Evaluating *Arabidopsis thaliana* ubiquitin1 promoter activity in *Solanum lycopersicum*.

Richard Gossens
Radboud University Nijmegen
r.gossens@student.ru.nl

ABSTRACT

Overexpression of genes involved in heat stress response may yield heat stress resistant plants. To bring genes into overexpression, a good constitutive promoter is key. The main question in this study was: *Is the Arabidopsis thaliana ubiquitin1 promoter constitutively active in Tomato (Solanum lycopersicum)?* Tomato plants transformed with an *A. thaliana* ubiquitin1 promoter driving expression of β-glucuronidase (GUS) were tested for expression. Six out of fourteen transformed explants showed expression of GUS, either in protein activity or transcript. This study shows that the *A. thaliana* ubiquitin1 promoter is not constitutively active in Tomato and instead a shows sporadic expression.

Keywords

Promoter activity, tomato, Solanum lycopersicum, heat stress, heat stress transcription factors.

INTRODUCTION

Physiological processes in plants can be studied by knocking out genes or overexpressing genes. The latter can be performed by inserting a gene of interest in plants. Constitutive promoters are frequently used to overexpress genes, since the effects are easily detectable in most tissues in most developmental stages[1]. The ubiquitin1 promoter (UBQ1) has been identified in the model plant species *Arabidopsis thaliana*, and is drives expression in every tissue and developmental stage[2]. This promoter can be used to bring genes in overexpression to study the physiological processes, in this case the heat stress response (HSR). Since the *A. thaliana* UBQ1 promoter has been well characterized this promoter it was used in our study. The function of the HSR in plants is to protect against the consequences of heat and induce acclimation. It is a complex response, which includes a variety of heat stress transcription factors (Hsfs) and heat stress proteins (Hsps) which prime the plant to heat stress. Hsfs are common in eukaryotes however, their gene family size can differ greatly. Plants Hsfs gene family size varies from 18 in *Solanum lycopersicum* to 34 in *Glycine max*[3]. Class A Hsfs contain a motif to interact with the transcriptional machinery. HsfA2 plays an important role in thermotolerance, a state of tolerance after acclimatization in a mild heat stress. Overexpression of HsfA2 has shown enhanced thermotolerance in *A. thaliana*[4]. Charng et al. (2006) showed HsfA2 to be essential in sustaining acquired thermotolerance. However it is not essential for induction of acquired thermotolerance. HsfA4 is shown to be related to oxidative stress response. A dominant mutant of HsfA4a has decreased ascorbate peroxidase 1 levels. Class B Hsfs contain a repressor domain and are known to have a repressing effect on HSR[6].

Mainly anthers, especially meiosis and pollen development have shown high sensibility to heat stress. In agriculturally relevant temperatures (32˚C/26˚C, day/night, during 30 hours) pollen grain viability decreased significantly[7]. For Tomato and other fruiting crops, this means that yield can be affected by to heat. Hsfs could be overexpressed in fruiting crops to enhance their thermotolerance. The central research question in this study was: *Is the A. thaliana ubiquitin1 promoter (UBQ1) constitutively active in S. lycopersicum?* It is thought that the *A. thaliana* UBQ1 promoter will be constitutively active in *S. lycopersicum*. Since uniform GUS-staining in *A. thaliana* was shown by UBQ1 driving GUS expression [2].

MATERIAL & METHODS

Kanamycin and GUS PCR

DNA extraction on T_o transformed plants was performed following Fulton et al. (1995). All samples were tested with a standard PCR, using primers for kanamycin resistance gene, GUS and EF1α (Table 1). After amplification of the DNA 3 µl

SRC 2016, November 30, 2016, The Netherlands.

Table 1 sequences of the used primers for PCR and qPCR.

	Forward	Reversed
GUS	TTAACTATGCCGGAATCCATCGC	AACGCTGACATCACCATTGGC
Kan	CAGACAATCGGCTGCTCTGATGC	CGTCAAGAAGGCGATAGAAGGCG
EF1α	CCTCCGTCTTCCACTTCAGGATG	GTCACAACCATACCAGGCTTGATC
EXP1	TTTGACCTCGCTATGCCTATGTTTC	CTAAGTTGAAGTAACGGAATCCATTGATG
TUB	TGGACAGTCTGGTGCTGGTAATAAC	TTCTCCGCTTCTTTACGAACAACATC

PCR product was mixed with 2 µl loading dye and this was loaded into a 1% agarose gel and run for 20 minutes at 100V.

