Localization of Activity of ASY1, TA29 and NTM19 promoters in tomato

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Abstract

The global warming has been affecting crop production in many countries. Studies demonstrate that the reduced production in heat stressed plants has as a main cause the impairment of pollen development. Our hypothesis is that pollen development can be protected against heat stress. To do so, tissue specific promoters were tested regarding their localization to be used to overexpress members of a family of transcription factors that orchestrate the cell response front the heat, the Heat Shock Factors (HSFs). The HSFs activate the transcription of Heat Shock Proteins (HSPs), which play many roles in protecting cells against heat. During heat stress, meiosis, microspore and mature stages of pollen development are differentially affected and therefore tissue specific promoters are required for being linked to the HSF which will be overexpressed. As in *Arabidopsis* or tobacco ASY1, TA29 and NTM19 promoters are active in pollen mother cell, tapetum and microspore respectively. In this work, we localized the activity of these three promoters in tomato plants through the histochemical GUS assay. Our results indicate that TA29 and NTM19 promoters are active in tapetum and the activity of ASY1 promoter couldn't be reported.

Key-words: heat stress, thermotolerance, tissue-specific promoter, HSF, overexpression.

Introduction

The Earth is experiencing increasing temperatures since the mid-nineteenth century. Although the causes of this phenomenon are unclear, data regarding predictions of climate change have been generated. Over the 20th century, the temperature of the Earth increased 0.6°C and the prediction for 2100 is an increase of 1.4 - 5.8°C [1].

Crop production has been shown to be impaired by the increased temperatures. Peng, S. *et al*. (2004) demonstrated that for each 1°C increase in minimum night temperature, the biomass production of irrigated rice in Philippines decreased by ≈10% in the dry season [2]. Another example of effect of high temperatures in crop yield includes the production of soybean and corn in US. Data based on climate variation and crop production from 1982-98 showed that for each 1°C increase in temperature, 17% of grain yield is reduced [3].

Although the effect of high temperatures are mostly studied in vegetative plant tissues [4], the main cause of reduced crop yield takes place in reproductive tissues, precisely in the pollen development [5]. By performing crosses between female and male plants exposed to controlled or high temperature conditions, Peet *et al*. (1998) [6] and Young *et al*. (2004) [7]

demonstrated that the effects of heat stress are more severe in pollen development than in female gametophyte, consequently disrupting seed production.

The stages of the male reproductive development are affected by heat stress differentially. Initially, diploid pollen mother cells undergo meiosis originating tetrads of haploid microspores. The microspores are released from the tetrad by enzymes secreted by the tapetum, a layer of cells that surrounds the locules containing the microspores. The tapetum is present only in the early stages of the pollen development and plays vital roles such as enzyme secretion, pollen wall development and nutritive support. Each haploid microspore undergo asymmetric mitosis originating the large vegetative cell, which will participate on the pollen tube growth, and the small generative cell, which undergoes to mitoses, originating two sperm cells [8]. The earlier stages of the pollen development are the most sensitive to high temperature [9], but tapetum cells can also abort due to heat stress causing male sterility [10].

The reduction of crop yield can be solved by the production of crops in which the pollen development is ensured under heat stress conditions, i.e. thermotolerant crops. The overexpression of Heat Shock Factors (HSFs), a family of transcription factors that orchestrates the heat stress response by activating the transcription of Heat Shock Proteins (HSPs), in pollen or anther tissues may enhance the tolerance to heat in these tissues if linked to tissue-specific promoters. HSPs have many functions during heat stress that protect the cell against heat, like stabilizing damaged proteins and facilitating their renaturation [8]. Increasing the expression of HSFs, HSPs will be more abundant in the cell and will therefore protect the heat sensitive tissue.

In this study, the activities of ASY1, TA29 and NTM19 tissue specific promoters were tested. ASY1 protein has a role in coordinating meiotic division in *Arabidopsis*, once it is closely associated with elements which participate on the chromosome axis formation [9]. TA29 protein is expressed in tapetum of *Arabidopsis* and it is likely to be a cell wall protein [10]. NTM19 protein is expressed in the unicellular microspore and might have a role in formation of intine and exine layer or asymmetrical mitoses in tobacco plants [11]. Considering that ASY1, TA29 and NTM19 promoters are active in pollen mother cell, tapetum and microspore respectively, we tested whether these promoters are active in the same tissues in tomato. Each promoter was linked to a vector containing the β-glucuronidase (GUS) gene and transformed into tomato plants. The GUS coloring assay revealed that both NTM19 and TA29 are active in the tapetum of tomato plants. The activity of ASY1 couldn't be localized by this assay.

