/10	0.015317	0.85000
29	Biosynthesis of various plant secondary metabolites	7/29	0.027452	0.48000
30	Isoquinoline alkaloid biosynthesis	2/6	0.125950	0.50000

31	Monobactam biosynthesis	2/8	0.204660	0.00000
32	Glucosinolate biosynthesis	9/65	0.244730	0.00000
33	Betalain biosynthesis	1/3	0.285650	1.00000
34	Phenylpropanoid biosynthesis	6/43	0.300980	0.21142
35	Indole alkaloid biosynthesis	1/4	0.361530	0.00000
36	Tropane, piperidine and pyridine alkaloid biosynthesis	1/9	0.636260	0.00000
37	Caffeine metabolism	1/10	0.675040	0.00000
38	Flavonoid biosynthesis	4/47	0.752440	0.10758
Metabolism of cofactors and vitamins				
39	Pantothenate and CoA biosynthesis	7/25	0.012161	0.22862
40	Vitamin B6 metabolism	4/12	0.030490	0.22857
41	Riboflavin metabolism	3/13	0.151530	0.18519
42	Nicotinate and nicotinamide metabolism	3/13	0.151530	0.23636
43	Thiamine metabolism	4/22	0.197820	0.00578
44	One carbon pool by folate	2/8	0.204660	0.54762
45	Lipoic acid metabolism	3/24	0.476100	0.01868
46	Folate biosynthesis	3/31	0.654940	0.05303
47	Ubiquinone and other terpenoid-quinone biosynthesis	4/47	0.752440	0.17771
Lipid metabolism				
48	Glycerophospholipid metabolism	5/38	0.376530	0.10378
49	Glycerolipid metabolism	2/21	0.670170	0.08076
50	alpha-Linolenic acid metabolism	1/26	0.947100	0.00000
51	Sphingolipid metabolism	1/27	0.952800	0.00000
52	Fatty acid biosynthesis	1/56	0.998340	0.00000
Metabolism of other amino acids				
53	D-Amino acid metabolism	4/7	0.003297	0.00000
54	Glutathione metabolism	7/26	0.015192	0.52630
55	Cyanoamino acid metabolism	6/29	0.078007	0.00000
56	beta-Alanine metabolism	4/18	0.114490	0.00000
57	Taurine and hypotaurine metabolism	1/5	0.429380	0.37500
Energy metabolism				
58	Sulfur metabolism	3/15	0.206940	0.06630
59	Carbon fixation in photosynthetic organisms	3/21	0.387710	0.09506
60	Nitrogen metabolism	1/12	0.740720	0.00000
Metabolism of terpenoids and polyketides				
61	Zeatin biosynthesis	5/21	0.062874	0.06098

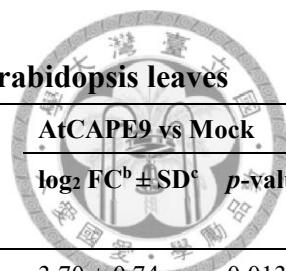


62	Monoterpenoid biosynthesis	2/7	0.164460	0.00000
63	Terpenoid backbone biosynthesis	2/30	0.844730	0.03751
Nucleotide metabolism				
64	Purine metabolism	16/73	0.002587	0.20997
65	Pyrimidine metabolism	7/41	0.135220	0.26962
Glycan biosynthesis and metabolism				
66	N-Glycan biosynthesis	1/35	0.981120	0.00000

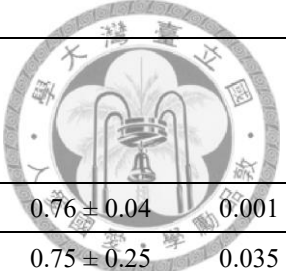
^a Match status = Indicates the number of metabolites found in each particular pathway.

^b *p*-value = The *p*-value was calculated from the enrichment analysis

^c Impact values = Represent the importance of each pathway calculated from the pathway topology analysis.

