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Khongorzul Odgerel

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Molecular genetics and metabolomic studies related to tuberization and metabolite composition of cultivated potatoes

Khongorzul Odgerel

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Doctoral School: Doctoral School of Plant Science

Name: Khongorzul Odgerel

Discipline: Plant biotechnology

Head of Doctoral School: Lajos Helyes DSc
Professor, Doctor of the Hungarian Academy of Sciences
MATE, Director General of Gödöllő Campus

Supervisor: Zsófia Bánfalvi DSc
Group leader, Doctor of the Hungarian Academy of Sciences
MATE, Institute of Genetics and Biotechnology

Co-supervisor: Anikó Veres Dr
Doctor, Associate Professor
MATE, Doctoral School of Plant Science

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Approval of School Leader Approval of Supervisor

.....

Approval of Co-supervisor

ABBREVIATIONS

ABA: abscisic acid
ANOVA: analysis of variance
BAP: benzylamino-purine
CARE: *cis*-acting regulatory elements
CIM: callus induction medium
CD: cell development
dNTP: deoxynucleotide triphosphate
ED: embryo development
FD: flower development
FrD: fruit development
GC-MS: gas chromatography-mass spectrometry
HP: Hópehely
IPTG: isopropyl β-D-thiogalactoside
LB: Luria broth medium
LD: leaf development
LD: long day
MS: Murashige-Skoog medium
MSTFA: N-methyl-N-(trimethylsilyl)-trifluoroacetamide
NAA: naphthyl acetic acid
PCA: principal component analysis
PD: pollen development
PEG: polyethylene glycol
PlantRegMap: The Plant Transcriptional Regulatory Map Platform
PLS-DA: partial least squares discriminant analysis
RD: root development
RM: rooting medium
RT: retention time in minutes
RT-PCR: reverse transcription PCR
RT-qPCR: reverse transcription-quantitative PCR
SIM: shoot induction medium
SOC: super optimal broth medium
SCD: seed and seed coat development
SD: short day
SD: stem development
TIC: total ion chromatogram
WL: White Lady
YEB: yeast extract beef medium
X-Gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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1. INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the most consumed food crops in the world. According to the Faostat, average potato consumption per capita in the world reached 32.3 kg in 2018 and it is 10.2% more than 10 years ago. Potato tuber is a source of great amounts of carbohydrates, proteins, as well as minerals and vitamins (B6 and C) and it is always considered to be a staple food.

With worldwide increasing demand, sustainable productivity is becoming a challenging issue of potato breeding because potato productivity is limited in many environmental conditions by its sensitivity to biotic and abiotic stresses such as pests, elevated temperature, drought, frost and salinity. Thus, early tuberization is an advantage because early tuberizing potatoes can complete their life cycle before stress becomes a serious constraint.

Tuber is formed from differentiation of the stolon tissue under tuber-inducing condition. The tuberization process takes place alongside with other growth or developmental processes in the plant such as foliage development, leaf expansion, flowering and sprout development. It involves the transport of biomolecules from the source organ (mature leaves) to the sink organ, the developing tuber. It is known that the source-sink relationship, which is influenced by various environmental factors, determines tuber development and tuber yield. It has not been known, however, how the canopy as a source can influence the metabolite composition of tubers and vice versa; does the developing tuber influence the metabolite composition of the source, the mature leaves? Thus, the first aim of our study was to answer these questions.

In *Arabidopsis* and other plant species, the ubiquitous gene *GIGANTEA* (*GI*) is involved in flowering, circadian clock control, chloroplast biogenesis, carbohydrate metabolism, stress responses, and volatile compound synthesis. In potato, only its role in tuber initiation has been demonstrated. However, based on findings in other plant species, we hypothesised that the function of GI in potatoes is not restricted only to tuberization. Thus, the second aim of our study was to test this hypothesis.

The protein encoded by the *BIG BROTHER* (*BB*) gene is referred as an ENHANCER1 OF DA1 (EOD1) which restricts cell proliferation and represses the organ growth. Since we found a positive correlation between the rate of canopy development and the time of tuber initiation repression of *BB* gene was considered to be a promising candidate for increasing leaf size and

influencing tuberization in potato. Thus, the third aim of our work was the suppression of the *BB* gene expression in potato.

2. OBJECTIVES TO ACHIEVE

1. Unravel the influence of vegetative organs on the primary polar metabolite composition of potato tubers
2. Unravel the effect of tuberization on the primary polar metabolite composition of leaves
3. Characterize the *GIGANTEA* (*GI*) and *BIG BROTHER* (*BB*) genes in *Solanum tuberosum* L. cv. 'Désirée'
   - study the organ-specific expression of the two *GI* genes, *StGI.04* and *StGI.12*, and *StBB*
   - study the effect of stress treatments on the expression of the two *GI* genes, *StGI.04* and *StGI.12*, and *StBB*
   - repress the expression of *StGI.04* and *StBB*
   - evaluate the effect of *StGI.04* and *StBB* repression on plant morphology and tuberization

3. LITERATURE REVIEW

3.1. Origin and importance of potato

The tuber crop potato (*Solanum tuberosum* L.) belongs to the Solanaceae family of flowering plants. Tuber formation is a unique developmental process, where stolon originates from the base of the main stem and continues to grow horizontally, swells under the inductive conditions and eventually develops into a mature tuber with many dormant axillary buds known as tuber "eyes". After a period of dormancy, tuber eyes sprout and develop to a new plant. Hence, tubers serve the dual role of storage organ as well as vegetative propagation system (XU et al. 1998).

Potato originated and was first domesticated in the Andes Mountain of South America. Currently, potatoes are grown in over 180 countries. In 2019, more than 17 million hectares of potatoes were harvested worldwide (https://www.statista.com). Potato is the fourth most important food crop in the world after rice, wheat and maize in terms of human consumption (https://www.fao.org/). According to the FAOSTAT, the world potato production is estimated at 388 million tons and an average potato consumption per capita reached 32.3 kg in 2018. Potato production provides not only food, also employment and income as a cash crop (SCOTT et al. 2000) and helps in increasing food availability, while contributing to a better land use ratio by raising the aggregate efficiency of agricultural production systems (GASTELO et al. 2014). From 1997 to 2007, the potato cultivation in developing countries increased by 25% due to high demand (https://cipotato.org).

Potato is considered to be an important supplier of beneficial components such as vitamins, proteins, minerals and carbohydrates, which benefit human body as nutrients supplementary and antioxidants (KING and SLAVIN 2013; BURLINGAME et al. 2009). For instance, 100 g baked white potato provides 390 kJ (93 kcal) energy mainly from carbohydrates, and very little of which is from fat and proteins. Currently about 2% of the world's dietary energy is achieved from potatoes (ZAHEER and AKHTAR 2016). Nutrient content depends on a number of factors, with variety being among the most important.

