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Purification of stilbene synthase from *Phaseolus vulgaris* and determination of its catalytic activity

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Stilbene compounds, secondary metabolites produced by plants in response to external environmental stimuli, are garnering increasing attention. Stilbene synthase (STS) plays a crucial role in their synthesis. This study focuses on the purification of stilbene synthase from *Phaseolus vulgaris* and the determination of its catalytic activity. The stilbene synthase gene obtained from the Phaseolus genome was expressed in large quantities by constructing engineered strains of *Escherichia coli*. Furthermore, the active proteins produced by the stilbene synthase gene in *Phaseolus* plants were measured by assessing the activity of stilbene synthase obtained from both His column and Q column purifications. In this study, the active stilbene synthase gene was initially identified from the Papilionoideae. Exploring stilbene synthase contributes to a deeper understanding of the relationship between plant stress resistance and plant evolution.

Key words: Stilbene synthase, *Phaseolus vulgaris*, His column, Q column, PvSTS.

INTRODUCTION

The phenyl propionic acid metabolic pathway, a distinctive metabolic route, serves as a common mechanism for generating secondary metabolic products in most plant species. Its primary goal is to produce flavonoid glycosides, anthocyanins and various polymeric lignin substances. Flavonoids assist plants in resisting UV damage, certain flavonoid glycosides and isoflavone glycosides act as inducers to promote the expression of nodulation genes in rhizobia bacteria, anthocyanins participate in the synthesis of plant pigments, and poly lignin provides structural support and functions as a range of plant antitoxins (Martínez-Márquez et al., 2016).

These compounds exhibit diverse pharmacological and biological activities. Currently, 23 families and 59 genera of plants (Table 1) are known to produce stilbene compounds (Pecyna et al., 2020). In the terpenoid metabolic pathway of plants, specifically the phenyl propionic acid pathway, stilbene synthase plays a crucial role in synthesizing stilbene derivatives from coenzyme A intermediates (such as p-coumaroyl CoA, cinnamyl CoA, etc.) and malonyl CoA as reactants. Thus, this enzyme is pivotal in the synthesis of stilbene compounds (Abe and

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Table 1. Distribution table of stilbene compounds in plant kingdom.

Number	Class	Family
1	Leguminoese	Arachis, Cassia, Sophora, Trifolium, Bauhinia, Ilex, Dalbergia, Pterolobium, Derris, Amorpha, Pterocarpus, Cajanus, Guibourtia, Laburnum, Pericopsis, Schotia, Haplormosia, Youacapoua, Centrolobium
2	Moraceae	Morus, Artocarpus, Cudrania, Chlorophora
3	Dipterocarpaceae	Hopea, Shorea, Balanocarpus
4	Vitacese	Vitis, Parthenocissus, Ampelopsis, Cissus, Rhoicissus
5	Pinaceae	Pinus, Picea
6	Polygonsceae	Polygonum, Rheum
7	Mytaceac	Eucalyprus, Angophora
8	Combretaceae	Combretum
9	Myristicaceae	Knema, Virola
10	Cyperaceae	Carex, Scirpus
11	Lamiaceme	Scutellaria, Salvia, Vitex
12	Anacardiaceae	Rhus
13	Saxifragacesc	Hydrangea
14	Phytolaccaceae	Petiveria
15	Liliaceac	Veratrum, Smilax
16	Fagaceae	Nothofagus
17	Betulaceae	Alnus
18	Urticaceae	Toxylon
19	Umbelliferae	Pleuraspermum, Ferula
20	Cupressaceae	Juniperus
21	Gnetaceae	Gnetum
22	Ericaceae	Gaylussacia
23	Poaceae	Festuca

Morita, 2010). Stilbene compounds, often synthesized in response to plant stress, are secondary metabolites produced by plants when exposed to external environmental stimuli and belong to the terpenoid class (Hanhineva et al., 2009). As research progresses, there is increasing attention on these bioactive compounds, and elucidating the key enzyme in stilbene compound synthesis, namely stilbene synthase, holds great significance.

The primary route for generating stilbene compounds is through the phenyl propionic acid metabolic pathway (Abe and Morita, 2010). In the synthesis pathway of stilbene compounds, three enzymes play a crucial role in transforming related organic matter from primary metabolites. Phenylalanine is first converted to Coenzyme A (CoA)-activated thionyl phenylpropionate. This process involves the deamination of phenylalanine by phenylalanine lyase (PAL) to form cinnamic acid. Subsequently, cinnamic acid undergoes catalysis by cinnamic acid 4-hydroxylase (C4H) to produce p-coumaric acid. Under the action of p-coumaric acid CoA ligase (4CL), a thioester bond is formed at the carboxyl group of p-coumaric acid, resulting in the production of p-coumaroyl CoA (Yu et al., 2005). The next step involves the catalysis of p-coumaroyl CoA and two malonic monoacyl-CoA by stilbene synthase (STS), leading to the synthesis of secondary metabolites known as stilbenes (Figure 1). Additionally, p-coumaroyl CoA and other compounds can enter the flavonoid and isoflavone synthesis pathway under the catalysis of chalcone synthase (CHS).

