




Reprogramming plant specialized metabolism by manipulating protein kinases

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Abstract Being sessile, plants have evolved sophisticated mechanisms to balance between growth and defense to survive in the harsh environment. The transition from growth to defense is commonly achieved by factors, such as protein kinases (PKs) and transcription factors, that initiate signal transduction and regulate specialized metabolism. Plants produce an array of lineage-specific specialized metabolites for chemical defense and stress tolerance. Some of these molecules are also used by humans as drugs. However, many of these defense-responsive metabolites are toxic to plant cells and inhibitory to growth and development. Plants have, thus, evolved complex regulatory networks to balance the accumulation of the toxic metabolites. Perception of external stimuli is a vital part of the regulatory network. Protein kinase-mediated signaling activates a series of defense responses by phosphorylating the target proteins and translating the stimulus into downstream cellular signaling. As biosynthesis of specialized metabolites is triggered when plants perceive stimuli, a possible connection between PKs and specialized metabolism is well recognized. However, the roles of PKs in plant specialized metabolism have not received much attention until recently. Here, we summarize the recent advances in understanding PKs in plant specialized metabolism. We aim to highlight how the stimulatory signals are transduced, leading to the biosynthesis of corresponding metabolites. We discuss the post-translational regulation of specialized metabolism and provide insights into the mechanisms by which plants respond to the external signals. In addition, we propose possible strategies to increase the production of plant specialized metabolites in biotechnological applications using PKs.

Keywords Medicinal plants, Protein kinases, MAP kinase, Specialized metabolites, Post-translational regulation, Signal transduction

INTRODUCTION

Being sessile, plants cannot evade biotic and abiotic stresses. Plants possess sophisticated physical (structural) and chemical defense systems to combat pathogens and survive in the harsh environment.

Biosynthesis of specialized metabolites in response to various biotic or abiotic stresses is a key component of chemical defense. Unlike primary metabolites, specialized metabolites are not usually involved in the basal development of plants, and excessive accumulation of such metabolites often hinders or inhibits plant growth and development. Therefore, healthy plants usually accumulate very low levels of specialized metabolites;

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however, the biosynthesis is triggered rapidly when plants are subject to biotic or abiotic stress. Plants produce more than 200,000 specialized metabolites (Pott et al. 2019). Although the chemical nature of specialized metabolites varies in plants, the majority of them originate from the isoprenoid, phenylpropanoid, terpenoid, or fatty acid pathway (Dixon 2001). Many specialized metabolites are cytotoxic, capable of killing pathogenic microbes or insects, and are, thus, considered as chemical weapons of plants.

Biosynthesis of many (if not all) defense-associated specialized metabolites is coupled with the plant immune system that responds rapidly to external stimuli. The plant immune systems can be divided into two types: pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl 2006). PTI and ETI are accomplished by a series of protein kinases (PKs) for signal perception and transduction. PKs are a class of enzymes that use the phosphate group of ATP to phosphorylate target proteins (substrates). PKs are universal in eukaryotes. Genome sequence analysis of 25 major plant species identifies more than 26,000 PKs (Lehti-Shiu and Shiu 2012). The *Arabidopsis* genome contains more than 1000 genes encoding PKs (Wang et al. 2007). All PKs have a functional domain, consisting of 250–300 amino acids, for phosphate group transfer (Xu and Nussinov 1998). The functionally conserved PKs do not share high sequence identity outside of the functional domain (Xu and Nussinov 1998).

PKs play critical roles in the perception of external stimuli, especially in the plant immune system. The conserved motifs in PAMPs are recognized as ligands by relevant pattern recognition receptors (PRRs) in host plant cells (Mogensen 2009). Plant PRRs are receptor kinases and receptor-like kinases (RLKs) present on the plant cell surface and have been well reviewed (Couto and Zipfel 2016). In brief, RLKs have no kinase domain or intracellular signaling domain and functionally depend on receptor kinases. Both receptor kinases and RLKs contain a ligand-binding domain. Based on the protein structure of the ligand-binding domain, PRRs are classified into four groups (Couto and Zipfel 2016; Zipfel 2014): the leucine-rich repeat (LRR)-containing PRRs that bind the peptides released by microbial pathogens, the lysine motif (LysM) PRRs that recognize carbohydrate-based ligands, such as fungal chitin or bacterial peptidoglycan (Desaki et al. 2018), and lectin-type PRRs with binding activity to extracellular ATP or bacterial lipopolysaccharides (LPS). As some pathogenic bacteria pierce plant cells to secrete virulence effector proteins directly into the plant cytoplasm, a class of intracellular nucleotide-binding domain leucine-rich

repeat receptor kinases (NB-LRRs) specifically bind to the effector proteins (Noman et al. 2019; Wang and Chai 2020). The stimulus signal recognized by PRRs is then transferred to the receptor-like cytoplasmic kinases (RLCKs) by direct phosphorylation (Li et al. 2014a; Cui et al. 2018; Liang and Zhou 2018). A branched signaling cascade characterized by rapid ion-flux changes is primed within minutes, leading to the alteration of extracellular pH and reactive oxygen species (ROS) and elevation of cytosolic Ca^{2+} level. In turn, Ca^{2+} -dependent protein kinase (CDPK) and mitogen-activated protein kinase (MAPK) signaling cascades drive the transcriptome and metabolome reprogramming, establishing defense response (Boller and Felix 2009; Seybold et al. 2014; Lee et al. 2015; Coppola et al. 2019).

