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HPPD GENE EXPRESSION IN RELATION TO VITAMIN E CONTENT IN SPRING BARLEY

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Abstract

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The enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD) has a very important role in the biosynthetic pathway of vitamin E. Its activity influences the final level of tocols in plant tissues. Seven barley cultivars with different vitamin E level were grown under control conditions and activity of HPPD gene was measured four, eight and twelve days after pollination of ear tissues. It was found that activity of HPPD gene corresponded with vitamin E content detected in grains (r = 0.77*). The relationship between the gene activity for HPPD eight and twelve days after pollination and vitamin E content was also confirmed for analyzed cultivars grown in the field conditions (r = 0.85*).

vitamin E, tocols, tocopherols, HPPD, 4-hydroxyphenylpyruvate dioxygenase, gene activity, spring barley

Vitamin E has a very important role in human nutrition. It protects fatty acids against damage of free radicals and reduces the risk of emerge of arteriosclerosis, cataract, stroke, heart attack and some forms of cancer (DellaPenna, 1999). Vitamin E has a positive influence on the reduction of blood cholesterol and could slow down progress of Parkinson disease (Eitenmiller and Lee, 2004). The demand for foodstuff with higher natural content of some vitamins or minerals has grown rapidly in the last few years. Vitamin E falls into this group due to its positive effect on human health.

A suitable tool for achieving such demand is breeding of new plant cultivars that contain higher levels of these compounds. Cereals are a good source of vitamin E for human diet. Among cereals the highest vitamin E content was detected in barley (Holasová et al., 1995; Prýma et al., 2007). That is the reason for breeding of new cereal cultivars, in the first instance of barley, with higher vitamin E content in tissues. The first hulles cultivars with high vitamin E content of barley with waxy endosperm were used by Wang et al. (1993).

Biosynthetic pathway of vitamin E is very complicated and nowadays twelve genes from this pathway are described (for review see e.g. Collakova and DellaPenna, 2003). One of the important enzymes in the biosynthetic pathway is 4-hydroxyphenylpyruvate dioxygenase (HPPD). HPPD is considered as one of the essential factors which could control activity of the whole biosynthetic pathway due to its strategic location. Higher expression of this gene leads to higher vitamin E content in leaves and seeds of some experimental plants, e.g. *Arabidopsis*, tobacco or soya (Tsegaye et al., 2002). Breeding of new barley cultivars could be facilitated by molecular marker which would be able to differentiate cultivars with high vitamin E content and cultivars with low vitamin E content.

The aim of this work was to found out a possible difference in regulation of gene expression of HPPD gene during grain filling among cultivars and to describe the relationship between expression of this gene and vitamin E cntent in grain.

MATERIALS AND METHODS

Barley cultivars (Tab. I) were chosen on the basis of their known tocols contents and compositions (Ehrenbergerová et al., 2006). Plants were grown in the pots under regulated conditions; during the first two months plants were under following conditions: 10 °C at night (12 hours), 14 °C during the day (12 hours). In the third month, the daylight was prolonged to 15 hours and the temperature

was increased to 18 °C and the temperature during the night was raised to 14 °C. After the pollination, the daylight was prolonged to 20 hours (20 °C) and the temperature at night was increased to 16 °C. Plants were watered regularly and fertilized three times with 1 M of MS salt. The samples for evaluation of gene expression were collected in the day of pollination (as a negative control) and the fourth, the eighth and the twelfth day after pollination.

I: *Vitamin E content of barley grains of cultivars and lines grown in Žabčice 2005 and in control conditions 2007 and founded normalized relative expressions.*

Cultivar/ line	Vitamin E content in grains		Normalized relative expression			
	Sum of tocols [mg/kg]		Days after pollination			
	Žabčice 2005	Control conditions 2007	4	8	12	Sum
Krona	49.96	44.76	0.74	0.18	0.26	1.18
Kompakt	51.70	44.07	2.21	0.44	0.70	3.35
KM 1771	46.76	20.12	0.03	0.16	0.33	0.51
Carina	47.51	53.60	2.61	0.54	0.40	3.55
Wabet	60.45	52.25	0.90	1.00	0.63	2.53
Wanubet	60.36	54.40	2.50	0.71	0.73	3.94
Washonubet	67.19	40.26	1.18	0.92	0.87	2.97

Total RNA was extracted from 50 mg of tissue from developing grains using Ambion RNAqueous Kit. DNA was cleaved by Turbo DNA free (Ambion). The first-strand of cDNA was prepared from 500 ng of total RNA using QuantiTec Reverse Transcription Kit (Qiagen). All reactions were done according to the standard protocols. Gene quantification was performed using Real time PCR (Biorad). A specific primer pair of the studied gene was designed based on the sequence presented in the Gene Bank database (HVAJ693) using Primer 3 software (F_1:5'CGGAGCCAGATACAGACGTT3'. R_1: 5'GGTCGGCCTGTCCCCTACTGGC3'). The reverse primer was designed in the intron splicing site (Kosař et al., 2006). Each reaction was performed in 5 µl of 1:10 (v/v) dilution of the first strand of cDNA (corresponded to 25 ng of isolated total RNA) in the total reaction volume of 20µl using SybrGreen PCR Kit (Qiagen). Reaction conditions for thermal cycling (IQ5 Biorad) were: starting with a denaturation step at 95 °C for 15 minutes, followed by 40 cycles of 94 °C for 30s, 59 °C for 30s, and 72 °C for 30s. The fragment of barley α-tubulin was amplified as an internal control for the relative amount of RNA (Surprunova et al., 2004).