Stilbene synthase is typically produced with stilbene compounds, but it has now been discovered that there may also be an enzyme similar to STS (PvSTS) in *Phaseolus vulgaris*. This plant is known to contain no stilbene compounds, so it is generally believed that this plant does not possess the physiological activity of stilbene synthase (Rosano and Ceccarelli, 2014). However, a genetic comparison has identified a protein in the plant's genome with a sequence similar to that of ordinary stilbene synthase. It is now necessary to prove whether the protein exhibits the activity of ordinary stilbene synthase.

MATERIALS AND METHODS

Construction of recombinant plasmid and expression of target protein

The first step is to construct a gene expression vector using Gibson

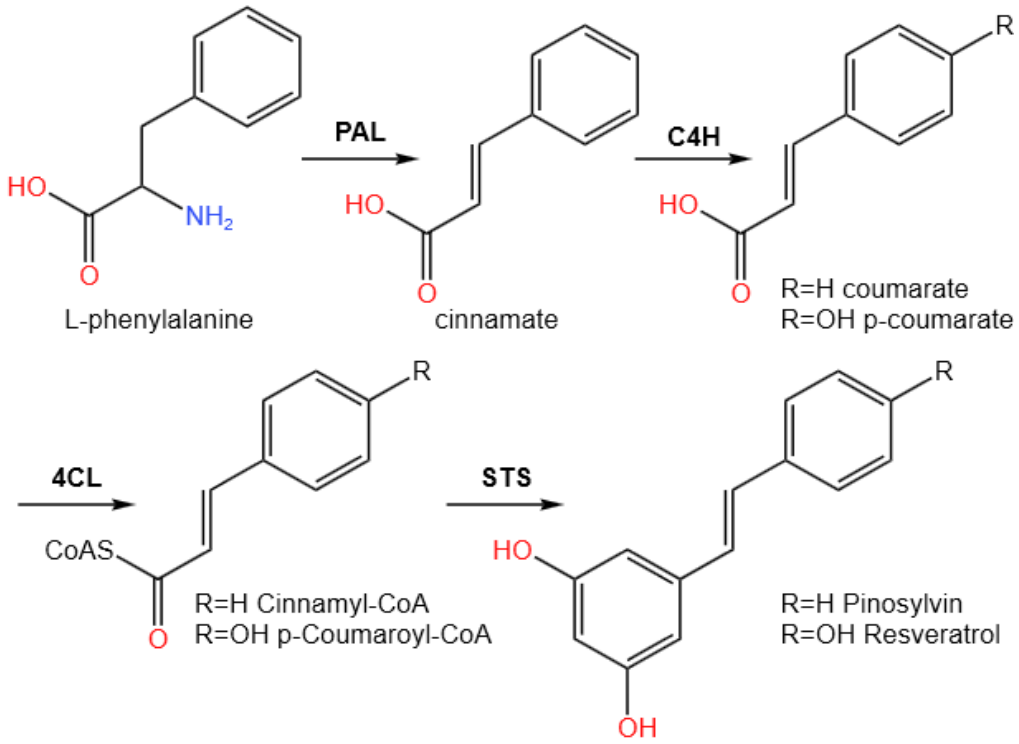

Figure 1. The metabolic process of stilbene compounds.

assembly (Figure 9a). The target gene was amplified by high-fidelity PCR with specific primers to obtain the target gene sequence containing homologous sequences with the plasmid vector. The obtained genes and plasmids were mixed and cultured in Gibson assembly master mix for homologous recombination (Figure 9b). The resulting recombinant plasmids were separated by electrophoresis, and the purified plasmid was obtained. After confirming the correct molecular weight through enzyme digestion electrophoresis, DNA sequencing was performed to confirm the successful construction of the recombinant plasmid (Beccaria and Cabooter, 2020).

The recombinant gene expression vector can be transformed into *E. coli* cells using the receptor transformation technique. The transformed *E. coli* cells were plated on a medium containing antibiotic, and the recombinant cells that successfully expressed the target gene were selected. Following this, single colony isolation was performed on the medium, and the purified strains isolated were tested through cleavage and PCR. The success of the transformation could be determined after electrophoresis (Hanhineva et al., 2009). The successfully transformed strains were selected for culture, and after reaching a certain extent of growth, IPTG was added to induce protein expression.