In addition to their functions in plant immunity, PKs play vital roles in response to abiotic stress. Unlike transcription factors (TFs), the kinases are not considered as the executors that directly reshape the transcriptomes and metabolomes in plants as they lack DNA-binding domains for altering gene expression. PKs target multiple TFs which directly bind to the target gene promoters to regulate their activities. The important roles of TFs in regulating plant specialized metabolites have been intensively studied in model plants and medicinal plants (Patra et al. 2013). However, how kinases modulate these regulators, particularly in plant specialized metabolism under biotic and abiotic stresses, remains poorly understood. It is, thus, imperative to study how PKs regulate specialized metabolism through TFs.

Medicinal plants produce a vast array of specialized metabolites with antimicrobial and/or antioxidant activities. Plant specialized metabolites are the major determinants of the medical and commercial values of medicinal plants (Hussein and El-Ansary 2019; Anand et al. 2019). Some specialized metabolites are used as drugs. More than 25% of clinical anthropic drugs are derived from medicinal plants (Wurtzel and Kutchan 2016). Well-known examples include the anti-malarial drug artemisinin from *Artemisia annua* (Weathers et al. 2006), as well as the anticancer drugs Taxol from *Taxus* spp. (Lenka et al. 2015) and vincristine and vinblastine from *Catharanthus roseus* (Dugé de Bernonville et al. 2017). However, while toxic to pests or microbes and inhibitory to animal cells, many specialized metabolites are also cytotoxic to plants (Isah 2019). Therefore, excessive synthesis of these metabolites may inhibit the growth and development of plants. In addition, specialized metabolism is energy-intensive, competing with resources for plant growth. The coupling of specialized metabolite biosynthesis and the immune system makes it possible for the plants to instantaneously synthesize

the toxic metabolites after sensing biotic stress without overly affecting growth and development (Gorlenko et al. 2020). Additionally, transcriptomic analysis of the medicinal plants, such as *Echinacea purpurea* (Tahmasebi et al. 2019), *C. roseus* (Verma et al. 2014; Pan et al. 2018), *Salvia miltiorrhiza* (Wenping et al. 2011), and *Valeriana officinalis* (Yeo et al. 2013), reveals that many protein kinase-encoding genes are differentially expressed in response to external stimuli.

Only a limited number of studies so far demonstrate the possible roles of PKs in regulating plant specialized metabolism. Even less known is the regulatory mechanisms that govern such processes. We, thus, compel to review and discuss the recent studies on (i) the possible relationship between PKs in the plant immune system and specialized metabolite biosynthesis, (ii) whether the production of different specialized metabolites in the same plant species requires the activation of different kinase signaling pathways, and (iii) whether the kinase signaling pathways are conserved across plants, especially medicinal plants. Additionally, we intend to provide convincing supports for using PKs to systematically modify the plant metabolic systems to increase the production of medicinally valuable specialized metabolites.

Stress-induced specialized metabolism in plants

Research into secondary metabolism dates back to 1910, when Albrecht Kossel, a Nobel laureate in chemistry, first created the term “secondary metabolites” to distinguish them from primary metabolites (Jones 1953; Hartmann 2008). Not until 1970s, the important functions of secondary metabolites in plants have been realized. “Secondary metabolites” are classified as molecules with no function in plant growth and development. These metabolites are usually synthesized at very low levels (less than 1% of total carbon) (Bourgaud et al. 2001) and rapidly induced when the environment is unfavorable (Ebel 1979). For example, leaf resins of *Larrea* spp. over-accumulate phenylpropanoid derivatives when exposing to ultraviolet (UV) radiation (Rhoades 1977). The abiotic stresses, such as drought, high salinity, excess light, and cold, trigger the production of anthocyanins in *Arabidopsis thaliana* (Christie et al. 1994; Cominelli et al. 2008; Nakabayashi et al. 2014; Isah 2019). Anthocyanin induction is more obvious during the biotic stresses by pathogenic microbes, pests, and herbivores (Bennett and Wallsgrove 1994; Ramakrishna and Ravishankar 2011; Ashraf et al. 2018). Upon pathogen infection, *A. thaliana* also produces other secondary metabolites, including indole and indole-sulfur compounds, glucosinolates, benzenoids,

terpenes, and fatty acid derivatives (reviewed in D’Auria and Gershenzon 2005). These metabolites induced by biotic or abiotic stress act as antimicrobial molecules or antioxidants. As more and more secondary metabolites are identified in plants, their important roles in plant growth and defense have also been gradually recognized. Some secondary metabolites are also produced by other organisms including bacteria and fungi (Turner 1971; Pietra 1997), the roles of these metabolites are far from secondary. The term “specialized metabolites” has, thus, emerged to replace secondary metabolites (Pichersky and Lewinsohn 2011; Dixon 2001; Kutchan 2001).

In plants, specialized metabolites are classified into terpenes, flavonoids, N-containing compounds (alkaloids), and phenylpropanoids according to their chemical structures (Wang et al. 2019). They are synthesized from the end products or intermediates of the TCA cycle, photosynthesis, and other processes. The biosynthesis of many plant specialized metabolites is induced by various biotic or abiotic stresses. The transcriptional regulation of biosynthesis of specialized metabolites in plants has been intensively investigated (Shoji and Yuan 2021; Cao et al. 2020; Meraj et al. 2020; Vom Endt et al. 2002; Yang et al. 2012; Patra et al. 2013). The majority of the TFs involved in the biosynthesis of specialized metabolites are from the families of R2R3 MYB, bHLH (basic helix-loop-helix), AP2/ERF (APETALA2/ethylene-responsive factor), WRKY, Zinc finger, and NAC factor (Cao et al. 2020; Shoji and Yuan 2021; Singh et al. 2021; Meraj et al. 2020; Yang et al. 2012). For example, during salt stress, MYB111 binds to the *cis*-elements in the promoter of *chalcone synthase* (*CHS*), *flavanone carboxylase* (*F3H*), *flavanol synthase 1* (*FLS1*), upregulates their transcripts which ultimately trigger the accumulation of the flavonoids (Li et al. 2019). Flavonoids act as antioxidants in plants (Pietta 2000). The absence of MYB111 in the *myb111* mutant dramatically induces the ROS level upon salt treatment (Li et al. 2019). A recent study found that *BHLH IRIDOID SYNTHESIS 3* (*BIS3*), a member of the bHLH TF family, transactivates iridoid pathway genes and positively regulates terpenoid indole alkaloid (TIA) biosynthesis in *C. roseus* upon the induction of jasmonic acid (JA) (Singh et al. 2021).

