doi: 10.17221/107/2015-CJGPB

Selecting Plants with Increased Total Polyphenol Oxidases in the Genus *Trifolium*

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Abstract

Jakešová H., Řepková J., Nedělník J., Hampel D., Dluhošová J., Soldánová M., Ošťádalová M. (2015): Selecting plants with increased total polyphenol oxidases in the genus *Trifolium*. Czech J. Genet. Plant Breed., 51: 155–161.

One of the aims in red clover (*Trifolium pratense*) breeding is to increase the polyphenol oxidase (PPO) activity, which may effectively reduce protein breakdown in silage and when cattle are fed by fresh clover. We analysed total PPO activity spectrophotometrically and on the level of gene expression using real-time quantitative PCR in single plants derived from an interspecific *T. pratense* × *T. medium* hybrid. Experiments were performed for two years and evaluated according to the general linear model with three factors (family, year, and cut). The analysis revealed considerable variability in total PPO activity between individuals and between families. Four families and two individuals with significantly higher PPO activity were selected. Their PPO activity ranged from 3.411 to 3.547 mkatal/min/g and from 4.041 to 5.731 mkatal/min/g, respectively, in comparison with the control variety Amos (2.370 mkatal/min/g). The majority of PPO transcripts were expressed by the two genes *PPO1/5* and *PPO2*. In some genotypes, the *PPO5* gene was expressed. Quantitative PCR confirmed the highest activity of *PPO* genes in seven hybrid plants with higher DNA contents corresponding to 30 chromosomes with 815 013 copies per plant. Our results indicate the suitability of combining two methods for improved selection: initial expression analysis to assess the PPO transcript level indicating gene activity and subsequent enzymatic assay.

Keywords: expression analysis; polyphenolic enzyme; protein quality; *Trifolium medium*; *Trifolium pratense*

Legume species are important both ecologically and agriculturally because of their ability to fix nitrogen via rhizobium symbiosis and as major protein sources for both humans and animals. The genus *Trifolium* L. is classified taxonomically into the tribe *Trifolieae* of the large and agronomically outstanding family *Fabaceae*. Red clover is an important forage legume that is widely cultivated in most temperate regions. It is grown in pastures for grazing, sown as a companion crop, and harvested for hay (Taylor & Quesenberry 1996).

In Europe and worldwide, red clover breeding is focused on improving such traits as persistency, resistance to biotic and abiotic factors, forage yield, and protein quality and stability. The benefits of protecting plant proteins from degradation in the rumen by means of polyphenol oxidases (PPOs) have been established. PPOs in higher plants are copper-containing enzymes that catalyse both hydroxylation of monophenols to *o*-diphenols and oxidation of *o*- and *p*-diphenols to *o*- and *p*-quinones (Mayer & Harel 1979). The catalytic action of PPO has an

doi: 10.17221/107/2015-CJGPB

enormous impact on the quality of fruit and vegetable crops and results in alterations to colour, flavour, texture, and nutritional value (Vamos-Vigyazo 1981). PPOs are present in significant quantities in red clover (Jones *et al*. 1995). Post-harvest chemical processes result in the formation of a cross-linked protein-phenol complex resistant to enzymatic digestion by proteases, and the PPO mechanism may effectively reduce protein breakdown in silage (Sullivan & Hatfield 2006) and when clover is fed green directly to cattle. These findings support the breeding of improved red clover varieties with increased PPO activity.

A *PPO* gene family in red clover resides as a cluster of five genes at least. Coding sequences of the *PPO1*, *PPO2*, and *PPO3* paralogous genes have been reported by Sullivan *et al.* (2004). Two other single-copy genes, *PPO4* and *PPO5*, were identified and characterized by Winters *et al.* (2009), who also revealed a subfamily comprising three variants of *PPO1*, *PPO1/2*, *PPO1/4*, and *PPO1/5*.

Our interest has been to assess PPO activity in hybrids between *T. pratense* and *T. medium* (Řepková *et al.* 2006). Cytological studies of the progeny using flow cytometric analysis have revealed plants with different DNA content as compared to the tetraploid *T. pratense* (Řepková *et al.* 2003). From the F_3 generation, repeated open-pollination within the population and with the *T. pratense* tetraploid variety Amos was performed for seven generations (1992–2015). The morphological, agronomic, and reproductive traits were evaluated during 2006–2012 (Jakešová *et al.* 2011). Analyses of total PPO were performed during 2012–2014 in newly bred plants named JEH1F, later the Pramedi variety. We focused on the relationship between total PPO activity and the expression analysis of *PPO* genes as a potential screening marker. The aim of these experiments was to develop a reliable system for selecting plants (individuals and populations) with significantly higher levels of PPO, useful for breeding.

