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Identification of suitable reference gene for quantitative transcription analysis (RT-qPCR) of *Fusarium culmorum* genes in infected barley plants

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Pathogenic fungi of the genus *Fusarium* negatively affect grain production and quality in cereal plants due to contamination of grain with trichothecene mycotoxins. The biosynthetic pathway of trichothecenes has been already described as well as the enzymes associated with this pathway that are encoded by the *Tri* genes (Boutigny et al., 2009). In studies aimed at resistance of cereals to *Fusarium*, the transcription of the *Tri* genes is determined with regards to the production of trichothecenes using quantitative real-time PCR (RT-qPCR). Normalization of RT-qPCR data for target genes requires selecting suitable reference genes (RGs). Housekeeping genes (HKGs), coding proteins involved in basic cellular processes, are often used as RGs for the normalization because the transcription of these genes exhibit stable levels under different experimental conditions (Thellin et al., 1999).

In studies of *Fusarium* genes transcription, three most commonly used RGs were *β-tubulin* (*β-tub*) (Covarelli et al., 2004; Ponts et al., 2007; Jiao et al., 2008; Subramaniam et al., 2015), gene for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Merhej et al., 2011) and gene for elongation factor (*EF*) (namely *EF2*—Boutigny et al., 2009). In studies of wheat– pathogen systems, *GAPDH* and *EF1A* (Hallen-Adams et al., 2011) and *β-tub* (Khan et al., 2006; Voigt et al., 2007) were used as reference genes. Harris et al. (2016) measured *F. graminearum* genes transcription during fungal infection in wheat, barley, and maize plants relative to the transcription of three *F. graminearum* RGs (*GAPDH*, *β-tub*, and *EF-1*). However, Kim and Yun (2011) evaluated numerous culture conditions, candidate genes, and algorithms to select the best RGs for RT-qPCR in *F. graminearum* and their results suggest that *GAPDH* is an overall unstable gene and other HKGs – such as *EF1B* and genes for cyclophilins (*CYP1*, *CYP2*)– were ranked among the least stable under some growth conditions. Only two HKGs (*EF1A* and *UBC*—gene for ubiquitin conjugating enzyme) displayed relatively stable transcription patterns under all conditions. Similarly, Havránková et al. (2011) showed that *β-tub* and *UBC* but not *EF2* were suitable RGs for evaluating transcription level of *Tri* genes for *in vitro* cultured *F. graminearum*. Based on their results, we evaluated the transcription of these three RGs in samples of two barley cultivars infected by *F. culmorum* and we confirmed *UBC* to be a suitable RG for the normalization of *Tri* genes transcription in a plant-pathogen system (Faltusová et al., 2015). In general, the plant-pathogen system contains DNA and RNA from both the host and the pathogen. The amount of the pathogen RNA in the sample may be different due to the increase of the amount of the pathogen during the course of the infection. Using a RG with transcription level not reflecting this increase could lead to a distortion of the results. The general inconsistency in the use of RGs in *Fusarium* studies led us to further verify the suitability of

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the *UBC* reference for monitoring the transcription of the *Fusarium* genes in barley. We have extended the experiments by using additional barley cultivars and ways of cultivation (in field or greenhouse) as well as subsequent processing of the harvested grain by malting. Malt samples were also supplemented by grain from plants treated with fungicides commonly used to treat *Fusarium* infection in cereals. These samples served for measurement with a small amount of the pathogen. The study thus provides a wide range of transcription values from a variety of infested samples obtained from different conditions.

