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In silico promoter analysis and expression of the *BIG BROTHER* gene in different organs of potato

Khongorzul ODGEREL[1] – Zsófia BÁNFALVI[1]

1: Hungarian University of Agriculture and Life Sciences, Institute of Genetics and Biotechnology, 2100 Gödöllő, Szent-Györgyi A. u. 4, Hungary, e-mail: Khongorzul.Odgerel@phd.uni-mate.hu,
0000-0002-8889-631X

Abstract: The ubiquitin E3 ligase *BIG BROTHER/ENHANCER OF DA1 (BB)* gene encoding a RING finger protein was identified as a central growth regulator in *Arabidopsis thaliana*. It was found that *BB* restricts cell proliferation and promotes leaf senescence. Besides of *Arabidopsis*, however, the role and regulation of *BB* in other plant species is only sparsely known. Supposing that the *BB* gene, like in *Arabidopsis*, has an important role in the development of potato we aimed to analyse a 3.0-kb promoter sequence of the potato *BB* gene, *StBB*, *in silico* and study the level of *StBB* expression by quantitative reverse transcription PCR in different organs. A total of 48 binding sites for 15 transcription factor (TF) families were predicted. Most of them were located in the -1.5-kb promoter region. The dominating family of TFs was DOF. It was found that 20 out of the 24 TFs with known functions are involved in developmental processes such as for example, the flower-, leaf-, stem- and root development or cell cycle regulation. In line with this finding, the *StBB* mRNA was detected in each organ tested with the largest amounts in petal and stamen. These results suggest a similar function of *StBB* in potato than that is of *BB* in *Arabidopsis*, i.e., restriction of organ overgrowth during development and limitation of the plant growth.

Keywords: *BIG BROTHER* gene, *cis*-acting regulatory elements, *Solanum tuberosum* L., transcription factors
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Introduction

Although plant growth is a complex, dynamic process, it is divided into the following phases: cell proliferation, elongation, and differentiation, which are regulated by both genetic (internal) and environmental (external) factors (Müller & Sheen, 2008; Bögre et al., 2008; Denay et al., 2017; Zluhan-Martínez et al., 2021). Hence leaves are photosynthetic organs, their size is pivotal for plant growth. During leaf development, the size is controlled by an organ-wide mechanism that coordinates the cell proliferation with cell expansion (Horiguchi et al., 2005). Numerous genes have been identified as cell proliferation regulators in *Arabidopsis thaliana* including the ubiquitin receptor *DA1–ENHANCER OF DA1 (DA1–EOD1)* module, which is identified as a negative regulator of leaf growth (Du et al., 2014; Vercruysse et al., 2019). DA1 is activated by the E3 ligases *BIG BROTHER/ENHANCER OF DA1 (BB)* and *DA2* (Peng et al., 2013; Xia et al., 2013; Dong et al., 2017; Vercruysse et al., 2019).

The RING finger protein *BIG BROTHER (BB)* was identified as a repressor of plant organ growth in 2006. The homozygous *bb-1* mutants, which lack *BB* mRNA, form larger petals and sepals, as well as thicker stems than wild-type (Disch et al., 2006)). Vanhaeren et al. (2016) found that single mutations such as *da1-1* and *bb/eod1-2* increase

the leaf size in *Arabidopsis*. The double mutation *da1-1_bb/eod1-2* cause the synergistic enlargement of both the first leaf pair and younger rosette leaves. Furthermore, depending on the *BB* expression, plants start to die prematurely. Thus, it was concluded that ectopic expression of *DA1* or *BB* restricts cell proliferation and promotes leaf senescence. Cattaneo and Hardtke (2017) reported that *bb2* loss-of-function mutations prolong the cell proliferation and uncouple cell proliferation from elongation in the root meristem. They evidenced that *BB* acts similarly in leaf (-like) organs and the primary roots. Downstream transcriptional effects of *DA1* and *BB* were also tested in the young, proliferating leaves within different induction time frames. It was found that both *DA1* and *BB* trigger molecular changes shortly after induction of their expression, but the expression of *BB* is higher than that of *DA1* and rapidly stimulates the expression of senescence markers.