Protein extraction and purification

After a period of cultivation, the culture medium was centrifuged, and the thalli were separated. Buffer was added and suspended, followed by ultrasonic crushing and filtration. The resulting protein solution was then separated and purified using a His column (Gaberc-Porekar and Menart, 2005). The presence of proteins was assessed through SDS-page electrophoresis (Figure 2). Subsequently, the protein solution obtained underwent treatment with osmosis and desalting before being separated and repurified using a Q-column. The location and purity of the proteins were determined by electrophoresis (Marbach and Bettenbrock, 2012) (Figure 3). Once the highly purified protein was obtained, it was concentrated and stored at a low temperature (Figure 4).

Determination of protein activity and LC-MS

The assay, involving an activity test with the obtained enzymes, demonstrated that PvSTS exhibits the protein activity typical of common STS. Previous research has outlined two reactions catalyzed by STS enzymes:

Reaction 1: p-Coumaroyl CoA and malonyl CoA were utilized as substrates, yielding Resveratrol as the main product and Naringenin chalcone as the by-product (Figure 10). Naringenin is formed spontaneously due to the unstable molecule of Naringenin chalcone.
Reaction 2: Caffeoyl CoA and malonyl CoA were used as substrates, resulting in Piceatannol as the primary product and Eriodictyol as the by-product (Figure 11).

Experiments were designed based on these principles to confirm whether PvSTS possesses the catalytic activity of STS. P-coumaryl CoA served as the foundational substrate, excess malonyl CoA was added as an excipient, and 10 μg of purified PvSTS was included for heat preservation. Following 24 h of incubation, the solution underwent filtration and separation to eliminate proteins and other macromolecular impurities. LC-MS detection was conducted. The samples were replicated three times, and the blank control group was maintained (Caruso et al., 2011). The spectral line corresponding to the product was measured and analyzed to determine the generation of the corresponding product, thereby assessing sample activity (Figures 5 to 8).

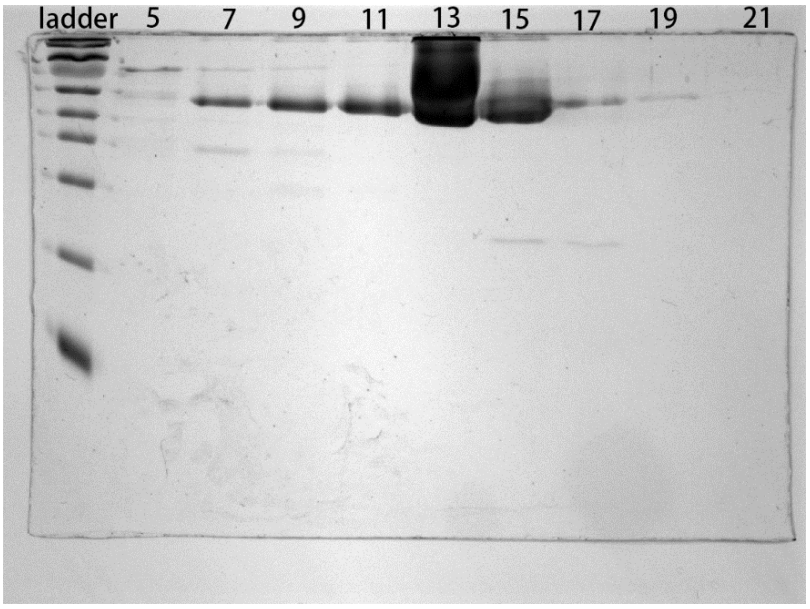

Figure 2. Electrophoretic results after His column.

