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BRIEF COMMUNICATION

Expression of two genes of paclitaxel biosynthetic pathway during germination of *Taxus baccata* zygotic embryos

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Abstract

The spatial and temporal expression of *dbat* and *dbtnbt* genes involved in the later steps of paclitaxel biosynthesis in relation to baccatin III and paclitaxel accumulation in *Taxus baccata* L. germinating embryos and seedlings was investigated. The steady-state of mRNA transcripts was measured by quantitative real time polymerase chain reaction (qRT-PCR), the content of taxanes was determined by HPLC. The spatial distribution of the metabolites was found to be in accordance with the transcript level of the respective genes. Higher content of mRNA transcripts in shoots of yew seedlings responded to higher content of taxanes in stems and needles. The highest increase in the transcript level of both genes was observed 8 d after placing the embryos on germination medium, before elongation of embryonic axis and emergence of the radicle. The pattern of temporal *dbat* expression was in line with the expression of the *dbtnbt* gene. The temporal distribution of the precursor baccatin III correlated well with paclitaxel, the final product of the pathway.

Additional key words: *dbat, dbtnbt*, qRT-PCR, yew.

Yew trees (*Taxus* spp.) is a natural source of taxane diterpenoids – all of approximately 400 well defined taxane diterpenoids are based upon a taxane skeleton with the phenylisoserine side-chain. Among them, paclitaxel and docetaxel are considered the commercially most important anticancer agents with unique mechanism of action (Schiff *et al*. 1979, Horowitz *et al*. 1986). The increasing demand for these drugs caused mass devastation of wild yew's populations and has led to explore alternative approaches for *Taxus in vitro* mass propagation. For the establishment of *Taxus in vitro* systems and commercial production of paclitaxel several approaches have been employed: stem-cutting methods (Chang *et al.* 2001), embryo cultures (Flores and Sgrignoli 1991, Chee 1995) or cell cultures (Fett-Neto and DiCosmo 1997). To achieve higher productivity the elicitation of free and immobilized cells is often used (Bonfill *et al*. 2007). The selection and long-term storage of cell lines with appropriate genetic, biochemical and physiological properties is also necessary (Škrlep *et al*. 2008).

One of the most promising means how to increase paclitaxel yield seems to be molecular engineering and genetic manipulation of the key steps of taxane biosynthesis. Except a few undefined steps the whole paclitaxel biosynthetic pathway was elucidated and 13 genes coding for the key enzymes were cloned and characterized (Croteau *et al.* 2006, Guo *et al.* 2006). According to Nims *et al.* (2006) the regulation of taxane biosynthetic pathway occurs at the level of mRNA and there is a correlation between the steady-state transcript abundance and the respective taxane accumulation. To our knowledge, there is no information on association between the transcript level of genes coding for the key enzymes of taxane biosynthesis and accumulation of corresponding metabolites in different parts of yew plant as well as during ontogeny. The aim of this work was to ascertain the pattern of spatial and temporal expression of

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Abbreviations: DBAT - 10-deacetylbaccatin-III-10β-*O*-acetyltransferase; DBTNBT - 3'-*N*-debenzoyl-2'-deoxytaxol-*N*-benzoyl-transferase; REU - relative expression units; RT-PCR - quantitative real time polymerase chain reaction.

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two genes coding for the enzymes of the later steps of paclitaxel biosynthesis: the *dbat* gene coding for 10-deacetylbaccatin-III-10β-*O*-acetyltransferase (DBAT) and the *dbtnbt* gene coding for 3'-*N*-debenzoyl-2'-deoxytaxol-*N*-benzoyltransferase (DBTNBT) and to correlate the gene expression pattern with intracellular accumulation of the corresponding metabolites baccatin III and paclitaxel by means of quantitative real time polymerase chain reaction (qRT-PCR).

Zygotic embryos were isolated from mature, 5.0 - 6.0 mm long and brown-colored seeds of fruits with fully developed red arils collected in September 2006 from *Taxus baccata* L. donor tree growing in the Botanical Garden of P.J. Šafárik University, Košice, Slovakia. The seeds were surface-sterilized and prepared for germination using the previously described methods (Bruňáková *et al*. 2004). For the experiment at least 200 embryos were used. RNA and taxanes were extracted from the samples containing embryos collected immediately after excision on day 0 and of germinating embryos and seedlings on days 4, 8, 12, 16, 20, 27, 34, 41 and 48. In order to assure reproducibility and homogeneity a bulk of at least 50 embryos or 5 - 10 seedlings was used for each analysis in the respective days.

