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Unleashing Plant Synthetic Capacity: Navigating Regulatory Mechanisms for Enhanced Bioproduction and Secondary Metabolite Discovery

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Abstract

Plant natural products (PNPs) hold significant pharmaceutical importance. The sessile nature of plants has led to the evolution of chemical defense mechanisms over millions of years to combat environmental challenges, making it a crucial and essential defense weapon. Despite their importance, the abundance of these bioactive molecules in plants is typically low, and conventional methods are time-consuming for enhancing production. Moreover, there is a pressing need for novel drug leads, exemplified by the shortage of antibiotics and anticancer drugs. Understanding how plants respond to stress and regulate metabolism to produce these molecules presents an opportunity to explore new avenues for discovering compounds that are typically under the detection limit or not naturally produced. Additionally, this knowledge can contribute to the advancement of plant engineering, enabling the development of new chassis for the biomanufacturing of these valuable molecules. In this perspective, we explore the intricate regulation of PNP biosynthesis in plants, and discuss the biotechnology strategies that have been and can be utilized for the discovery and production enhancement of PNPs in plants.

Introduction

Natural products (NPs), exhibiting a diverse array of biological activities, have been widely utilized for pharmaceutical applications, represented by a number of front-line medicines [1,2]. Plants have long served as significant sources of natural products, with the historical use of plant natural products (or secondary metabolites, or specialized metabolites; PNPs) for medicinal purposes dating back to early human civilizations [3,4]. As we transition into the modern era of drug development, numerous PNPs have been discovered for their promising bioactivities [2,3]. However, the extremely low abundance of PNP in nature often poses a challenge, hindering accessibility and the ability to meet the substantial market demand, as illustrated by the decades-long process of discovering and developing Taxol [5]. More recently, after the golden stage of NP discovery, there has been a noticeable decline in the identification of novel structures from nature [6]. However, the demand

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for extraordinary structures, crucial for drug development, is on the rise due to multiple challenges including escalating antibiotic resistance, global pandemics, complex diseases, rare diseases, regulatory hurdles [2,7].

Production wise, approaching the modern genetic era, synthetic biology has presented numerous opportunities and innovative strategies to tackle both sourcing and discovery challenges [8]. This is exemplified by the large and increasing number of examples using microbial cell factories to produce PNPs [9,10]. In tandem, diverse biotechnology approaches have been carried out for production enhancement and accumulation of desired products in plants [11,12]. Traditional breeding is a direction employed by plant breeder to obtain the varieties for increased yield of PNP productions, but the process is time-consuming, natural variation dependency and result unpredictable [11]. Molecular breeding is another direction utilized for metabolic reprogramming through genetic modifying the expression of crucial regulatory genes in the biosynthetic pathways [12]. In addition, the decade-long advancement of CRISPR/Cas9 genome editing technologies has introduced new features and prospects for plant engineering [13]. Discovery wise, genome mining strategies have proven effective in revealing novel structures from microbial samples, such as soil bacteria and human microbiota [14], but rarely plant.

Consequently, the question arises: How can we effectively harness and engineer plants to address the current challenges in the discovery and production of PNPs? When we explore the reasons behind the ability of plants to produce such a diversity of PNPs, it's essential to recognize that plants are sessile organisms. Being immobile, they are consistently exposed to a wide range of environmental stresses, compelling them to evolve mechanisms for rapid recognition and response to environmental changes. Exploring the molecular mechanisms on the regulation of PNP synthesis in plants could provide valuable inspiration. In this review, we discuss the potential and possibility of harnessing plant regulation of PNP synthesis to address the challenges in PNP discovery and production, using the plant as the chassis.

PNPs often function as defense molecules.

A number of specialized PNPs were produced in plants to deploy chemical defense upon exposure to biotic stress, which can be divided into two categories: phytoanticipins and phytoalexins [15,16]. Phytoanticipins are constitutively synthesized in plants for protection prior to infections from pathogens. To date, most of the bioactive compounds that were discovered in plants belong to this group [15]. Phytoalexins are defined as low molecular weight phytochemicals that are produced in plants to fight against invading pathogens through metabolism disruption, growth inhibition or maturation restriction [15]. For instance, phytoalexin camalexin was induced rapidly upon infection by *Botrytis cinerea* or *Alternaria brassicicola*, which displays antibacterial and antifungal activities to inhibit the pathogens growth [17]. Treatment with phenolic phytoalexin compounds, i.e., quercetin, scopoletin and scoparone, on oranges can significantly reduce green mold disease incidence caused by the fungus *Penicillium digitatum* [18].