**Table 3. List of 35 up-regulated metabolites induced by AtCAPE9 in Arabidopsis leaves**

No.	Average RT ^a (min)	Average m/z	Adduct	Metabolite Name	AtCAPE9 vs Mock	
					log ₂ FC ^b ± SD ^c	p-value
1	3.47	470.1430	[M-H]-	10-Formyl-7,8-dihydrofolic acid	3.70 ± 0.74	0.013
2	1.23	241.0324	[M-H]-	2-(4,6-Dichloro-2-methyl-1H-indol-3-yl)ethylamine	2.05 ± 0.70	0.037
3	1.70	267.0994	[M-H]-	Ser-Tyr	1.53 ± 0.43	0.025
4	2.99	217.1202	[M-H]-	Ser-Leu	1.45 ± 0.55	0.045
5	2.79	201.1246	[M-H]-	Ala-Ile	1.25 ± 0.32	0.021
6	1.08	146.0805	[M+H] ⁺	N-hydroxy-pipecolic acid	1.20 ± 0.46	0.045
7	2.26	104.1063	[M] ⁺	Choline	1.18 ± 0.37	0.031
8	2.46	349.2362	[M+H] ⁺	(1S,4aR,5S)-5-[(E)-5-Methoxy-3-methyl-5-oxopent-3-enyl]-1,4a-dimethyl-6-methylidene-3,4,5,7,8,8a-hexahydro-2H-naphthalene-1-carboxylic acid	1.14 ± 0.13	0.004
9	5.26	310.1126	[M+Na] ⁺	lycorine	1.11 ± 0.20	0.011
10	5.07	557.2346	[2M+H] ⁺	Meranzin hydrate	1.04 ± 0.31	0.028
11	5.19	263.1658	[M-H]-	Dinor-12-oxophytodienoic acid	1.02 ± 0.31	0.029
12	3.50	377.1468	[M+H] ⁺	Riboflavin	0.97 ± 0.36	0.044
13	1.20	559.2847	[2M+H] ⁺	Ramosetron	0.93 ± 0.26	0.024
14	7.21	305.1342	[M+Na] ⁺	4-(2,7-Dihydroxy-6-methylheptan-2-yl)-3-hydroxybenzoic acid	0.93 ± 0.22	0.018
15	4.69	309.1285	[M+H] ⁺	Toddalolactone	0.87 ± 0.12	0.006
16	6.08	359.2379	[M+H] ⁺	Ala-Ile-Arg	0.86 ± 0.34	0.049
17	2.03	367.1067	[M-H]-	1,3,5-Trihydroxy-4-(((2E)-3-(3-hydroxy-4-methoxyphenyl)prop-2-enoyl)oxy)cyclohexanecarboxylic acid	0.83 ± 0.23	0.025
18	6.15	260.1956	[M+H] ⁺	Lys-Leu	0.83 ± 0.18	0.016
19	1.54	225.1134	[M-H]-	Tuberonic acid	0.82 ± 0.32	0.049
20	1.19	360.1038	[M+H] ⁺	Clovamide	0.80 ± 0.26	0.034
21	1.62	268.1028	[M+H] ⁺	Adenosine	0.79 ± 0.25	0.032
22	4.92	339.2151	[M+H-H ₂ O] ⁺	(2E,4E)-12-Hydroxy-13-(hydroxymethyl)-14-methoxy-3,5,7-trimethyl-14-oxotetradeca-2,4-dienoic acid	0.79 ± 0.22	0.024
23	4.92	321.2055	[M+H]-	(2E,4E)-12-Hydroxy-13-	0.77 ± 0.20	0.022