MATERIAL AND METHODS

Clover materials and experiment design. Newly bred materials of the *T. pratense* × *T. medium* hybrid named JEH1F were tested for total PPO activity on the level of families and individuals. Family testing included 3 randomly selected families (9/1, 35/19, 49/10) in the F_8/F_9 generation and 31 families (9/1, 57/2, 67/3, 51/4, 48/5, 5/6, 15/7, 3/8, 41/9, 49/10, B/11, 7/12, 7/13, E/14, 11/15, 21/16, 35/19, 55/21,15/23, 31/25, 40/27, 41/28, 55/29, 30/30, A33/32, 67/33, 21/34, C/35, 31/36, 41/37, A47) in the F_9/F_{10} generation. The trials were established in randomized complete blocks either in four (F_8/F_9) or three (F_9/F_{10}) replicates with 5 m^2 plots. The entries were sown in May 2010 (F_8/F_9) and in June 2013 in different Nurseries at Hladké Životice (F_9/F_{10}). All trials lasted three years including the year of establishment.

On the level of individuals, 72 plants with increased DNA contents (data not shown) were selected from a larger number of families grown in the nurseries of F_8/F_9 (20 plants from families 9/1, 67/3, 51/4, 48/5, 5/6, 5/7, 7/13, E/14, 15/24, 41/29, 50/30, 67/34) and $F_9/_{10}$ (52 plants from six families 9/1, 67/3, 51/4, 5/6, 3/8, 67/33) generations and tested for PPOs. Forty-seven individual plants of the *T. pratense* tetraploid variety Amos and *T. medium* clone 10/8 were used as controls; they were grown at the same time and in the same field in the course of given years.

Sixty individual plants in the F_9/F_{10} generation from six families were tested for PPOs using real-time quantitative polymerase chain reaction (qPCR). These were the same plants used for total PPO activity analysis and additional eight plants with a sufficient amount of tissue for molecular analysis but not for total PPO activity screening. The *T. pratense* variety Amos and *T. medium* 10/8 were included as controls. In addition, seven individual JEH1F plants of the F_8/F_9 generation originating from six families (9/1, 5/6, 15/7, 3/8, 55/30, E 14/40) with the known and highest DNA content were included into qPCR analysis. For the purpose of PPO screening, plants were sampled during the second year of growth, either at bud-setting or early-flowering stage, when the first cut is usually taken. For population (family) testing, approximately 300 g of green matter was sampled from each family as well as from each replication separately. For testing of individual plants in the nurseries, the whole plant was cut. The same plants were analysed by qPCR. Three F_8/F_9 tested families were harvested from three cuts in four replications in 2011 separately and from the first cut in 2012. Thirty-one F_9/F_{10} tested families were harvested from the first cut in 2014. Green matter was oven dried at 50°C and milled for analyses. In addition, fully expanded leaves were removed from individual plants, frozen in liquid nitrogen, and stored at –80°C for subsequent molecular analyses.

Enzymatic assay of tyrosinase (polyphenol oxidase). The determination of total PPO activity fol-

doi: 10.17221/107/2015-CJGPB

lowed the method of Gregory and Bendall (1966) and Kyung No *et al.* (1999) with modifications. All samples used for this study were extracted with methanol and filtered. Polyphenol oxidase activity using L-tyrosine as the substrate was assayed spectrophotometrically, as described previously. Catalytic enzyme concentrations were expressed as the amount of enzyme mixture (1 g sample) which converts 1 mol of substrate (tyrosine) per unit time (minutes) at defined conditions (pH 6–7, laboratory temperature 20°C). Catalytic enzyme concentration was calculated from an increase in optical absorbance at 492 nm divided by the amount of time (30 min), including sample dilution. The increase in optical absorbance was expressed as the amount of reacted tyrosine on the basis of the conversion factor (pure tyrosine solution had an average optical absorbance of 0.940). The analyses were performed in given years (2011, 2012, and 2014).