The antimicrobial activity of phytoalexins makes them potential agents for therapy and disease controlling [19]. For example, the phytoalexin resveratrol from grapevine exhibits anticarcinogenic and antitumor activities to inhibit platelet aggregation and the growth of

various cancer cells as a cytostatic or a cytotoxic agent [20,21]. Maslinic acid, a triterpenoid phytoalexin product from olives, has been evidenced to exert antitumor, antidiabetic, antioxidant, cardioprotective and neuroprotective bioactivities, implying its potential as a nutraceutical [22]. The indole phytoalexin brassinin and its derivatives exhibit strong antiproliferative effect to inhibit the growth of human cancer cell lines *in vitro* [23].

Although PNPs have been used for medicinal purposes even before written history, their true biological functions and the regulatory mechanisms of the corresponding biosynthetic process in plant are not well understood. To explore into the question on how to precisely engineer plants as the future chassis, we need to first answer the questions on why and how plants produce these molecules *via* intricate signaling processes, including elicitation perception, signaling transduction, post-translation modification, defense gene expression, and *de novo* synthesis of enzymes [20].

Plants harness intricate mechanisms regulating production of PNPs.

Two responsive systems, pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI), are responsible for recognizing biotic stress and activating defense protections (Fig. 1) [24,25]. In the PTI immune protection, pattern-recognition receptors (PRRs) act as the first barrier on perception of pathogen invasions, which form dynamic PRR regulatory complexes with co-receptors and receptor-like cytoplasmic kinases (RLCKs). The activated RLCKs can then activate mitogen-activated protein kinase (MAPK) cascades through phosphorylation and thereafter activate downstream signaling components of transcription factors (TFs) [26]. ETI is a more advanced intracellular protection model recognizing the susceptibility components in the host (referred to as effectors) which are delivered by pathogens [24,25]. A set of specialized proteins, i.e., nucleotide-binding leucine-rich repeat receptors (NLRs), have been identified in plants to perceive and interact with these effectors to activate TFs for further immune responses [26,27]. TFs act as essential regulators to modulate gene expressions to induce the biosynthesis of defensive hormones and PNPs [28,29].

PNPs are categorized into three major groups based on their chemical structures: terpenoids, phenolics and nitrogen-containing compounds [30]. Although extensive efforts have been made to understand the regulatory mechanisms on biotic stress perception, relatively fewer studies have been carried out to specifically understand how the biosynthesis of phytoalexins are induced and regulated in response to biotic infections. To date, the only and best-known example of a fully characterized regulatory mechanism is the biosynthesis of camalexin, an indole alkaloid phytoalexin exhibiting strong inhibition to the proliferation of various cancer cells [31]. The production of camalexin is known to be induced upon the perception of bacterial infection. In *Arabidopsis* for example, when infected by pathogens, two LRR-RLKs, FLAGELLIN-INSENSITIVE 2 (FLS2) and ELONGATION FACTOR-TU RECEPTOR (EFR), can recognize the signature elicitors of flagellin epitope (flg22) and elongation factor-Tu epitope (elf18), respectively. These receptors then recruit a co-receptor, BRI1-associated receptor kinase (BAK1), and a Botrytis-induced kinase 1 (BIK1) to form dynamic complexes [26,27]. The BIK1, once form the complex, can activate downstream MAPK cascades, including MPK3, MPK4 and MPK6, to transduce the signaling and

activate TFs WRKY30 and WRKY33 via phosphorylation. These activated TFs then modulate the expressions of *CYP71B2/B3, CYP71A13*, and *PAD3* genes, ultimately promoting the biosynthesis of camalexin [32-34]. We believe that the biosynthesis of most PNPs is regulated through similar routes, but the corresponding receptors, signaling cascades, and TFs are largely unknown. By exploring the intricate perception and regulatory mechanisms, we can unlock a repertoire of engineering tools to selectively boost or induce the production of specific PNPs.

PNP production can be induced or enhanced through elicitation of the immune system.

