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Effect of salicylic acid on the expression of a gene involved in the biosynthesis of hyperforin in St. John's wort (*Hypericum perforatum*)

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Abstract

To address the role of the gene *HpPKS1* in the biosynthesis of hyperforin in St. John's wort, we analyzed the effect of different concentrations of salicylic acid on the expression of this gene via qRT-PCR method. To do this, the calli resulted from the leaf explants of *in vitro* grown plants were treated under three concentrations of salicylic acid (0, 100 and 250 µM) and the expression pattern of the gene *HpPKS1* was evaluated after 24, 48 and 72 hours. The results demonstrated that with increasing the concentration of salicylic acid, the expression of the gene showed a significant increment 24 and 48 hours after treatment; while after 72 hours a decrease was observed in all concentrations. Furthermore, in all time durations, the expression of *HpPKS1* was increased with raising the concentration of salicylic acid, so that the maximum activity of the gene was witnessed at 250 µM salicylic acid.

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Introduction

St. John's wort has been known and used as a medicinal plant from ancient romans era as a cure for mental disorders such as anxiety, insomnia, anger, migraine, fatigue, fibrositis, neurological and sciatic pains, gastritis and respiratory diseases (Filandrinos *et al*., 2006). Due to these and other known and unique features, this plant has been used in modern medicine. These features are mostly related to hypericin and hyperforin (Medina *et al*., 2006). Studies show that the extract of St. John's wort is a standard antidepressant drug in the treatment of mild to moderate depression (Sanches *et al*., 2002).

During recent decades, a drastic resistance has been raised against application of chemical drugs due to their adverse reactions and hence the extraction of medicinal substances from natural resources has been intensified (Arnholdt, 2000). The cultivation of medicinal plants in order to access active ingredients is restricted because of seasonal and climatic conditions, water availability and pests and diseases (Arikat *et al*., 2004). On the other hand, population growth, urbanization and unlimited gathering of medicinal plants from nature have led to excessive exploitation of these natural resources (Sharma *et al*., 2010). Furthermore, the complex structure of most of these secondary metabolites is a reason for their artificial (chemical) synthesis to be impossible or if possible, unaffordable. Plant cells are the only sources of these products. Cell culture has been proposed as an alternative for gathering these plants from nature to produce secondary metabolites (Scragg, 1986). Production of secondary metabolites can be improved through optimization of culture media. This has been achieved in *Hypericum* species (Gadzovska *et al*., 2005). One of these efforts is using elicitors. Elicitors are molecules which elicit plant cells to produce more metabolites. Some chemicals such as salicylic acid and methyl jasmonate have been used as elicitors. The mechanism by which these elicitors act is complicated, because these can affect many physiological and biochemical systems in the plant and may exert diverse changes in the cells. It has been revealed that these elicitors are received via membrane receptors and influence the expression of the genes involved in the biosynthesis of secondary metabolites through signal transduction mechanisms (Hasanloo *et al*., 2009).

Salicylic acid is known as a component of a key signal in the activation of plant defense responses. These reactions may lead to an increase in biosynthesis and accumulation of diverse plant secondary metabolites. Hence, this compound has been utilized to increase different metabolites such as alkaloids, terpenoids, flavonoids, phenolic compounds and phytoalexins in plants (Mueller *et al*., 1993).

Gene expression analysis is an important way to study how living organisms respond to their surrounding environment. Some of genes react rapidly to the environmental clues and some show a gradual change. These changes in their expression are known as an index for plant behavior in relation to its environment (Hazen *et al*., 2003). Based on our knowledge, there is no report about the effect of salicylic acid on the expression of *HpPKS1* in *Hypericum perforatum*.

Materials and methods

Plant materials

The seeds of *Hypericum perforatum* cv. New Stem were purchased from Richters Herbs Inc., Canada. In order to get sterile plantlets, the seeds were sterilized and cultured on MS (Murashige, and Skoog, 1962) medium and after vernalization for a week, kept in 16/8 photoperiod, 25/22 ±3°C day/night temperature and light intensity of 70 $\mu Mm^{-2}s^{-1}$ for 6 weeks. Leaf explants from these *in vitro* plants were cut into 3-4 mm stripes and put on the MS medium solidified with agar and containing 1 mg/L BAP and 0.5 mg/L IAA in darkness at 25°C. After four weeks, grown calli were cultured in the same condition. Resulted calli were cut into 2-3 mm pieces and were treated on the same medium containing different concentrations of salicylic acid (0, 100 and 250 µM) and kept in darkness at 25°C. For RNA extraction, calli were sampled after 24, 48 and 72 h after treatment, frozen in liquid nitrogen and maintained at -80°C.