To identify the connection between the individual factors and larger regulatory pathways, expression of *BB* was investigated by a combination of promoter deletion analysis and a phylogenetic footprinting approach. It was shown that removing 150 bp from the 5′ non-transcribed promoter sequence resulted in a 40% increase in petal size in the transgenic lines. Alignment of the isolated *BB* coding sequence from *A. thaliana* and seven other species from the Brassicaceae family showed a high degree of conservation within all genera (Breuninger & Lenhard, 2012).

Besides of *Arabidopsis*, however, the role and regulation of *BB* is only sparsely known. In *Saltugilia*, four candidate genes, including *BB*, underpinning of flower size were identified and down-regulation of *BB* in synthetic polyploids of *Nicotiana tabacum* with increased corolla tube size was demonstrated (Landis et al., 2017, 2020). According to our knowledge, however, no study on *BB* gene in potato has been reported thus far.

Potatoes are the fourth most consumed crop in the world, behind rice, wheat and corn (Birch et al., 2012) and the consumption is even increasing especially in Asian countries (http://www.fao.org/faostat). Therefore, productivity is still a key issue of potato breeding.

In our previous study, we found a positive correlation between the growth rate of the leaves and the time of tuber initiation (Odgerel & Bánfalvi, 2021). Supposing that the *BB* gene is involved in the regulation of leaf growth also in potato, the aim of our study was the analysis of the *BB* promoter sequence in silico and the level of *BB* expression in different organs of *Solanum tuberosum* cv. 'Désirée'. Here we show that the promoter region of the potato *BB* gene (*StBB*) carries several *cis*-regulatory elements (CAREs) for transcription factors (TFs) involved in the regulation of plant development and *StBB* is expressed in each organ of *S. tuberosum* cv. 'Désirée'.

Materials and Methods

Plant materials and growth conditions

Potato (*Solanum tuberosum* L.) cv. 'Désirée' plants were propagated *in vitro* in solid (0.8% agar) RM culture medium (MS medium without vitamins; (Murashige & Skoog, 1962)) supplemented with 2% (w/v) sucrose. Plants were grown in a growth room at 24°C with 16-h day/8-h dark cycles at a light intensity of 75 μmol m^{-2} s^{-1}. Plantlets were continuously cultured from stem segments in fresh medium every month. One-month-old plantlets were transferred into the sterile soil A200 (Stender GmbH, Schermbeck, Germany) and grown in a greenhouse under standard conditions with a 10-12 h light/26-28°C and 10-12 h dark/18-22°C cycle. In order to provide optimal growth condition in the greenhouse, plants were treated with fungicides at the be-

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ginning of acclimatisation and weekly with the pesticide Mospilan (Nippon Soda Co., Ltd., Tokyo, Japan). The plants were watered twice a week. For the *StBB* expression analysis, 'Désirée' root, stolon, tuber, stem, petiole, leaf, sepal, petal and stamens of greenhouse-grown plants were used. Tubers were harvested at the end of the vegetation period.

In silico DNA sequence analysis of the StBB promoter

Three thousand-bp sequences upstream from the translational start sites of the *S. tuberosum* Group Phureja *BB* gene (chromosome 11, from 37009829 bp to 37012829 bp, reverse complement) was retrieved from the Potato Genomic Resource Spud DB (http://solanaceae.plantbiology.msu.edu/).
The binding sites of transcription factors (TFs) were predicted by the plant regulatory data and analysis platform The Plant Transcriptional Regulatory Map (http://plantregmap.gao-lab.org/). The latest, upgraded version PlantTFDB v5.0 (Tian et al., 2019) was used at a threshold p-value $\leq 1e^{-5}$.