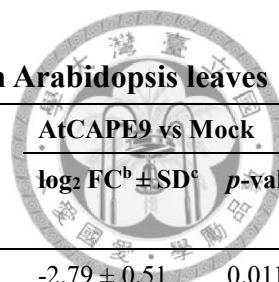


			2H ₂ O)+	(hydroxymethyl)-14-methoxy-3,5,7-trimethyl-14-oxotetradeca-2,4-dienoic acid		
24	4.75	268.1012	[M+H] ⁺	Adenosine	0.76 ± 0.04	0.001
25	3.42	323.0900	[M-H] ⁻	4'-Hydroxywarfarin	0.75 ± 0.25	0.035
26	4.75	289.1332	[M+H] ⁺	2-(4-Phenylphenyl)-N-(pyridin-2-yl)acetamide	0.75 ± 0.22	0.028
27	1.71	214.9967	[M-H] ⁻	[(2-Chlorobenzyl)sulfanyl]acetic acid	0.74 ± 0.18	0.018
28	1.11	134.0472	[M-H] ⁻	Adenine	0.74 ± 0.28	0.044
29	4.82	145.0873	[M-H] ⁻	2-(3-Methylbutoxy)acetic acid	0.73 ± 0.24	0.034
30	4.51	378.1292	[M+Na] ⁺	3,4-dihydro-papaveraldine	0.72 ± 0.28	0.047
31	1.20	573.2659	[M+Na] ⁺	Convallatoxin	0.72 ± 0.19	0.023
32	2.11	206.0420	[M+H] ⁺	Xanthurenic acid	0.66 ± 0.14	0.015
33	1.30	335.1091	[M+H] ⁺	Worenine	0.64 ± 0.22	0.038
34	3.82	374.1197	[M+H] ⁺	5-Hydroxyrosglitazone	0.63 ± 0.21	0.035
35	6.45	721.3673	[M+FA-H] ⁻	DGMG 18:3	0.61 ± 0.19	0.031

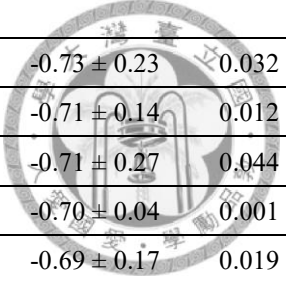
^a RT = Retention time.

^b FC = Fold change, mean value of peak area obtained from AtCAPE9 treatment group/ mean value of peak area obtained from mock treatment group.

^c SD = Standard deviation

**Table 4. List of 36 down-regulated metabolites induced by AtCAPE9 in Arabidopsis leaves**

No.	Average RT ^a (min)	Average m/z	Adduct	Metabolite Name	AtCAPE9 vs Mock	
					log ₂ FC ^b ± SD ^c	p-value
1	5.11	363.0675	[M+Na] ⁺	Esculin	-2.79 ± 0.51	0.011
2	1.67	273.1229	[M+H] ⁺	Sotalol	-1.74 ± 0.37	0.015
3	5.11	527.1562	[M+Na] ⁺	Erlose	-1.32 ± 0.25	0.012
4	5.11	543.1323	[M+H] ⁺	Rhusflavanone	-1.32 ± 0.26	0.013
5	1.68	119.0485	[M+H-2H ₂ O] ⁺	(3,4-Dihydroxyphenyl)ethanol	-1.14 ± 0.24	0.015
6	5.11	325.1111	[M+H-H ₂ O] ⁺	D-Turanose	-1.06 ± 0.31	0.027
7	7.37	478.2956	[M-H] ⁻	1-Oleoyl-sn-glycero-3-phosphoethanolamine	-1.05 ± 0.27	0.022
8	7.22	540.3303	[M+FA-H] ⁻	LPC 16:0	-1.01 ± 0.08	0.002
9	1.26	124.0388	[M+H] ⁺	4-Pyridinecarboxylic acid	-0.99 ± 0.23	0.018
10	5.25	303.0760	[M+Na] ⁺	N-(1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)-2-phenylacetamide	-0.99 ± 0.10	0.003
11	1.18	341.0861	[M+H] ⁺	Esculin	-0.95 ± 0.32	0.035
12	5.11	145.0488	[M+H-2H ₂ O] ⁺	D-Psicose	-0.91 ± 0.27	0.027
13	5.11	97.0278	[M+Na] ⁺	Propionic acid	-0.91 ± 0.19	0.015
14	4.73	241.0023	[M-H-H ₂ O] ⁻	(E)-1-Hydroxy-2-methylbut-2-enyl diphosphate	-0.90 ± 0.25	0.025
15	5.11	127.0383	[M+H] ⁺	1,3,5-Benzenetriol	-0.90 ± 0.26	0.027
16	3.41	433.1172	[M+H] ⁺	apigetrin	-0.89 ± 0.18	0.014
17	5.11	109.0278	[M+H-H ₂ O] ⁺	5-(Hydroxymethyl)-2-furaldehyde	-0.88 ± 0.23	0.022
18	4.10	477.0653	[M-H] ⁻	Neoglucobrassicin	-0.88 ± 0.16	0.011
19	5.11	360.1497	[M+NH ₄] ⁺	Leucrose	-0.84 ± 0.15	0.011
20	1.31	461.1341	[M+Na] ⁺	Sec-O-glucosylhamaudol	-0.80 ± 0.29	0.041
21	2.52	175.0254	[M-H] ⁻	Vitamin C (Ascorbate)	-0.78 ± 0.21	0.023
22	1.14	201.0533	[M+H-H ₂ O] ⁺	3-Acetyl-8-methoxycoumarin	-0.77 ± 0.10	0.006
23	2.46	683.2267	[2M-H] ⁻	D-(+)-Trehalose	-0.75 ± 0.18	0.018
24	1.61	124.0388	[M+H] ⁺	4-Pyridinecarboxylic acid	-0.75 ± 0.24	0.033
25	1.16	101.0591	[M+H] ⁺	3,3-Dimethylacrylic acid	-0.74 ± 0.12	0.008



