Elicitors from the endophytic fungus *Trichoderma atroviride* promote *Salvia miltiorrhiza* hairy root growth and tanshinone biosynthesis

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Abstract

Biotic elicitors can be used to stimulate the production of secondary metabolites in plants. However, limited information is available on the effects of biotic elicitors from endophytic fungi on their host plant. *Trichoderma atroviride* D16 is an endophytic fungus isolated from the root of *Salvia miltiorrhiza* and previously reported to produce tanshinone I (T-I) and tanshinone IIA (T-IIA). Here, the effects of extract of mycelium (EM) and the polysaccharide fraction (PSF), produced by *T. atroviride* D16, on the growth and secondary metabolism of *S. miltiorrhiza* hairy roots are reported. The results indicated that both EM and PSF promoted hairy root growth and stimulated the biosynthesis of tanshinones in hairy roots. EM slightly suppressed the accumulation of phenolic acids, while PSF had no significant influence on the accumulation of these compounds. When comparing the effects of EM versus PSF, it was concluded that PSF is one of the main active constituents responsible for promoting hairy root growth, as well as stimulating biosynthesis of tanshinones in the hairy root cultures. Moreover, the transcriptional activity of genes involved in the tanshinone biosynthetic pathway increased significantly with PSF treatment. Thus, PSF from endophytic *T. atroviride* D16 affected the chemical composition of the host plant by influencing the expression of genes related to the secondary metabolite biosynthetic pathway. Furthermore, treatment with PSF can be effectively utilized for large-scale production of tanshinones in the *S. miltiorrhiza* hairy root culture system.

Key words: Endophytic fungi, hairy roots, phenolic acids, *Salvia miltiorrhiza*, tanshinones, *Trichoderma atroviride*.

Introduction

Plants synthesize a large array of natural products or secondary metabolites which are important for them to survive and flourish in the natural environment. At the same time, different types of secondary metabolites, such as saponins and essential oils, were produced by plants throughout long-term co-evolution as a defence response mechanism against pathogens, probably including endophytic fungi (Macias et al., 2007). Therefore, as biotic elicitors, pathogens and constituents of microbial cells, especially the carbohydrate or polysaccharide fractions, can be used to induce or stimulate the production of secondary metabolites in plant cell and tissue cultures (Zhao et al., 2005). This strategy has been successfully used to enhance secondary metabolite production in plant tissue cultures. For example, the content of thiarubrine A was enhanced 3-fold in *Ambrosia artemisiifolia* hairy root cultures through the utilization of autoclaved cell wall filtrates from the fungus *Protonyces gravidus*, a pathogen of *A. artemisiifolia* (Bhagwath and Hjortsø, 2000). Similarly,
biotic elicitors from Claviceps purpurea were included in Azadirachta indica hairy root cultures, leading to a 5-fold increase in the production of azadirachtin (Satdive et al., 2007). Additionally, Jung et al. reported that bacterial elicitors stimulated the biosynthesis of scopalamine in adventitious hairy root cultures of Scopolia parviflora via the inhibition of H6H (hydroxycamine 6β-hydroxylase) expression (Jung et al., 2003).

Endophytic fungi are microorganisms residing in internal tissues of living plants, which do not cause any immediate or overt negative effects (Rodriguez et al., 2009). Fossil records indicate that endophytic fungi have been associated with plants for at least 400 million years (Krings et al., 2007). During this extended period of co-existence and through evolutionary processes, endophytic fungi have formed a range of different relationships (symbiotic, mutualistic, commensalistic, and parasitic) with varied hosts in response to host genotype and environmental factors (Singh et al., 2011). Both field and laboratory studies have demonstrated that at least some plant species in natural habitats require endophytic fungi for stress tolerance and survival (Rodriguez and Redman, 2008). The fitness benefits conferred by endophytic fungi include their ability to produce a number of bioactive products and/or promote biosynthesis of host plant secondary metabolites, which enable host plants to resist external biotic and abiotic stresses (Zhao et al., 2011b; Ren and Dai, 2012). Additionally, some of these secondary metabolites can be used by humans as beneficial medicine. Therefore, endophytic fungi can be considered a powerful means of stimulating the accumulation of plant secondary metabolites for human medicinal use. Moreover, exploring the effects of endophytic fungi on plant secondary metabolism will facilitate the production of targeted drugs through bioengineering.