RNA isolation and real-time quantitative PCR. Total RNA was isolated from 100 mg of leaves from individual plants using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Genomic DNA was removed using a Turbo DNA free kit (Life Technologies, Carlsbad, USA). The concentration and purity of total RNA were determined by NanoDrop spectrophotometry and agarose gel electrophoresis. Total RNA was reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA). The gene-specific primers are summarized in Table 1. PPO1/2, PPO1/5 and PPO4 primers were used in accordance with Winters *et al.* (2009). The other primers were derived from sequences deposited at the GenBank: *Tp PPO1/4* (FJ587214), *Tp PPO2* (AY017303), *Tp PPO3* (AY017304), and *Tp PPO5* (EF183484.1). Primers for each *PPO* gene were designed for distinct sections of all *PPO* gene sequences so that cross-amplification was minimized. Also, the PCR amplification product from each *PPO* gene was distinguishable by different length which was always checked after RT-PCR amplification by agarose electrophoresis. The equality of all cDNA 500ng/µl samples was checked by the housekeeping gene ubiquitin-conjugating enzyme E2 (UBC2), which was evaluated as one of the most constitutively expressed reference genes in red clover tissues (Khanlou & Van Bockstaele 2012). Inspection of UBC2 expression was done prior to PPO analyses and all samples expressed UBC2 within the C_t range of 3 cycles. All *PPO* gene expression results were subsequently normalized to the expression of UBC2. For the purpose of absolute quantification, a specific product from PCR amplification of each target gene (5 min at 95°C, 30 cycles of 30 s at 95°C and 30 s at 60°C and 30 s at 72°C, 5 min at 72°C) was inserted into the pCR 2.1 vector and cloned into OneShot Chemically Competent *E. coli* (TA Cloning Kit, Life Technologies). Inserted sequences were verified by sequencing. Cloned purified plasmids were linearized and quantified using NanoDrop. Conversion from the concentration of double-stranded DNA to copy number was performed while considering the average molecular weight of a base pair (650 Da), total length of plasmid with inserted fragment, and

Table 1. Name and sequence for polyphenol oxidase (PPO) primers designed for *PPO* genes and used for real-time quantitative PCR analysis

Gene	Name	Sequence 5'–3'
PPO1/2	PPO1/2-F	CAATCGTTGACCTAAACTATAACAGAA
	PPO1/2-R	GTGTCACCGCCGCGATAA
PPO1/4	PPO1/4-F	CCAACAGCAAGACCAATAGACAATT
	PPO1/4-R	ATCTCTTCCGGCGGCATAGAAAT
PPO1/5	PPO1/5-F	CACAACGCCCTTCCAGAAA
	PPO1/5-R	TCACAACCGAATCCAAAACA
PPO2	PPO2-F	TCCATTCAGTTGTTGTCCACCAA
	PPO2-R	TAAATGTGCAGCTCGTCTTACC
PPO3	PPO3-F	CCTCCCAGTGCAGAACAACA
	PPO3-R	CGCGATAAGGGCTTCCATGAA
PPO4	PPO4-F	ACGAAGGTGGCGTAGATGAC
	PPO4-R	CATTTCCATGGTGAGCGTAA
PPO5	PPO5-F	ACACAACGTCCTTCCAGAAAATT
	PPO5-R	ACTCGATCCCATCTATCACCAAA

doi: 10.17221/107/2015-CJGPB

Avogadro's number 6.022×10^{23}. Subsequently, 10-fold serial dilutions of linearized plasmid were used as a standard for absolute quantification, ranging from 10^7 to 10^3 copies. qPCR reactions were performed in a final volume of 20 µl containing 1000 ng of cDNA template and 8 pmol of each of the forward and reverse primers using SYBR® Select Master Mix according to the manufacturer's instructions (Life Technologies). qPCR runs were performed on 96-well optical plates in triplicates using the 7500 Fast RT-PCR System (Applied Biosystems). Amplification cycle conditions were as follows: 2 min at 50°C and 2 min at 95°C followed by 40 cycles of 3 s at 95°C and 30 s at 60°C. Amplification results from standard dilution series were used for constructing the standard curves. The resulting equations were then used to calculate the precise number of target cDNA molecules in samples tested within the same reaction plate as was the standard.