Elicitation *via* supplementing specific biotic elicitors in plant has been demonstrated as an effective strategy to enhance the production of bioactive defense molecules (Table 1) [35,36]. There are two types of biotic elicitors: exogenous- and endogenous-elicitors (Fig. 1) [37]. Exogenous elicitors are compounds originally from microorganisms or pathogens, such as signaling peptide, fungal and bacterial lysates, yeast extracts and pathogen polysaccharides [37]. For instance, the productions of phenylpropanoid and naphtodianthrone were rapidly accumulated in *Hypericum perforatum* cell suspensions once treated with fungal elicitors *Fusarium oxysporum* and *Botrytis cinerea* [38]. In another example, biosynthesis of curcumin is promoted in tumeric plants (*Curcuma longa* L.) after sprayed with chitosan elicitor solution [39]. More recently, a comprehensive analysis coupling gene co-expression network analysis with genome analysis in wheat revealed the induction or enhanced expression of a large number of biosynthetic genes [40], with one set later identified as synthesizing a novel isoflavone phytoalexin named triticein [41]. This work highlights the potential of genomics-based methods to uncover novel PNPs by harnessing the vast available pathogen-induced transcriptome data.

Endogenous elicitors, on the other hand, are defined as small molecules produced by plant hosts in response to biotic invasions, such as plant hormones and their derivatives or analogs [37]. In broccoli sprouts, the concentrations of several bioactive metabolites, including vitamin C, flavonoids and indole glucosinolate, were increased after treatments with hormone elicitors of salicylic acid (SA) or methyl jasmonate (MeJA) [42]. Productions of cichoric acid, caftaric acid, and chlorogenic acid were elevated in *Echinacea purpurea* L. hairy root cultures after supplemented with elicitor gibberellic acid (GA_3). The highest amount of these products was detected in the culture with moderate GA_3 concentration in comparison to those with other GA_3 concentrations (lower and higher), implying that the induction of secondary metabolism is dose dependent on the elicitors [43]. Likewise, the induction of monocot terpenoid phytoalexins through both exogenous (e.g., glycosphingolipid derivatives, lytic enzymes, biotrophic hyphae) and endogenous elicitors (e.g., jasmonates [JA], ethylene, SA) has been thoroughly reviewed [44].

Despite the success achieved in enhancing the production of certain PNPs through biotic elicitation, there are challenges and potential issues that need to be addressed. Firstly, the application of elicitors may induce environmental stress on plant growth, resulting in negative side effects, such as reduced growth rate and biomass. Also, treatment with biotic elicitors may not solely induce the production of specific PNPs of interest. Instead, it may trigger the downstream biosynthesis of many other PNPs in addition to the desired

metabolites. This complexity adds challenges to the isolation and purification process, making them more intricate and expensive. Furthermore, the availability of biotic elicitors for diverse plant species and the optimal concentration of elicitors for stimulating the biosynthesis of specific PNP in plants present additional challenges as well.

Advancement of genetic manipulation tools brought new opportunities to engineer plants for PNP production.

Recent advancement of genetic manipulation techniques has provided new opportunities to engineer plants, allowing for the accumulation of desired PNPs through modifying the regulatory and biosynthetic pathways (Fig. 1). One general and direct forward method is to overexpress one or several corresponding biosynthetic genes. For instance, expression of grapevine stilbene synthase (*STS*) genes (*Vst1, Vst2* or *VqSTS6*) under constitutive or inducible promoters in tobacco, rice, barley, wheat, and grape could produce high amount of stilbene and increased plant resistance to pathogens [45]. This example also suggests that it is not always necessary to overexpress native biosynthetic genes; genes from other plants with similar functions can also be utilized to enhance the production of corresponding products in different plants. Another strategy to enhance the production of a bioactive metabolite of interest in plants involves reducing the production of undesirable compounds through blocking the corresponding biosynthetic pathway. RNA interference (RNAi) is an efficient approach used to alter gene expression and enzyme activity for metabolic reprogramming in plants [46]. For example, artemisinin content was significantly increased (3.4-fold) in *A. annua* after silencing the expression of squalene synthase (SQS), an essential enzyme catalyzing a competitive sterol pathway to artemisinin biosynthesis, using RNAi method [47]. CRISPR/Cas9 mediated genome editing is another powerful tool for genetic modification and metabolic engineering [48]. In a particular example, silencing of carotenoid cleavage cioxygenase 8 (*CCD8*) gene through CRISPR/Cas9 in tomato (*Solanum lycopersicum*) resulted in content reduction of orobanchol, with the amount of carotenoid increased [49]. By applying a robust multiplex CRISPR/Cas9 system carrying six sgRNA cassettes for five genes in γ-aminobutyric acid (GABA) shunt pathways, Li et al., created multiple tomato knock-out mutants, including single, double, triple, and quadruple mutants [50]. All mutants, except the *gaba-1* single mutant, have shown increased GABA contents, among which the quadruple mutant accumulated the highest amount of GABA (19-fold) than the wild-type plant [50].