Plant PKs and specialized metabolites

In addition to transcriptional regulation, post-translational regulation, such as phosphorylation, is evident in many metabolic and developmental pathways. Phosphorylation activates a series of signaling cascades that regulate plant responses to biotic and abiotic stresses.

An increasing number of studies have shown that many TFs are the substrates of PKs. Phosphorylation can regulate the activity, interactions, and subcellular localization of target proteins (Lee and Yaffe 2016). Based on subcellular localization, PKs can be divided into two broad categories: the ones associated with cell wall or plasma membrane via transmembrane domain(s), and those that resided in the cytosol, nucleus, or connect with organelles. Most of the available studies focus on the roles of PRRs, cell wall-associated kinases (WAKs), and mitogen-activated protein kinases (MAPKs) since they are critical in signaling perception and transduction (Erb and Reymond 2019). MAPKs can target the TF regulators and TFs to regulate the expression of defense-associated genes (Ng et al. 2018). MAPKs are also one of the most studied kinases in medicinal plants (Asai et al. 2002; Xu et al. 2008; Taj

et al. 2010; Raina et al. 2012; Paul et al. 2017). Hence, we focus on the PRRs, WAKs, and MAPKs (Fig. 1), and highlight some kinases with direct roles in specialized metabolism (Table 1).

Cell wall-associated kinases

WAKs are related to cell wall-associated defense response, such as strengthening the cell integrity by triggering the biosynthesis of cellulose and phytoalexin (Hu et al. 2017). The *Arabidopsis* WAK family has five members. In monocot, e.g., rice, the WAK family has more than 100 members. Recent studies have reported the roles of WAKs in fungal resistance and the primary or specialized metabolism in model species (Diener and Ausubel 2005; Harkenrider et al. 2016; Zhang et al. 2017). The newly identified ZmWAK-RLK1 in maize

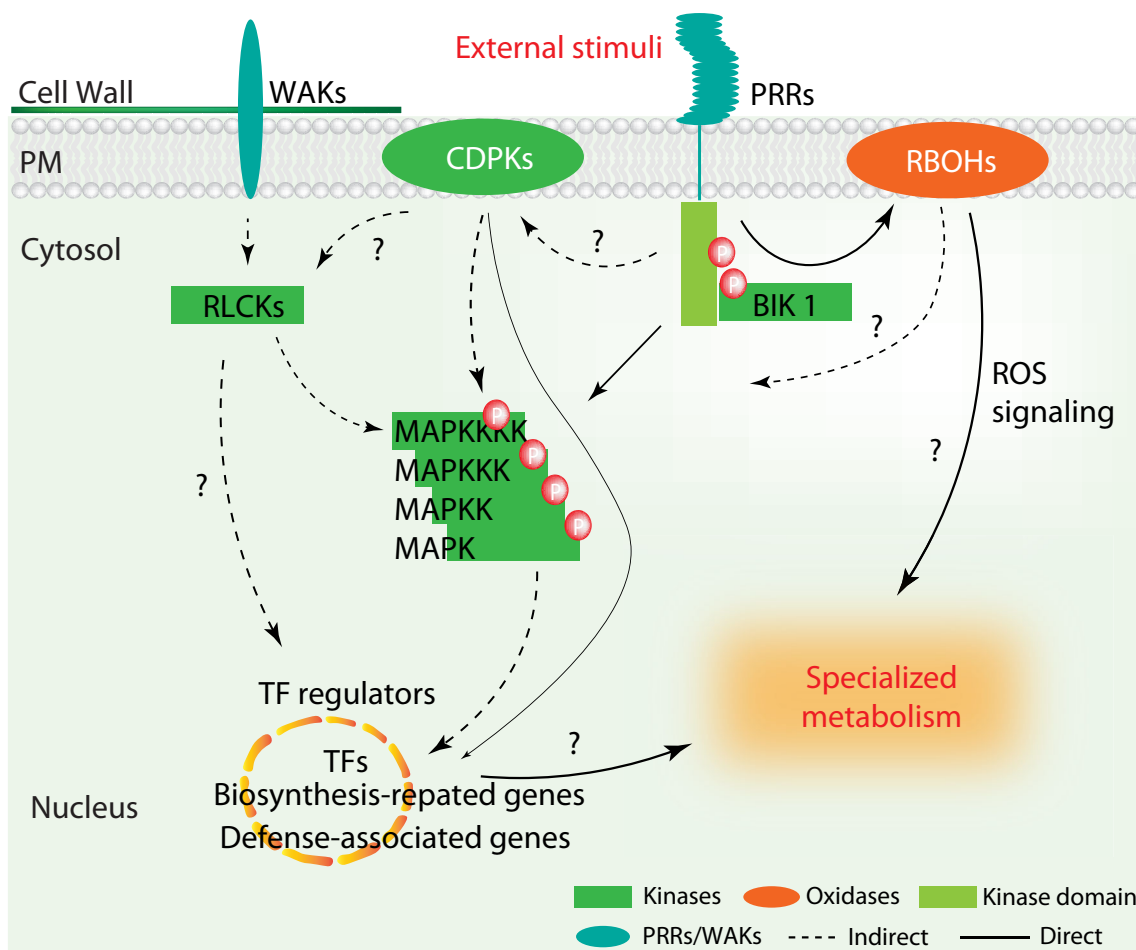


Fig. 1 A potential role of PKs in regulating specialized metabolism in medicinal plants. External stimuli are sensed by membrane-located kinases, such as wall-associated kinases (WAKs) and pathogen-associated molecular patterns (PAMPs) recognition receptor-like kinases (PRRs). The perception activates downstream kinases including cytosolic receptor-like kinases (RLCKs) and MAP kinase cascade, which eventually triggers the transcriptomic and metabolic reprogramming. PRRs may also connect with NADPH oxidases (RBOHs) or Ca²⁺-dependent kinases (CDPKs) to induce ROS/Ca²⁺-dependent signaling cascades, that may contribute to the regulation of specialized metabolism. TFs transcription factors; PM plasma membrane