Statistical analysis. The measured data for total PPO activity were analysed using a general linear model with three factors (family, year, and cutting). First, the global hypothesis regarding PPO equality of all clover families was tested. When this was rejected, statistically significant different families were found by pairwise comparisons using the least significant differences.

The same approach was also used to evaluate PPO activity in the *T. pratense* × *T. medium* hybrid and parental species. All calculations were done using GenStat 16 at a 5% significance level. Absolute quantification of qPCR for each gene is expressed as the mean value per plant ± standard deviation.

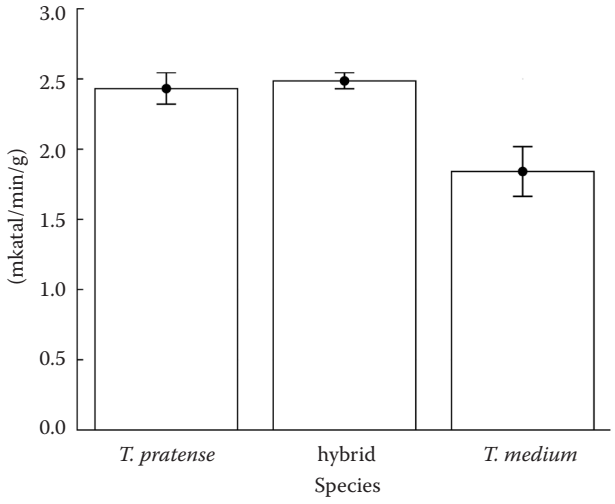

Figure 1. Evaluation of polyphenol oxidase activity results and their standard errors for hybrid plant breeding material of *Trifolium pratense* × *T. medium* and the parental species in F_8/F_9 and F_9/F_{10} generations

RESULTS

Screened populations. Determined values of total PPO activity were compared between the families of JEHF1 hybrid material and *T. pratense* or *T. medium* in both years. The statistical values of predictions and least significant differences of predictions for total PPO activity were calculated. PPO activity in the hybrid material was higher (2.486 ± 0.0557 mkatal/min/g) compared to that in *T. pratense* (2.431 ± 0.1103 mkatal/min/g) but this difference was not significant (Table 2, Figure 1). Significantly higher total PPO activity was observed within both *T. pratense* and hybrids compared to *T. medium* (1.841 ± 0.1772 mkatal/min/g).

In respect to differences between the JEHF1 families tested in 2011 and 2012 (F_8/F_9), their total PPO activity showed a higher level than did the *T. pratense* variety Amos. This difference was not significant, however. In the F_9/F_{10} generation, there were significant differences ($P < 0.05$) in PPO activity between the families. Four of them (9/1, 11/15, 30/30, and 21/34) had significantly higher total PPO activity compared to the Amos variety, while two families (48/5, 55/29) had significantly lower total PPO activity than that of the Amos variety (Table 3). Detailed analysis in 2011 summarizing total PPO activity in three cuts (3.4, 3.5 and 3.9 mkatal/min/g) confirmed no effect from cutting.

Screened individuals. In the F_8/F_9 generation of hybrid individuals, there were no statistically significant differences in total PPO activity. Although several plants showed higher total PPO activity (67/3/26, 48/5/9, 5/6/50), none of them was significantly higher than the *T. pratense* variety Amos. In the F_9/F_{10} generation, there were two plants showing significantly

Table 2. Statistical evaluation of polyphenol oxidase (PPO) activity in *Trifolium pratense* × *T. medium* hybrid and parental species in F_8/F_9 and F_9/F_{10} generations

Sample	Prediction (mkatal/min/g)	SE*
Hybrid	2.486[a]	0.0557
T. pratense	2.431[a]	0.1103
T. medium	1.841[b]	0.1772

*Standard errors of predictions ($P = 0.05$); differences between means having different superscripts are statistically significant

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Table 3. Statistical evaluation of polyphenol oxidase (PPO) activity in families of the *T. pratense* × *T. medium* hybrid and parental species represented by the Amos variety

F_9/F_{10} family	Prediction (mkatal/min/g)	Difference from Amos
K 48/5	1.350[a]	–1.215
K 55/29	1.453[a]	–1.112
Amos	2.370[b]	0.000
K 11/15	3.411[c]	1.032
K 30/30	3.447[c]	1.068
K 9/1	3.531[c]	1.152
K 21/34	3.547[c]	1.168

Differences between means having different superscripts are statistically significant

higher total PPO activity (9/1/10, 67/3/15) (Table 4). Unfortunately, the majority of plants had significantly lower total PPO activity than that of Amos.

