Pesticide Biochemistry and Physiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Suitable reference genes for accurate gene expression analysis in *Papaver rhoeas* under 2,4-D herbicide stress

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ARTICLE INFO

Keywords:
RT-qPCR
Weed
Reference genes
Auxinic herbicide
Gene expression
Herbicide resistance

ABSTRACT

Resistance to 2,4-D (2,4-diclorophenoxyacetic acid) herbicide is increasing in various dicotyledonous weed species, including *Papaver rhoeas*, a weed infesting Southern European wheat crops. Non-target-site resistance to this herbicide is governed by a range of genes involved in herbicide stress response. To enable reliable measurement of gene expression levels in herbicide-resistant and susceptible plants it is necessary to normalize qPCR data using internal control genes with stable expression. In an attempt to find the best reference genes, the stability of seven candidate reference genes was assessed in plants resistant and susceptible to 2,4-D, subjected or not to herbicide stress. Using three statistical algorithms (geNorm, BestKeeper and NormFinder), the overall results revealed that glyceraldehyde-3-phosphate dehydrogenase, actin and ubiquitin were the most stable reference genes. The normalization expression levels of *GH3* (indole-3-acetic acid amido synthetase) and *GST3* (glutathione S-transferase) which are two genes up-regulated following 2,4-D treatment, were determined to verify the stability of these selected reference genes. A sudden increase in *GH3* and *GST3* expression was already detected 5 h after herbicide application, confirming their involvement in plant response to 2,4-D. The validation results confirmed the applicability and accuracy of these reference genes. This study identified and validated reference genes in the non-model weed species *P. rhoeas* and these will facilitate gene expression analysis studies aimed at identifying functional genes associated with non-target-site resistance.

1. Introduction

Quantitative polymerase chain reaction (qPCR) has become the most commonly used method for gene expression quantification. The accuracy of qPCR techniques is challenged by many sources of variation, including sampling errors, template quality and amplification efficiency [1]. The reliability of RT-qPCR results is highly dependent on the reference genes chosen [2]. The expression level of a reference gene needs to remain constant under varying experimental parameters such as treatments used, organs sampled and experimental pressures applied [1]. For herbicide resistance studies, reference gene sets should consist of genes for which expression has been proven stable before and after herbicide application, and among resistant and susceptible plants [3]. Moreover, some reference genes that had been reliably validated in one weed species are not well suited for use in others. Prior validation is therefore required when a reference gene is suggested to be used in a new weed species or in a new experimental condition.

To date, studies aimed at evaluating reference genes with a stable expression under herbicide application have only been reported for a few grass weeds: *Alopecurus myosuroides* Huds [4], *Lolium* spp. [5], *Echinochloa phyllopogon*[6] and *Avena fatua*[7]. Besides these, there are no investigations to validate reference genes for qPCR in *Papaver rhoeas* and moreover, in general there is a lack of genomic and genetic resources for this species. *P. rhoeas* is a troublesome weed infesting wheat fields in Southern Europe that has evolved resistance to ALS (acetolactate synthase) inhibitors [8–10] and to the herbicide 2,4-D [11].

ALS is a key enzyme in the biosynthesis of branched amino-acids. The use of ALS inhibitors has increased rapidly worldwide because of their excellent selective control of major broadleaf weeds at low dosages, but they are also the herbicide class that is most prone to select for resistance in weeds [12]. The broad use of ALS inhibitors against *P. rhoeas* in wheat fields has led to the evolution of resistant populations. As a consequence, the use of auxinic herbicides is increasing and these products are now registered as pre-mixes or tank mixtures with ALS inhibiting herbicides, not only to expand the spectrum of weeds controlled, but also to mitigate weed resistance evolution to the more resistance-prone ALS inhibitor chemistry. Given that 2,4-D resistant crops will soon be on the market, this might lead to an increase of resistant

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http://dx.doi.org/10.1016/j.pestbp.2017.09.008
Received 3 March 2017; Received in revised form 7 September 2017; Accepted 16 September 2017

weeds [13].

To date, non-target-site resistance (NTSR) mechanisms, which include reduced herbicide uptake, reduced translocation and detoxification, are recognized as being the only type of resistance mechanism to 2,4-D (herbicide group O) [14,15] but the molecular mechanisms of NTSR remain to be elucidated. A recent paper by Goggin et al. [16] has shown that 2,4-D resistance in wild radish plants is due to a possible auxin transport impairment. Similar mechanisms might also be the cause of 2,4-D resistance in other cases [11,17].

Polar auxin transport in plants is facilitated by auxin transporting ATP-binding cassette (ABC) transporters that are often associated with other regulatory proteins and auxin membrane facilitators (PINs) to form effective auxin transport complexes in the plasma membrane [18]. It is likely that mutations in members of this auxin transport apparatus can impair the transport of auxin herbicides such as 2,4-D [19] as described in Goggin et al. [16]. Therefore, 2,4-D resistance can be due to differences in the gene expression level of one or several genes involved in the transport pathway. For this reason, identifying and validating reference genes that enable reliable measurement of gene expression levels following herbicide treatment in *P. rhoeas* is relevant for the subsequent elucidation of genes involved in NTSR.

The first aim of this study is to identify suitable reference genes for normalization of gene expression in *P. rhoeas* under 2,4-D herbicide treatment. The second aim is to validate a set of these reference genes by assessing the effect of 2,4-D application on the expression of two genes: *GH3* (indole-3-acetic acid amido synthetase) and *GST3* (glutathione S-transferase). *GH3* transcripts are known to increase abundantly when plants are treated with auxinic-like herbicides such as 2,4-D [20–22]. The main function of GH3 proteins are their involvement in auxin-regulated growth and development of plants [22]. GSTs are a family of enzymes initially known for their ability to detoxify herbicides by catalyzing their conjugation with glutathione [23]. However, *GST* expression has been shown to be up-regulated by a variety of toxic exogenous compounds (xenobiotics), without necessarily playing a role in their degradation [24]. Plant *GST* genes have been shown to be auxin-inducible [23].