RNA extraction, reverse transcription PCR (RT-PCR) and reverse transcription – quantitative PCR (RT-qPCR)

Total RNAs from plant tissues were extracted by using the method of Stiekema et al. (1988). One μg of RNA was reverse transcribed into first-strand cDNA using the Maxima H minus First Strand cDNA Synthesis Kit with dsDNase (Thermo Scientific Molecular Biology, Waltham, MA, USA). RT-PCR was conducted in T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) for checking the cDNA quality using a primer pair for the cytoskeleton component gene *ACTIN*, a commonly used reference gene for RT-qPCR in potato (Nicot et al., 2005). RT-qPCR assays were performed using a Light Cycler-96 thermal cycler (Roche Diagnostics GmbH, Mannheim, Germany) and the Luminaris Color HiGreen Flourescein qPCR Master Mix (Thermo Scientific Molecular Biology, Waltham, MA, USA). Data were analysed with the Light Cycler-96 Software version 1.1 (Roche Diagnostics GmbH, Mannheim, Germany). Expression analysis of the *StBB* gene was carried out using the primer pair StBB Fw and StBB R. Parallel reactions to amplify *ACTIN* and *EF1*α, the commonly applied reference genes (Nicot et al., 2005), were used to normalize the amount of template. Relative expression was calculated based on both reference genes. Non-reverse transcriptase control without enzyme mixture and a negative control without cDNA were always included, with three technical replicates in each experiment. All primers were designed using the NCBI primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and their sequences are presented in Table 1.

Results

In silico prediction of TFs binding to the StBB promoter

The prediction of transcriptional binding sites by The Plant Transcriptional Regulatory Map (PlantRegMap) resulted in identification of 48 binding sites for 29 TFs in the *StBB* promoter region. These TFs belonged to 15 families. Eighteen out of the 48 binding sites served for the DOF family TFs. The other dominating families were BBR-BPC, bHLH, M-type MADS and MIKC-MADS (Table 2). Location of the TF binding sites with the indication of TF families is presented in the top of Figure 1.
The predicted TFs are involved in a wide range of biological processes and respond to different internal and external stimuli (Table 2). Nevertheless, we found that 20 out of the 24 TFs with known functions are related to developmental processes as for example, the

Table 1: Primer sequences used in RT-qPCR

Name	Forward sequence ($5' - 3'$)	Reverse sequence ($5' - 3'$)
StBB	TCCATCAGCACCAATCCATAC	CATGCTCCTCGATTCCAGATAC
Actin	TGGACTCTGGTGATGGTGTG	GGTTTCAAGTTCCTGTCTGT
EF1	GACAAGCGTGTTATTGAGAGG	CACAGTGCAGTAGTACTTAGTG