Expression analysis. The mean content of PPO transcripts expressed by each transcribed gene is presented in Table 5. The results are expressed as the number of copies produced by a particular gene. The lowest concentration of essentially 0 gene copies was found for *PPO1/2*. The highest concentration was found for *PPO2* followed by *PPO1/5*. These two genes express the majority of PPO enzymes in the tested families in F_9/F_{10}. Concerning individual families, the highest level of PPOs was evaluated in the 5/6 family, and gradually diminishing values were observed in the 9/1, 51/4, 67/3, 3/8, and 67/33 families. The second highest PPO activity found by qPCR in

Table 4. Statistical evaluation of polyphenol oxidase (PPO) activity in individuals of *T. pratense* × *T. medium* hybrid and parental species represented by the Amos variety

Individuals $F_9/_{F10}$	Prediction (mkatal/min/g)	Difference from Amos
9/1/14	0.617[a]	–1.948
5/6/8	0.627[a]	–1.938
67/33/29	0.797[a]	–1.768
67/3/4	0.857[a]	–1.708
67/33/28	0.897[a]	–1.668
67/33/4	0.917[a]	–1.648
Amos	2.379[b]	0.000
9/1/10	4.041[c]	1.662
67/3/15	5.731[c]	3.352

Differences between means having different superscripts are statistically significant

Table 5. Polyphenol oxidase (PPO) activity in leaves of *Trifolium* for five *PPO* genes (number of copies/1000 ng cDNA)

	Mean ± SD*							Total
	PPO1/2	*PPO1/4*	*PPO1/5*	*PPO2*	*PPO3*	*PPO4*	*PPO5*	
Family JEH F9/F10								
9/1	0	34 ± 35	8 843 ± 7 451	57 532 ± 11 0634	562 ± 527	279 ± 515	803 ± 650	68 053
67/3	0	108 ± 112	18 246 ± 20 984	16 318 ± 18 960	914 ± 639	2 598 ± 4 670	1 211 ± 1 179	39 395
51/4	0	115 ± 40	18 165 ± 4 497	30 396 ± 42 642	673 ± 715	2 157 ± 1 541	1 631 ± 632	53 138
5/6	1 ± 2	2 807 ± 5 931	22 189 ± 34 303	28 245 ± 65 819	8 729 ± 12 335	6 714 ± 7 292	2 982 ± 4 624	71 666
3/8	0	43 ± 34	8 374 ± 905	23 665 ± 4 550	288 ± 66	2 460 ± 681	961 ± 245	35 791
67/33	0	730 ± 937	21 010 ± 17 673	1 862 ± 2 380	858 ± 2093	7 042 ± 12 770	2 135 ± 1 949	33 638
Average	0	640	16 138	26 336	2 004	3 542	1 621	
JEHF1 F8/F9	45 ± 102	1 259 ± 2 493	145 794 ± 238 745	645 717 ± 725 476	920 ± 1309	3 921 ± 5 337	17 325 ± 36 063	815 013
Amos	80 ± 74	6 084 ± 7 201	80 020 ± 68 809	552 016 ± 359 371	264 ± 32	6 066 ± 3 527	20 997 ± 7 757	665 527
T. medium 10/8	0	11 667 ± 16 479	40 ± 69	1 797 073 ± 1 648 816	5 738 ± 8 592	945 ± 1637	66 ± 78	1 815 529

*Mean values per plant ± standard deviation

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the 9/1 family corresponds with the highest total PPO content as determined by spectrophotometry. qPCR confirmed the highest PPO activity in seven JEHF1 plants with higher DNA contents corresponding to 30 chromosomes. Total PPO activity (815 013 copies per plant) exceeded that in the 5/6 family (71 666 copies per plant) by more than 11-fold, and total PPO activity of the *PPO2* gene (645 717 copies per plant) exceeded that of the 5/6 family with 28 245 copies per plant by more than 20-fold. Total PPO activity of the Amos variety also differed significantly from that of the analysed JEH F_9/F_{10} families. In addition to the high activity of the *PPO1/5* and *PPO2* genes, the activity of *PPO5* was also significantly higher in comparison with other analysed genotypes. Total PPO activity of *T. medium* was dependent mainly on the *PPO2* activity.