2. Materials and methods

2.1. Plant materials and 2,4-D application

The *Papaver rhoeas* population used in the study (11–93) was collected from Italian wheat fields in the province of Foggia where control with 2,4-D and ALS inhibitors had failed. A screening test conducted in the greenhouse confirmed that 75% of the plants were resistant to the auxinic herbicide 2,4-D. Mature capsules were harvested and seed samples cleaned and stored at room temperature. Seed germination was conducted as described in Scarabel et al. [9]. After 10 days, seedlings were transplanted into plastic boxes (24 cm × 30 cm × 9 cm) filled with a substrate containing 60% silt-loam soil, 15% sand, 15% perlite and 10% peat by volume and placed in a greenhouse at Legnaro, north eastern Italy (45°21′N, 11°58′E). The experiment was performed during spring when natural temperature and light conditions were optimal for *P. rhoeas* growth.

When the plants reached the six-eight leaf stage, they were treated with 2,4-D (2,4-D LV 600, Dow Agrosciences, 600 g l^{-1}, solution) at the recommended Italian field rate corresponding to 360 g a.i. ha^{-1}. Herbicide was applied using a precision bench sprayer delivering 300 l ha^{-1}, at a pressure of 215 kPa and a speed of 0.75 ms^{-1}, with a boom equipped with three flat-fan hydraulic nozzles (Teejet 11002). Susceptible plants from a reference population (11–36 l) were used as a control for herbicide application efficacy.

Plant phenotypes were visually assessed four weeks after herbicide application. Dead and survived plants were classified as susceptible and resistant, respectively. All seedlings were grown, sprayed and collected at the same time and in the same conditions.

To identify genes with stable expression among experimental conditions (herbicide-treated *versus* untreated) and among phenotypes (resistant *versus* susceptible to 2,4-D), a time-course experiment was conducted. One leaf from each plant was collected at each of three different times: before the herbicide application, 5 h and 24 h after herbicide application, encompassing the most crucial period after herbicide application when expression of non-target-site–based resistance genes enables resistant plants to survive herbicide application. The leaf material was immediately frozen in liquid nitrogen and stored at − 80 °C until RNA extraction.

2.2. RNA isolation and cDNA synthesis

Total RNA extraction was performed using the Inivitrap® spin plant RNA mini kit (Stratec Biomedical AG, Germany) according to the manufacturer's instructions. The lysis buffer used was DCT and prior to elution, the RNA samples were treated with DNase I (RNase free) (New England Biolabs Inc., Massachusetts, USA) in order to eliminate trace contaminating DNA. Total RNA concentration of each sample was measured at 260 nm using the NanoDrop 2000 UV–Vis Spectrophotometer (NanoDrop products, Wilmington, USA). Only RNA samples having an A260/A280 ratio value of 1.8 to 2.2 and A260/A230 ratio between 2 and 2.2 were used for subsequent analysis. Total RNA integrity was verified by electrophoresis in 1% (w/v) agarose gels under denaturing conditions, also confirming the absence of gDNA.

Total RNA was extracted from 6 susceptible and 6 resistant plants at three times (before, 5 h and 24 h after herbicide application), yielding 36 RNA samples. cDNA synthesis was performed in duplicate for RNA samples that had passed the quality controls. Reverse transcription (RT) reactions were performed simultaneously for all samples. cDNA was synthesized from 1 µg of total RNA using the ImProm-II™ Reverse Transcriptase (Promega, Wisconsin, USA) with an oligo$(dT)_{15}$, following the manufacturer's instructions. Reactions were immediately stored at − 20 °C until further use as template in subsequent qPCR.

2.3. Candidate reference genes and primer design

Eight candidate reference genes reported as stable under herbicide stresses in weeds [4,5,25] and other abiotic stresses [26] were selected for analysis in this study. These candidate genes are involved in biosynthesis pathways [glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), type 2A protein phosphatase-2 (*PP2A*)], cytoskeletal structure [beta-tubulin (*TUB*) and actin (*ACT1*)], protein metabolism [elongation factor 1α (*ELF1α*), ubiquitin (*UBI*)], mRNA translation regulation [nuclear cap-binding protein subunit 20 (*CBP20*)] and ribosomal structure [internal transcribed spacer2 (*ITS2*)].

As no sequences of these genes for *Papaver rhoeas* were deposited in GenBank, multiple alignments were constructed with the homologous genes of other plants, using MEGA6 software [27] in order to obtain a partial coding DNA sequence (100–700 bp) on which to design specific primers for RT-qPCR short amplicons. A first set of primers was therefore manually designed to the conserved regions of the genes and specific amplification from cDNA was checked by PCR followed by electrophoresis on 2% (w/v) agarose gels. All the amplicons of the expected size were purified with NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel Gmbh & Co., Germany) following the manufacturer's instructions, sequenced and edited with FinchTV 1.4 software. Primers were synthesized by Invitrogen and amplicons sequencing was performed by BMR Genomics (Padova, Italy). To confirm the gene sequences obtained, they were compared with the BLAST search algorithm provided by NCBI.

Subsequently, specific qPCR primers were designed on the sequenced *P. rhoeas* fragments using the Pearl Primer free software [28] sets with the following conditions: amplicon size of 90–120 bp, a primer length of 25 ± 3 nucleotides, a melting temperature (Tm) of 64 °C–70 °C, a GC content between 40% and 60%, GC clamp at 3′. All