Potato biodiversity is vast, with more than 4000 known varieties (BURLINGAME et al. 2009). Cultivated potatoes are highly heterozygous due to their outbreeding nature. It is known that polyploids are biologically interesting because of their complexities as well as because they are economically important in many cases as major crops (DA SILVA and SOBRAL 1996). Modern

potato cultivars are tetraploids (2n = 4x = 48) and due to this genetic heterogeneity, the large number of target traits and the specific requirements of commercial cultivars, potato breeding is challenging (SLATER et al. 2014). However, many current commercial cultivars suffer from a number of production and quality issues. Therefore, productivity is a key issue of potato breeding. The productivity, however, depends on stress sensitivity of plants and potato is considered to be a sensitive crop for adverse condition that significantly reduces yield. Thus, there has been numerous research aiming to understand the physiological, biochemical and genetic basis of potato plant for improving potato production. Recent significant advances in molecular genetics and the analysis of highly complex quantitative traits can be exploited by potato breeders to accelerate genetic gains, thus enabling more improvements in potato cultivars.

3.2. Metabolite composition of potato tubers

Potatoes serve as a substantial portion of the world's subsistence food supply and their nutritional status depend on their metabolic composition. Potato tubers are approximately 80% water and 20% solids and this can vary by cultivar (PILLAI et al. 2013). Potato tubers contain small amount of simple sugars and great amount of complex carbohydrates and proteins, as well as minerals and vitamins and other secondary metabolites which have roles in tuberization and responses to the environment. Since potato has a vast biodiversity with over 5,000 different cultivars and more than 100 wild potato species (BURLINGAME et al. 2009), vast amount of phytochemical studies have been investigated the metabolite composition of tubers using diverse techniques and methods. For instance, several studies examined the phytochemical diversity in tubers of different cultivars using a gas chromatography-mass spectrometry (GC-MS), ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS), inductively coupled plasma mass spectrometry (ICP-MS) and determined polar and nonpolar nutrients including amino acids (alanine, asparagine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, etc.), organic acids (caffeic acid, citric acid, 2,3-dihydroxypropanoic acid, fumaric acid, etc.), sugars (fructose, glucose, sucrose, polysaccharides, etc.), fatty acids (linolenic acid, palmitic acid, stearic acid, etc.) and sugar alcohols e.g., mannitol, sorbitol, xylitol, lactitol and maltitol (DOBSON et al. 2010; URI et al. 2014; CHAPARRO et al. 2018; FUKUDA et al. 2019; CLAASSEN et al. 2019; ODGEREL and BÁNFALVI 2021).

Baked potato with skin provides a greater percentage of nutrients than peeled potato. In general, 100 g of baked potato (97 calories) contains 15% of the recommended daily amounts of vitamin B6, 16% of potassium, 9% of magnesium, 6% of iron, and 4% of pantothenic acid (GIBSON and KURILICH 2012; NAVARRE et al. 2016). Among the different genotypes of potato tubers, yellow-fleshed cultivars have the highest level of vitamin C and pigmented potato genotypes have significantly higher total phenolic content and antioxidant activity (KÜLEN et al. 2013).

Potato tuber also contains bioactive compounds including polyphenolics (e.g., chlorogenic acid, methylumbelliferones, and the flavonoids apigenin, rutin, and kaempferol 3-O-rutinoside), terpenes (e.g., the carotenoids lutein and neoxanthin), polyamines (e.g., kukoamines), and alkaloids (e.g., calystegines, solanine, chaconine, tomatine), amides (e.g., oleamides), coumarins (e.g., 4-methylumbelliferone), which have demonstrated activity against cancer (PICCIONI et al. 2012; CHAREPALLI et al. 2015), heart disease (HUANG et al. 1988; AHMAD et al. 1993), diabetes (HABTEMARIAM 2011; ZHOU et el. 2012) and obesity (MIYASHIT et al. 2007).

The two main glycoalkaloids in tuber, α-solanine and α-chaconine, are to protect plants from the stresses of other organisms and they are reported to be toxic to central nervous and digestive system of animals and humans (FRIEDMAN et al. 1997). HA et al. (2012) showed that matrix-assisted laser desorption/ionization mass spectrometric imaging (MALDI-MSI) tools are suitable to detect steroidal glycoalkaloids in potato tuber tissues. Later, WAN et al. (2022) demonstrated that electromembrane extraction (EME) combined with liquid chromatography coupled with mass spectrometry (LC-MS/MS) has a great potential to extract and purify these toxic compounds from tubers. Moreover, nuclear magnetic resonance (NMR) analysis can reveal the almost entire metabolome of potato tuber. DO PRADO APPARECIDO et al. (2022) characterized the metabolites from aqueous extract of potato, cassava, sweet potato, taro and yam, and found that the amino acids (alanine, arginine, asparagine, phenylalanine, glutamine, histidine, isoleucine, tyrosine, threonine, tryptophan, and valine), organic acids (*γ*-aminobutyrate, malate), and other compounds (allantoin, choline) were present in the highest level in potato tubers.

At the very early stages of tuberization, the apoplasmic mode switches to a symplasmic unloading transport mode mediated by plasmodesmata. This transition is accompanied by reduced cell wall invertase and increased sucrose synthase activities (LI and ZHANG 2003). The increase in sucrose synthase activity correlates with the accumulation of starch in storage organs of many

plant species (STEIN and GRANOT 2019). The sucrose degradation leads to starch biosynthesis in plastids. The average starch content of potato is 10-18%. Potato starch is an essential source of energy in a well-balanced diet, but it also fulfils a functional role in various food and non-food applications. It consists of two α-(1,4)-d-glucose polymers, lightly branched amylose (18%-21% of starch dry mass) and highly branched amylopectin (79%-82% of starch dry mass) (GOMAND et al. 2010). In sprouting tubers, the starch is degraded by the combined actions of amylases, starch phosphorylases and debranching enzymes and the resulted sugars are used as an energy source for the new generation of potato plants (reviewed by SONNEWALD and SONNEWALD 2014).

3.3. Regulation of potato tuber development

Potato is usually propagated using seed tubers. Seed tubers produce sprouts in their eyes, which develop into shoots. The stems, foliage, stolons, roots, inflorescences and the next generation of tubers are formed from these shoots (STRUIK 2007). In tuber-inducing condition, e.g. short day (SD), the stolons ceases to grow further and the tip begins to swell to form the tuber. This process is called tuberization (JACKSON 1999). HANNAPEL et al. (2007) divided the tuberization process into the following phases: sprout development, plant establishment, tuber initiation, tuber bulking, and tuber maturation. Timing of these growth stages and tuber size varies depending upon genetic factors as well as environmental factors such as temperature, photoperiod, soil type, availability of moisture, and storage conditions of seed tubers. Tuberization is often considered to be the most sensitive stage that limits climate-associated geographical distribution and actual yield of potato. Hence, an understanding of the tuberization process is even more important in the face of the changing global climate (DUTT et al. 2017).