RNA extraction

Total RNA extraction kit (Jena Bioscience, Germany) was used for RNA extraction based on the kit manual. To remove possible DNA contamination, treatment with DNase I was done. Quality and quantity of RNA were evaluated with electrophoresis and spectrophotometry.

Gene expression study

For cDNA synthesis, 1 µg RNA was treated with MMuLV Revert Aid (Fermentas, Poland) based on the company protocol using Oligo-dT_{18} primers. To study the expression of *HpPKS1* gene, its specific primers (forward: TGTACGTCTCATCCAGTCAGC and reverse: ACACCACCGTAACAGCCTAAG) as the target gene and *GAPDH* gene specific primers (forward: ATGGAC CATCAAGCAAGGACTG and reverse: GAAGGCCATT CCAGTCAACTTC) as the internal control gene were used. For qRT-PCR, a Real-Time PCR machine (Step One, ABI, USA) was used. PCR steps were as one cycle of 94°C for 10 min and 45 cycles of 94°C for 15s and 60°C for 45 s. Melting curve analysis was carried out from 60°C to 95°C with read intervals of 1 min. Analysis of qRT-PCR data based on two biological and three technical replications was performed through the method $2^{\Delta Ct}$ (Wang *et al*., 2015).

Results and discussion

Results showed that in all three sampling times, with increasing salicylic acid concentration, the expression of the gene *HpPKS1* was increased. Fig. 1, 2 and 3 show this trend in 24, 48 and 72 hours after treatment. As it can be seen in fig. 1., 24 h after treating the samples, the expression indices of the gene were 1028, 1986 and 2789 for the treatments 0, 100 and 250µM salicylic acid, respectively. A similar trend was observed after 48 hours (fig. 2.). Interestingly, after 72 hours, the relative expression of the gene *HpPKS1* for the three concentrations showed the indices 1879, 1000 and 2566, respectively, which resembled the same trend of the previous sampling times. *HpPKS1*is a gene encoding a key enzyme playing role in hyperforin biosynthesis and its increase has been in accordance with the increase of hyperforin in St. John's wort.

The role of *HpPKS1* in this plant has not yet been revealed, but due to high accumulation of its transcript in the upper parts of the St. John's wort shoots, it is probable that this gene is acting in accumulation of the compounds against herbivores (Karppinen, 2010).

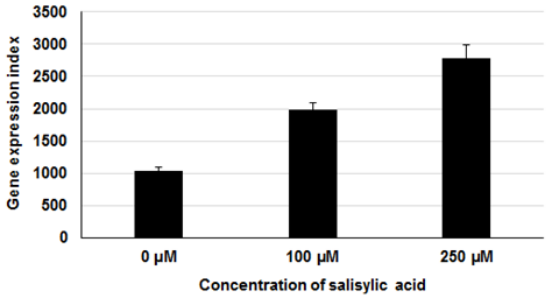

Fig. 1. The expression of *HpPKS1* gene 24 hours after treatment with different concentration of salicylic acid.

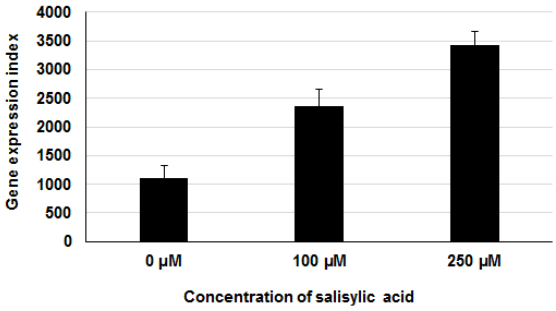

Fig. 2. The expression of *HpPKS1* gene 48 hours after treatment with different concentration of salicylic acid.

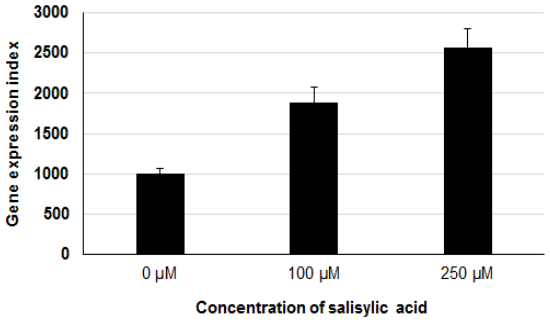

Fig. 3. The expression of *HpPKS1* gene 72 hours after treatment with different concentration of salicylic acid.