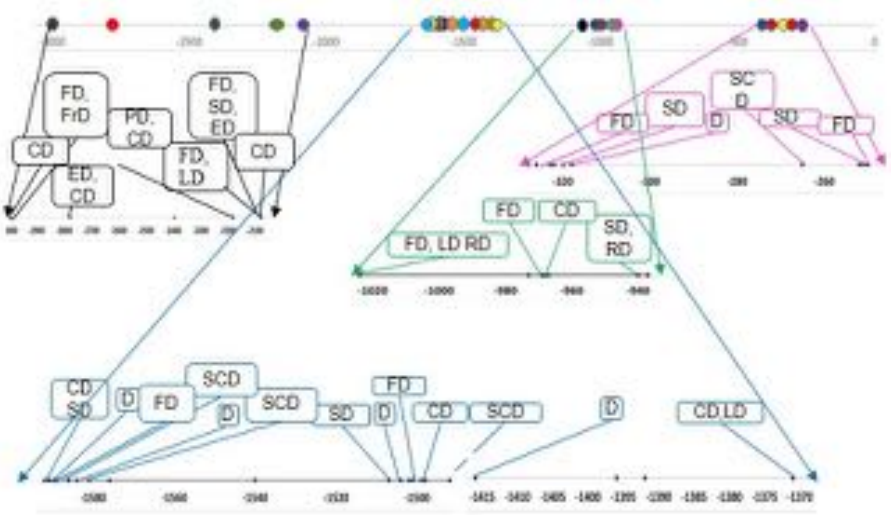

Figure 1: Predicted binding sites of TFs in the promoter region of the *S. tuberosum* Group Phureja *StBB* gene. Thin line represents the promoter region from the translation start site to -3000 bp. Round shapes in different colour represent predicted TF families: red, DOF; light blue, BBR-BPC; yellow, C2H2; green, M-type MADS; purple, MIKC-MADS; pink, B3; light green, LBD; grey, HB other; white, WRKY; orange, GRAS; dark green, ERF; black, RAV; brown, bHLH; red circle, MYB; squash, MYB-related. TFs involved in developmental processes are illustrated by boxes. The coloured arrows indicate zooming in the different regions of the promoter. Abbreviations: CD, cell development; D, development; ED, embryo development; FD, flower development; FrD, fruit development; LD, leaf development; RD, root development; SD, stem development; SCD, seed and seed coat development; PD, pollen development.

flower-, leaf-, stem- and root development or cell cycle regulation (Fig. 1).

Identification of the main CAREs in the StBB promoter

The binding sequences and locations of TFs with known functions are listed in Table 2. The BARLEY B RECOMBINANT/BASIC PENTACYSTEINE (BBR/BPC) is a plant-specific transcription factor family (Meister et al., 2004). BBR/BPC TFs bind to a GA-rich motif and this motif was found at around -1.5 kb in the *BB* promoter. BPC6 and BPC1, which bind to this motif, both respond to ethylene. In addition, BPC1 is involved in the regulation of plant development (PlantRegMap prediction).

The CACGTG motif representing the binding site of the basic helix-loop-helix (bHLH) family proteins was identified at the very distal end of the *StBB* promoter. The second largest class of the plant TFs, the bHLH fam-

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Table 2: Transcription factors with known functions binding to the *StBB* promoter