With grafting experiments, researchers found that after perception of inductive photoperiodic conditions in leaves, some sort of signals are produced and transmitted from scion to the underground stolons (reviewed by EWING and STRUIK 1992). Leaves are source organs, while stolons and tubers are considered as sink organs. This source-sink relationship determines the tuber development, which is regulated by various factors including biomolecules, and environmental factors (ZIERER et al. 2021). LI et al. (2016) reported that well-watered (90% soil water content) condition combined with sufficient nitrogen (0.2 g N kg^{-1} added to soil) caused the increasing yield mainly by enhancing the source capacity (total leaf area and leaf life span) of plants. Besides, photoperiod-dependent pathways and temperature effects are crucial to the tuberization process

(LAFTA and LORENZEN 1995; MARTÍNEZ-GARCÍA et al. 2001). In particular, SD, cool temperature, and low rates of nitrogen supply promote tuberization, whereas tuber formation is delayed by long days (LDs), high temperature, and low carbon:nitrogen ratio in leaves (RODRÍGUEZ-FALCÓN et al. 2006).

Similar to flower induction, tuber formation is affected by phytohormones. High levels of gibberellic acids (GAs) are correlated with the inhibition of tuberization, whereas low levels are associated with induction both at the site of perception (the leaf) and in the target organ, the stolon apex. In addition, GA levels have an important role in the short-term adaptive responses of potato plants to photoperiods. Abscisic acid (ABA) and sucrose also can influence tuberization as ABA stimulates tuberization by counteracting GA, while sucrose regulates tuber formation by influencing GA levels (MARTÍNEZ-GARCÍA et al. 2001; XU et al. 1998). Cytokinins (CKs) have been considered to favor tuberization under inductive conditions; tuber initiation needs the CKs for the inhibition of cell elongation and the promotion of lateral growth or swelling as well as starch accumulation. Furthermore, transformation of stolon cells to tuber cells may also be facilitated by the presence of CKs (reviewed by HANNAPEL 2017 and DUTT et al. 2017). Besides of these, cyclopentenone (10-oxo-11-phytodienoic acid) produced by 9-LIPOXYGENASE (9-LOX) activity in young tubers, can be the precursor of tuberonic and jasmonic acids (JAs) which regulate the tuber growth (HAMBERG 2000). Nevertheless, phytohormones alone are not sufficient to facilitate efficient development of tuber and their action is influenced by assimilates, primarily by sucrose (reviewed by ZIERER et al. 2021).

The effects of biomolecules including proteins, genes and RNAs regulating or associated with tuberization process were widely studied in the past decades. As it is described above, tuberization is controlled by phloem-mobile signals that arise from the leaf. BATUTIS and EWING (1982) demonstrated that the photoreceptor PHYTOCHROME B (PHYB) is involved in tuber induction in relation with photoperiod. Later, JACKSON et al. (1998) showed that reduced levels of *StPHYB* in transgenic antisense *S. tuberosum ssp. andigena* plants enabled them to tuberize in both SD and LD conditions. Thus, they concluded that the StPHYB protein plays a role in inhibiting tuberization in LDs in *S. tuberosum ssp. andigena*, a strict SD plant for tuberization. Further work revealed that in addition to StPHYB, StPHYF plays an essential role in the LD-mediated suppression of tuberization by forming a complex with StPHYB (ZHOU et al. 2019).

Of the several transcription factors (TFs) in the TALE superclass (proline–tyrosine–proline loop), the two main groups in plants are the KNOTTED-LIKE HOMEOBOX (KNOX) and BELL (BEL) types. BEL1 and KNOX TFs have been shown to interact in a tandem complex to regulate the expression of target genes. In potato, StBEL5 and its KNOX protein partner designated as POTATO HOMEODOMAIN 1 (POTH1) regulate tuberization by targeting genes that control growth. The heterodimer of StBEL5 and POTH1 binds to a tandem TTGAC-TTGAC motif that is essential for regulating transcription (CHEN et al. 2004). SHARMA et al. (2014) demonstrated that *StBEL5* has transcripts that move long distances in the plant and enhance tuberization and root growth. Transport of the *StBEL5* mRNA is facilitated by RNA-binding proteins of the POLYPYRIMIDINE TRACT-BINDING PROTEIN (PTB) family, which bind to the 3′ UTR (untranslated region) of the mRNA and this binding allows its movement and increases RNA stability (CHO et al. 2015). Besides of StBEL5, StBEL11 and StBEL29, which in contrast to BEL5 suppress tuberization, are also phloem mobile as well as *POTH1* mRNA, which is prevented also by PTB (GHATE et al. 2017).

Photoperiodic control of tuberization and flowering regulation are partially the same. Under favorable conditions, a florigen or tuberigen is produced in the leaf vascular bundles and transported to the shoot apical meristem or to the underground stolon tips to induce floral or tuberization transition, respectively. The major florigen was confirmed to be the FLOWERING LOCUS T (FT), a small globular protein, while a FT-like paralog, a member of the SELF-PRUNING family (SP), *StSP6A* was found to be the major component of tuberigen (NAVARRO et al. 2011). FT interacts with the leucine zipper (bZIP) TF, FD, via a 14-3-3 protein to form the florigen activation complex (FAC) whereas a FD-like TF and a 14-3-3 protein together with StSP6A form the tuberigen activation complex, TAC (TEO et al. 2017). The FD protein is also shown to interact with the flowering repressor TERMINAL FLOWER-1 (TFL1), homologous to CENTRORADIALIS (CEN), to form a transcriptional inhibitory complex repressing the same floral identity genes that are induced by FT and, as a paralog of it, StCEN blocks activation of the *StSP6A* gene by StFD-like in potato (ZHANG et al. 2020).

Another SP family member, StSP5G, shows opposite role to that of StSP6A in tuberization. CONSTANS (CO) is reported to play roles in regulation of both flowering and tuberization. Potato plants overexpressing *StCO* tuberize later than wild-type under a weekly inductive photoperiod. In addition, StCO affects the mRNA levels of *StBEL5* and *StSP6A*. CYCLING DNA-BINDING

WITH ONE FINGER 1 (cycling DOF1; CDF1), MADS box and ABA RESPONSIVE ELEMENT-BINDING FACTORs (ABFs) TFs have also been shown to be involved in the regulation of tuberization. StCDF1 regulates tuberization and plant life-cycle length by acting as a mediator between the circadian clock gene *GIGANTEA (GI)* and *StSP6A*. StCDF1 down-regulates *StCO1/2,* which suppresses the transcription of *StSP5G*, enabling expression of the mobile *StSP6A* signal and resulting in the induction of tuber development at the stolon termini. Expression of *StCDF1* and *StSP6A* is regulated by StBEL5 (reviewed by KONDHARE et al. 2020). Recently, it was found that *StCDF1* has its own natural antisense transcript (*StFLORE*) with antiphasic gene expression over the circadian cycle, while in turn *StFLORE* is regulated by StCDF1. Furthermore, *POTH1* is also regulated by StCDF1 indicating a complex regulatory circuitry of tuber development (RAMÍREZ GONZALES et al. 2021).

Overexpression of *StSP6A* gene in potato results in impaired shoot growth but accelerated tuberization. Tubers of these plants promote the formation of new daughter tubers instead of sprouts, indicating that StSP6A has a strong sink-forming capacity and transports assimilates more efficiently to belowground sinks compared to wild-type plants (LEHRETZ et al. 2019; 2021). It was also shown that StSP6A interacts with SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTER 11 (StSWEET11) in phloem companion cells of stem and stolon and blocks sucrose efflux into the apoplasm contributing thereby to increased sucrose delivery toward the stolon (ABELENDA et al. 2019). Nevertheless, modelling the physiological relevance of sucrose export showed that the nature of this effect is non-linear (VAN DEN HERIK et al. 2021).