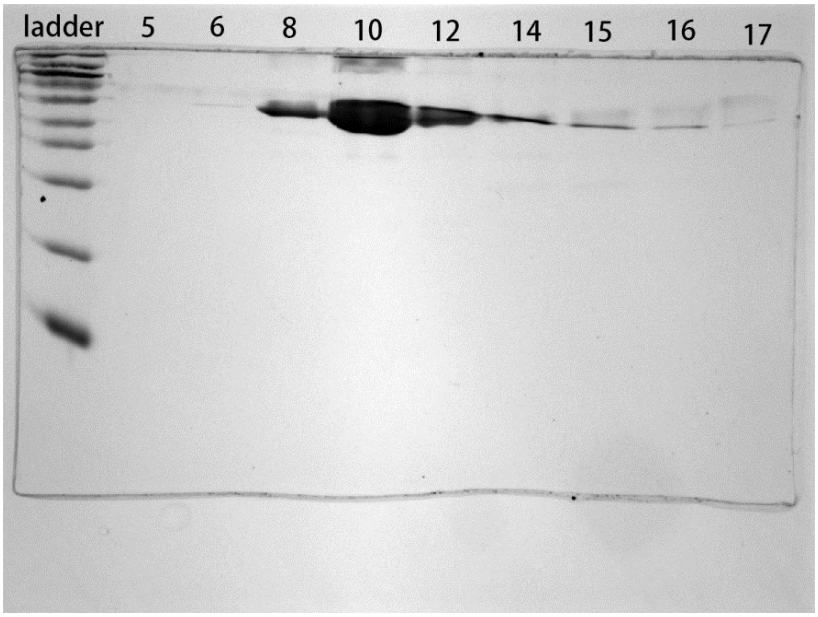

Figure 3. The result of electrophoresis after Q column.

RESULTS AND DISCUSSION

During the purification process, it is essential to assess the protein content and purity through electrophoresis. In these experiments, a series of images (Figures 2 to 4) were obtained to validate the presence of the protein.

The results indicate that the purified protein exhibits high purity with minimal impurities, making it suitable for protein activity detection (Figures 5 to 8). Subsequent analysis revealed the presence of the expected products in sufficient quantities, confirming that PvSTS possesses the general catalytic activity characteristic of STS proteins.

In conclusion, the experiments demonstrate that PvSTS exhibits the catalytic activity typical of common STS proteins.

The objective of this experiment was to investigate whether PvSTS exhibits the catalytic activity typical of

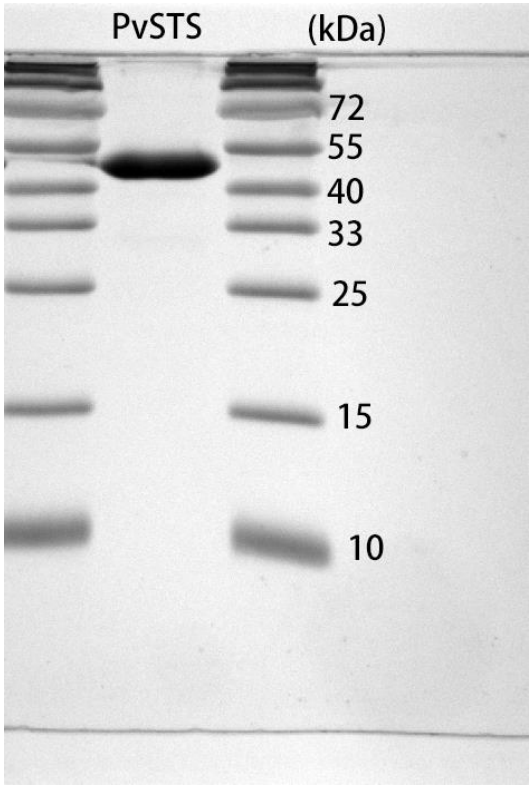

Figure 4. The result of electrophoresis after purification.