Table 1 Kinases involved in specialized metabolism

Kinase	Kinase family	Plant species	Specialized metabolite	References
MKK9	MAPKs	<i>Arabidopsis thaliana</i>	Camalexin	Xu et al. (2008)
MPK3, MPK6	MAPKs	<i>A. thaliana</i>	Camalexin	Mao et al. (2011)
MAPKKK α	MAPKs	<i>A. thaliana</i>	Camalexin	Ren et al. (2008)
MEKK1	MAPKs	<i>A. thaliana</i>	Camalexin	Ren et al. (2008)
MPK3, MPK6	MAPKs	<i>A. thaliana</i>	Indole glucosinolates	Xu et al. (2016)
SmMAPK3	MAPKs	<i>Salvia miltiorrhiza</i>	Tanshinone	Xie et al. (2020)
SmSnRK2.6	SnRKs	<i>S. miltiorrhiza</i>	Phenolic acid	Jia et al. (2017)
CrMPK3	MAPKs	<i>Catharanthus roseus</i>	Monoterpenoid indole alkaloids	Raina et al. (2012)
CrMPKK1	MAPKs	<i>C. roseus</i>	Monoterpenoid indole alkaloids	Paul et al. (2017)
MdSOS2L1	CIPKs	Apple	Procyanidin biosynthesis and malate metabolism	Hu et al. (2016b)
MdHXK1	Hexokinase	Apple	Anthocyanins	Hu et al. (2016a)
OsMPK3, OsMPK4, OsMPK6	MAPKs	Rice	Phytoalexin	Kishi-Kaboshi et al. (2010)
<i>MhSnRK1</i>	SnRKs	<i>Malus hupehensis</i>	Ripening-related metabolism	Wang et al. (2012)
<i>PpSnRK1a</i>	SnRKs	Peach	Ripening-related metabolism	
<i>AaSnRk2.6</i>	SnRKs	<i>Artemisia annua</i>	Artemisinin	Zhang et al. (2015)
AKIN10	SnRKs	<i>A. thaliana</i> etc	Terpenes	Robertlee et al. (2017)
MAPKK1 (NtJAM1)	MAPKs	Tobacco	Nicotine	De Boer et al. (2011)
NtMPK4	MAPKs	Tobacco	Nicotine	Liu et al. (2021)

affects fungal resistance by altering the level of benzoxazinoids (BXs) (Yang et al. 2019). BXs are derived from indole-3-glycerophosphate (IGP) in the shikimate pathway. Mutation of *ZmWAK-RLK1* leads to a decrease of BXs in maize compared to that in the wild-type plants after fungal infection. However, it is not known how *ZmWAK-RLK1* regulates BX biosynthesis. As the perception of stimuli is conserved in the higher plants, WAKs may have a similar function in medicinal plants.

So far, no study of WAKs in medicinal plants has been reported, but transcriptomic analysis has identified the possible involvement of WAKs in response to stress and metabolism. *Rheum australe* is an endangered medicinal plant that produces anthraquinones and stilbenoids. *R. australe* grown at high altitudes in the Himalayas exposes to low temperature and high UV radiation. Anthraquinones are the precursors of anticancer drugs and have antioxidant activity (Campos-Martin et al. 2006). Stilbenoids act as antioxidants that can undergo intramolecular cyclization under UV radiation (Chong et al. 2009). Transcriptomic analysis reveals that the MAPK cascades, CDPKs, and WAKs are upregulated in the plants grown in natural conditions compared to those in growth chambers (Mala et al. 2021).

Plasma membrane-located PKs

The majority of PKs located in the cell wall, plasma membrane, or extracellular parts are PRRs. An *Arabidopsis* PRR, FLAGELLIN-SENSITIVE 2 (FLS2), activates BOTRYTIS-INDUCED KINASE 1 (BIK1) upon pathogen infection (Lu et al. 2010). BIK1 is a receptor-like cytoplasmic kinase (RLCK) that directly phosphorylates the respiratory burst oxidase homolog protein D (RBOHD). RBOHD is an NADPH oxidase involved in triggering the accumulation of ROS (Kadota et al. 2014; Li et al. 2014a). RBOHD and its homologs produce ROS through an NADP-malic enzyme (ME)-catalyzed reaction. ME converts L-malate to pyruvate and produce carbon dioxide and NADPH (Detarsio et al. 2003). Pyruvate and glyceraldehyde-3-phosphate are the starting molecules of the methylerythritol-4-phosphate (MEP) pathway (Rodríguez-Concepción and Boronat 2002). The mevalonate (MVA) and MEP pathways produce all plant terpenoids, the largest family of specialized metabolites (Yazaki et al. 2017). RBOHs accept the electrons from NADPH and generate superoxide. Superoxide can spontaneously form hydrogen peroxide in plants. Both superoxide and hydrogen peroxide are ROS, acting as toxic molecules as well as secondary messengers. The

plant cells synthesize antioxidants, some of which are specialized metabolites, such as flavonoids, phenolic acid, and carotenoids, to scavenge over-accumulated ROS (Chapman et al. 2019). Meanwhile, ROS signaling may crosstalk with kinase signaling and hormone signaling to regulate defense response (Waszczak et al. 2018).