26	1.67	179.0695	[M+H] ⁺	Coniferyl aldehyde	-0.73 ± 0.23	0.032
27	5.11	180.0859	[M+H] ⁺	D-(+)-Glucosamine	-0.71 ± 0.14	0.012
28	1.28	422.0258	[M-H] ⁻	Glucoiberin	-0.71 ± 0.27	0.044
29	1.18	150.0576	[M+H] ⁺	Methionine	-0.70 ± 0.04	0.001
30	2.00	324.0938	[M+H] ⁺	Lycorine hydrochloride	-0.69 ± 0.17	0.019
31	2.89	263.1379	[M+H] ⁺	Phe-Pro	-0.66 ± 0.16	0.020
32	5.11	99.0436	[M+H- H ₂ O] ⁺	Levulinic acid	-0.64 ± 0.14	0.015
33	4.10	563.1434	[M-H] ⁻	5-Hydroxy-2-(4-hydroxyphenyl)-4-oxo-3-(β-D-xylopyranosyloxy)-4H-chromen-7-yl 6-deoxy-α-L-mannopyranoside	-0.62 ± 0.17	0.023
34	3.55	250.1466	[M+H] ⁺	2-amino-10-methanesulfinyldecanoic acid	-0.62 ± 0.24	0.047
35	4.17	725.2245	[M+H- H ₂ O] ⁺	Kaempferol-3-Rhamnoside-4"-Rhamnoside-7-Rhamnoside	-0.60 ± 0.13	0.015
36	2.69	582.2174	[M+H] ⁺	5,7-dihydroxy-8-(1-(2-hydroxy-6-methoxyquinolin-3-yl)-3-(4-methylpiperazin-1-yl)-3-oxopropyl)-2-phenyl-4H-chromen-4-one	-0.59 ± 0.16	0.024

^a RT = Retention time.

^b FC = Fold change, mean value of peak area obtained from AtCAPE9 treatment group/ mean value of peak area obtained from mock treatment group.

^c SD = Standard deviation

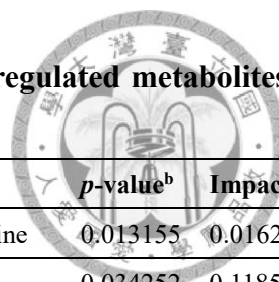


Table 5. Enriched metabolic pathways Associated with AtCAPE9-upregulated metabolites in Arabidopsis leaves

Pathway Name	Match Status ^a	Metabolites	<i>p</i> -value ^b	Impact ^c
Purine metabolism	2/73	Adenosine; Adenine	0.013155	0.01627
Riboflavin metabolism	1/13	Riboflavin	0.034252	0.11852
Zeatin biosynthesis	1/21	Adenine	0.054889	0.00000
alpha-Linolenic acid metabolism	1/26	12-OPDA	0.083822	0.12805
Glycine, serine and threonine metabolism	1/33	Choline	0.085221	0.00000
Glycerophospholipid metabolism	1/38	Choline	0.097641	0.02862

^a Match status = Indicates the number of metabolites found in each particular pathway.

^b *p*-value = The *p*-value was calculated from the enrichment analysis

^c Impact values = Represent the importance of each pathway calculated from the pathway topology analysis.