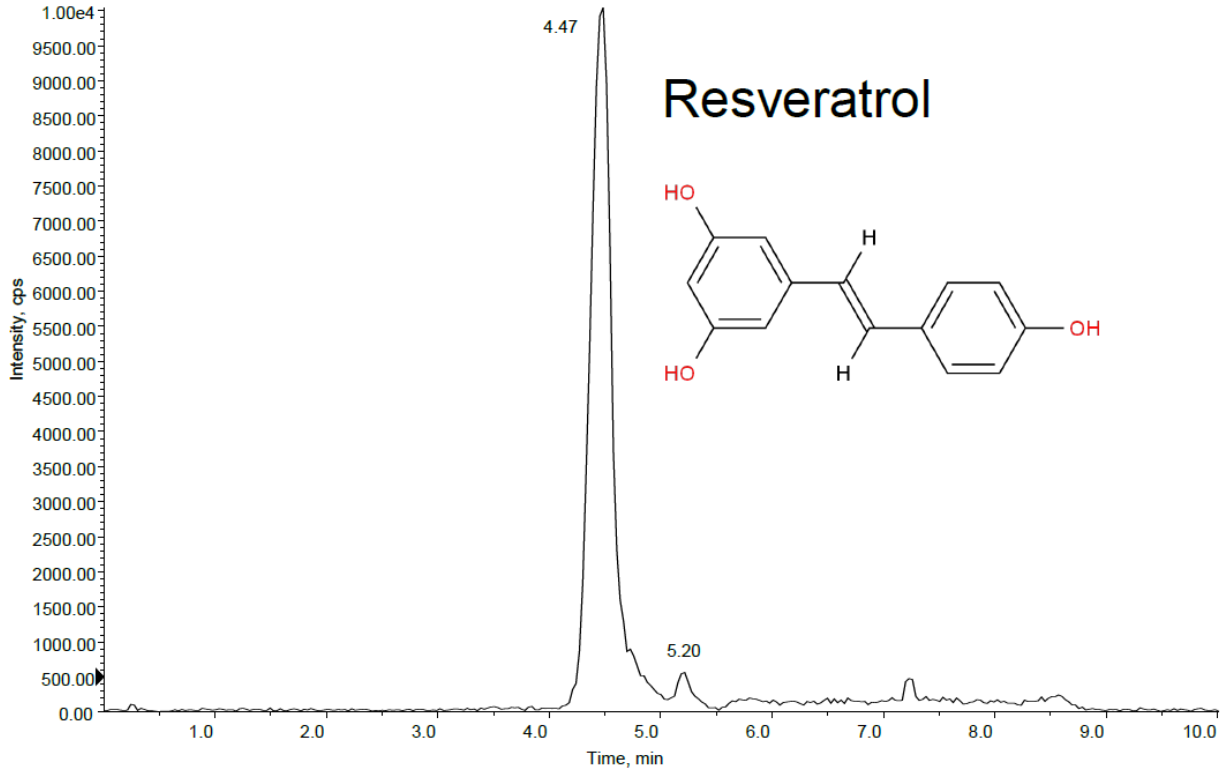

Figure 5. The result of resveratrol of LC-MS after reaction 1.

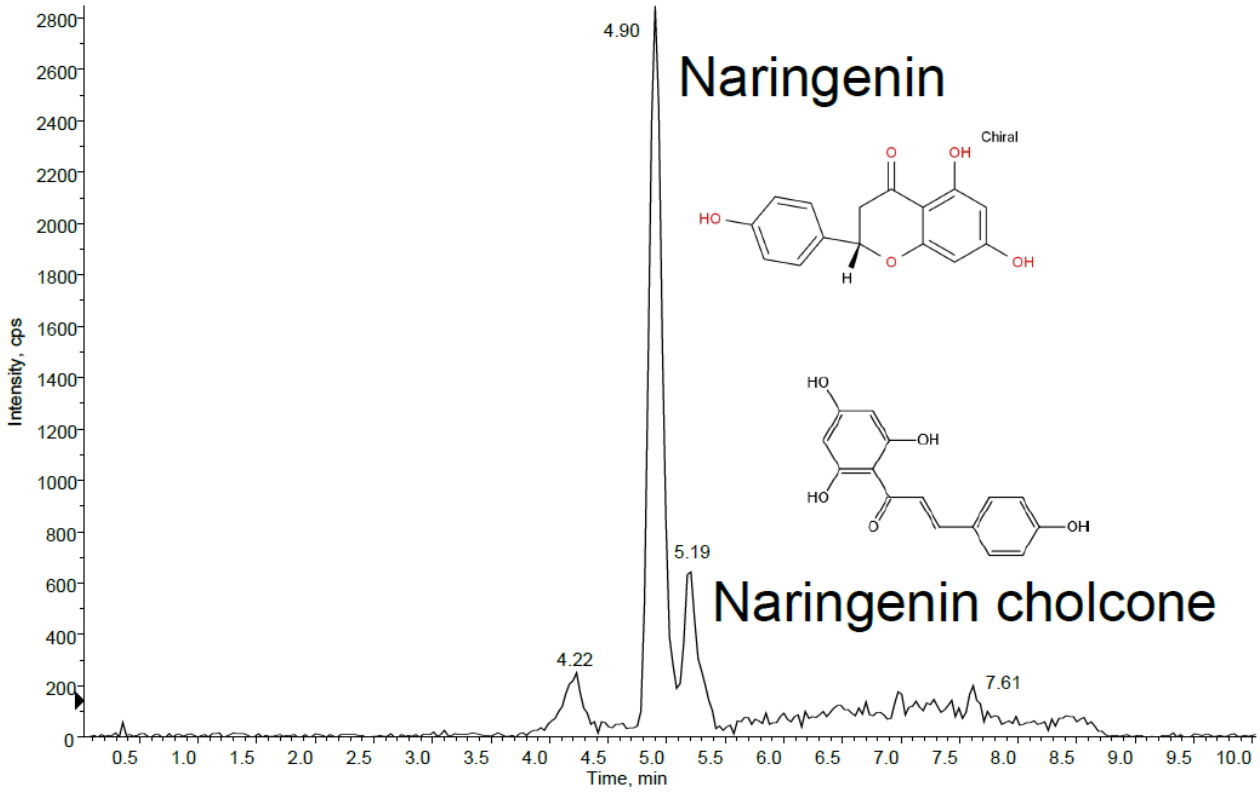

Figure 6. The result of naringenin of LC-MS after reaction 1.