Hairy root is a type of transformed root induced by the infection of wounded plant tissue with the soil bacterium Agrobacterium rhizogenes bearing the root-inducing (Ri) plasmid (Saito, 1993). Hairy roots can produce similar levels of metabolites to those seen in the whole plant, with the advantages of fast growth rates (in hormone-free media), genetic stability, and biochemical stability. Due to these characteristics, the use of hairy roots has been widely explored in plant science research, having the advantage of a convenient, stable, and efficient plant tissue culture system for the production of valuable metabolites present in the roots of the wild-type plant (Srivastava and Srivastava, 2007). Salvia miltiorrhiza Bunge (Labiatae), which is known as Danshen in China, is an important and well-known traditional Chinese medicinal plant. Its rhizomes, which contain two groups of biologically active compounds, caffeic acid-derived phenolic acids and various tanshinones belonging to diterpene quinones, have been widely used for the treatment of cardiovascular diseases and menstrual disorders, as well as for the prevention of inflammation (Zhou et al., 2005). The hairy root culture of S. miltiorrhiza has been established as a useful platform for metabolic engineering research that can supersede the whole plant and as a potential means for the production of phenolic acids and tanshinones (Zhi and Alfermann, 1993; Chen et al., 1999).

In a previous study (Ming et al., 2012), an endophytic fungus, identified as Trichoderma atroviride D16, was isolated from the root of S. miltiorrhiza. This endophyte was capable of indigenously producing tanshinone I (T-I) and tanshinone IIA (T-IIA) in rich nutritional medium under shake-flask conditions. However, it was observed that a substantial decrease in the production of tanshinones occurred in the in vitro cultured endophyte following repeated subculturing. The role played by T. atroviride in the survival of the host plant may help explain this phenomenon. To the authors’ knowledge, there have been no reports regarding the effects of plant-derived bioactive compound-producing endophytic fungi elicitors on the secondary metabolism of their host plants. In this study, the effects of extract of mycelium (EM) and the polysaccharide fraction (PSF) isolated from T. atroviride on the root growth and biosynthesis of phenolic acids and tanshinones in S. miltiorrhiza hairy root cultures were investigated in order to understand the role of T. atroviride in host plant survival.

Materials and methods

Hairy root culture

The S. miltiorrhiza hairy roots used in this work were derived after infecting the plantlets with an Ri T-DNA-bearing A. rhizogenes bacterium (C58C1). Stock cultures of the hairy roots were maintained on solid, hormone-free half-strength B5 medium with 7.5 g l⁻¹ agar and 20 g l⁻¹ sucrose, at 25 °C in the dark. All experiments were carried out in shake-flask cultures with 250 ml Erlenmeyer flasks on an orbital shaker set at 25 °C and 180 rpm. Each flask contained 100 ml of liquid half-strength B5 medium and was inoculated with 1.0 g fresh weight of roots from 3-week-old shake-flask cultures. The hairy roots were harvested from the shake flasks by filtration and washed three times with distilled water, blotted dry with paper towels, and then dried at 50 °C in an oven until a constant dry weight (DW) was observed.

Extract of mycelium preparation and induction

The endophytic fungus T. atroviride D16 isolated from the root of S. miltiorrhiza has been deposited in the China General Microbiological Culture Collection Center (CGMCC) in Beijing, China, with the collection number 4712 (Ming et al., 2012). Trichoderma atroviride D16 was inoculated into 250 ml Erlenmeyer flasks, each containing 100 ml of liquid half-strength B5 medium. Biomass was removed by filtration after incubation on rotary shakers at 28 °C and 180 rpm for 10 d. After washing three times in distilled water, mycelia were suspended in distilled water, autoclaved at 121 °C for 40 min, and then filtered through three pieces of filter paper under vacuum. The filtrate obtained was the extract of mycelium (EM) and this was stored at 4 °C in a refrigerator after it was autoclaved at 121 °C for 15 min.