The expression of *dbat* and *dbtnbt* genes was performed by qRT-PCR with SYBR Green I detection of the amplicons. As no appropriate internal reference gene with stable expression in different plant tissues or during ontogeny was found in our preliminary experiments (data not shown), the data were normalized to total RNA accurately quantified by the *Quant-iT™ RiboGreen®* RNA kit (*Molecular Probes*, Eugene, OR, USA). The relative amount of the gene transcripts was evaluated by the method of standard curve. The RNA extraction, quantification and qRT-PCR were performed according to previously described method (Katkovčinová *et al*. 2008). For taxane determination the plant material was lyophilized and extracted with dichloromethane, dissolved in methanol and analyzed by HPLC.

Freshly isolated embryos were 1.5 - 2.0 mm long, torpedo-shaped and white-colored. The embryos became greenish 8 d after the excision and placing on germination media. The stage of germination was completed by the elongation of embryonic axis and the radicle differentiation between days 8 - 12. During the post-germination stages most of the seedlings developed into vigorous plantlets with a single primary root and a leafy stem (Fig. 1*A-I*).

To gain more information on the site of the highest paclitaxel biosynthetic activity, the expression patterns of *dbat* and *dbtnbt* genes in different parts of yew plants were made in relation to the accumulation of corresponding metabolites. The expression analysis of 48-d-old seedlings showed that the genes were expressed in all plant parts (Table 1). The study of spatial distribution of baccatin III and paclitaxel in the same samples revealed a good correlation with the transcript levels of the genes; higher level of mRNA transcripts in the shoots corresponded with higher content of these metabolites in stems and needles. The maximum amount of baccatin III was detected in the stems whereas paclitaxel was mainly accumulated in the needles.

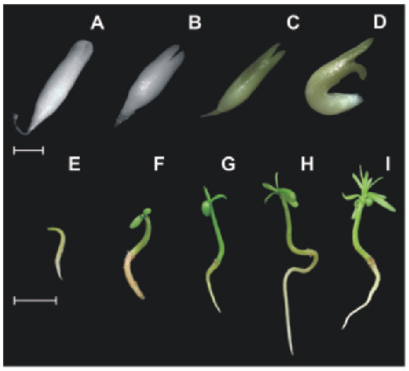

Fig. 1. Germination of *T. baccata* L. zygotic embryos (*A* - *C*) and seedling development – post-germination stages (*D* - *I*); *A* - freshly excised white, torpedo-shaped embryo (day 0), *B* - 4-d-old white colored embryo with more distinguishable cotyledones, *C* - greenish 8-d-old embryo, *D* - 12-d-old embryo, radicle differentiation and elongation of embryonic axis signalize the completion of germination stage (*bar* for *A* - *D* = 0.5 mm); *E* - *I* - seedlings developing into vigorous plantlets with a single primary root and leafy stem (reported on days 16, 27, 34, 41 and 48; *bar* for *E* - *I* = 10 mm).

To determine temporal expression of *dbat* and *dbtnbt* genes the time-course of the amount of mRNA was analyzed in zygotic embryos on day 0 and in 4-, 8-, 12-, 16-, 20-, 27-, 34-, 41- and 48-d-old seedlings. The qRT-PCR revealed the presence of mRNA transcripts even in freshly excised embryos (0.3 REU for *dbat* and 0.1 REU for *dbtnbt*). The highest increase in transcript level of the genes was observed in 8-d-old greenish embryos (32.0 REU for *dbat*; 127.0 REU for *dbtnbt*). After the elongation of embryonic axis and differentiation of

Table 1. Relative expression units (REU) of the *dbat* and *dbtnbt* genes and the content of corresponding metabolites baccatin III and paclitaxel in 48-d-old seedlings of *Taxus baccata.* Means ± SD (2 - 3 measurements of a bulk of roots, stems and needles of 10 seedlings were done).