Although less explored, there are reported examples of modifying transcription factors (TFs) to successfully alter plant metabolism, resulting in the accumulation of metabolites of interest. For example, tobacco transgenic plants expressing an *Arabidopsis* TF *AtMYB12* accumulated high amount of flavonols and exhibited enhanced resistance against insect pests *Spodoptera litura* and *Helicoverpa armigera* [51]. In poplar, up-regulation of a TF *MYB115* activated expression of proanthocyanidin (PA) biosynthetic genes, thereby increasing accumulation of PAs, and enhancing plant resistance to the fungal pathogen *Dothiorella gregaria* [52]. Overexpression of a *Artemisia annua* WRKY TF *AaWRKY17* increased the production of artemisinin and enhanced plant resistance to *Pseudomonas syringae* [53].

Genetic manipulation has been demonstrated as a prominent strategy to enhance the production of bioactive compounds in plants. However, certain limitations impede its widespread application. Particularly, current crop transformation approaches are time-consuming, leading to slow generation of transgenic plants, and the transformation rates are notably low. The relatively limited understanding of the regulation and biosynthesis of plant metabolism often leaves it unclear which targets to engineer for modifying metabolism and enhancing the production of target secondary metabolites - whether it's a biosynthetic gene, regulatory gene, or perception gene.

New opportunities to use plant as the chassis for PNP discovery and production.

Plant PRRs consists of a large group of receptor-like kinases (RLKs) and receptor-like proteins (RLPs), which play vital roles on perception of extracellular invasion signaling, and thereby activating downstream protection signaling for biosynthesis of NPs [29]. To date, very few PRRs have been demonstrated to recognize specific pathogen invasions for immune activation [29]. Functional characterization of new PRRs involving in diverse defense pathways could be a potential strategy for activating biosynthesis of novel PNPs in response to biotic stress.

Likewise, PNP biosynthetic genes are generally regulated by TFs, which act as key mediators connecting PRRs signaling and downstream immune responses [54]. Transcription factors, including WRKY, NAC, AP2/ERF, MYB, bZIP, and bHLH, have been reported to be involved in biotic stress responses for promoting biosynthesis of defense molecules [54,55]. However, our understanding on plant signaling transduction and regulatory mechanisms from PRR perception to specific TFs is limited. Functional investigation on TFs in response to biotic stress can provide new insights on engineering plants to produce unknown or desired PNPs. Furthermore, the PNP biosynthetic pathways are typically regulated by several different TFs under stress conditions. Thus, co-expressing multiple TFs could be an effective way to enhance biosynthesis of target PNPs. Beyond transcriptional modifications, there is a growing emphasis on chromatin-level regulation, with epigenetic modifications emerging as a promising strategy for altering plant metabolism [56,57].

Additionally, post-translational modifications, a key mechanism regulating gene expression in plants and consequently plant metabolism, should be taken into account [58]. If the post-translational modification enzymes (e.g, kinases, methyltransferases) modifying PNP biosynthetic proteins are characterized, they could be promising targets to be utilized to alter plant metabolism. For example, NUDX23 was recently identified in post-translational regulation of *PSY* and *GGPPS* genes for carotenoid biosynthesis by co-migrating with PSY and GGPPS proteins in a complex. Silencing NUDX23 and overexpressing it significantly reduced and enhanced carotenoid production, respectively [59].

To sum up, a comprehensive characterization of new biological elements, such as promoters, receptors, TFs, and other regulatory components involved in plant immune responses and downstream metabolism regulations, will significantly advance plant engineering to enhance or induce production of desired PNPs or novel PNPs. It is time to revisit plants to understand why and how plants produce PNPs of diverse structures and biological activities. This

understanding will empower us to overcome existing challenges in PNP discovery and production, unlocking the full potential of plants' pharmacopoeia.