The EM dose was expressed via the total carbohydrate content determined by the phenol-sulphuric acid method using sucrose as a standard. EM was added into the liquid half-strength B5 medium of 3-week-old shake-flask-cultured hairy roots until the total carbohydrate concentration reached 40, 150, and 300 mg l⁻¹, respectively. Control treatments were added to fresh liquid half-strength B5 medium. The hairy roots with various concentrations of EM treatment were collected at various time intervals (0, 6, 12, and 18 d).

Polysaccharide fraction preparation and induction

The PSF was prepared from EM (50 litres). The solution of mycelium water-soluble extract was concentrated under vacuum at 60 °C
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by a rotary evaporator to a suitable volume and mixed with 4 vols of 95% ethanol, then kept at 4 °C for 48 h. Following this, the solution was centrifuged at 10,000 g for 10 min, and the precipitate from ethanol dispersion was collected. After lyophilization, the precipitate (16.19 g) was further subjected to deproteinization with Sevag reagent (chloroform–n-butanol 4:1, v/v), and small molecule impurities were removed by dialysis. PSF with a mol. wt. >2000 Da remained in the dialysis tube. After lyophilization, PSF (7.18 g) was stored in a desiccator at room temperature. PSF was added into the liquid half-strength B5 medium of 3-week-old shake-flask-cultured hairy roots at concentrations of 30, 60, and 180 mg l⁻¹. Control treatments were added to fresh liquid half-strength B5 medium. Hairy roots were collected at various time intervals (0, 3, 6, 12, 18, and 24 d).

HPLC analyses

The hairy roots were dried at 50 °C in an oven until a constant weight was obtained, and then were ground into powder and extracted with methanol (30 mg of roots ml⁻¹) under sonication for 60 min. The methanol extract was applied to the high-performance liquid chromatography (HPLC) system for the analysis of the secondary metabolites. This analysis was performed on an Agilent-1100 system as previously described using a ZORBAX SB-C18 chromatographic column (250 mm ×4.6 mm, 5 μm) at 30 °C with a H₂O (+0.5% HCOOH) (A)/acetonitrile (B) gradient (Ming et al., 2012). Rosmarinacidic (RA), salvianolic acid B (SAB), dihydroshinone I (DT-I), T-I, cryptotanshinone (CT), and T-IIA in the methanol extract of hairy roots were identified by comparison with the available standards. The reference standards were obtained from Chengdu Mansite Pharmaceutical Co. Ltd. [Chengdu, Sichuan Province], PR China.

RNA isolation and real-time quantitative PCR analysis

PSF was added into the liquid half-strength B5 medium of 3-week-old shake-flask-cultured hairy roots at a concentration of 60 mg l⁻¹. Controls were treated with fresh liquid half-strength B5 medium. Hairy roots were collected at various time intervals (0, 3, 6, 12, 18, and 24 d) and stored at ~80 °C. Total RNA was extracted from S. miltiorrhiza hairy root samples using TRIzol Reagent (Invitrogen) according to the manufacturer’s protocol. The quality and concentration of RNA were confirmed by ethidium bromide-stained agarose gel electrophoresis and spectrophotometric analysis.

Total RNA was reverse transcribed using the PrimeScript™ RT reagent Kit (Takara, Japan) to generate cDNA according to the manufacturer’s instruction. Primers with the following sequences, HMGKR (5′-AGGCTTCTGACCGGATAA-3′) and HMGKK (5′-GAATCTGCATCCCTCCAC-3′), DXRK (5′-CCATGAGCAGTTCTTATG-3′) and DXRK (5′-GGATGATCTTCTCCACGG-3′), GGGPSK (5′-CGAGAACTGCTAAGGGA-3′) and GGGPSK (5′-GTTCTGCTATGTGCAATGTA-3′), CPSK (5′-TGCAAGAGATTCGCCTAC-3′) and CPSK (5′-CTTGTGCTCATAAGGGA-3′), and KSLK (5′-CATGTCGAACAAGGGA-3′) and KSLK (5′-AATCTCACAAGGATGTGAC-3′), were used as described previously to detect the gene expression of smHMG, smDAR, smGGPS, smCP, and smKSL, respectively (Kai et al., 2011). Primers identifying the 18S gene, 18SF (5′-CCATGAGCAGCTAGTAAAG-3′) and 18SR (5′-GTCAAAAGGACGACGAT-3′), were used as a reference gene to normalize cDNA loading (Kai et al., 2011). The realtime PCR amplification was performed in a 384-well plate Roche LC480 thermocycler (Roche Diagnostics) with SuperReal PreMix kit (TIANGEN, China). Each reaction contained a mixture of 1 μl of diluted cDNA, 0.2 μl of forward primer (10 μM), 0.2 μl of reverse primer (10 μM), 5 μl of SYBR Green PCR Master Mix (TIANGEN, China), and 3.6 μl of RNase-free H₂O. The reaction mixture was incubated for 15 min at 95 °C, and for 40 cycles of 10 s at 95 °C and 30 s at 60 °C. The relative gene expression was quantified using the comparative CT method.