In the field experiment, spring barley cultivars (Lilly, Tolar, Gladys, Sebastian, Henrike, Sladar, Signora, Radegast, Aktiv), were inoculated with *F. culmorum* isolate B (conidial suspension with a density of 0.8×10^7 spores in 1 ml) by using a hand sprayer in Crop Research Institute, Prague, at the mid-flowering stage (BBCH 65). The whole barley spikes were sampled concurrently from control non-infected and infected plants at 14, 21 and 28 dpi for all cultivars at the same time of day (54 samples in total) in three independent replicates. Greenhouse experiments were carried out in Agrotest Fyto, Ltd., Kroměříž. Six spring barley cultivars (Krasnodarskij 95, Amulet, Malz, Nordus, Diplom and Radegast) were grown in free soil. At the beginning of anthesis (BBCH 61–65), trials were inoculated with *F. culmorum* by spraying [strain (W. G. Sm.) Sacc. KM16902; concentration of 0.5×10^6 conidia of *F. culmorum* in 1 ml of inoculum, spray dose of 200 l/ha]. Whole barley spikes were sampled individually before infection (control) and after 4, 7, 14 and 21 dpi at the same time of day (30 samples in total) in three independent replicates. The spikes were immediately frozen in liquid nitrogen and stored at −80 °C. The spring barley cultivars Bojos, Malz and Xanadu were grown for subsequent malting in the field (Agrotest Fyto, Ltd., Kroměříž) under conditions of both natural (control) and artificial infection with *F. culmorum* [strain (W. G. Sm.) Sacc. KM16902, concentration was same as above] in two consecutive years (2011 and 2012). One half of control plants and of plants used for infection were treated with the fungicides Hutton (active ingredients: prothioconazole, spiroxamine and tebuconazole) in growth stage BBCH 39 and Prosaro (active ingredients: prothioconazole and tebuconazole) in growth stage BBCH 65. The inoculation with *F. culmorum* was carried out at growth stage (BBCH 61–64). The grain samples were representatively selected (600 g per replicate) at full maturity from all experimental variants (each containing 2 replicates) for malting. Laboratory malting was subsequently carried out from a pooled sample using two different malting systems. In a first malting experiment, grain samples of Bojos and Malz (grown in 2011) were malted in the micromalting plant M-3BX (RAVOZ; Agrotest Fyto, Ltd., Kroměříž). For the second experiment, two batches of samples of Malz and Xanadu were malted in the automatic micromalting equipment KVM in the Research Institute of Brewing and Malting, PLC Brno (batch A: plants cultivated in 2011; batch B: plants cultivated in 2012). 36 samples in total (1st experiment) and 24 samples in total for both A and B (2nd experiment) were taken: 1) before malting (only in the 1st experiment), 2) after 24 h (in germination stage I), 3) after 72 h (germination stage II) and 4) after kilning in three biological replicates.

Genomic DNA was isolated from homogenized frozen barley grains (0.1 g) by using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The content of pathogen DNA (i.e. the amount of pathogen) in the DNA isolated from the samples was established by qPCR using a TaqMan MGB probe Fc 92s1 5′FAM 3′ (AAAGAAGTTGCAATGTTAGTG) and two primers Fc 92s1-F (TTCACTAGATCGTCCGGCAG) and Fc 92s1-R (GAGCCCTCCAAGCGAGAAG) as described in Leišová et al. (2006) specific for *F. culmorum* sequence on chromosome III.

RNA was isolated through phenol− chloroform extraction using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA). The isolated and purified RNA was reverse-transcribed using TaqMan® Reverse Transcription Reagents in a Verity® thermal cycler (Applied Biosystems, Inc., USA). The reaction mixtures and thermal conditions were set up according to the manufacturer's instructions. The primers used to amplify the cDNAs of the *UBC* by RT-qPCR were UBC-PS.1 (TCCCCTTACTCTGGCGGTGTC) and UBC-PS.2 (TTGGGGTGGTAGATGCGTGTAGT) (Lysøe et al., 2006). The reaction mixture composition is given in appendix A.1. The amplification was performed in StepOnePlus™ thermal cycler (Applied Biosystems, Inc., USA) in triplicate for each of the three biological replicates (thermal profile is given in appendix A.2). The specificity of the PCR products was checked by both dissociation curve (appendix B– Fig. 3) and electrophoretic analysis. *Fusarium* DNA amount and *UBC* transcription data in all non-infected samples were at an undetectable level. The PCR efficiency was determined using calibration curves made with two-fold dilutions of a cDNA. Data of pathogen DNA quantification and *UBC* transcription measured as Cq (quantification cycle) were related to each other. Pearson correlation coefficient r was calculated for the relationship between the amount of the pathogen and the *UBC* transcription data. A p-value calculator for correlation coefficients was used thereafter (Soper, 2017).

The transcription of *UBC* was measured by RT-qPCR with efficiencies ranging from 1.990 to 1.997. *UBC* transcription data were correlated with pathogen amount in grains of infected barley plants and in malt made from infected barley grains (appendix B– Figs. 1 and 2). The determination coefficient of the regression curves R^2 ranged between 0.705 and 0.941. The calculation of Pearson correlation coefficient r showed a highly significant correlation ($p < .0001$) for all experiments. The correlation coefficient r was 0.8398 and 0.8788 for field and greenhouse cultivated barley plants, respectively. In the case of malted barley grain samples, the correlation coefficient was 0.8351 for the first malting experiment. The second experiment with malted barley grains showed a correlation coefficient of 0.9663 and 0.9705 for experiment A and B, respectively. We thus confirmed the *UBC* gene as a suitable RG for normalization of *Fusarium culmorum* genes transcription in infected barley due to the positive correlation between *UBC* transcription and increase of fungal DNA in infected barley samples. As mentioned in Kumar et al. (2011), several authors have suggested the necessity of selecting a valid reference before gene transcription profiling, even for closely related organisms with different tissues and different treatments of interest. Based on our results, besides testing the stability of the RG in a pathogen under different conditions, we also recommend testing whether this RG has the ability to reflect changes in pathogen content in infected plant material.

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