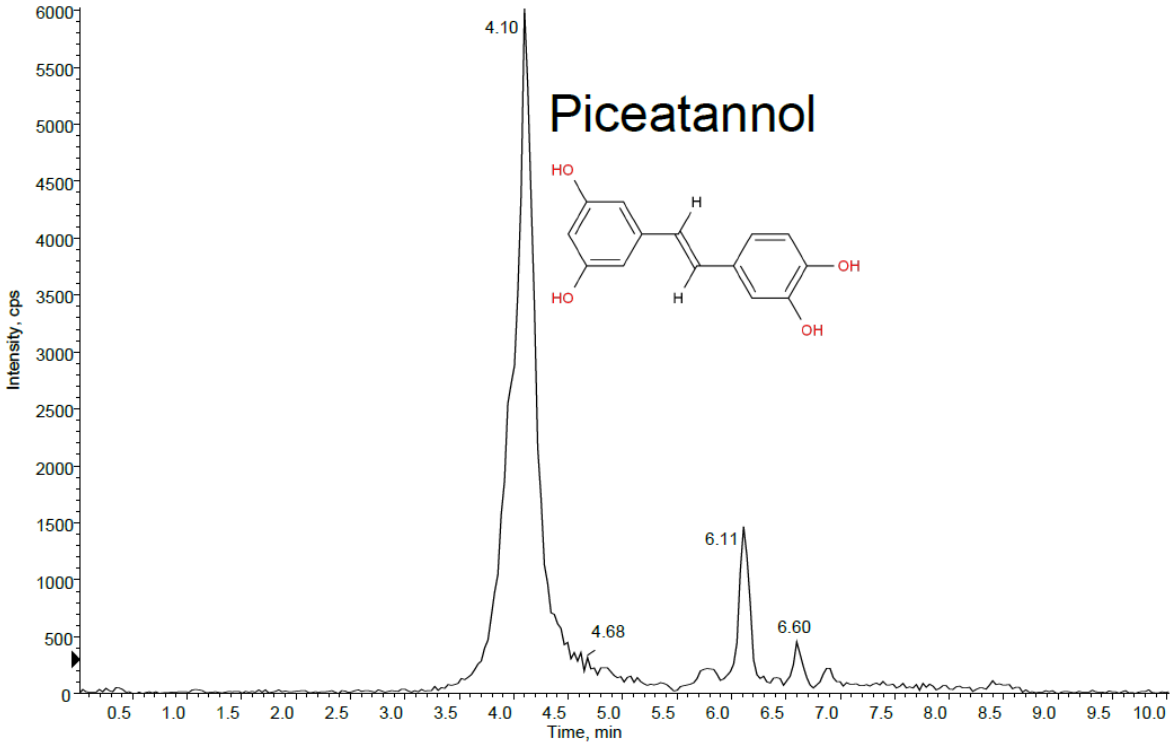

Figure 7. The result of piceatannol of LC-MS after reaction 2.