CHINCINSKA et al. (2008) studied the role of sucrose transporter SUT4 in potato and found that StSUT4 controls circadian gene expression potentially, by regulating sucrose export from leaves. Furthermore, it affects the clock-regulated genes such as *StFT, StCO* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*StSOC1*). It was also shown that inhibition of *StSUT4* led to decreased length of internodes, early flowering and early tuberization and higher tuber yields (CHINCINSKA et al. 2008; 2013). Another sucrose transporter, StSUT1, is expressed both in loading phloem of leaves and sink tubers and it is essential for long-distance transport of sucrose and tuber development acting as a phloem exporter transferring sucrose from the sieve elements into the apoplasm (KÜHN et al. 2003).

MARTIN et al. (2009) observed that the level of micro-RNA172 (StmiR172) in potato was higher under tuber-inducing SD than under non-inductive LD. Plants overexpressing *StmiR172*

showed increased levels of *StBEL5* mRNA. They identified and cloned a potato *APETALA 2-LIKE* (*AP2-like*) gene, *RELATED TO APETALA2 1* (*StRAP1*), that contains a *StmiR172* target site. An inverse correlation between the abundance of *StRAP1* transcript and *StmiR172* in several organs of wild-type plants and down-regulation of *StRAP1* in *miR172*-overexpressing leaves and plants, in which *StPHYB* was silenced, was observed. These observations suggest that this miRNA induces the degradation of *StRAP1* mRNA. It was concluded that StmiR172 acts downstream of the tuberization repressor PHYB and upstream of the tuberization promoter BEL5 to regulate tuber induction and it is also a long-distance regulator of tuberization (MARTIN et al. 2009). Interestingly, miR172 is controlled by the phloem mobile micro-RNA156 (StmiR156), which determines the transition from juvenile to adult developmental stages. Overexpression of StmiR156 in potato caused severe morphological alterations and did not support underground tuber formation. Instead, it induced the formation of aerial stolons and tubers from axillary meristems under SD condition. These results led to the hypothesis that StmiR156 acts as a negative regulator of tuberization in leaves by suppressing StmiR172, but may have an additional function in stolons (BHOGALE et al. 2014; KONDHARE et al. 2021).

DUTT et al. (2017) proposed a model for overall molecular regulation of potato tuberization under SD condition (Fig. 1). In this figure, all the main biomolecules involved in potato tuberization process: negative regulators StPHYB, StSUT4, StRAP1, StCO, StSP5G and positive regulators StSP6A, POTH1, StBEL5, StmiR172, StCDF1 and plant growth regulators ABA, CK, JA are included.

Eventually, potato tuberization is a complex process that integrates endogenous and environmental signals to ensure proper timing of organ formation. While a number of regulatory metabolic and hormonal responses, and protein–protein interactions that regulate potato tuberization have been described, a detailed spatial and temporal analysis of their cell and tissue specificity is required.

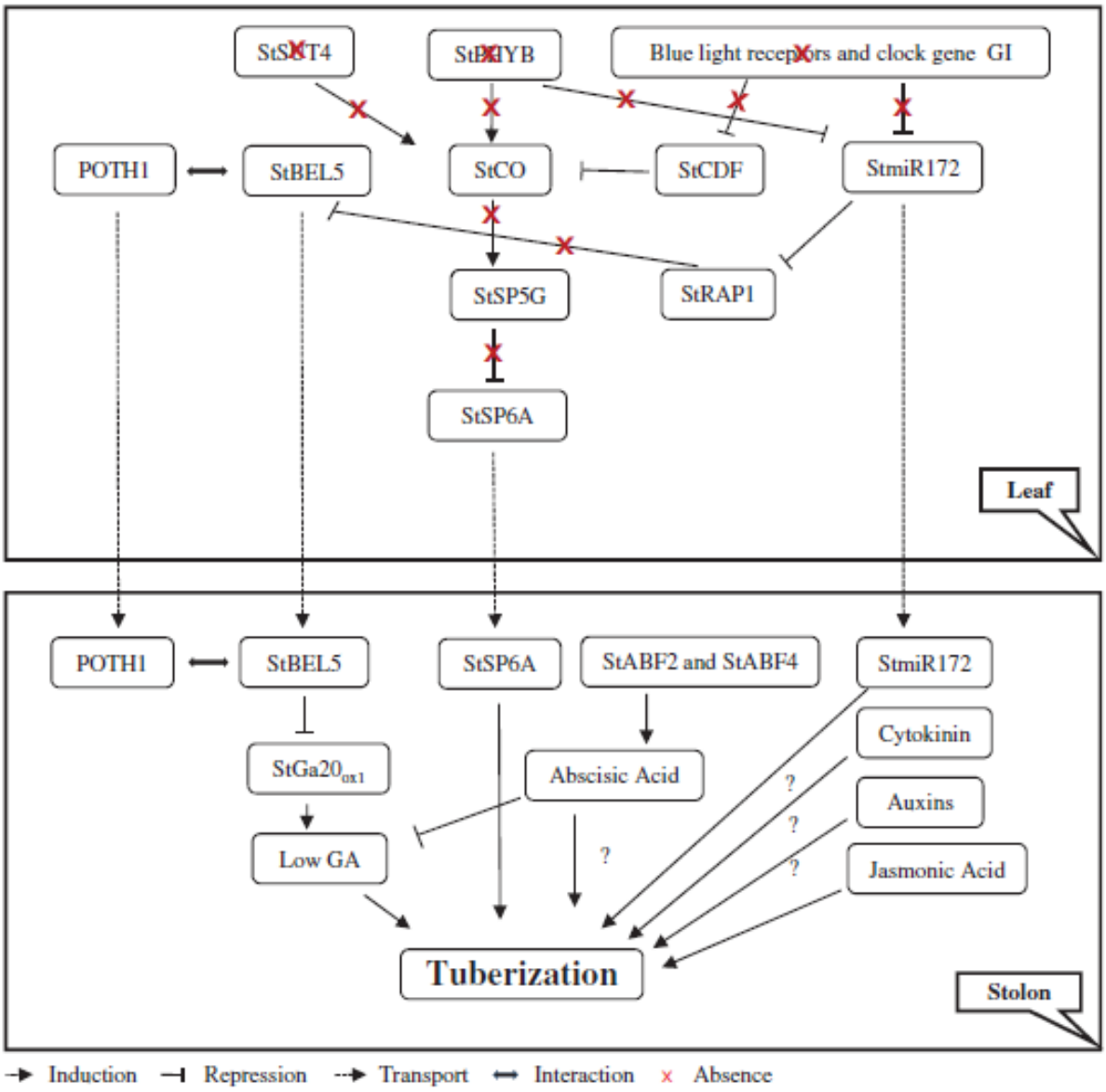


Figure 1. Molecular regulatory networks of potato tuberization under short-day condition (copied from DUTT et al. 2017)

3.4. Function and regulation of the *GIGANTEA* (*GI*) gene in plants

GIGANTEA (GI) is a plant-specific, circadian clock-regulated, nuclear protein. The most extensively studied physiological function of GI is in flowering. It was shown in several plant species that GI regulates flowering time through the photoperiodic pathway. It is indicated by the *gi Arabidopsis* mutants that GI acts in the flowering pathway because *gi* mutants flower late under LD conditions. GI forms a complex with the FLAVIN-BINDING KELCH-REPEAT F-BOX 1

(FKF1) protein and up-regulates the expression of *CONSTANS* (*CO*) by degrading the *CO*-repressor, CYCLING DOF FACTOR 1 (CDF1). CO measures the duration of daytime and activates *FLOWERING LOCUS T* (*FT*), encoding the mobile peptide florigen, and *TWIN SYSTER OF FT* (*TSF*) under LD conditions (reviewed by MISHRA and PANIGRAHI 2015). Recently, it has been shown that the GI-FKF1-CDF1-CO module is employed even by mango in regulating its temperature dependent flowering (PATIL et al. 2021).