GUS-staining on transformed plant tissue

Leaf, root, anther and fruit tissue of 14 pUBQ1::GUS-Kanamycin transgenic T0 plants were stained with GUS. Five anther sizes to represent growth stages were harvested (2mm, 4mm, 6mm, 8mm and full flower). The tissue with GUS-staining solution was incubated overnight at 37˚C. Stained tissues were destained using 70% ethanol and incubation at 60˚C for 3 hours, during destaining the ethanol was refreshed several times. The tissues subsequently, were screened for presence of GUS-stains with the use of a dissecting microscope.

Quantification of anther RNA

RNA was isolated from transformed plants following Simms et al. (1993). After isolation, RNA integrity and quantity was evaluated on gel and by Nanodrop spectrophotometer. DNA degradation was performed by using 1 µg RNA sample, 1U of DNAseI, 2 µl 10X DNAseI buffer and DEPC-treated water was used to fill up to 20 µl total volume. The reaction mixture was incubated at 37˚C for 30 minutes, afterwards 1 µl of 50mM EDTA solution was added and incubated at 65˚C for 10 minutes. The RNA product was converted into cDNA using the iScript cDNA Synthesis Kit. GUS, as a gene of interest and EF1α, EXP1 and TUB, as reference genes, were quantified using quantitative PCR (qPCR) with the appropriate primers (Table 1). PCR efficiency was averaged with samples of the same reaction, for each biological sample the relative quantification (RQ) was calculated ($RQ = \frac{1}{PCR\ efficiency^{Cq}}$) for all used primers[1]. The RQ of the three reference genes, EF1α, EXP1 and TUB, has been averaged to acquire a normalization factor (NF). The RQ of the GUS samples was then divided by the corresponding NF to obtain the normalized relative quantification (NRQ); $NRQ = \frac{RQ}{NF}$.

RESULTS

Kanamycin and GUS PCR

To test whether the used plants where successfully transformed a PCR was performed with primers for the inserted genes. Three independent PCRs were performed to amplify the kanamycin resistance gene and genotype plants based on the presence of the kanamycin resistance gene, however these results were contradicting.

To clarify this, another PCR was performed to get unambiguous results. This was a PCR for GUS and EF1α. EF1α is an endogenous gene which was used to verify whether DNA was extracted successfully, thus functioning as a control PCR (Figure 1). In this gel samples UB1-4, UB1-13, UB1-18, UB1-30, UB1-34, UB1-20, UB1-31, UB1-12 and UB1-3, showed amplified GUS product. EF1α amplification succeeded in all samples, indicating successful DNA extraction.

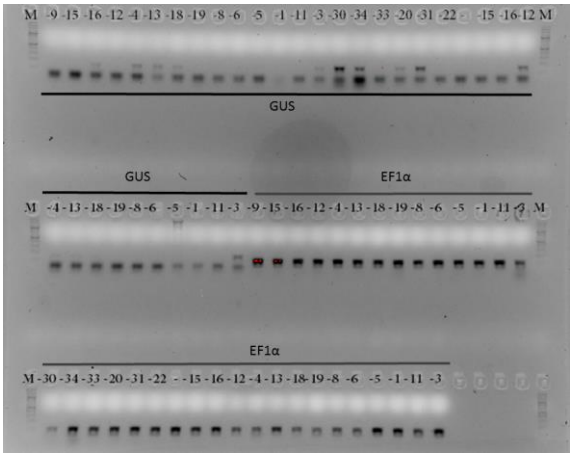

Figure 1 GUS + EF1a PCR; M indicates the marker, - indicates a negative control and –X (e.g. -9) indicate samples.

GUS-staining of transformed plant tissue

The activity of the UBQ1 promoter was tested with a GUS-staining. Performed GUS-stainings showed GUS expression in anther tissue, style, root, fruit and leave tissue. However, GUS-staining when observed was found patchy. Staining was found concentrated in a limited part of a root and was found in patches of fruit tissue and in the veins of the leaf (Figure 2).