Data analysis

All experiments, including both control and different treatments of hairy root cultures, HPLC analysis, and semi-quantitative real-time PCR, were performed in triplicate. The results are presented as their mean values and standard deviations (SD). The error bars in the figures represent the standard deviation in biological triplicates. The statistical significance of the differences in root growth and the accumulation of phenolic acids and tanshinones was analysed by one-way analysis of variance (ANOVA) with SPASS software, and the term significant has been used to denote the differences for which P < 0.05. The statistical significance of differences in gene transcripts was analysed by one-sample t-test.

Results

Effects of extract of mycelium and polysaccharide fraction on the biomass of S. miltiorrhiza hairy roots

Both in elicited and in control shake-flask cultures, the biomass of S. miltiorrhiza hairy roots displayed a steady and linear growth trend during the biomass growth period, but was more rapid in the treated groups (Fig. 1). The use of EM (Fig. 1A) and PSF (Fig. 1B) resulted in a significant increase in biomass accumulation from day 12. The effects of both EM and PSF on the growth of hairy roots, when comparing their three respective concentrations, displayed no significant difference. The effects of PSF were slightly different compared with EM treatment: on day 18, the biomass of hairy roots with PSF treatment was ~60% greater than the control, while the biomass of hairy roots subjected to EM treatment was ~40% greater than the control. Therefore, the effects of PSF on hairy root biomass were more pronounced than those of EM treatment on day 18 (P < 0.05). Moreover, the biomass promotion by PSF on day 24 was also more apparent than that on day 18 (P < 0.05).

Effects of extract of mycelium and polysaccharide fraction on the accumulation of phenolic acids in S. miltiorrhiza hairy roots

The effects of EM and PSF on the accumulation of phenolic acids in S. miltiorrhiza hairy roots are shown in Fig. 2. With EM treatment, the accumulation of RA in hairy roots was slightly stimulated on day 12, but was mildly suppressed on day 18 (Fig. 2A). On the other hand, the accumulation of SAB was moderately suppressed on days 12 and 18 (Fig. 2C). However, in hairy roots with PSF treatment, the contents of both RA and SAB showed no significant changes on days 6, 12, 18, and 24 (Fig. 2B, D).

Effects of extract of mycelium and polysaccharide fraction on the accumulation of tanshinones in S. miltiorrhiza hairy roots

As shown in Fig. 3, both EM and PSF treatment caused a substantial alteration in the abundance of the four tanshinones. The accumulation of DT-I, T-I, CT, and T-IIA was notably stimulated at all EM doses applied to the hairy root culture at
18 d (Fig. 3A, C, E, G). Amazingly, the dramatic stimulation of DT-I, T-I, CT, and T-IIA was also observed with treatment with 30, 60, and 180 mg l⁻¹ PSF at 24 d (Fig. 3B, D, F, H). Among the four tanshinone species, DT-I and CT were most dramatically stimulated by EM and PSF. The content of DT-I in hairy roots with treatment with 300 mg l⁻¹ EM was ~35-fold that of the control on day 18 (1.338 mg g DW⁻¹ versus 0.039 mg g DW⁻¹) (Fig. 3A) and the content of CT with treatment with 150 mg l⁻¹ EM was ~83-fold that of the control on day 18 (3.061 mg g DW⁻¹ versus 0.037 mg g DW⁻¹) (Fig. 3E). Similarly, the content of DT-I in hairy roots with treatment with 180 mg l⁻¹ PSF was ~23-fold that of the control on day 18 (1.216 mg g DW⁻¹ versus 0.052 mg g DW⁻¹) (Fig. 3B) and the content of CT with treatment with 180 mg l⁻¹ PSF was ~66-fold that of the control on day 18 (3.496 mg g DW⁻¹ versus 0.053 mg g DW⁻¹) (Fig. 3F). Additionally, the content of T-I with treatment with 300 mg l⁻¹ EM (Fig. 3C), 0.247 mg g DW⁻¹, was ~3-fold that of the control culture on
day 18, 0.091 mg g DW⁻¹, and the content of T-IIA (Fig. 3G) with treatment with 40 mg l⁻¹ EM, 0.234 mg g DW⁻¹, was ~5-fold that of the control culture on day 18, 0.046 mg g DW⁻¹. Moreover, the content of T-I with treatment with 30 mg l⁻¹ PSF (Fig. 3D), 0.602 mg g DW⁻¹, was ~5-fold that of the control culture on day 18, 0.123 mg g DW⁻¹, and the content of T-IIA (Fig. 3H) with treatment with 40 mg l⁻¹ PSF, 0.434 mg g DW⁻¹, was ~11-fold that of the control culture on day 18, 0.040 mg g DW⁻¹.