Family	Name	Position	Matched sequence	Function*
BBR-BPC	BPC6	-1589 -1540	ACTTTTTTCTCTCTCTCTCTC TTCATCTTCTCTCTCTCATGC	Response to ET
	BPC1	-1591 -1416 -1502	AGGAGAGAGAGAGAGAAAAAAGTG AAGAAAGGAAGAATAATAAAGAGA GAGAAAAAAGAAGAAGAAGAG	Response to ET, regulation of development
bHLH	PIF3	-2996	GTCCACGTGG	De-etiolation, GA and far-red light signalling, regulation of anthocyanin metabolism
	SPT	-2994	ACCACGTGG	Circadian rhythm, response to cold and red light, fruit and carpel development, negative regulation of seed germination
	PIF3	-2997	GGTCCACGTGGT	Response to CK, cell growth
C2H2	IDD1	-265	AATTAGAAGACAAAAAT	Regulation of GA signalling, seed germination and maturation
	REF6	-1371	GAAAACAGAGTG	Response to BR, cell growth, flowering, histone modification, leaf development
DOF	DOF1.5	-1584	AGAGAGAGAAAAAAGTGAAAA	Seed coat development
	DOF1	-323 -1581 -973 -1504	AAAAGGGAAAAGCAAAGAAAA GAGAGAAAAAAGTGAAAAAAA CTGAACAAAAAGGAAAACAAA AGGAGAAAAAAGAAGAAGAAG	Response to chitin
	DOF5.9	-320 -1507 -251	AACAAAAGGGAAAAGCAAAGA CAAAGGAGAAAAAAGAAGAAG AACCAAGCCAAAAAGGAAAAT	Phloem or xylem histogenesis
	OBP3	-1586 -318	TTCACTTTTTTCTCTCTCTCT TTTGCTTTTCCCTTTTGTTAA	Photomorphogenesis
	OBP1	-1498 -967	TTCTCTCTTCTTCTTCTTTTT ATTCCCTTTGTTTTCCTTTTT	Response to AUX and SA, cell wall modification, positive regulation of cell cycle
GRAS	GAI	-1589 -1492	GAGAGAGAGAGAGAAAAAAG AAGAAGAAGAGAGAACAACT	Regulation of N utilization, protein catabolism, seed dormancy and ROS, response to ET, ABA, SA, JA, far-red light and salt, negative regulation of GA signalling, seed germination, phloem transport
HB_other	KNAT1	-1592	TTTTCTCTCTCTCTCTCCTC	Cell fate specification, xylem and phloem development
LBD	LBD18	-940	ATTCTGCCGGTTTTTATGG	Xylem development, lateral root formation
M-type_ MADS	AGL20	-1590 -1501 -969 -326	CTTTTTTCTCTCTCTCTCTCC TCTCTTCTTCTTCTTTTTTCT TCCCTTTGTTTTCCTTTTTGT TTTTTTTCTTTGCTTTTCCCT	Translocation, response to cold and GA, positive regulation of flower development
MIKC_ MADS	PI	-250	GCCAAAAAGGAAAA	Specification of floral organ identity
	AGL15	-2078	CTTTCCACATTTAGGAATT	Somatic embryogenesis, negative regulation of SD photoperiodism and seed maturation, flowering, negative regulation of floral organ and fruit abscission, cellular response to AUX
	AG	-2080	CACTTTCCACATTTAGGAA	Leaf development, maintenance of floral organ identity
	AP3	-2077	TCCTAAATGTGGAAA	Specification of floral organ identity
MYB	MYB124	-2778	CGTAAACGCTCCACA	Embryo sac development, guard cell differentiation
MYB_ related	MYBL2	-1392	CACCTCCTTATCTTC	Response to salt, AUX, JA and Cd
RAV	RAV1	-1024	GTGGTAATTTCTGTTGA	Response to BR, negative regulation of flower development, leaf and lateral root development
WRKY	WRKY2	-2181	GGGTCAAC	Pollen development, longitudinal axis specification, establishment of cell polarity

*Abbrevations: ABA, abscisic acid; AUX-auxin; BR, brassinosteroid; Cd, cadmium; CK, cytokinin; ET, ethylene; GA, gibberellic acid; JA, jasmonic acid; N-nitrogen; ROS, reactive oxygen species; SA, salicylic acid; SD, short day

ily proteins are involved in ethylene and gibberellin signalling pathways and are identified as positive regulators of carpel and fruit development, light signalling, flavonoid biosynthesis, anthocyanin metabolic process and repression of seed germination (Feller et al., 2011).

The binding sites of C2H2 family zinc finger proteins including IDD1 and the RELATIVE OF EARLY FLOWERING 6 (REF6) were found at -265 bp and -1371 bp, respectively. IDD1 is involved in gibberellin signalling, seed germination and maturation (Feurtado et al., 2011), while REF6 responds to brassinosteroids and regulates the cell growth, flowering and leaf development (C. Li et al.,

2016).

Eighteen CAREs recognised by seven DOF TFs were predicted, however, some of them were overlapping and only five CAREs were unique. The predicted DOFs are involved in different biological processes. DOF1.5 is involved in seed coat development, DOF1 responds to chitin and DOF5.9 has a role in phloem or xylem histogenesis. The OBP-type DOF family TF, OB3, is involved in photomorphogenesis, while OBP1 is involved in cell wall modification and cell cycle regulation and respond to auxin and salicylic acid. The core sequence recognised by DOFs is the AAAG motif (Yanagisawa & Schmidt, 1999) and this motif or its reverse sequence CTTT was present in all predicted DOF family TF binding sites.