DISCUSSION

Red clover plants with high PPO activity are beneficial not only for their protein quality in silage and green fodder but also for the environment, owing to increased nitrate absorption by ruminants and decreased nitrogen loss to the environment through the animals' urine (Winters *et al.* 2009). The browning reaction has been thought to alter the polyunsaturated fatty acid profile of such livestock products as milk and meat (Lee 2014; Gadeyne *et al.* 2015). From this viewpoint, red clover is better than other protein sources.

In this study, spectrophotometric determination was used to assess total PPO activity in clover plants. Our study confirmed a high variability in total PPO activity among individuals and among families derived from the interspecific hybrid *T. pratense* × *T. medium*. Both positive and negative differences were observed, and in some cases these were significant. PPO activity was higher in hybrid material compared to *T. pratense*, although this difference was not significant, and total PPO activity was significantly higher compared to *T. medium*. The total PPO activity differed more in individuals than in families. The reason may be due to targeted selection of hybrid plants with increased DNA content by flow cytometry. The majority of PPO transcripts were expressed by two genes (*PPO1/5* and *PPO2*) in the JEH and JEHF1 families. High activity was found also for the *PPO5* gene in JEHF1 plants. PPO transcripts in *T. medium* were dependent mainly on the *PPO2* gene activity. Products of *PPO2* were most abundant in the Amos variety and *T. medium*. No unambiguous correlation was revealed between PPO activity as determined by enzymatic assays and PPO transcripts found by expression analyses. *PPO* gene expression could not result directly in PPO activity, most of PPOs are in a latent form. There could even be a contradiction between total PPO activity and the expression activity of *PPO* genes. This may be due to the existence of two forms of PPOs in red clover chloroplasts: an active form which makes up 5–10% of the total PPO protein and which shows full activity, as well as a latent form which makes up the vast majority of the PPO protein and requires activation (Lee 2014). The plant has two mechanisms to increase the PPO activity: induction (enhancing the expression of PPO genes) and activation (conversion of latent to active enzyme). Numerous PPO induction factors have been confirmed across many different species, including: pathogen invasion (Li & Steffens 2002), attack of herbivorous insects (Steinite *et al.* 2004), and influence of wounding (Stewart *et al.* 2001). For red clover PPO, specifically, Lee *et al.* (2009) correlated the degree of cell damage with increased PPO activity. This is of agricultural importance in relation to forage cutting and wilting during silage making. Our experiment did not confirm any effect from cutting. PPO activity was stable in harvested plants across three cuts in 2011. This gives evidence for a balanced PPO level during the growing period. To evaluate the effect of different environmental factors will need further experiments.

Our finding concerning variability in PPO expression corresponds with other expression analyses. Differential expression profiles of the *PPO* genes (*PPO1*, *PPO2*, and *PPO3*) have been reported by Sullivan *et al.* (2004) in red clover and Thipyapong *et al.* (1997) in tomato (*Lycopersicon esculentum* Mill.). Differences in gene expression were revealed between red clover and zigzag clover.

PPO content could be a useful criterion in breeding for improved protein quality. Our results indicate the suitability of combining two methods for improved selection: (1) expression analysis for the absolute PPO transcript assessment indicating gene activity, for which a small amount of leaf tissue is sufficient; and (2) subsequent assay of PPO activity. Selected plants grown under stress conditions could increase their PPO activity during interaction with pathogens.

To conclude, evaluation of PPO activity enabled us to select plants with increased PPO activity, that can be utilized in advanced plant breeding. Meanwhile, we can avoid those plants which have low levels of PPO activity. This information is important for clover breeding efforts to improve protein quality.

doi: 10.17221/107/2015-CJGPB

Expression analyses for two genes (*PPO2* and *PPO1/5*) could be useful as a screening criterion for screening individuals with high PPO activity.

Acknowledgments. Financial support for this work from Ministry of Agriculture of the Czech Republic (Project No. QI111A019) is gratefully acknowledged.

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Received for publication August 3, 2015
Accepted after corrections November 24, 2015

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