When comparing the effects of EM and PSF on tanshinone biosynthesis, the contents of tanshinones in hairy roots exhibited a steady and linear increase during the period of treatment with both EM and PSF. Although the increased levels of DT-I and CT with PSF treatment were not greater than those with EM treatment, the absolute contents of DT-I and CT in hairy roots with EM and PSF treatment exhibited no difference. Moreover, both the increased levels and absolute contents of T-I and T-IIA with PSF treatment were
much greater than that with EM treatment. When extending the treatment time to 24 d, the effects of PSF on tanshinone biosynthesis were similar to those on day 18.

Transcriptional response of the tanshinone biosynthetic pathway to the induction of polysaccharide fraction in S. miltiorrhiza hairy roots

To investigate the effects of PSF on the tanshinone biosynthetic pathway at the transcriptional level, the expression of genes involved in tanshinone biosynthesis (Fig. 4A) was studied by real-time quantitative PCR. As shown in Fig. 4B, during the hairy root culture period, 1-deoxy-d-xylulose 5-phosphate reductoisomerase (DXR), geranylgeranyl diphosphate synthase (GGPPS), copalyl diphosphate synthase (CPS), and kaurene synthase-like (KSL) mRNA levels were observed to be gradually stimulated, reaching peak levels on day 24, and their maximum mRNA transcript level reached 5-, 7-, 8-, and 7-fold that of the control, respectively. The transcription levels of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), a rate-limiting enzyme involved in the mevalonate (MVA) pathway, significantly increased on day 12 and day 24. On day 24, the HMGR mRNA transcript level was 8-fold that of the control (Fig. 4B).

Discussion

As reported previously (Ming et al., 2012), the endophytic fungus T. atroviride D16 can indigenously produce T-I and T-IIA, with a substantial decrease occurring in the production of tanshinones in the in vitro cultured endophyte following repeated subculturing. In this study, the experimental results indicated that EM and PSF from endophytic T. atroviride exhibited obvious effects on root growth and secondary metabolism in S. miltiorrhiza hairy roots. Both EM and PSF could promote hairy root growth (Fig. 1) and stimulate the biosynthesis of tanshinones in hairy roots (Fig. 3). However, EM could slightly suppress...
the accumulation of phenolic acids, while PSF had no significant influence on this accumulation (Fig. 2). When comparing the effects of EM with PSF, it is concluded that PSFs are one of the main active constituents responsible for promoting hairy root growth and stimulating biosynthesis of tanshinones in S. miltiorrhiza hairy root cultures. Moreover, PSF significantly increased the transcriptional levels of genes involved in the tanshinone biosynthesis pathway (Fig. 4). So far, dozens of endophytic fungi have been found to produce plant-derived bioactive compounds (Zhao et al., 2011b). As far as is known, there have been no reports regarding the effects of these endophytic fungal elicitors on the secondary metabolism of their host plants.