	dbat [REU]	*dbtnbt* [REU]	baccatin III [μg g^{-1}(d.m.)]	paclitaxel [μg g^{-1}(d.m.)]
Roots	2.47 ± 0.14	1.52 ± 0.29	26.24 ± 9.02	13.23 ± 2.02
Stems	18.00 ± 0.04	9.60 ± 0.83	67.42 ± 1.00	46.85 ± 1.80
Needles	10.10 ± 1.82	37.80 ± 5.42	50.31 ± 6.04	73.88 ± 8.87

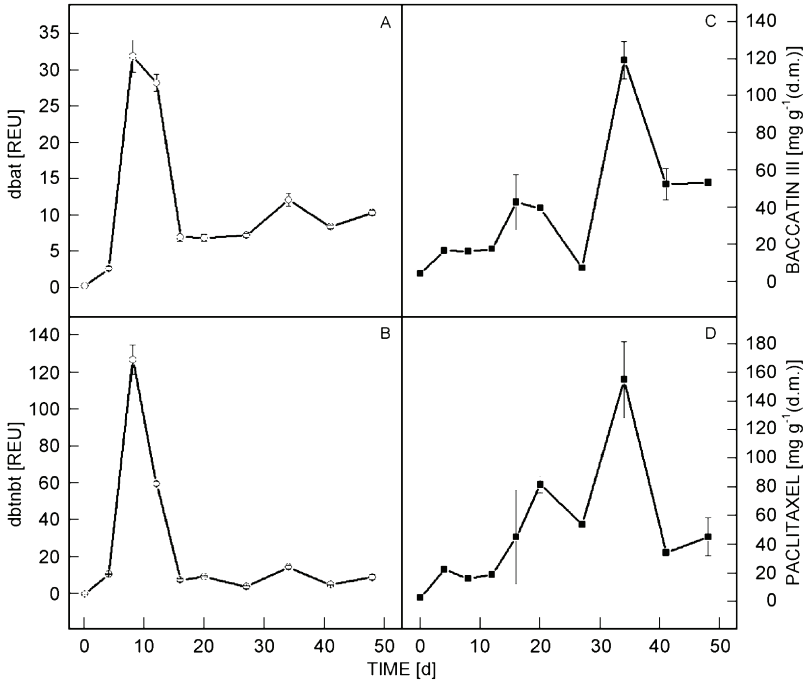

Fig. 2. Temporal gene expression and accumulation of corresponding metabolites; *A,B* - the steady-state levels of mRNA transcripts of *dbat* and *dbtnbt* genes; *C,D* - the expression patterns of baccatin III and paclitaxel. All parameters were measured during germination of *T. baccata* L. zygotic embryos (days 0, 4, 8) and post-germination stages of *T. baccata* L. seedlings (days 12, 16, 20, 27, 34, 41 and 48). Means ± SD; 1 - 3 measurements of a mix of at least 50 embryos and 5 - 10 seedlings were done in the respective days.

radicle observed on day 12, the gene expression sharply decreased up to day 16 (7.05 REU for *dbat* and 7.74 REU for *dbtnbt*) and then varied from 7.0 to 18.5 REU (*dbat*) and from 3.9 to 17.3 REU (*dbtnbt*). The pattern of temporal *dbat* expression corresponded with the expression of the *dbtnbt* gene (Fig. 2*A,B*). The content of baccatin III and paclitaxel analyzed in the same samples ranged from 4.3 to 119.3 μg g^{-1}(d.m.) for baccatin III and from 2.8 to 155.0 μg g^{-1}(d.m.) for paclitaxel. During germination the accumulation of taxanes slightly increased; the maximum content was observed on day 34 in the phase of fully-developed plantlets and was followed by approximately 3-fold-decrease detected on day 41. The temporal distribution of the precursor baccatin III was in positive correlation with paclitaxel, the final product of the pathway (Fig. 2*C,D*). At the level of metabolite accumulation no immediate response to increasing gene expression was evident in germinating embryos.

Using total RNA isolated from all parts of seedlings including roots, stems and needles, the expression of *dbat* and *dbtnbt* was determined quantitatively by qRT-PCR which allowed an exact detection of very small amounts of mRNA transcripts. Since the expression of house-keeping genes shows variability during ontogeny (Goncalves *et al.* 2005) as well as in different parts of a plant, an accurate quantification of total RNA was used to assign equal amounts of RNA in all analyzes. Our results proved that *dbat* and *dbtnbt* genes are expressed in all plant parts but higher amount of their transcripts was detected in the shoots. Similar tissue expression pattern was observed also for other genes catalyzing the earlier steps in the paclitaxel pathway (Liao *et al.* 2004, Kai *et al.* 2004, 2005, 2006).