Salicylic acid up-regulates defense-related genes through the regulatory protein, non-expresser of pathogenesis related gene 1 (NPR1), which is a key

mediator molecule related to systemic acquired resistance (SAR) in monocots and dicots (Dong, 2004). The NPR1 regulates SAR and plays a role in salicylic acid signal transduction that leads to activation of pathogenesis-related genes. NPR1 enters nucleus, but does not attach directly to DNA and needs TGA transcription factors to accomplish its mission (Pieterse and Van loon, 2004). These factors attach to an activating sequence 1-as in the promoter of the gene pathogenesis-related (PR). NPR1 can enhance the ability of some TAG proteins to DNA and hence affect the PR gene expression (Durrant and Dong, 2004). Based on Hayat *et al* (2010), salicylic acid can increase the resistance against pathogens and production of pathogenesis related proteins even in the absence of any pathogen.

As it is illustrated in fig. 4., in the elicitor free medium, the expression indices of the gene *HpPKS1* after 24, 48 and 72 hours are 1028, 1109 and 1000, respectively, which show a relative constancy and reveals that there is a basic expression of the gene even without exposing the plants to salicylic acid. It is obvious that there is no time course change in the expression of the gene. This is while in the samples treated with 100 µM salicylic acid, the expression the gene has been increased after 24 and 48 hours almost two times. However, after 72 hours, a decreasing trend was observed (fig. 5.). The same result came out in the samples treated with 250 µM salicylic acid (Fig. 6.).

It can be postulated that this decrease in the expression of the gene *HpPKS1* is due to inactivation of salicylic acid after 72 hours. Salicylic acid can be inactivated in two ways: combining with another compound or metabolic inactivation. It has been found that plants have the ability of producing o-glucosides or salicylic acid glucose esters. Plants can metabolically inactivate salicylic acid through excessive hydroxylation of the aromatic ring (Raskin, 1992). Further, salicylic acid has the capacity of conjugating to other molecules (Hayat *et al*., 2010). Therefore, gradually inactivation of this elicitor during time can be an explanation for the observed decrease in the expression of the *HpPKS1* gene after 72 hours.

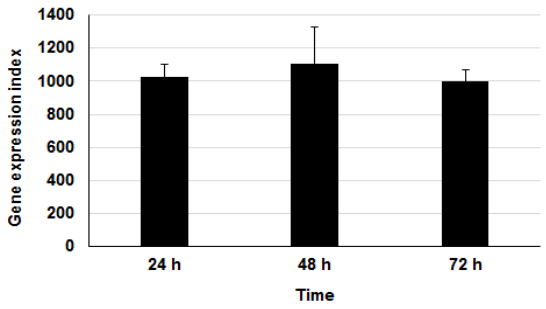

Fig. 4. Expression pattern of *HpPKS1* gene in control samples in different time courses.

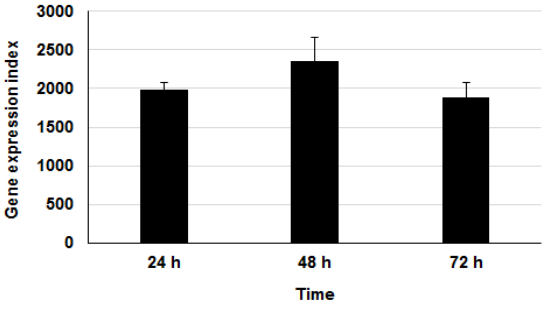

Fig. 5. Expression pattern of *HpPKS1* gene in 100 µM salicylic acid treated samples in different time courses.

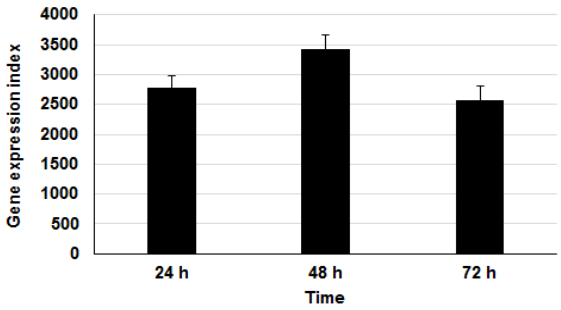

Fig. 6. Expression pattern of *HpPKS1* gene in 250 µM salicylic acid treated samples in different time courses.

The results of an experiment carried out by Sirvent and Gibson (2002) showed that among the concentrations of 1, 2.5 and 5 mM salicylic acid in St. John's wort meristem cultures, 1 mM concentration led to an increase in hyperforin, while higher concentrations did not cause any further increase. Moreover, this increase was accompanied with a decrease in plant weight and height which can be considered as a defense response in the presence of salicylic acid.

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