The GAc/gAAA core motif that previously proposed to be the binding site of the GRAS family proteins, which play role in nitrogen utilisation, hormone and red-light response, seed germination and dormancy (Hakoshima, 2018) is located at -1492 bp and -1589 bp upstream from the translation start site of the *StBB* gene. The HB-other and LBD family TFs, KNAT1 and LBD18, both play a role in xylem development. In addition, KNAT1 is important for cell fate specification, while LBD18 is involved in lateral root development (Liebsch et al., 2014; Lee et al., 2009).

The M_type_MADS TFs have four binding sites, while the MIKC_MADS TFs have only two binding sites in the *StBB* promoter as three out of the four predicted sites are overlapping. These TFs belong to the large group of TFs, the MADS-domain family. The MADS-domain proteins are involved in diverse plant developmental processes including embryogenesis, flower development, maintenance of floral organ identity, flowering time, response to cold and gibberellic acid (Theißen et al., 2016; Borner et al., 2000). According to PlantRegMap AGAMOUS like-20 (AGL20s) responds to cold and gibberellic acid and regulates flower development as well. AGAMOUS like-15 (AGL15) is found to be the regulator of somatic embryogenesis and negative regulator of short day photoperiodism, seed maturation, floral organ and fruit abscission. AG15 responds to auxin. AGAMOUS (AG) is involved in leaf development and maintenance of floral organ identity. The binding sites of AGL15 and AG are overlapping and are located at around -2 kb in the *BB* promoter.

Binding sites of MYB and MYB-related family proteins, which regulate various developmental processes and salt and drought stress responses (X. Li et al., 2019), were detected at two different sites in the *StBB* promoter region, at -2278 bp and -1392 bp. The CARE recognised by RAV1, which belongs to RAV TF family, was found at -1024 bp. RAV1 negatively regulates the flower, leaf and root development and responds to brassinosteroids (Hu et al., 2004).

Although WRKY is a large family of TFs it was represented only by WRKY2. The binding site at -2181 bp carries the characteristic GGTCAA motif found also in the WRKY2 binding site of tomato, a relative of potato (PlantRegMap prediction).

Expression of the StBB gene in different organs of S. tuberosum cv. 'Désirée'

Expression of *StBB* in root, stolon, tuber, stem, petiole, source- and sink leaf, petal, sepal and stamen is shown in Figure 2. Expression of *StBB* was detected in all organs tested. The highest level of expression was found in petal followed by the reproductive organ, stamen. *StBB* mRNA level in petal was 7.5-fold higher than in root and stem. Medium level of *StBB* expression was detected in stolon, tuber, source- and sink leaves and in sepal. The lowest *StBB* transcript levels were found in root and stem.

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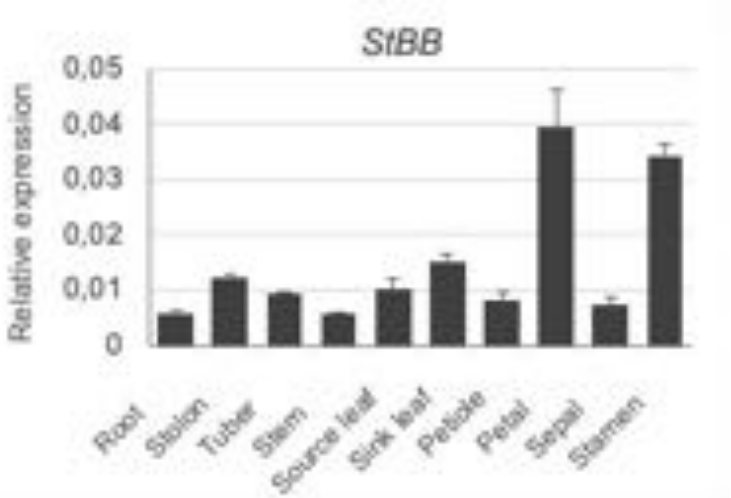

Figure 2: Expression profile of the *StBB* gene in different organs of *S. tuberosum* cv. 'Désirée' determined by RT-qPCR. Bars indicate mean relative expression values of *StBB* gene compared to the mean expression values of *ACTIN* and $EF1\alpha$ + SE ($n = 3$ technical replicates). Samples were collected from organs of 3-5 plants.