Until now, no specific organ or tissue was identified to be responsible for paclitaxel biosynthesis. The presence of a plastid-specific targeting sequence in several enzymes of paclitaxel biosynthetic pathway (Hefner *et al.* 1998, Wildung and Croteau 1996) is consistent with the observation that mono-, di- and tetraterpenes are biosynthesized in plastids (McCaskill and Croteau 1999, Turner *et al.* 1999). Our finding that the highest content of paclitaxel was found in the needles is in agreement with the knowledge that paclitaxel is present in all plant parts but preferentially accumulates in the bark and needles (Fett-Neto and DiCosmo 1992). According to our results baccatin III accumulated in the stems rather than in the needles. Although the analysis of our results showed a trend toward a relationship between gene expression and subsequent taxane accumulation, opposed findings on tissue and organ specific taxane accumulation in various *Taxus* spp. and an extensive variation of the content should also be taken into account (Wickremesinhe and Arteca 1994, Zhiri *et al.* 1994).

Germination is a complex process requiring a coordinated spatial and temporal expression of many genes which products are involved in numerous metabolic reactions associated with respiration, enzymatic activities, RNA and protein synthesis. Use of qRT-PCR revealed that *dbat* and *dbtnbt* are genes whose

mRNA level varies during germination of zygotic embryo and post-germination stages of seedling development. The mRNA amount significantly increased during the later stages of germination, before elongation of embryonic axis and radicle differentiation. Following this event the gene expression decreased but still maintained at the level which was higher than in the freshly excised embryos.

Although the biochemical changes occurring in the megagametophyte and embryos of germinating conifer seeds have been studied at different levels, little is known about the transcriptional regulation of the gene expression during germination and seedling development. Nowadays, an attention is paid mainly to genes associated with the process of germination. Number of genes whose mRNA abundance changes over the course of embryo development was identified in *Pinus* spp. (Goncalves *et al*. 2005, Canas *et al.* 2006). In *Pseudotsuga menziesii* the transcript level of the gene coding for the cystein protease culminated during the germination stage whereas the expression of genes for several chaperonins, luminal- or chlorophyll-binding proteins showed peaks during the post-germination stage (Tranbarger and Misra 1995).

Taxanes are secondary metabolites which are involved in the defense-mechanisms of yew against various herbivores and pathogens. During germination and post-germination growth the quantity of paclitaxel and baccatin III significantly varied which could be explained by the observation of Wu *et al.* (1999) who reported that accumulation of paclitaxel in cells leads to possible feedback repression and product degradation. In spite of this the amount of baccatin III and paclitaxel had an increased tendency over all stages of embryo development. Similarly Gallardo *et al.* (2001) reported a strong increase in myrosinase and two jasmonate-inducible myrosinase-binding proteins involved in the protection of *Arabidopsis* seedlings against various pests by the end of germination process. As we have shown previously in the non-elicited undifferentiated callus cultures (Katkovčinová *et al.* 2008), no evident relationship was found between temporal gene expression and accumulation of corresponding metabolite in differentiated tissues of *T. baccata* seedlings.

In conclusion, the results reported here indicate for the first time that *dbat* and *dbtnbt* genes involved in later steps of taxane biosynthesis are expressed in early stages of ontogeny of *T. baccata* with a specific pattern of spatial and temporal expression typical for genes involved in the biosynthesis of metabolites of plant defense-mechanisms. The sudden increase in the gene expression probably assigns an important amount of mRNA transcripts for enzymes catalyzing the biosynthesis of taxanes which protect the seedlings in early stages of development. Paclitaxel and its precursor baccatin III exhibited the same accumulation pattern although the steady-state of their amount varied significantly during germination and post-germination growth. The transcript level of *dbat* and *dbtnbt* genes in different organs of the seedlings positively correlated with the spatial distribution of corresponding metabolites. However, the temporal expression of the genes did not fit with the accumulation pattern of the metabolites. Detailed knowledge on the course of gene expression in relation to accumulation of the respective metabolite would give more opportunities to manipulate taxane production *in vitro*.

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