Sucrose nonfermenting-1 kinases

Sucrose nonfermenting-1 kinases (SnRKs) are first identified in yeast (*Saccharomyces cerevisiae*). SnRKs can be divided into SnRK1, SnRK2, and SnRK3 subfamilies; members of the three subfamilies are involved in nitrogen, sucrose, and lipid metabolism (reviewed in Halford and Hey 2009). Overexpression of *MhSnRK1* in tomato (*Solanum lycopersicum* L.) triggers fruit ripening (Wang et al. 2012). Overexpression of *PpSnRK1 α* , a homolog of *MhSnRK1* in peach (*Prunus persica* (L.)), also induces early fruit ripening in tomato (Yu et al. 2018). Yeast two-hybrid assay shows that SnRK1 interacts with the TF SIRIN (Yu et al. 2018), a MADS-box TF that regulates fruit ripening (Vrebalov et al. 2002). Ripening is a vital developmental process that results in a series of metabolic changes in fruits. Plants synthesize alkaloids and tannins during the early phase of the ripening process. These metabolites act as antifeedants which make the ripen fruits less favorable for animals (Prasanna et al. 2007). These antifeedant molecules are then replaced by sucrose and other specialized metabolites, such as lycopene, when fruits ripened.

SnRKs also regulate the biosynthesis of other specialized metabolites. The SnRK2.6, a member of the SnRK2 subfamily, regulates the levels of phenolic acids and tanshinoes in *Salvia miltiorrhiza* (Wang and Wang 2011). SnRK2.6 interacts with and activates the ABA-responsive TF SmAREB1 (ABA-responsive element ABRE-binding protein 1). Transgenic plants overexpressing *SmSnRK2.6* have similar phenolic acid levels as the plants overexpressing *SmAREB1* (Wang and Wang 2011). SnRKs also control terpene biosynthesis by regulating the activity of 3-hydroxy-3-methylglutaryl Coenzyme A Reductase (HMGR), the key rate-limiting enzyme in the mevalonic acid (MVA) pathway (Li et al. 2014b). The *Arabidopsis* SnRK1 AKIN10 negatively regulates the activity of HMGR by phosphorylation (Robertlee et al. 2017).

Mitogen-activated protein kinases

The MAPK cascade is an ancient signaling pathway prevalent in eukaryotes. The protein components in the cascade can be classified into four groups based on their

positions in the pathway from downstream to upstream: MAP kinases (MAPKs), MAPK kinases (MAPKKs), MAPKK kinases (MAPKKKs), and MAPKKK kinases (MAPKKKKs). The upstream MAP kinases (such as MAPKKKs) can sequentially activate downstream kinases (MAPKKs), but not vice versa. Some MAPKKs have a conserved MAPK-docking motif K/R-K/R-K/R-X1–6-L-X-L/V/I at the N-termini but no transmembrane domain (Jiang and Chu 2018). Some MAPKs have a common C-terminal docking domain (CD) ([LH][LHY]Dxx[DE]xx[DE]EPxC; x represents any amino acid) that functions as a docking site for MAPKKs, phosphatases, and other protein substrates (Group et al. 2002). MAPKs function as signaling intermediates, which need to be triggered by the upstream receptor signals and perceived by downstream response components. The upstream receptor signals are derived from a series of membrane-associated kinases, such as PRRs. The downstream response components include enzymes, TFs, and PKs (Nakagami et al. 2005; Taj et al. 2010; Bigeard and Hirt 2018). MAPK signaling is involved in many biotic and abiotic stress responses and has been well reviewed (Taj et al. 2010; Nakagami et al. 2005; He et al. 2020). Accumulating evidence also suggests the vital roles of MAPKs in the biosynthesis of specialized metabolites. Here we discuss the post-translational regulation of important plant specialized metabolites by MAPKs.

Anthocyanins

Flavonoids are one of the most extensively studied plant specialized metabolites with antimicrobial and antioxidants activities. Anthocyanins are a family of flavonoids accumulated in flowers, fruits, and vegetables. Anthocyanin biosynthesis is mainly controlled by three families of TFs, the R2R3 MYBs, bHLH, and WD-repeat proteins that form the MYB-bHLH-WD40 (MBW) complex. In *Arabidopsis*, the R2R3 MYB TFs MYB75 (PAP1), MYB90 (PAP2), MYB113, and MYB114 (Gonzalez et al. 2008; Borevitz et al. 2000), the bHLH TFs TT8, GLABRA 3 (GL3), and ENHANCER OF GLABRA 3 (EGL3), and the WD40 TF TTG1 are positive regulators of anthocyanin (Gonzalez et al. 2008; Patra et al. 2013). Recent studies using *Arabidopsis* elucidate both positive and negative regulatory roles of MAPKs in anthocyanin biosynthesis. Light-induced anthocyanin biosynthesis is positively regulated by MPK4. Light rapidly activates MPK4, which in turn interacts with and phosphorylates MYB75 to enhance anthocyanin accumulation in *Arabidopsis*. Phosphorylation of MYB75 by MPK4 increases its stability but not alters its binding to the target gene promoters (Li et al. 2016). The *Arabidopsis* MAPKK6 is a

negative regulator of anthocyanin biosynthesis. The *mapkk6* mutant accumulates anthocyanin in leaves, and the expression of *MYB75* is higher in the mutant compared to wild type (Wersch et al. 2018).