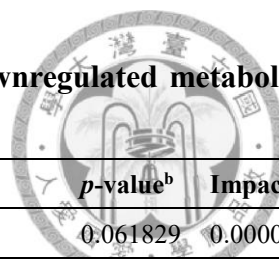


Table 6. Enriched metabolic pathways Associated with AtCAPE9-downregulated metabolites in Arabidopsis leaves

Pathway Name	Match Status ^a	Metabolites	<i>p</i> -value ^b	Impact ^c
Propanoate metabolism	1/19	Propanoate	0.061829	0.00000
Ascorbate and aldarate metabolism	1/20	Ascorbate	0.064997	0.13265
Starch and sucrose metabolism	1/22	alpha,alpha-Trehalose	0.071306	0.00960
Glutathione metabolism	1/26	Ascorbate	0.083822	0.00000
Biosynthesis of various plant secondary metabolites	1/29	L-Methionine	0.09312	0.00000
Cysteine and methionine metabolism	1/47	L-Methionine	0.14734	0.12916
Amino sugar and nucleotide sugar metabolism	1/52	D-Glucosamine	0.16193	0.00000
Glucosinolate biosynthesis	1/65	L-Methionine	0.19893	0.00000

^a Match status = Indicates the number of metabolites found in each particular pathway.

^b *p*-value = The *p*-value was calculated from the enrichment analysis

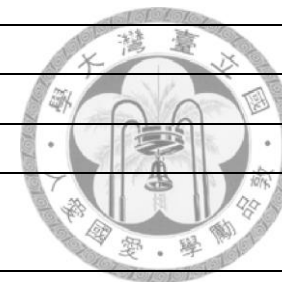
^c Impact values = Represent the importance of each pathway calculated from the pathway topology analysis.

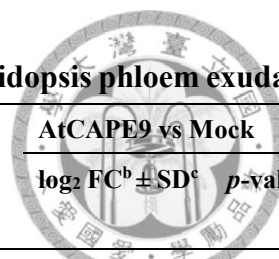
Table 7. Metabolites regulated by AtCAPE9 and linked to plant immunity

Metabolite Name	Plant Immune	References
Up-regulated		
N-hydroxy-pipecolic acid (NHP)	SAR	[72]
Dinor-12-oxophytodienoic acid (12-OPDA)	Local pathogen resistance Stomatal closure	[149]
Riboflavin	SAR Local pathogen resistance	[157]
Down-regulated		
Psicose	Local pathogen resistance	[150]
Ascorbic acid	Local pathogen resistance	[159]
Trehalose	SAR Local pathogen resistance	[151, 160]
Coniferyl aldehyde	Resistance to pests & pathogens	[153]

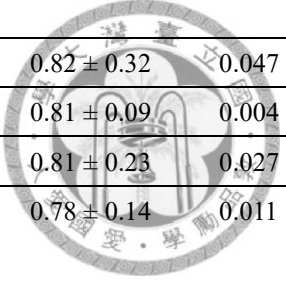
^a FC = Fold change, mean value of peak area obtained from AtCAPE9 treatment group/ mean value of peak area obtained from mock treatment group.

^b SD = Standard deviation.