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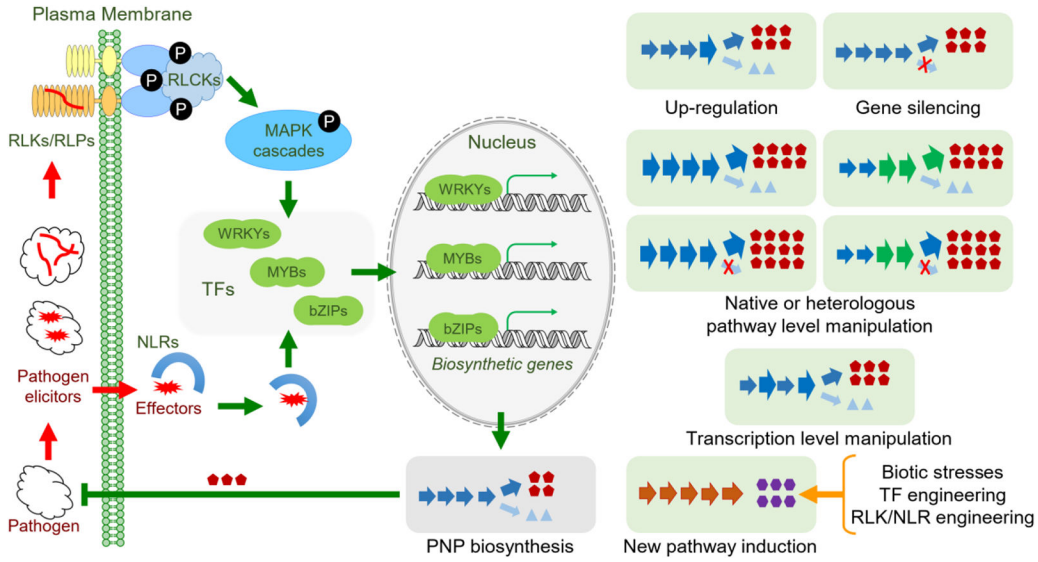

Figure 1.

Schematic representation on the induction of PNP production in response to biotic stress, with an overview of genetic manipulation strategies aimed at enhancing PNP production and facilitating the discovery of novel PNPs. RLK/RLPs: receptor-like kinase and proteins; RLCKs: receptor-like cytoplasmic kinases; MAPK: mitogen-activated protein kinase; TFs: transcription factors; NLRs: nucleotide-binding leucine-rich repeat receptors.

Table 1.

Examples for enhancement of nature products in plant *via* elicitation approach

Elicitor	Nature Product	Plant Species	Reference
Jasmonic and salicylic acid	Stilbene and flavonoids	*Vitis. Vinifera*	Xu et al., 2015
Chitosan	Curcumin	*Curcuma longa* L.	Sathiyabama et al., 2016
Yeast extract	Vinblastine and vincristine	*Catharanthus roseus*	Maqsood et al., 2017
Dextran	Phenylpropanoid and Flavonoids	*Solanum lycopersicum* L	Lu et al., 2019
Jasmonic acid	Phenolic and flavonoid compounds	*Hibicus sabdariffa* Linn	Jirakiattikul et al., 2021
Chitin	Camptothecin	*Nothapodytes nimmoniana* (J.Graham) Mabb	Keshavan et al., 2022
Cronobacter sakazakii	Phenolic compounds	*Dionaea muscipula* J. Ellis	Makowski et al., 2020
Escherichia coli	Diosgenin content	*Helicteres isora* L	Shaikh et al., 2020
Fusarium oxysporum	phenylpropanoid and naphtodianthrone	*Hypericum perforatum* L.	Gadzovska et al., 2015
Penicillium oxalicum	Artemisinin	*Artemisia annua* L	Zheng et al., 2016
Chaetomium globosum and *Paraconiothyrium brasiliense*	Paclitaxel	*Corylus avellana* L	Salehi et al., 2019
Alternaria panax and *Mesorhizobium amorphae*	Ginsenoside	*Panax ginseng* C.A. Meye	Le et al., 2018; Hao et al., 2020