Material and Methods

In order to carry out our studies in promoter activity, we used a miniature *Lycopersicon esculentum* cultivar, the Micro-Tom. This cultivar displays a very dwarf phenotype, characterized by short stature due to very short internodes, small fruits, dark green leaves, , short life cycle and high transformability. Mutations in the SELF-PRUNING and DWARF genes are responsible for this phenotype [15].

Micro-Tom plants harboring each promoter linked to the GUS gene were kindly provided by Hanjing Li (Radboud University of Nijmegen). Promoter::GUS constructs were generated by inserting 2 kb upstream of the first codon of the ASY1, TA29 or NTM19 tomato protein coding sequence to the pKGWFS7 vector which contains the GUS gene. The construct was transformed into Micro-Tom plants via *Agrobacterium*.

From each transgenic Micro-Tom plant, buds with different sizes (1-7 mm) were collected. The buds were measured and cut with a razor blade into 4-7 pieces depending on its size or macerated in GUS staining solution. Sepals and petals were removed from bigger sized buds. The pieces were immediately immersed in GUS solution (0,4% Triton X-100, 50 mM Na-phosphate buffer pH 7.2, 2 mM Ferricyanide, 2 mM Ferrocyanide, 1mM X-Gluc and H_2O) and incubated for overnight at 37 °C in the dark. In order to dehydrate the bud pieces, the GUS solution was replaced for 70%, 80%, 90% and 100% ethanol remaining at each ethanol concentration for one hour.

The activity of the promoter is detected by the stained blue color at the tissue where the enzyme is functional. In order to have refine visualization at the cellular level, the anther pieces were embedded in Technovit 7100 resin [16] and sectioned in 10-12 µm slices with the microtome.

Results

The objective of this work is to localize the activity of tissue specific promoters in order to link the most appropriate one to a HSF and increase its expression, enhancing pollen protection against heat. Three promoters were chosen due to their specific activity in tapetum, pollen mother cell or microspore in tobacco or *Arabidopsis* plants. To know if these promoters present the same localization of activity in tomato, and therefore are suitable for overexpressing a HSF, GUS coloring assay was performed with anthers of plants harboring promoter::GUS constructs.

GUS coloring could not report ASY1 promoter activity

Buds sized from 1mm were harvested from tomato plants transformed with pASY1::GUS construct. In GUS solution, the slices of these buds became dark orange and no blue color was identified in all buds slice. Moreover, no pollen mother cells were found in GUS solution where the buds were macerated.

Although the staining of pollen mother cells was unsuccessful, stained tetrads were reported when buds with different sizes were macerated in GUS solution (**Figure 1A and 1B**). Stained tetrads were also reported in plants that didn't have the construct (negative control), excluding the possibility of the blue color be a result of GUS enzyme activity (**Figure 1C and 1D**). A stronger staining in the junction of the four cells due to callose deposition can be noticed in all the stained tetrads.