Camalexin

Camalexin is an indole alkaloid phytoalexin, acting as an antimicrobial molecule in *Brassicaceae* species (Glawischnig 2007). Camalexin biosynthesis is regulated by the WRKY TF WRKY33 and the R2R3 MYBs, MYB34, MYB51, and MYB122 (Birkenbihl et al. 2012; Frerigmann et al. 2015). WRKY33 directly binds to the promoter of *Phytoalexin deficient 3 (PAD3)*, a camalexin biosynthetic gene (Zhou et al. 1999). WRKY33 is the target of MPK3/MPK6 (Mao et al. 2011). The upstream of MPK3/MPK6 is MAPKK (MAPKK α) and MAPKKK(-MEKK1), as the expression of the constitutively active form of MAPKK or MAPKKK promotes the camalexin biosynthesis (Ren et al. 2008). In addition, the pathogen-responsive Calcium-dependent protein kinase5 (CPK5) and CPK6 in *Arabidopsis* also regulate the camalexin biosynthesis by phosphorylating WRKY33 (Zhou et al. 2020). Phosphorylation of WRKY33 by MPK3/6 increases its transactivation, whereas CPK5/CPK6 enhances DNA binding of WRKY33 (Zhou et al. 2020), suggesting that CPKs and MAPKs may function cooperatively (Zhou et al. 2020; Yang et al. 2020). The orthologs of MPK3/MPK4/MPK6 in rice also function in regulating the phytoalexin biosynthesis (Kishi-Kaboshi et al. 2010). In addition, MPKK9 and MPK4 are also involved in the regulation of camalexin biosynthesis in *Arabidopsis*. Overexpression of the wild-type or the phospho-mimic MAPKK9 induces camalexin pathway gene expression and camalexin accumulation in *Arabidopsis*. This MPKK9-induced camalexin biosynthesis requires the activation of the two downstream kinases, MAPK3/MAPK6 (Xu et al. 2008). Another study shows that MPK4 exists as a nuclear complex with WRKY33 and MAP Kinase Substrate 1 (MKS1) in the absence of pathogens. Upon challenged with pathogens, MPK4 is activated and phosphorylates MKS1, leading to the release of WRKY33 from the complex, which in turn activates camalexin biosynthesis (Qiu et al. 2008).

Monoterpenoid indole alkaloids

C. roseus produces over 130 monoterpenoid indole alkaloids (MIAs), of which vinblastine and vincristine are important chemotherapeutic agents (Neuss and Neuss 1990; Qu et al. 2019). MIA production is triggered by pathogen infection, wounding, UV-B, and methyl jasmonate (MeJA), the methyl ester of the

defense hormone jasmonic acid (JA) (Pan et al. 2018; Gomi 2020). MIA biosynthesis is transcriptionally regulated by a network of TFs, including the AP2/ERFs ORCAs (ORCA2/ORCA3/ORCA4/ORCA5/ORCA6), bHLH TFs (CrMYC2, BIS1/BIS2/BIS3, and RMT1), the WRKY TF (CrWRKY1), GATA-family TF (CrGATA1), and bZIP factors (GBF1 and GBF2) (Liu et al. 2019; Menke et al. 1999; Paul et al. 2017, 2020; Van Der Fits and Memelink 2000; Van Moerkercke et al. 2015, 2016; Zhang et al. 2011; Patra et al. 2018; Li et al. 2013; Suttipanta et al. 2011; Singh et al. 2020, 2021). The ORCAs, BISs, CrMYC2, and CrGATA1 are positive regulators, whereas RMT and GBFs are negative of MIA biosynthesis. Phosphorylation plays a role in MIA accumulation in *Catharanthus*. MeJA induces the accumulation of MIAs in *C. roseus* by activating the MAPK signaling cascade. Transient overexpression of *CrMPK3* in *C. roseus* leaves upregulates the transcripts of genes associated with MIA biosynthesis and increases the production of MIAs, including serpentine, vincristine, vindoline, and catharanthine (Raina et al. 2012). CrMPK3 and its close homolog CrMPK6 are substrates of CrMAPKK1, the homolog of *Arabidopsis* MAPKK9 (Paul et al. 2017). CrMAPKK1 significantly enhances the transactivation activity of CrMYC2, ORCA3, ORCA4, or ORCA5 on the MIA pathway gene promoters in plant cells. In addition, overexpression of CrMAPKK1 significantly induces MIA pathway gene expression and increases MIA accumulation in *C. roseus* hairy roots (Paul et al. 2017).

Indole glucosinolates

Glucosinolates are a group of thioglucosides found in the Brassicaceae family. The group includes the tryptophan-derived indole glucosinolates (IGS) and methionine-derived aliphatic glucosinolates (AGS). Glucosinolates and the derivatives serve as defense molecules against insect herbivores and pathogens. R2R3 MYBs (MYB34, MYB51, and MYB122), bHLH TFs (MYC2, MYC3, and MYC4), and ERF6 regulate IGS biosynthesis in *Arabidopsis*. The MAP kinases MPK3/MPK6 are involved in IGS biosynthesis in response to pathogen infection. ERF6 is directly phosphorylated by MPK3/MPK6 to activate IGS biosynthesis. In addition, MYB51 and MYB122 are also involved in the MAPK signaling pathway (Xu et al. 2016; Yang et al. 2020).

Nicotine

Nicotine is the major alkaloid accumulated in tobacco. Similar to the MIA regulation in *Catharanthus*, the JA-responsive ERF189, ERF199, ERF221, and NtMYC2 are the major regulators of nicotine biosynthesis in tobacco.