GI is involved in circadian clock control, while the expression of *GI* itself is regulated by the circadian clock and peaks 8-10 hours after dawn. GI interacts with several clock proteins. It also functions in the process of light signalling. It appears to be a positive regulator of *PYTOCHROM B* (*PHYB*) signalling. The *gi* mutant *Arabidopsis* seedlings possess long hypocotyls under blue light indicating that GI has a role also in the blue light signalling. Furthermore, GI functions in chloroplast biogenesis and chlorophyll accumulation; the *gi* mutants are characterised by increased chlorophyll level. GI has a direct connection with the sucrose metabolism. Increased starch content was observed in *Arabidopsis* and rice *gi* mutants. GI interacts with TREHALOSE-6-PHOSPHATE SYNTHASE 8 and this interaction may have a direct influence on the carbohydrate metabolism (reviewed by BRANDOLI et al. 2020).

Genetic mapping identified the *GI* locus on chromosome 1 in *Arabidopsis*. DNA sequencing revealed that the *GI* gene consists of 14 exons and encodes for a protein of 1,173 amino acids. Besides of flowering and circadian clock control, GI has pleiotropic functions owing to its involvement in diverse processes such as hypocotyl elongation, vegetative growth, chlorophyll accumulation, sucrose- and hormone signalling, starch accumulation, transpiration, herbicide-, cold- and drought tolerance, miRNA processing and floral scent emission (reviewed by MISHRA and PANIGRAHI 2015; JOSE and BÁNFALVI 2019; BRANDOLI et al. 2020).

GI functions in conferring salt and freezing tolerance to *Arabidopsis* and *Brassica nigra*. In contrast, in *Brassica rapa* and poplar plants, down-regulation of *GI* enhances salt tolerance. *GI* expression is induced in response to drought stress and in combination with miRNA172 suppresses *WRKY44*, a TF participating in sugar metabolism. GI plays an inhibitory role during oxidative stress by downregulating the expression of *SUPEROXIDE DISMUTASE* (*SOD*) and *ASCORBATE PEROXIDASE* (*APX*) (reviewed by JOSE and BÁNFALVI 2019). The latest results show that GI influences not only the abiotic stress responses but is involved also in response to pathogen defence

and it confers susceptibility to plants during spot blotch attack by regulating the salicylic acid signalling pathway (KUNDU and SAHU 2021).

BRANDOLI et al. (2020) reported a novel role of GI in *Petunia hybrida*. Flowers of the *GI* silenced lines emitted 20% less volatiles on fresh weight basis over 24 hours and showed changes in the scent profile. The relative abundance of the *trans*-cinnamic acid derivatives, whose precursor is phenylalanine, showed alterations especially in the morning.

The rhythmic pattern of *GI* expression is altered in the *EARLY FLOWERING 3* (*ELF3*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) mutants as well as in *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) overexpressing *Arabidopsis* plants (FOWLER et al. 1999). The activities of clock associated proteins TIME FOR COFFEE (TIC), LIGHT-REGULATED WD1 and 2 (LWD1 and LWD2) are required for the repression of the *GI* transcription in the morning (HALL et al. 2003; WU et al. 2008). Furthermore, the PSEUDO RESPONSE REGULATORS (PRRs) are also involved in regulation of *GI* expression (NAKAMICHI et al. 2007; KAWAMURA et al. 2008).

LU et al. (2012) demonstrated that CCA1 represses the *GI* expression by binding to the *GI* promoter. The CCA1 binding motif is closely related to the so-called EVENING ELEMENT (EE) detected in the promoters of those genes, which are expressed late in the day. The consensus sequence of EE is AAAATATCT (HUDSON and QUAIL 2003). This motif is also recognised by the MYB-related TFs including LHY and REVEILLE 8 (REV8) (ALABADI et al. 2002; RAWAT et al. 2011). BERNS et al. (2014) identified three EEs in the *Arabidopsis GI* promoter located between -1.5 and -1.25 kb upstream to the translation start site. In cold-responsive promoters, EEs are often present together with ABA RESPONSE ELEMENT LIKE (ABREL) elements with the core sequence of ACGTG (MIKKELSEN and THOMASHOW 2009). BERNS et al. (2014) demonstrated that ABRELs are present also in the *Arabidopsis GI* promoter in three copies between -1.8 and -1.5 kb and contribute to the light inducibility of *GI* transcription. Thus, it was concluded that EEs and ABRELs are essential in combination to confer a high amplitude diurnal pattern of *GI* expression in *Arabidopsis*. The night time repression of *GI* transcription is attributed to the evening complex constituting of ELF3, ELF4 and LUX ARRHYTHMO (LUX) at the LUX binding site with the core sequence of GATACG (NUSINOW et al. 2011; HELFER et al. 2011). Besides light, *GI* expression is also regulated by temperature as warmer temperature of 28°C up-

regulates *GI* transcript level in comparison to the cooler temperature of 12ºC (PALTIEL et al. 2006).

GI genes have been detected and isolated from several plant species from monocots to dicots and found to show diurnal cycle of expression and conserved function in terms of flowering time determination and circadian clock regulation (reviewed by MISHRA and PANIGRAHI 2015). *GI* has ubiquitous expression in all the organs and several plant tissues including vascular bundles, mesophyll, apical shoot meristem and root and in all stages of plant growth, however, at different levels (FOWLER et al. 1999; LUO et al. 2011; SAWA and KAY 2011; TANG et al. 2017).

In potato, involvement of *GI* in initiation of tuberization was demonstrated by KLOOSTERMAN et al. (2013). According to the proposed model based on studying the wild Andean landrace *S. tuberosum* Group Andigena, a strict SD plant for tuberization, GI influences tuber formation as a partner of FKF1. Like in *Arabidopsis*, the GI-FKF1 complex can bind StCDF1 and target it for degradation by the proteasome. Since StCDF1 induces the transcription of *SELF-PRUNING 6A* (*SP6A*), GI is an indirect repressor of tuberization. Recently, searching for the *A. thaliana GI* homologue, KARSAI-REKTENWALD et al. (2022) found two *GI* transcript variants in potato with 83.73% identity located on chromosome 4 and 12 and designated them *StGI.04* and *StGI.12*. *In silico* characterisation and expression analysis of the two genes revealed that their regulation, at least in part, is different in the potato cultivar "Désirée".