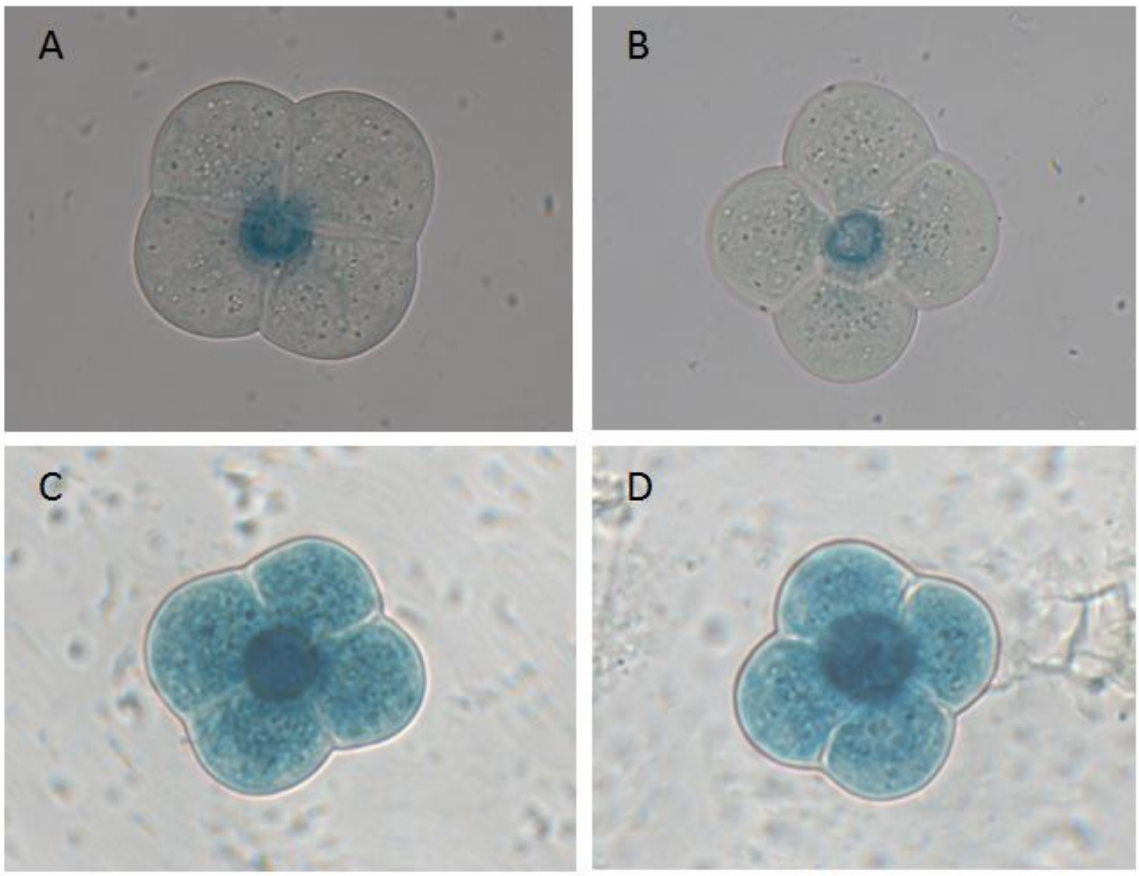

Figure 1: Stained tetrads of ASY1::GUS transformed plants and negative control. (A) Tetrad from a 2.5 mm bud ASY1::GUS transformed. (B) Tetrad from a 3 mm bud ASY1::GUS transformed. (C, D) Tetrads of non-transformed buds. Magnification: 40X (A, B) and 10X (C, D).

TA29 promoter is active in tapetum

As expected, the localization of activity of the TA29 promoter in tomato is similar to its activity in tobacco. The blue stained area surrounding the anther locule corresponds to tapetum cells (**Figure 2**). Although the tapetum appear not homogenously stained, no immature pollen grains were blue stained, confirming the specificity of this promoter. Some diffusion of the blue precipitate to the anther cells can be noticed due to the time of exposure to the substrate. Stomial cells, which degenerate to allow the anther opening and pollen release, were also positively stained in some cases (**Figure 2C**).