MAPK signaling is also involved in nicotine biosynthesis. Transient overexpression of the JA-factor stimulating MAPKK1 (NtJAM1) with the AP2/ERF ORC1 (ERF221) significantly activates the nicotine pathway gene promoters in tobacco cells (De Boer et al. 2011). NtORC1 is a homolog of CrORCA3. In *Arabidopsis*, MPK4 regulates light-induced biosynthesis of anthocyanin (Li et al. 2016) and pathogen-induced camalexin (Qiu et al. 2008). A recent study shows that NtMPK4, a homolog of AtMPK4, regulates nicotine biosynthesis by activating ERF221. Conditional overexpression of *NtMPK4* in hairy roots upregulates nicotine pathway gene expression and increases nicotine accumulation. In addition, co-expression of *NtMPK4* with *ERF221* significantly activates the nicotine pathway gene promoter compared to expressing *ERF221* alone (Liu et al. 2021).

Tanshinone

The functions of MAPKs seem to be conserved in medicinal plants that produce unique specialized metabolites. In *Salvia miltiorrhiza*, MAPK seems to be associated with the biosynthesis of tanshinone (Xie et al. 2020). *S. miltiorrhiza* is a traditional herb in Chinese medicine. The roots of *S. miltiorrhiza* are rich in salvanolic acid and tanshinone. Tanshinone has significant medicinal potentials and is shown to inhibit the infection and replication of the viruses SARS-CoV-2 and CL^{pro} (Park et al. 2012). Genome-wide analysis has revealed *S. miltiorrhiza* genome harbors 18 MAPKs, 9 MAPKKs, 83 MAPKKKs, and 6 MAPKKKKs. Yeast two-hybrid assay shows that SmMAPK3 interacts with SmMYB36, a TF that promotes the biosynthesis of tanshinone in the hairy roots of *S. miltiorrhiza* (Ding et al. 2017).

Artemisinin

A. annua is a traditional Chinese herb that produces the sesquiterpene lactone artemisinin with anti-malarial activity. Artemisinin biosynthesis is positively regulated by the abscisic acid (ABA)-responsive basic leucine zipper TF AabZIP1, which binds to the promoter of genes involved in artemisinin biosynthesis (Zhang et al. 2015). The ABA-responsive Artemisinin biosynthesis promoting kinase 1 (AaAPK1), a SnRK2 in *A. annua*, interacts with and phosphorylates AabZIP1 to regulate the biosynthesis of artemisinin (Zhang et al. 2018).

Reprogramming plant specialized metabolism by manipulating PKs

The studies on different metabolic pathways collectively suggest that protein phosphorylation occurs in many specialized metabolic pathways. PKs are vital parts of the gene regulatory networks that control the biosynthesis of plant specialized metabolites in response to various biotic and abiotic factors. In addition, as defense response and specialized metabolite biosynthesis are well connected, many PKs involved in plant defense response are potential regulators of specialized metabolism. Furthermore, homologous kinases regulate structurally diverse specialized metabolites in different plant species. PKs, thus, can serve as potential tools to reprogram metabolic pathways in plants. Constitutively, active kinases have been used to activate metabolic pathways to overproduce certain specialized metabolites in plants. For example, the transgenic *Arabidopsis* lines expressing the constitutively active forms of MAPKKK α and MEKK1 (Δ MAPKKK α and Δ MEKK1), driven by the DEX-inducible promoter, rapidly accumulate camalexin after the induction by DEX (Ren et al. 2008). Identification of candidate kinases, modification of the phosphorylation sites, and generation of transgenic plants overexpressing active kinase(s) are attractive approaches to reprogram metabolic pathways in plants. By secondary mutagenesis and genetic screening, plants that are tolerant to toxic metabolites with little growth inhibition can be identified (Fig. 2).

The modulation of metabolites can alternatively be achieved by overexpressing the native versions of kinases. Overexpression of *CrMAPKK1* elevates the levels of MIAs in *C. roseus* (Paul et al. 2017). Overexpression of apple Hexokinase 1 (MdHXX1) triggers the accumulation of anthocyanin biosynthesis in apple calli and fruits (Hu et al. 2016a). The level of anthocyanin induced by overexpressing MdHXX1 is much higher than that of calli overexpressing the TF MdbHLH3 (Hu et al. 2016a). The MdHXX1-mediated phosphorylation of MdbHLH3 occurs at the Ser³⁶¹ site enhances the transcriptional activity of MdbHLH3. Loss of phosphorylation by substituting the amino acid Ser³⁶¹ to Ala³⁶¹ in MdbHLH3 lowers the anthocyanin production in transgenic calli (Hu et al. 2016a). As activation of MdHXX1 requires glucose, whereas a constitutively active form of MdHXX1 may promote the production of anthocyanins without glucose treatment. The hexokinases are conserved across the plant kingdom, overexpression of HXX1 may also reshape the metabolism in other species. However, high hexokinase activity results in reduced fruit and seed size (Menu et al. 2004), and overexpression hexokinase has inhibitory effects on