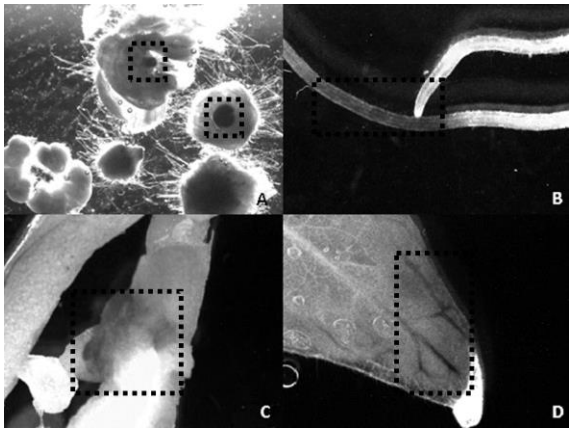

Figure 2 GUS staining in (A) anther, (B) root, (C) fruit, (D) leaf tissue indicated by dotted boxes

Quantification of GUS transcript

To test and quantify the activity of the UBQ1 promoter qPCR was performed (Figure 3). Samples that showed a Cq at least 5 lower than the No Template Control (NTC) were selected as positives for expression of GUS. The NTC did not contain any DNA template, therefore it functions as a control of unspecific amplifications. The NTC showed exponential amplification of DNA after 29 cycles, indicating that UB1-34, UB1-30, UB1-13, UB1-3, UB1-16, UB1-15 contained GUS transcript. UB1-4 did not reach quantification in any cycle.

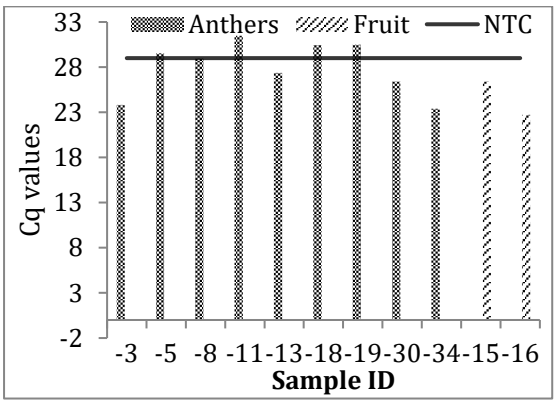

Figure 3 Quantitative cycles (Cq) of the qPCR.

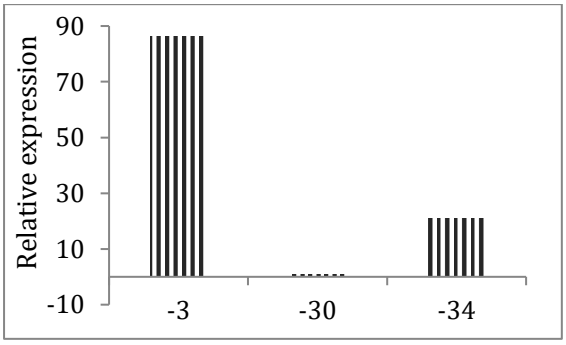

Figure 4 Relative expression found in anthers of transgenic plants. The relative expression is based on the ratio of the NRQ of the samples.

Figure 4 displays the relative expression found in three samples, sample UB1-3, UB1-30 and UB1-34. UB1-3 has the highest relative expression, the expression is found 86-fold of UB1-30 and UB1-34 has 21-fold the transcript found in UB1-30.

DISCUSSION

Sample UB1-4 and UB1-34 are successfully transformed and GUS is being expressed. Our observed stainings in these plants were found patchy and rare in contrast to the observations in *A. thaliana* by Holtorf et al. (1995). Expression of GUS is found in fruit, leaves, roots and anthers however, the expression was observed in only a few of successfully transformed plants.

In table 2 a summary off the results of Staining experiments, PCR and qPCR is given and shows positively tested samples. From the GUS-staining and GUS-qPCR experiments it can be concluded that the ubiquitin1 promoter does yield some expression, however to conclude it's constitutive like the hypothesis is undue. Thus to answer the main research question: *Is the Arabidopsis ubiquitin1 promoter (UBQ1) constitutively active in Solanum lycopersicum?* No, not in the explants used in this study. However, promoter activity was quantifiable in anther tissue. Therefore, the promoter might be useful to maintain pollen viability in heat stress by locally overexpressing Hsfs in anthers. Perhaps the *A. thaliana* promoter is taxonomically too different for good uniform expression in *S. lycopersicum*. A similar experiment has been performed with a maize ubiquitin promoter in rice in which expression exceeded 7x expression levels of 35S[11]. This could lay in the fact that rice and maize both are *Poaceae*. *A. thaliana* and *S. lycopersicum* are genetically more distinct species.

Table 2 Tested successfully transformed samples per method.