3.5. Function and regulation of the *BIG BROTHER (BB)* gene in plants

During leaf development, the size is controlled by an organ-wide mechanism that coordinates the cell proliferation with cell expansion (HORIGUCHI 2006). 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. 2020). 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. 2020).

BIG BROTHER (BB) is a RING finger protein, which 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. (2017) 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 and 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.

3.6. Effects of abiotic stresses on potato

As one of the major food crops, enhancing potato productivity is important for food security of an increasing population. However, abiotic and biotic stresses including extreme temperature, drought, soil salinity, insects and diseases, are always considered to be the negative impacts for sustainable production. Abiotic factors are fundamental components of the environment and affect many aspects of plant physiology and cause widespread changes in cellular processes. Many of the changes are adaptive responses that lead to increased stress resistance and are therefore potential targets for crop improvement (reviewed by ZHANG et al. 2022).

The inadequate rainfall, excessive levels of salts in the soil, limited fresh-water resources lead to drought stress that prevent plant growth and productivity in many regions all over the world. Drought affects plant photosynthetic processes at the canopy, leaf or chloroplast level and sink organs. Globally, drought will decrease potential potato yield by 18-32% in the projected period of 2040-2069 (reviewed by OBIDIEGWU et al. 2015).

KONDRÁK et al. (2012) investigated the responses in leaves of *Solanum tuberosum* cv. 'White Lady' and yeast *trehalose-6-phosphate synthase* (*TPS1*) transgenic lines under the condition of 70% and 30% soil water content, respectively. They detected in total of 379 genes with altered expression including some TF genes and level of fructose, galactose, glucose and starch were changed in leaves under drought stress.

Polyethylene glycol (PEG) is generally used as drought stress inducer. The PEG-induced stress decreases the number of new leaves, leaf protein and chlorophyll content and root growth in some potato genotypes, while increases malondialdehyde (an important indicator of lipid peroxidation) concentration (LIU et al. 2019; GERVAIS et al. 2021). It was demonstrated that drought stress regulates proline accumulation and *Δ-1-PYRROLINE-5-CARBOXYLATE SYNTHASE* (*P5CS*) and *PYRROLINE-5-CARBOXYLATE REDUCTASE* (*P5CR*) gene expressions in potato leaves (KONDRÁK et al. 2012; LIU et al. 2019).

If sensitive genotypes of potato faces with drought stress during their tuber initiation and tuber bulking period, they will produce significantly fewer stolons, lower and small-sized tubers as well as lower tuber yield and number, while drought tolerant genotypes produce higher tuber yield and number compared to sensitive ones (ALICHE et al. 2020; GERVAIS et al. 2021). The MYB family is one of the largest TF families in plants, regulating various developmental processes and stress responses in potato (DUBOS et al. 2010). Drought and salt stress induce several MYB member genes' expression in different tissues of potato plant (LI et al. 2019).

Besides the drought stress, salinity causes severe damage to potato production and productivity due to osmotic stress-induced ion toxicity. This leads to physiological changes in the plant, including nutrient imbalance, impairment in detoxifying reactive oxygen species (ROS), membrane damage, and reduced photosynthetic activities. Several physiological and biochemical phenomena, such as the maintenance of plant water status, transpiration, respiration, water use efficiency, hormonal balance, leaf area, germination, and antioxidants production are adversely affected. The salinity response and tolerance include complex and multifaceted mechanisms that

are controlled by multiple proteins and their interactions (reviewed by CHOURASIA et al. 2021) For instance, under salinity stress, great amount of reduction in tuber yield was recorded due to inhibition of initial sprouting, plant development and tuberization stage (GHOSH et al. 2001; ZHANG et al. 2005; GUARAV et al. 2017).

It is known that drought and salinity stress have unique and overlapping signals and they induce hyperosmotic (increased extracellular osmolarity that leads to cell dehydration) and oxidative (damage to cellular components such as membrane lipids, proteins, and nucleic acids, and metabolic dysfunction) stress in plant cells. The hyperosmotic stress initiates both ABA-dependent and ABA-independent signaling, which in turn elicits many adaptive responses in plants (reviewed by ZHU 2016; ZHANG et al. 2022). The ABA treatment, drought and salt stresses activate several enzymes, for instance tyrosine decarboxylase, tyrosine hydroxylase and L-DOPA decarboxylase, in potato leaves. These activated enzymes result in significant increase in alkaloid contents of potato such as dopamine and norepinephrine (SWIEDRYCH et al. 2004).

ABA is a key hormone in regulating plant responses to abiotic environmental stresses and the ABA signal transduction leads to the activation/repression of stress related genes (KUMAR et al. 2019). ABA signalling is mediated by a few key regulators such as SUCROSE NONFERMENTING 1 (SNF1)-RELATED PROTEIN KINASES 2s (SnRK2s), ACGT-containing ABA response elements (ABREs). SnRK2 is considered to be a key mediator of stomatal closure and other plant responses to salt, drought and several other hyperosmotic stress-inducing conditions (FUJII et al. 2007). Nevertheless, hundreds to thousands of genes are transcriptionally regulated by drought, high salinity and cold stress via ABA signalling (ZHU 2016).

Plants are easily affected by temperature variations, and high temperature (heat stress) and low temperature (cold stress) affect plant development and reduce the yields. Like other stresses, plant response to extreme temperature is controlled by a variety of mechanisms, including physiological, biochemical, and molecular regulator mechanisms. For instance, biochemical changes, transcript levels of the key enzymes of starch degradation and the accumulation of soluble sugars (glucose and fructose) were significantly increased, while sucrose content was decreased in the leaves of 5-week-old potato plants after 12 hours of cold treatment (ORZECHOWSKi et al. 2021). In molecular mechanisms, many genes and TFs related to plant stress resistance have been identified in plants. Member of the ethylene response factor (*ERF*) family, C-repeat binding factors (*CBF*s)

are temperature stress-related genes in potato. In particular, the expression levels of *StCBF3* and *StCBF4* in the leaves, stems, and roots are significantly increased under high-temperature conditions (LI et al. 2020). Furthermore, mostly heat shock proteins (HSPs) are involved in heat response in plants. HSPs can be grouped into five families based on their molecular weight and sequence homology (WATERS 2013). ZHAO et al. (2018) identified 48 putative *HSP20* genes (*StHSP20*s) in potato and found that the relative expression levels of 14 of them were significantly up-regulated under heat stress.