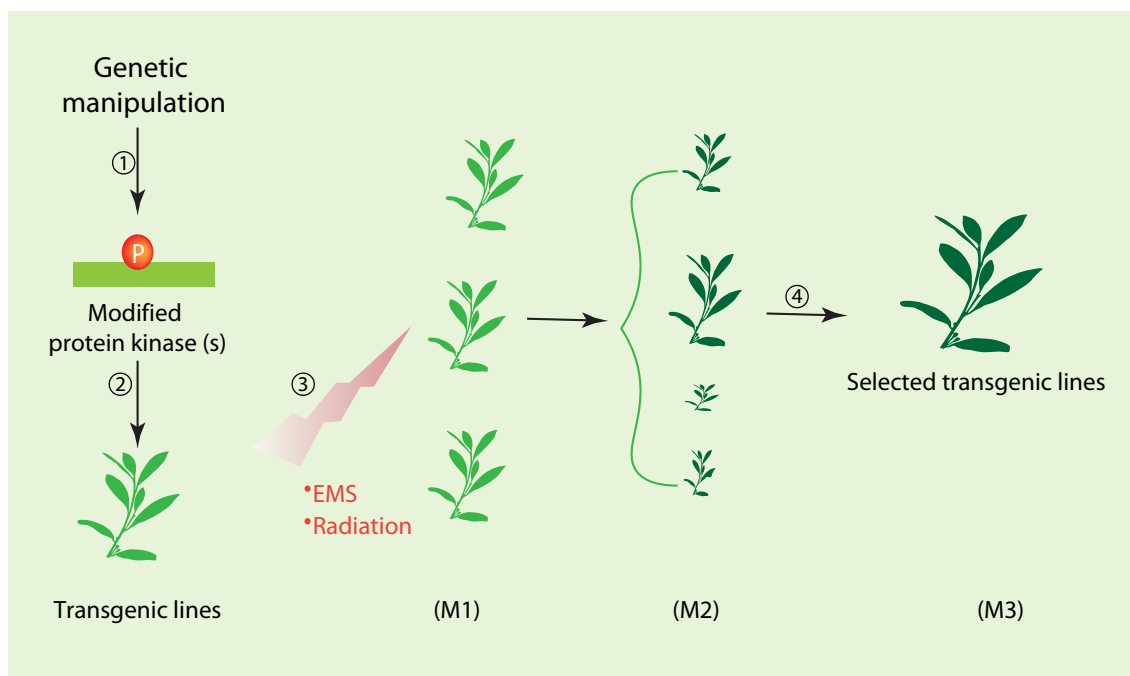


Fig. 2 A strategy to screen for a transgenic plant that resists increased toxic metabolites. Modification of a kinase (1) in transgenic plants (2) by overexpressing or gene-editing (knock-in mutagenesis) an engineered kinase that is either constitutively activated (phosphomimic) or with enhanced stability. "P" represents the phosphorylation of a protein kinase resulting in modified activity. The transgenic plants will be screened for increased metabolite accumulation and normal growth. In case the increase of metabolite accumulation causes growth inhibition, a secondary mutagenesis (3) using chemical means, such as ethyl methanesulfonate (EMS) or irradiation, will be employed to generate a mutant population (M1) with various growth phenotypes (plants with varying sizes in M2 population), from which a line (M3) with high metabolite accumulation and normal growth will be selected for further breeding

plant development (Dai et al. 1999; Kelly et al. 2012), suggesting the necessity of secondary mutagenesis to screen for a transgenic plant without growth defects or achieving conditional expression using inducible system (Borghini 2010).

Kinases may also negatively regulate specialized metabolism. Overexpression of the activated version of *MKK9* in *Arabidopsis* results in less anthocyanin under low nitrogen condition compared to the wild-type and the transgenic line expressing the native version of *MKK9* (Luo et al. 2017). When a toxic or undesirable metabolite needs to be reduced, ectopic expression of a kinase with negative regulatory activity, or knockout of a kinase with positive activity using genome editing technology, will likely achieve the objectives.

CONCLUSION

Specialized metabolites produced by plants, especially medicinal plants, have been important sources of drugs, drug precursors, and dietary supplements. While plants use these metabolites to protect themselves from biotic and abiotic stresses, the natural toxicity of specialized metabolites can inhibit growth and development. The

production of specialized metabolites can be increased to some extent by modifying the pathway enzymes or regulatory proteins associated with their biosynthesis in plants. However, systematically reprogramming specialized metabolism signaling pathways in plants can be an equally effective or even a better strategy.

The kinase-initiated cascades responsible for signal perception and transduction seem to share many similarities, although the specialized metabolites produced by different plants vary significantly. For example, *Arabidopsis*, *S. miltiorrhiza*, tobacco, and *C. roseus* recruit the MAPK cascade to activate the biosynthesis of structurally diverse camalexin, tanshinone, and MIAs (Xie et al. 2020; Raina et al. 2012; Xu et al. 2008; De Boer et al. 2011; Paul et al. 2017). Therefore, creating transgenic lines targeting PKs may lead to altered metabolic outcomes in many medicinal plant species. Based on the assumption that kinase functions are conserved across the plant kingdom, it is possible to engineer the endangered medicinal plants which lack sufficient genomic information. However, as stable transformation protocol is not available for many medicinal plants, transient-based reprogramming (cite the new mol plant paper), hairy roots, cell cultures, or modified *Agrobacterium*-mediated gene delivery and

virus-based gene silencing are effective alternatives (Ma et al. 2020; Maher et al. 2020). A recent study demonstrates a versatile and efficient *Agrobacterium* and RNA viral replicon-based transient expression system that can be used to reprogram developmental and hormone signaling pathways in a wide range of plant species (Torti et al. 2021). Such an approach can potentially be used for PKs to reprogram specialized metabolism in plants.

Other PKs, such as NAD kinase (NADK) and targets of rapamycin kinase (TOR), play critical roles in regulating plant development. However, the roles of these kinases in regulating plant specialized metabolism have not been well studied. NADK is the only known enzyme that phosphorylates NAD(H) (reviewed in Waller et al. 2009). NADK regulates NADP(H)-dependent metabolism, including the xanthophyll cycle and fatty acid β -oxidation in plants (Waller et al. 2009). Furthermore, the emerging studies suggest a high potential for plant improvement by targeting TORs (reviewed in Xiong and Sheen 2014; Ryabova et al. 2019). Deciphering the functions of NADK and TOR in plant specialized metabolism requires more investigations.

PKs are key regulators of plant growth and defense. Metabolic reprogramming of medicinal plants is of great value for the modern society. Creating designer plants with higher yield of specialized metabolites but minor growth penalty by targeting PKs is a proven alternative to those achieved by targeting TFs and enzymes.

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Declarations

Conflict of interest All the authors declare no conflict of interest.

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