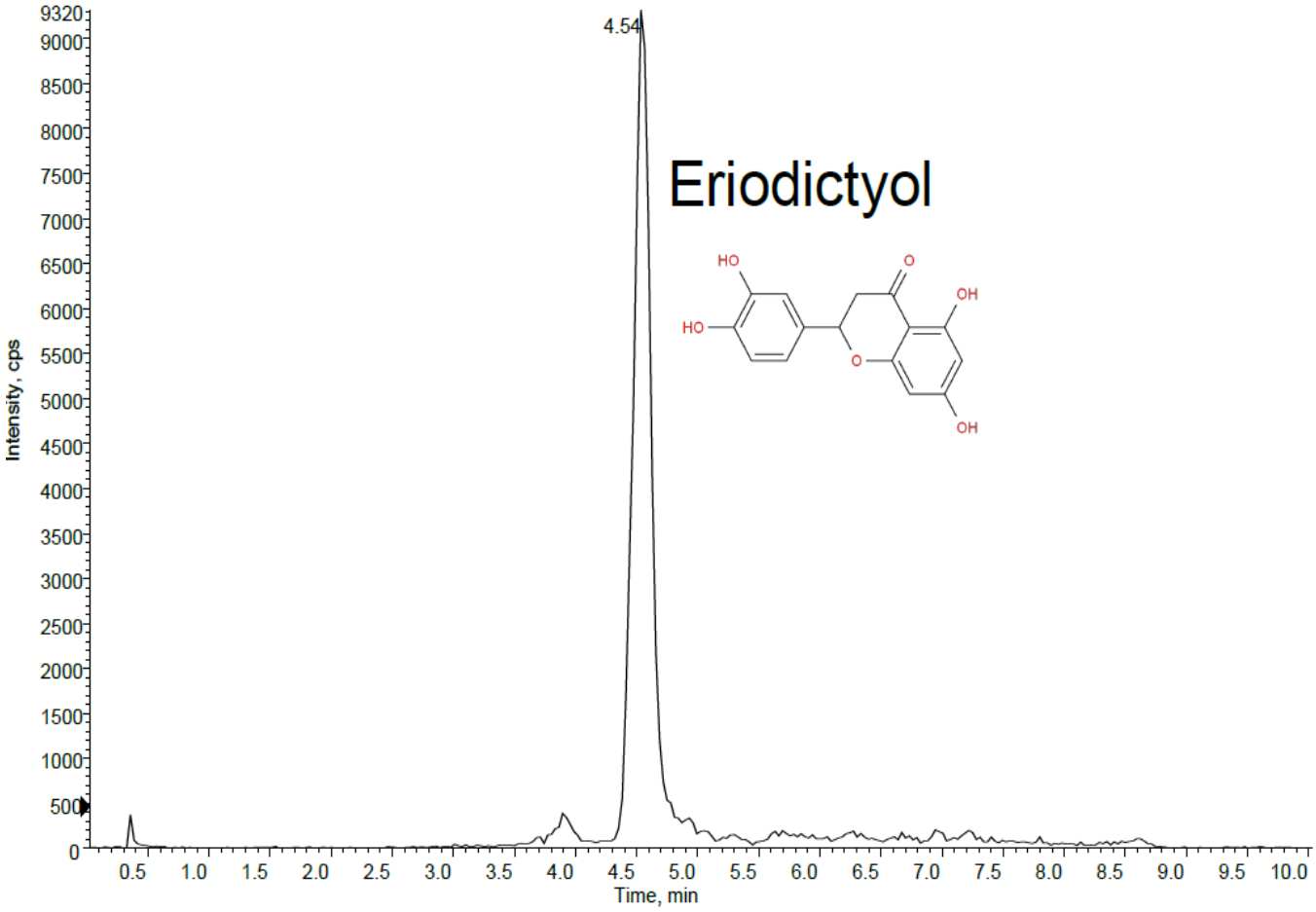

Figure 8. The result of eriodictyol of LC-MS after reaction 2.

ordinary stilbene synthases. However, as the bean plant itself could not naturally produce this protein, it necessitated genetic engineering for expression and extraction. The study is organized into three main phases.

1. Protein expression: The gene sequence encoding the protein was isolated and then recombined with the gene expression vector of engineered bacteria to construct a recombinant plasmid. Subsequently, the recombinant plasmid was introduced into the engineered bacteria for cultivation and expression (Spriestersbach et al., 2015).
2. The engineered bacteria underwent crushing and centrifugation to extract proteins for separation. The extracted proteins were then purified using a His column and a Q column, resulting in protein samples with sufficient purity for activity determination (Lu et al., 2008).
3. The protein was mixed with the corresponding substrate for the reaction. After a specified duration, the presence of the expected product was determined using LC-MS (Li and Elledge, 2012).

Although no derivatives of STS have been found in *P. vulgaris*, this study suggests that the bean still possesses the enzyme needed to produce the compound. This observation may indicate that this enzyme is common in plants and plays an irreplaceable role in physiological processes beyond the synthesis of stilbene compounds (Marbach and Bettenbrock, 2012). As a crucial compound in plant stress response, stilbene derivatives also underscore the significance of STS series proteins in related reactions.

The discovery of PvSTS with general stilbene synthase bioactivity in *P. vulgaris* demonstrates that the absence of stilbene compounds does not equate to the absence of stilbene synthase. This suggests that the protein, which remains biologically active in plants, may be involved in other vital life processes (Zhao et al., 2010).

The consistency in the function and sequence of stilbene synthase across different plants also indicates a degree of conservation. This suggests that this protein may play more crucial and essential physiological roles in plants beyond the synthesis of stilbene compounds.