Sample	GUS-staining	GUS-PCR	GUS-qPCR
UB1-3		X	X
UB1-4	X	X	
UB1-12		X	
UB1-13		X	X
UB1-15			X
UB1-16		X	X
UB1-18		X	
UB1-20		X	
UB1-30		X	X
UB1-31		X	
UB1-34	X	X	X

Similarly further efforts can be made to isolate the *S. lycopersicum*, or other *Solanacea*, ubiquitin promoter and have it drive expression of a gene of interest. The use of a constitutive promoter to test whether overexpression of Hsfs enhances thermotolerance of a plant can be questioned. The use of such a promoter will result in expression of Hsfs in every developmental stage and every tissue. Which means that, during its entire life cycle the plant will invest resources to thermotolerance in all tissues, which itself might decrease yield. Recent work shows that reproductive tissues are more susceptible to heat stress then vegetative tissues[12]. Thus instead of using a constitutive promoter, a heat-inducible promoter might be used to drive Hsf gene expression. In rice six highly heat inducible genes have been identified[1]. For instance OsHsfB2cp which showed high expression in the panicle under heat stress. It can be hypothesized that this expression, found mainly in the panicle, will be allocated the likewise in *S. lycopersicum* and therefore may provide an adequate promoter for local Hsf overexpression. Similarly endogenous tomato promoters might be studied for their expression patterns and evaluated on their activity throughout the plant or in anthers specific.

ROLE OF THE STUDENT

The research was performed at the department of Molecular Plant Physiology under supervision of Ivo Rieu and Hanjing Li who developed the theoretical outline and thought of methods to test the hypothesis. All practical work of the research project was conducted and designed by Luuk Hobbelen and Richard Gossens in equal proportions during an undergraduate internship. Technical assistant Peter de Groot, taught us the used methods and techniques. The paper was written by Richard Gossens.

REFERENCES

1. Hernandez-Garcia, C. M. & Finer, J. J. Identification and validation of promoters and cis-acting regulatory elements. *Plant Sci.* **217-218,** 109–119 (2014).
2. Holtorf, S., Apel, K. & Bohlmann, H. Comparison of different constitutive and inducible promoters for the overexpression of transgenes in Arabidopsis thaliana. *Plant Mol. Biol.* **29,** 637–646 (1995).
3. Miller, G. & Mittler, R. Could heat shock transcription factors function as hydrogen peroxide sensors in plants? *Ann. Bot.* **98,** 279–288 (2006).
4. Ogawa, D., Yamaguchi, K. & Nishiuchi, T. High-level overexpression of the Arabidopsis HsfA2 gene confers not only increased themotolerance but also salt/osmotic stress tolerance and enhanced callus growth. *J. Exp. Bot.* **58,** 3373–3383 (2007).
5. Charng, Y. -y. *et al.* A Heat-Inducible Transcription Factor, HsfA2, Is Required for Extension of Acquired Thermotolerance in Arabidopsis. *Plant Physiol.* **143,** 251–62 (2006).
6. Scharf, K. D., Berberich, T., Ebersberger, I. & Nover, L. The plant heat stress transcription factor (Hsf) family: structure, function and evolution. *Biochim Biophys Acta* **1819,** 104–119 (2012).
7. Bita, C. E. *et al.* Temperature stress differentially modulates transcription in meiotic anthers of heat-tolerant and heat-sensitive tomato plants. *BMC Genomics* **12,** 384 (2011).
8. Fulton, T. M., Chunzoongse, J., Tanksley, S. D., Breeding, P. & Hall, E. Microprep Protocol for Extraction of D N A from Tomato and other Herbaceous Plants. **13,** 207–209 (1995).
9. Simms, D., Cizdziel, P. E. & Chomczynski, P. TRIzol ™: A New Reagent for Optimal Single-Step Isolation of RNA. *Focus (Madison).* **15,** 99–103 (1993).
10. Rieu, I. & Powers, S. J. Real-time quantitative RT-PCR: design, calculations, and statistics. *Plant Cell* **21,** 1031–3 (2009).
11. Cornejo, M. J., Luth, D., Blankenship, K. M., Anderson, O. D. & Blechl, a E. Activity of a maize ubiquitin promoter in transgenic rice. *Plant Mol. Biol.* **23,** 567–581 (1993).
12. Bokszczanin, K. L. & Fragkostefanakis, S. Perspectives on deciphering mechanisms underlying plant heat stress response and thermotolerance. *Front. Plant Sci.* **4,** 315 (2013).
13. Rerksiri, W., Zhang, X., Xiong, H. & Chen, X. Expression and promoter analysis of six heat stress-inducible genes in rice. *Sci. World J.* **2013,** (2013).