4. MATERIALS AND METHODS

4.1. Materials

4.1.1. Plant materials

Table 1. Plant materials used for the experiments

№	Potato variety	Origin
1.	*Solanum tuberosum* L. cv. 'Désirée'	Fritz Lange KG, Bad Schwartau, Germany
2.	*Solanum tuberosum* L. cv. 'White Lady'	Potato Research Centre, Keszthely, Hungary
3.	*Solanum tuberosum* L. cv. 'Hópehely'	Potato Research Centre, Keszthely, Hungary
4.	aGI lines – *GI.04*-repressed derivatives of *Solanum tuberosum* L. cv. 'Désirée'	Plant Physiology Group, Institute of Genetics and Biotechnology, MATE
5.	aBB lines – *BB*-repressed derivatives of *Solanum tuberosum* L. cv. 'Désirée'	This work

4.1.2. Bacterial strains

Table 2. Bacterial strains used for the experiments

№	Strain name	Genotype	Reference
1.	*Escherichia coli* DH5α	F^- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1	HANAHAN (1983)
2.	*Agrobacterium tumefaciens* LBA4404	Ach5 (RIF R) Ti pAL4404 (strepr) Octopine	HOEKEMA et al. (1983)

4.1.3. Plasmids and vectors

Table 3. Plasmids and vectors used for the experiments

№	Name	Type	Reference
1.	pCP60	Binary vector for *A. tumefaciens*-mediated plant transformation	RATET (CNRS, Paris, France, unpublished)
2.	pGEM-T Easy	Cloning vector	KNOCHE and KEPHART (1999)
3.	pRK2013	Self-transmissible helper plasmid	DITTA et al. (1980)
4.	pGEM-T Easy::aBB	pGEM-T Easy carrying a 210-bp fragment of *StBB*	This work
5.	pCP60::aBB	pCP60 carrying a 210-bp fragment of *StBB*	This work

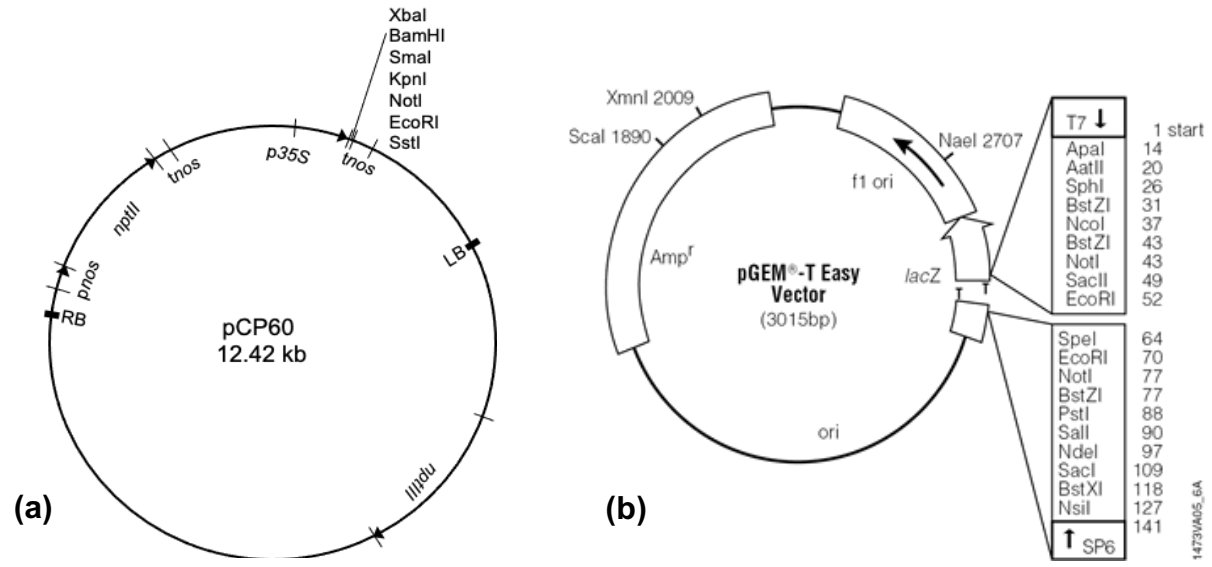


Figure 2. Schematic maps of the plasmids. (a) pCP60 (b) pGEM®-T Easy

4.1.4. PCR primers

Table 4. Nucleotide sequence of primers

№	Name	Forward sequence (5'-3')	Reverse sequence (5'-3')
1.	GI250	*GAATTCAATTGGAAGCACACCTAA	GGTACCGGCCAAATCTGAAGCATCTAA
2.	GI101	GTACGTGCACTCAGCATATCA	GCAGGACCATGGATACCATTTA
3.	GI.04spec	GTACGTGCACTCAGCATATCA	GCAGGACCATGGATACCATTT
4.	GI.12spec	TGGCTTCTTCAAGCACAAGGT	GCGGTAATTTGATCCTTCCGC
5.	BB210	ACATCAAAGCGGTGAAGCAAA	GGTACCACAGAACACGTGGTACAAAGC
6.	StBB	TCCATCAGCACCAATCCATAC	CATGCTCCTCGATTCCAGATAC
7.	ACTIN	TGGACTCTGGTGATGGTGTG	GGTTTCAAGTTCCTGTCTGT
8.	EF1α	GACAAGCGTGTTATTGAGAGG	CACAGTGCAGTAGTACTTAGTG
9.	P5CS	CGATCCACAATCAGAGCTAATTC	GCAGTCATACCACCTCTTCCA
10.	GWD	CCCACGATCTTAGTAGCAAA	TTAGCTCCAACCATTTCACT
11.	HSP20-44	GAGAATGTGAAAATGGAGGAA	ATTAATAGCTTTCACCTCAGGC

* The restriction enzyme recognition sites *Eco*RI and *Kpn*I are underlined. All primers were designed using the NCBI primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Oligoes were purchased from Integrated DNA Technologies, Leuven, Belgium.

4.1.5. Chemicals and antibiotics

Chemicals and antibiotics were purchased from the manufacturers Reanal, Sigma, Thermo Scientific, Merck, Duchefa, Sanofi, etc.

4.1.6. Enzymes and kits

Table 5. Enzymes

№	Name	Manufacturer
1.	Dream Taq Polymerase	Thermo Scientific, Waltham, MA, USA
2.	Deoxyribonuclease	Thermo Scientific, Waltham, MA, USA
3.	Ribonuclease	Thermo Scientific, Waltham, MA, USA
4.	*Eco*RI	Thermo Scientific, Waltham, MA, USA
5.	*Kpn*I	Thermo Scientific, Waltham, MA, USA
6.	T4 DNA ligase	Promega, Madison, WI, USA

Table 6. Kits

№	Name	Manufacturer
1.	pGEM-T Easy cloning kit	Promega, Madison, WI, USA
2.	Maxima H minus First Strand cDNA Synthesis kit	Thermo Scientific, Waltham, MA, USA
3.	Luminaris Color HiGreen Flourescein qPCR Master Mix kit	Thermo Scientific, Waltham, MA, USA

4.1.7. Growth media

According to the laboratory protocol (SAMBROOK et al. 1989), LB, SOC and YEB media with appropriate antibiotics were used for the growth of bacteria. Rifampicin 100 mg/l, kanamycin 25 mg/l were added to the *A. tumefaciens* growth medium and kanamycin 10 or 25 mg/l or ampicillin 100 mg/l were added to the *E. coli* growth medium. Blue-white selection for the recombinant pGEM-T Easy plasmids was carried out in the presence of 20 mg/l 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-Gal) and 100 mM isopropylthio-β-galactoside (IPTG) in the medium.