Transcription activity was evaluated as a normalized relative expression (RE) calculated with real-time PCR efficiency correlation according to method of Pfaffl (2001). The sample with the highest expression level was considered as the internal calibrator. The efficiency of all reactions was calculated from the direction of calibration curve (Rasmussen et al., 2001). Each sample was examined in triplicate.

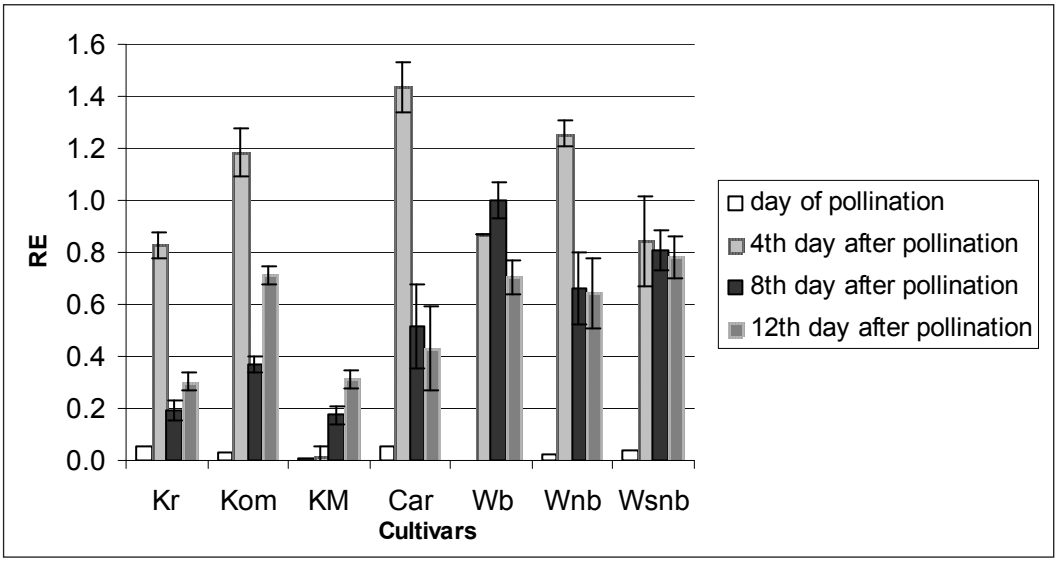
Vitamin E content was measured by a methodology described by McLaughlin and Weihrauch (1979) and Prýma et. al. (2000).

RESULTS AND DISCUSSION

By majority of cultivars the highest activity of the HPPD gene was detected four days after pollination (Fig. 1).

Relative expression of the HPPD gene evaluated in analyzed samples from control conditions showed relationship with vitamin E content in grains with the correlation coefficient 0.71). The expression of the HPPD gene in cultivars with the low content of vitamin E was descending (e.g. *Krona, KM 1771*) during sampling whereas the expression of this gene in cultivars with the high level of vitamin E (*Wabet, Wanubet*) was identical or descending very slowly. Higher gene expression was found in the cultivars with higher level of vitamin E than in the cultivars with lower vitamin E content. There was found a relationship between the activity of the gene on the eighth and the twelfth day after pollination and content of vitamin E, typical for the cultivars in the field conditions (Ehrenbergerová et al., 2006). This relationship was statistically significant and the correlation coefficient was 0.85*. In control conditions, sum of the relative expressions from the fourth to the twelfth day after pollination corresponded with the content of vitamin E in grains ($r = 0.77^*$, $r_s = 0.71$). The differences in vitamin E content between field and control conditions could be explained by different sensitivity of cultivars to environment impact.

The lowest vitamin E content was detected by line *KM 1771* (20.12 mg/kg) whereas in cultivar *Wanubet,* three times higher vitamin E content (54.40 mg/kg) was detected in control conditions. Cultivar *Wanubet* had eight time higher sum of relative activity than line *KM 1771.* These results confirmed conclu-



Means of the relative gene expression ± SE quantification is based on Ct (threshold cycle) values that were normalized using the Ct value corresponding to a barley housekeeping gene for α- tubulin. Cultivars/ lines: Kr – Krona, Ko – Kompakt, Car – Carina, Wb – Wabet, Wnb – Wanubet, Wsnb – Washonubet. All samples were examined in triplicate.