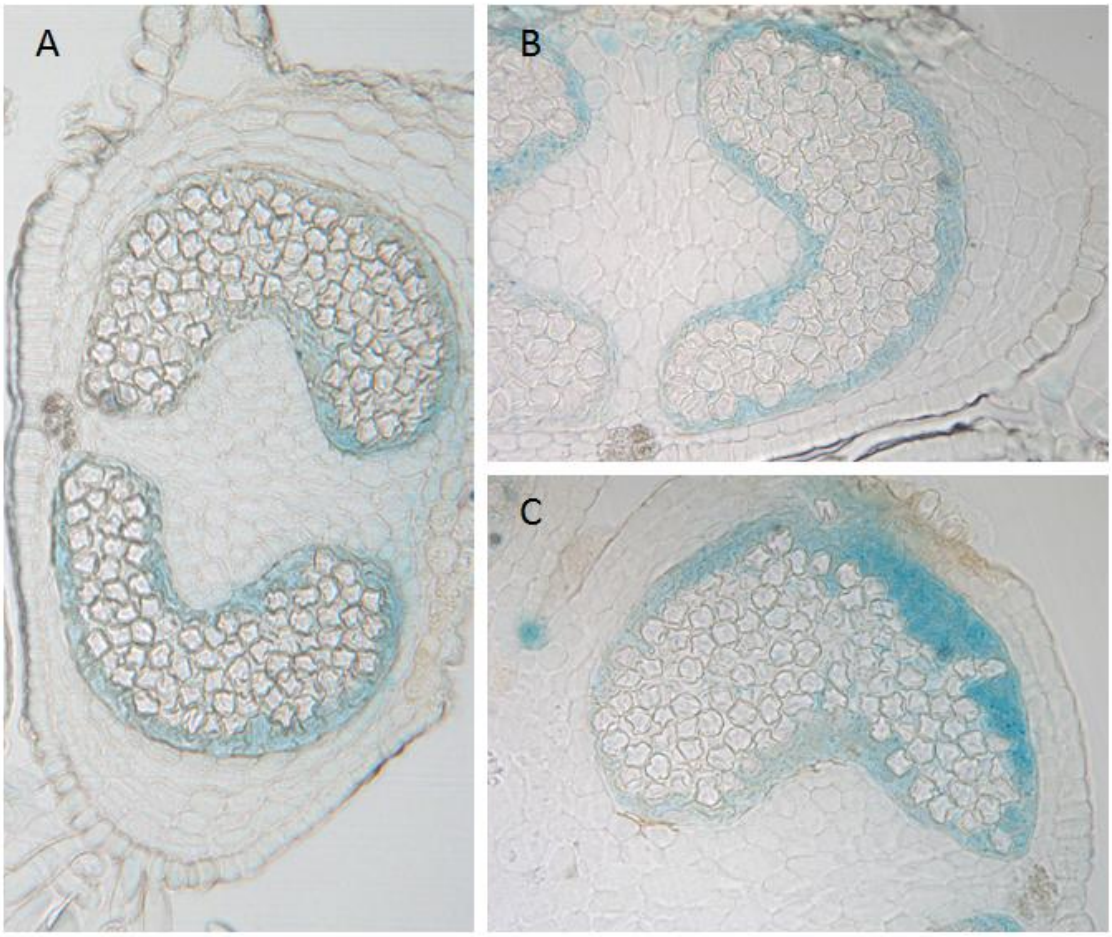

Figure 2: Sections of tomato anthers harboring TA29::GUS construct. (A, B and C) Blue stained tapetum. Magnification: 10X (A) and 25X (B and C).

NTM19 promoter activity differs in tobacco and tomato

We expected the NTM19 promoter to be active exclusively in microspores. However, as the results of TA29 promoter transformed plants, tapetum cells were blue stained. The fresh cut reveals a stronger blue staining surrounding the anther locules as well as some diffusion of the blue precipitate (**Figure 3A**). In thinner sections microscopically analyzed, it is clearer that only tapetum cells are blue stained (**Figure 3B and C**). Four and 4.5 mm buds were strongly stained while 5 mm buds were not (**Figure 3D**), suggesting that this promoter can be temporarily active.