**Table 8. List of 39 up-regulated metabolites induced by AtCAPE9 Arabidopsis phloem exudates**

No.	Average RT ^a (min)	Average m/z	Adduct	Metabolite Name	AtCAPE9 vs Mock	
					log ₂ FC ^b ± SD ^c	p-value
1	1.06	146.0817	[M+H] ⁺	N-hydroxy-pipecolic acid	2.17 ± 0.83	0.045
2	4.37	382.1554	[M+Na] ⁺	n-homoveratroylhomoveratrylamine	1.35 ± 0.35	0.021
3	2.92	327.1578	[M+H] ⁺	1-Propanone, 1-[2,3-dihydro-4-hydroxy-2-(1-hydroxy-1-methylethyl)-5-benzofuranyl]-3-phenyl-	1.31 ± 0.27	0.014
4	6.66	520.3374	[M+H] ⁺	3-Hydroxy-2-((9Z,12Z)-octadeca-9,12-dienoyloxy)propyl 2-(trimethylazaniumyl)ethyl phosphate	1.30 ± 0.36	0.025
5	2.81	296.1484	[M+H] ⁺	2-[(2-Ethylbutanoyl)amino]-4,5-dimethoxybenzoic acid	1.21 ± 0.45	0.044
6	4.91	270.1100	[M+H] ⁺	N-(2-Phenylethyl)-1,3-benzodioxole-5-carboxamide	1.16 ± 0.25	0.015
7	2.75	218.1507	[M+H] ⁺	2-Cyclohexyl-N-phenylacetamide	1.13 ± 0.26	0.017
8	5.15	365.1523	[M+H] ⁺	MMV659004	1.11 ± 0.32	0.027
9	6.35	295.1153	[M+H] ⁺	7-methoxy-6-(1,2,3-trihydroxy-3-methylbutyl)chromen-2-one	1.07 ± 0.28	0.022
10	6.35	281.1363	[M+Na] ⁺	Octyl .alpha.-ketoglutarate	1.04 ± 0.26	0.021
11	6.35	133.0640	[M+H-H ₂ O] ⁺	3-Ethylbenzoic acid	1.02 ± 0.29	0.026
12	6.35	119.0848	[M+H] ⁺	DL-2,4-Diaminobutyric acid	1.01 ± 0.25	0.020
13	2.78	180.0870	[M+H] ⁺	D-(+)-Glucosamine	0.99 ± 0.29	0.028
14	5.39	379.2065	[M+Na] ⁺	(2E,4E)-12-Hydroxy-13-(hydroxymethyl)-14-methoxy-3,5,7-trimethyl-14-oxotetradeca-2,4-dienoic acid	0.98 ± 0.18	0.011
15	3.49	377.1432	[M+H] ⁺	(-)-Riboflavin	0.93 ± 0.22	0.019
16	6.35	107.0846	[M+H-H ₂ O] ⁺	3-Vinyl-7-oxabicyclo[4.1.0]heptane	0.91 ± 0.24	0.022
17	4.43	239.1258	[M+H] ⁺	1-(3-Ethyl-2,4-dihydroxy-6-methoxyphenyl)-1-butanone	0.88 ± 0.31	0.038
18	6.36	135.0793	[M+H] ⁺	3,4-Dimethylbenzaldehyde	0.88 ± 0.21	0.019
19	6.98	454.2906	[M+H] ⁺	1-Palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine	0.83 ± 0.12	0.007



20	3.91	313.1523	[M+H] ⁺	Phe-Phe	0.82 ± 0.32	0.047
21	3.06	148.0414	[M+H] ⁺	Phthalimide	0.81 ± 0.09	0.004
22	3.14	191.0329	[M+Na] ⁺	vanillic acid	0.81 ± 0.23	0.027
23	2.75	136.0778	[M+H- H ₂ O] ⁺	DL-Octopamine	0.78 ± 0.14	0.011
24	3.10	117.1012	[M+H] ⁺	N-(3-Aminopropyl)acetamide	0.78 ± 0.31	0.049
25	4.91	298.0946	[M+H] ⁺	N'-[(1E)-(5-Chloro-2-hydroxyphenyl)methylene]-2-morpholin-4-ylacetohydrazide	0.77 ± 0.25	0.032
26	4.31	262.0344	[M+H] ⁺	2,6-Dichloro-N-(3-methoxypropyl)benzamide	0.76 ± 0.20	0.023
27	3.14	139.0377	[M+H] ⁺	3,4-Dihydroxybenzaldehyde	0.75 ± 0.13	0.010
28	7.02	496.3376	[M+H] ⁺	1-Palmitoyl-sn-glycero-3-phosphocholine	0.74 ± 0.14	0.011
29	6.30	189.1629	[M+H] ⁺	7,8-Diaminononanoic acid	0.74 ± 0.26	0.039
30	4.00	293.0962	[M+Na] ⁺	Benzyl glucopyranoside	0.72 ± 0.13	0.010
31	5.00	353.1941	[M+H] ⁺	vincarine	0.70 ± 0.13	0.011
32	4.43	207.1364	[M+H- 2H ₂ O] ⁺	2-Oct-7-enylpentanedioic acid	0.69 ± 0.11	0.008
33	1.45	121.0639	[M+H- H ₂ O] ⁺	5-Norbornene-2-carboxylic acid	0.68 ± 0.12	0.010
34	1.17	284.0975	[M+H] ⁺	Guanosine	0.64 ± 0.25	0.045
35	5.41	294.1088	[M+H] ⁺	N-(2,2,2-Trifluoro-1,1-diphenylethyl)acetamide	0.64 ± 0.26	0.049
36	1.16	152.0556	[M+H] ⁺	Guanine	0.64 ± 0.20	0.031
37	6.13	389.2273	[M+Na] ⁺	5(S),6(R)-Lipoxin A4 methyl ester	0.63 ± 0.12	0.012
38	4.26	223.1318	[M+H] ⁺	3-Ethoxy-4-isobutoxybenzaldehyde	0.61 ± 0.19	0.031
39	6.13	211.0861	[M+H] ⁺	5,6,7,8-Tetrahydro-4H-cyclohepta[b]thiophene-2-carbohydrazide	0.60 ± 0.08	0.006

^a RT = Retention time.