Discussion

Analysis of promoters and CAREs is important for genetic engineering of crops. In this study, CAREs in the *StBB* promoter of potato were identified. A 3-kb region upstream from the translation start site was tested *in silico* and 48 TF binding sites of 15 TF families were predicted. We assume that the 3-kb fragment carries all the important regulatory elements. This assumption is based for example, on the publication of Lang et al. (2007), who studied the promoter of the *SBgLR* gene in *S. tuberosum* and showed that a 2.3-kb DNA sequence upstream from ATG contains all regulatory motifs that are likely to be required for the high-level of gene expression, specifically, in pollen. In another study, it was demonstrated that the majority of the discovered common motifs in the promoters of *GLUCAN ENDO-1,3-BETA-GLUCOSIDASE* genes of *S. tuberosum* cv. DM 1-3 516 R44 are concentrated between +1 and -500 bp of the transcription start site (Kebede & Kebede, 2021).

In a previous study, Breuninger and Lenhard (2012) analysed a region located at 1035 bp upstream from the *BB* start codon for identification of upstream regulators that promote or inhibit *BB* expression in *A. thaliana*. Based on a promoter deletion assay by complementing of a *bb* mutant with the *BB* cDNA fused to the *BB* promoter region they found that with the exception of the distal 100 bp the other part of the fragment contains important positively acting promoter elements. Searching for binding sites for TFs in the PLACE database full matches were found to the AUXIN REPONSE FACTOR (ARF) binding site (TGTCTC) and to that of a MYB TF. However, using a luciferase assay it turned out that the ARF is most likely not functional in the *BB* promoter of *Arabidopsis*. Using the PlantRegMap prediction tool, we identified three TFs responding to auxin, namely OBP1, AGL15 and MYBL2. Besides of the MYB-related MYBL2, the MYB TF family protein MYB124 also was predicted to have a binding site in the *StBB* promoter at -2778 bp (Table 2). Nevertheless, additional experiments are needed to decide whether the predicted CAREs are functional or not in the *StBB* promoter.

In the current study, binding sites of 20 TFs

involved in developmental processes such as flower, fruit, leaf, stem and root development or cell cycle regulation were predicted to be located in the *StBB* promoter, which is in line with the expression of *StBB* gene in each tested organ, i.e., root, stolon, tuber, stem, source leaf, sink leaf, petiole, petal, sepal and stamen. Disch et al. (2006) also detected *BB* mRNA in all organs with highest amounts in proliferating tissues including shoot, root and floral meristems, vasculature, young organs and developing embryos of *Arabidopsis*. Disch et al. (2006) examined not only the *BB* expression level in *Arabidopsis* but also tested a series of genotypes that expressed increasing amounts of *BB* mRNA from the endogenous promoter ranging from 0% to 600% of the wild-type level and concluded that *BB* is both necessary and sufficient to limit *Arabidopsis* floral organ size, floral biomass accumulation and stem thickness. We detected the highest level of *StBB* expression in petals followed by the reproductive tissue, stamen. Thus, we hypothesise that the function of *StBB* in potato may be similar to that found for *BB* in *Arabidopsis*, i.e., restricting organ overgrowth and especially, the overgrowth of petal and stamen.
On the basis of current research of *in silico* and expression analysis, *StBB* could be a promising target for potato crop improvement as repression of *StBB* may result in accelerated plant growth and early tuber bulking. A future analysis of *StBB* will be required to understand how the level of *StBB* expression is determined and how *StBB* influences organ growth at the molecular level.

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