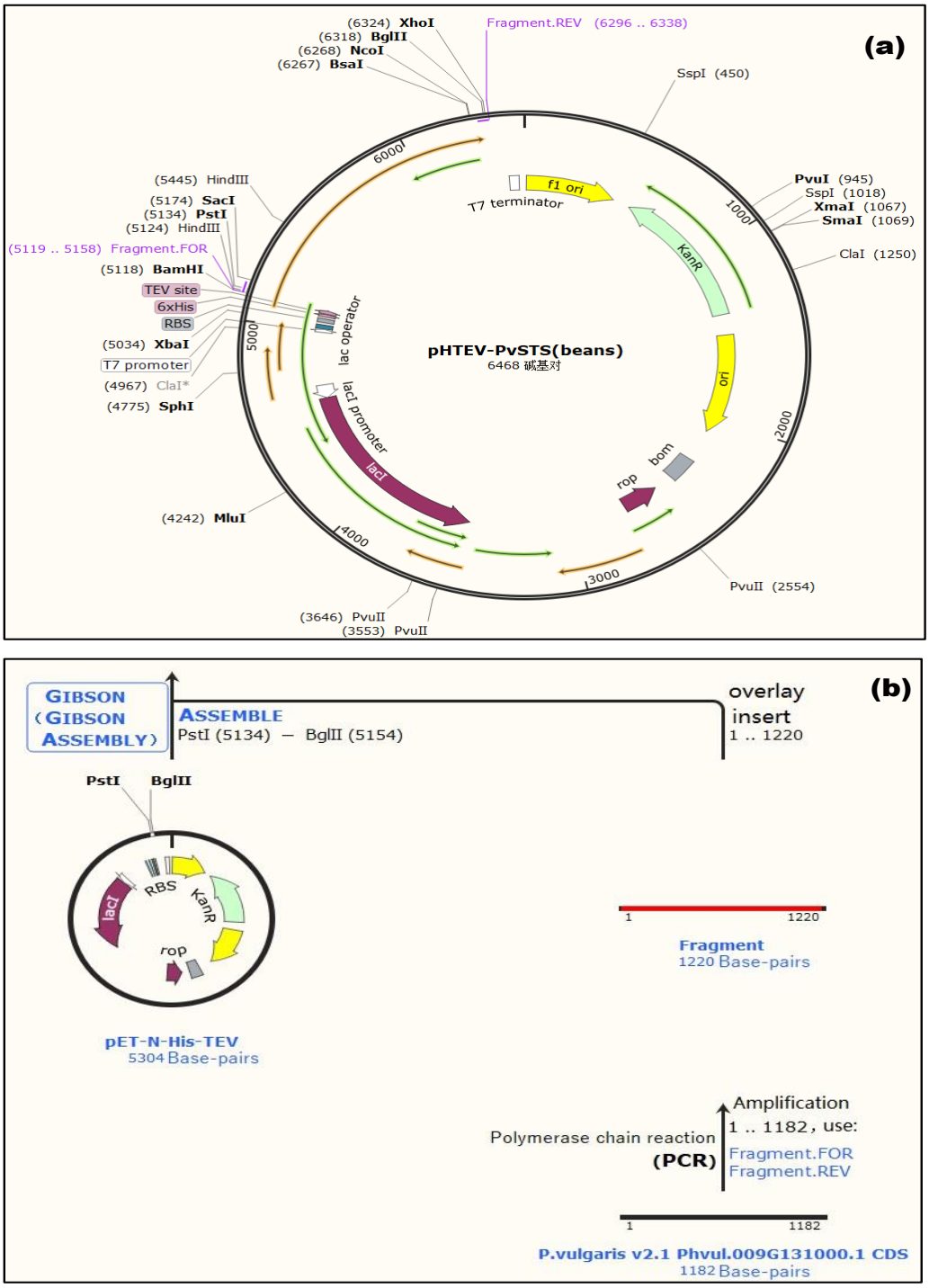

Figure 9. The principle of construction of recombinant plasmid. (a) Constructing a gene expression vector with Gibson assembly; (b) Homologous recombination.

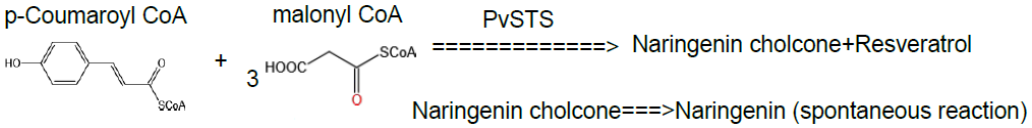

Figure 10. The reaction of p-Coumaroyl CoA and malonyl CoA.

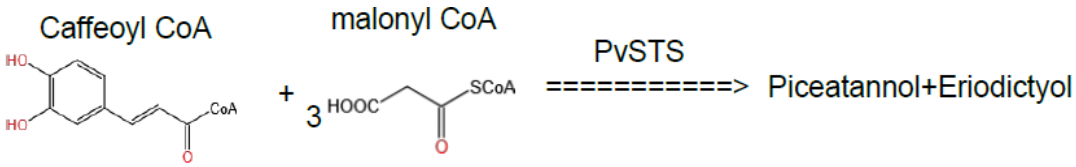

Figure 11. The reaction of caffeoyl CoA and malonyl CoA.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

THE DATA AVAILABILITY STATEMENT

All data, models, and code generated or used during the study appear in the submitted article.

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