^b FC = Fold change, mean value of peak area obtained from AtCAPE9 treatment group/ mean value of peak area obtained from mock treatment group.

^c SD = Standard deviation

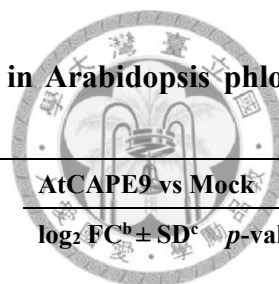


Table 9. List of 1 down-regulated metabolite induced by AtCAPE9 in Arabidopsis phloem exudates

No.	Average	Average	Adduct	Metabolite Name	AtCAPE9 vs Mock	
	RT ^a (min)	m/z			log ₂ FC ^b ± SD ^c	p-value
1	7.08	129.0537	[M+H- H ₂ O] ⁺	Adipic acid	-0.58 ± 0.23	0.048

^a RT = Retention time.

^b FC = Fold change, mean value of peak area obtained from AtCAPE9 treatment group/ mean value of peak area obtained from mock treatment group.

^c SD = Standard deviation

Table 10. List of 2 candidate mobile signaling molecules up-regulated by AtCAPE9

No.	Leaves					Phloem Exudates				
	Average RT ^a (min)	Average m/z	Metabolite Name	AtCAPE9 vs Mock		Average RT ^a (min)	Average m/z	Metabolite Name	AtCAPE9 vs Mock	
				log ₂ FC ^b ± SD ^c	p-value				log ₂ FC ^b ± SD ^c	p-value
1	1.08	146.0805	N-hydroxy-pipecolic acid	1.20 ± 0.46	0.045	1.06	146.0817	N-hydroxy-pipecolic acid	2.17 ± 0.83	0.045
2	3.50	377.1468	Riboflavin	0.97 ± 0.36	0.044	3.49	377.1432	Riboflavin	0.93 ± 0.22	0.019

^a RT = Retention time.

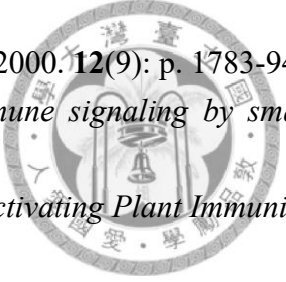
^b FC = Fold change, mean value of peak area obtained from AtCAPE9 treatment group/ mean value of peak area obtained from mock treatment group.

^c SD = Standard deviation



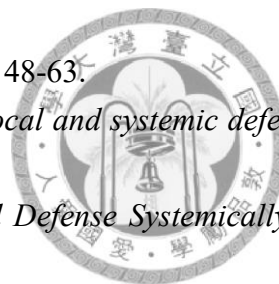
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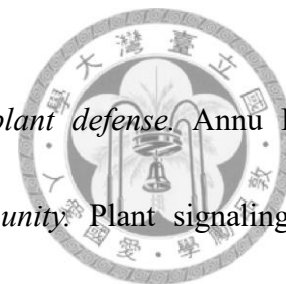
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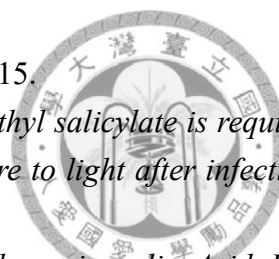
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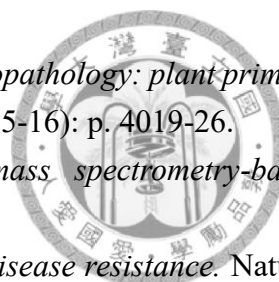
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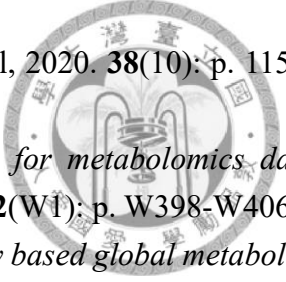
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