1: *Normalized relative expression (RE) of the HPPD gene in grains of seven barley cultivars (according Pfaffl, 2001)*

sion of Tsegaye et al. (2002) about important role of the HPPD gene in the biosynthetic pathway of vitamin E. It is also clear that some further steps in the biosynthetic pathway limit the vitamin E content in plants (Tsegaye et al., 2002; Falk et al., 2003; Falk et al., 2005). The relative activities of HPPD gene were consistent with the results of Falk et al. (2004) that the highest vitamin E content in cereals could be detected in early stage of milk ripeness.

It is possible to estimate the final content of vitamin E in grain from the expression profile of this gene during grain filling (r = 0.77*). It was proved that the phase of flowering is very important for the final vitamin E content and a possible decrease of the HPPD gene activity in this phase has a negative impact to the final content of vitamin E in grain. Unfortunately, method of evaluation of gene expression is quite labourious and we can not recommend it as a selection criterion for breeding.

SOUHRN

Vliv exprese genu pro HPPD na obsah vitaminu E v ječmeni jarním

Cílem pokusu bylo nalézt vztah mezi aktivitou genu pro 4-hydroxyfenlypyruvát dioxygenázu a výsledným obsahem vitaminu E v obilce u ječmene jarního. Sedm odrůd s rozdílným obsahem vitaminu E bylo pěstováno v kontrolovaných podmínkách ve fytotronu. Vzorky byly odebírány z vyvíjejících se klasů čtvrtý, osmý a dvanáctý den po opylení. Z vyvíjejících se obilek byla izolována RNA, která byla převedena na cDNA. Hodnocení normalizované relativní exprese bylo provedeno na přístroji IQ5 (Biorad).

Zjištěná relativní exprese z jednotlivých odběrů byla vztažena k celkovému obsahu vitaminu E u zkoumaných odrůd a linií. Nejvyšší aktivita genu pro HPPD byla detekována čtyři dny po opylení a korespondovala s výsledným obsahem vitaminu E v zrnu (r = 0,70). V dalších odběrech aktivita genu pro HPPD klesla a tento pokles byl výraznější u odrůd s nižším obsahem vitaminu E než u odrůd s vysokým obsahem vitaminu E. Aktivita genu zjištěná osmý a dvanáctý den po opylení korelovala s obsahem vitaminu E zjištěným v polních podmínkách (r = 0,85*). Suma relativních expresí za celou dobu sledování byla ve vztahu s výsledným obsahem vitaminu E v zrnu (r = 0,77*, r_s =0,71). Rozdíl mezi sumou relativní exprese genu mezi odrůdou s nejvyšším obsahem vitaminu E (*Wanubet*) a nejnižším obsahem vitaminu E (*KM 1771*) byl osminásobný, zatímco rozdíl v obsahu vitaminu E byl pouze trojnásobný. To nasvědčuje tomu, že některé z dalších kroků v biosyntetické dráze limitují obsah vitaminu E v rostlinách.

Uvedenou metodou lze předpovědět výsledný obsah vitaminu E v zrnu (r = 0,77*), pro její složitost ji však nelze doporučit jako selekční kritérium pro šlechtitele.

vitamin E, tokoly, tokoferoly, HPPD, 4-hydroxyfenylpyruvát dioxygenáza, genová exprese, ječmen jarní

Abbreviations: HPPD – 4-hydroxyphenylpyruvate dioxygenase, MS salt – Murashige and Skoog medium, RE – normalized relative gene expression.

SUMMARY

The aim of this trial was to find a relationship between HPPD gene activity and the final vitamin E content in barley grain. Seven cultivars with different vitamin E content were grown under control conditions. Samples were taken from developing grains four, eight and twelve days after pollination. RNA was isolated from the grain tissues and it was transferred to cDNA. Evaluation of normalized relative expression was performed on IQ5 (Biorad).

The found relative expression of HPPD gene was compared with the final vitamin E content of examined cultivars in control and field conditions. The highest activity of HPPD gene was detected 4 days after pollination and it corresponded with the final vitamin E content in grains ($r = 0.70$). In the following samples the activity of HPPD decreased but this decrease was more obvious in cultivars with low vitamin E content. The HPPD gene activity on the eighth and the twelfth days after pollination correlated with vitamin E content typical for this group of cultivars grown in field conditions ($r = 0.85^*$). Sum of relative expressions from all three samplings was in the relationship with the final vitamin E content in grain ($r = 0.77^*$, $r_s = 0.71$). The difference between the sum of relative activity of the cultivar with the highest vitamin E content (*Wanubet*) and the lowest vitamin E content (*KM 1771*) was eightfold whereas the difference between vitamin E content was only triplicate. It indicates that there are some other steps in the biosynthetic pathway which limit the final vitamin E content in plants.

With method of evaluation of gene expression it is possible to predict the final vitamin E content in grain but due to its elaborateness we can not recommend it as a selection criterion for breeders.

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