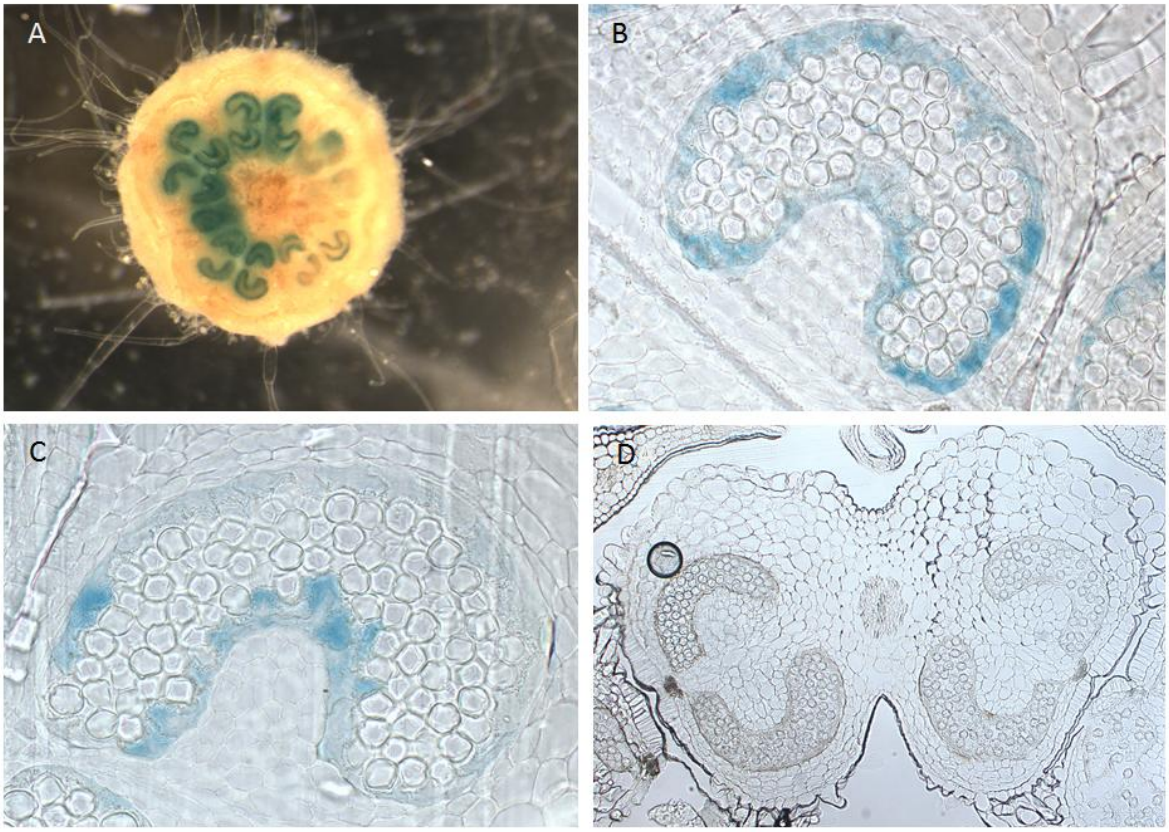

Figure 3: Fresh cut sections of tomato anthers harboring the NTM19::GUS construct. (A) Fresh cut of a 4.5mm bud. (B) Blue stained tapetum of a 4mm bud. (C) Blue stained tapetum of a 4.5 mm bud. (D) Unstained tapetum of a 5 mm bud. Magnification: 2.5X (A), 40X (B and C) and 10X (D).

Discussion

The development of thermotolerant crops has been a target of interest during the last decades due to the increasing temperatures caused by the global warming. Such crops can be developed by overexpressing the transcription factors that are involved in the response to heat, the HSFs. As the pollen development is the main cause of reduced production of crops, we tested tissue specific promoters regarding its localization of activity in order to link them to the HSF to be overexpressed and protect the heat sensitive tissue.

The activity of ASY1 promoter couldn't be reported by GUS assay. It is likely that this promoter is active during meiosis, which is a very early stage of the pollen development. Therefore, very small buds were collected to perform GUS assay. Although it wasn't possible to notice any blue staining in the fresh sections due to tissue oxidation, blue stained tetrads were found in GUS solution where buds were macerated. As these tetrads were also found in negative controls, the blue precipitated may not be a result of the enzyme activity and does not report ASY1 promoter activity as well.

False positives can occur due to the activity of a GUS enzyme produced by plant itself or other enzyme capable of processing X-Gluc substrate. GUS-like activities were reported in many reproductive organs of *Lycopersicon eseulentum* plants by Hu, C. *et al*. (1990) [17]. Plegt and Bino (1989) [18] reported GUS intrinsic activity in sporogenous cells in the same tomato specie. Although no activity was found at meiotic stages, the author reported blue

precipitation in later stages of pollen development. Therefore, the stained tetrads could be a result of GUS intrinsic activity or the processing of X-Gluc by another enzyme.

The activity of TA29 promoter was localized at the tapetum cells as expected. Therefore, this promoter is a strong candidate to be linked to a HSF. Overexpressing a HSF in tapetum might protect this tissue against heat stress by increasing the expression of HSPs. Once the tapetum is less damaged by heat, it can provide nutrients and enzymes that may ensure the pollen development under heat.

The NTM19::GUS construct showed to be active in tatetum cells as the TA29 promoter. NTM19 promoter was chosen because it is active in microspore cells of tobacco. If this pattern was repeated in tomato, this promoter attached to a HSF could protect the microspores against the heat damages. As this activity wasn't observed, this promoter cannot be used to ensure microspore development under heat. However, being active in tapetum, NTM19 promoter can ensure pollen development the same manner as TA29 promoter. Both promoters can be used to be linked to a HSF and protect tapetum.

The differential activity of NTM19 promoter in tobacco and tomato is an interesting finding. Probably, the promoter region of the NTM19 genes in tabacco and tomato possesses different elements that drive their activity in respective species. To find out which elements are these, promoter deletion analysis could be performed by linking fragments of different sizes to the GUS enzyme.

The GUS assay is very suitable for reporting localization of promoter activity for being a relatively simple assay and due to the high stability of the blue precipitate. However, the levels of activity are equally relevant for the purpose of these experiments. Quantitative PCR should be performed with both treated and untreated transformed plants, what will provide data about promoter stability. The combination between localization and level of activity data of each promoter is crucial for choosing the best promoter to be attached to the HSF to be overexpressed. Therefore, more studies should be performed in order to choose the promoter which will be linked to the HSF to protect pollen development.

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