There are a few reports about fungi promoting plant growth by producing auxins. One plant growth-promoting endophytic fungus, Penicillium citrinum, isolated from sand dune flora was reported to produce the plant growth hormone gibberellin (Khan et al., 2008). Interestingly, the fungus T. atroviride was also reported to enhance biomass production and promote lateral root growth through an auxin-dependent mechanism in Arabidopsis thaliana in another study (Contreras-Cornejo et al., 2009). However, with the treatment of PSF isolated from endophytic T. atroviride, the growth of S. miltiorrhiza hairy roots was also promoted in the present study (Fig. 1B). Therefore, in regards to the mechanisms for the promotion of plant growth, other pathways may exist besides those that are auxin dependent. In the authors’ opinion, the putative mechanism for the plant growth promotion by PSF from T. atroviride might be that PSF elicited the local and systemic defence responses of S. miltiorrhiza hairy roots and then further stimulated the biosynthesis of plant growth-promoting substances in hairy roots.

Phenolic acids and tanshinones are two groups of the main biologically active compounds in the traditional Chinese medicinal plant Danshen (Zhou et al., 2005). One report indicated that tanshinones had much stronger antimicrobial activity than phenolic acids, with DT-I and CT being the strongest antimicrobial components among the tanshinones in S. miltiorrhiza hairy roots (Zhao et al., 2011a). This may explain why, in the present study, PSF from T. atroviride had no significant influence on the accumulation of phenolic acids (Fig. 2), but could dramatically stimulate the biosynthesis of tanshinones (Fig. 3), especially DT-I and CT. Thus it is likely that when T. atroviride colonized the tissue of the S. miltiorrhiza root, the PSF on the surface of the mycelium elicited the chemical defence responses of the root and stimulated the accumulation of tanshinones, especially DT-I and CT, to inhibit the fungal growth.

Tanshinones, a group of abietanoid diterpenes, constitute a major class of bioactive compounds in S. miltiorrhiza roots, with proven therapeutic effects and pharmacological activities (Wang et al., 2007). The biosynthesis of tanshinones (Fig. 4A) is complicated, in particular due to the participation of a variety of rate-limiting enzymes (Ma et al., 2012). The two common precursors of plant terpenoids, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), are synthesized through two different pathways in separate cellular compartments: one pathway is the MVA pathway occurring in the cytoplasm, and the other pathway involves the non-MVA or 2-C-methyl-d-erythritol phosphate (MEP) pathway occurring in the plastids. HMGR is an important enzyme in the MVA pathway, while DXR is a key enzyme in the MEP pathway. GGPPS catalyses the condensation reactions of IPP and DMAPP to form GGPP, a precursor of diterpenes, which is an important step in the synthesis of tanshinones. So far, CPS and KSL are the most downstream genes known in the tanshinone biosynthetic pathway. In order to clarify the effect of PSF on tanshinone synthesis specifically, the transcript levels of HMGR, DXR, GGPPS, CPS, and KSL were investigated utilizing real-time quantitative PCR (Fig. 4B). Transcriptional levels of the genes DXR and HMGR were stimulated, with stimulation of DXR being more robust. This is in agreement with the opinion that the biosynthesis of tanshinones mainly occurs via the MEP pathway, with dependence on the cross-talk between the MEP and the MVA pathways (Ma et al., 2012). Gene expression levels of GGPPS, CPS, and KSL were also observed to increase gradually with PSF treatment. These results indicated that PSF stimulates many of the genes involved in the biosynthesis of tanshinones and then promotes the accumulation of tanshinones in S. miltiorrhiza hairy roots. The question of how PSF stimulates these genes and which signal transduction pathway is involved in the process will need to be clarified in future studies.

In the present study, the results suggested that PSF from T. atroviride D16 could elicit chemical defence responses in the host plant with the purpose of confronting pathogens. PSF affected the chemical composition of the host plant by influencing the expression of related genes involved in the secondary metabolite biosynthesis pathway. Therefore, PSF can be used as a potent elicitor for stimulating tanshinone production in S. miltiorrhiza hairy root cultures. Thus, treatment with PSF may be considered as an effective approach for the large-scale production of tanshinones in S. miltiorrhiza hairy root culture systems. However, PSF, which mainly contains some types of polysaccharides, is also crude. Which type of polysaccharide is the active constituent responsible for promoting hairy root growth and stimulating the biosynthesis of tanshinones in the hairy root culture is still unclear and will be clarified in future studies.

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References