For plant culture Murashige-Skoog medium (MS) (MURASHIGE and SKOOG 1962), rooting medium (RM - MS without vitamins containing 2% (w/v) sucrose and 0.8% agar), callus induction medium (CIM - MS medium supplemented with 1.6% glucose, 5 mg/l naphthyl acetic acid (NAA), 2.5 mg/ml benzylamino-purine (BAP), 250 mg/l cefotaxime, 50 mg/l kanamycin) and shoot induction medium (SIM - MS supplemented with 1.6% glucose, 1 mg/l zeatin riboside, 5 mg/ml NAA, 0.2 mg/ml gibberellin, 250 mg/l cefotaxime, 50 mg/l kanamycin) were used.

4.2. Methods

4.2.1. Plant growth conditions

Potato plants were cultivated *in vitro* in RM culture medium in a culture room at 24°C under a photoperiod of 16 h/8 h day/night cycle at a light intensity of 75 µmol m^{-2} s^{-1}. Plants with apical bud were continuously sub-cultured in RM medium for every 4 weeks or propagated from stem segments carrying a single auxillary bud. One-month-old *in vitro* plants were transferred into the sterile soil A200 (Stender GmbH, Schermbeck, Germany) and grown further under the greenhouse conditions with a photoperiod of 14 h day/10 h night and temperature regime of 20-28°C. In winter, the ambient light conditions were supplemented with artificial lightening by sodium lamps. Optimal growth conditions were provided by watering the plants twice a week. Plants were treated with fungicides at the beginning of acclimatisation and weekly with the pesticide Mospilan.

For grafting and metabolite profiling, greenhouse-grown plants, leaves and tubers of 'Hópehely' (HP) and 'White Lady' (WL) were used. For the gene expression analysis, root, stolon, tuber, stem, petiole, leaf, sepal, petal and stamens of 'Désirée' plants grown in the greenhouse were used. Tubers were harvested at the end of the vegetation period. For the abiotic stress treatments, one composite leaf of each three individual plants per treatment was collected from 6-8-week-old plants grown in pots.

4.2.2. Grafting and plant phenotyping

Three consecutive grafting experiments were carried out. For each experiment, 15-20 plants of the two potato cultivars were grafted by the splice grafting method. Stock plants were decapitated 2-3 cm above the root shoot joint and served as rootstocks without leaves. Scions were prepared by cutting the plants with a diagonal cut through the internodal part of the stem at the same distance above the ground as for the rootstocks. The scions were fitted to the stock and wrapped with rubber clips. For four days after grafting, the plants were incubated in a plastic box with a lid under high humidity in darkness at 24˚C. The non-grafted and grafted plants were grown further under greenhouse conditions for one week and then transferred to pots with a 14 cm diameter top and 14 cm depth. Six weeks after grafting, the plants, together with the soil, were carefully tipped out of the pots, checked for tuber formation and re-planted. The five largest leaves from each plant were harvested, and their area was measured to estimate canopy development. At

the end of the vegetation period, the tubers were harvested, visually evaluated for shape and colour and measured to obtain fresh weight yield.

4.2.3. Metabolite extraction and profiling

Six weeks after grafting, three to four sets of leaves, each containing three leaf disks 1 cm in diameter originating from three individual plants, were prepared, frozen in liquid nitrogen and stored at -70˚C until they were used for metabolite extraction. After harvesting, four to five sets of tubers, each containing three tubers approximately 1.5-2.0 cm in diameter, were prepared and washed well with deionised water. Approximately 0.1-0.2 cm-thick freshly cut radial slices taken from the centres of the tubers were cut into small pieces, frozen in liquid nitrogen and stored at -70˚C. Both the leaf and tuber samples were ground into fine powder in a mortar with a pestle. Metabolite extraction and profiling were performed as described in detail by NIKIFOROVA et al. (2005). Ribitol (0.2 mg/ml) was added to the extract as an internal standard. N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was used for derivatisation, and the samples were analysed in a quadrupole-type gas chromatography-mass spectrometry (GC-MS) system (Finnigan Trace/DSQ, Thermo Electron Corp., Austin, TX, USA) equipped with a 30 m capillary column (Rxi-5 ms, 0.25 mm ID, 0.25 μm df, Restek) in TIC (total ion chromatogram) mode. Mass spectra were recorded at 0.8170 scans/sec with a m/z 50-650 scanning range. Thermo Scientific Xcalibur software was used for exporting the spectra and searching the NIST 11 mass spectral database. In addition, the sugars were identified based on a comparison of the retention time and mass spectrum to an authentic standard that was analysed under identical conditions.

4.2.4. Cloning and *Agrobacterium* mediated transformation of potato leaves

Antisense *BIG BROTHER* (aBB) plants were generated by amplifying a 210-bp fragment of the *S.tuberosum* E3 ubiquitin ligase BIG BROTHER-like (*StBB*-LOC102597766), using the primer pair BB210 (Table 4) from 'Désirée' genomic DNA isolated according to SHURE et al. (1983). The PCR reaction volume was 25 μl, including 100 ng of genomic DNA, 0.5 μl forward primer (10μM), 0.5 μl reverse primer (10μM), 0.5μl dNTP mix (10mM), 2.15 μl 10x Dream Taq buffer and 0.15 μl Dream Taq enzyme (5 U/μl). Denaturation was at 95°C for 5 min, and the reaction mixture was incubated for 34 cycles at 95°C for 30 sec, at 57°C for 30 sec and at 72°C for 90 sec. The product was elongated at 72°C for 10 min. The PCR fragment was analysed and

visualised by ethidium bromide on a 1% agarose gel and ligated into the pGEM-T Easy vector following the manufacturer's protocol. After ligation, the plasmid DNA was transformed into high efficiency competent *E. coli* DH5α cells and the recombinant plasmids were selected using blue-white selection on LB medium supplemented with ampicillin, X-Gal and IPTG. Plasmid DNA was isolated from white colonies with the miniprep method (SAMBROOK et al. 1989). The presence of the cloned 210-bp *StBB* fragment (aBB) was verified by PCR and DNA sequencing at BIOMI Ltd., Gödöllő, Hungary. To re-clone the aBB fragment into the binary vector pCP60 the pGEM-T Easy::aBB plasmid DNA was digested with the restriction enzymes *Eco*RI and *Kpn*I using the method of the producer and ligated in antisense orientation into pCP60 as a *Kpn*I-*Eco*RI fragment between the constitutive *CaMV35S* promoter and the nos terminator. The plasmid DNA was then transformed into *E. coli* DH5α. Transformed cells were selected on LB plates supplemented with kanamycin. Plasmid DNA was isolated from kanamycin resistant colonies and the presence of the aBB fragment in pCP60 was tested by PCR. The pCP60::aBB plasmid was introduced into *A. tumefaciens* by tri-parental mating. Three bacterial strains participating in the mating mixture is called triparental mating or triparental bacterial conjugation (WISE et al. 2006; Fig. 3). In our experiment, the helper strain *E. coli* carried the self-transmissible plasmid pRK2013, the donor *E. coli* carried the plasmid to be mobilized, which was pCP60::aBB, and the recipient was the *A. tumefaciens* strain LBA4404. Transconjugants were selected on YEB+kanamycin plates. The presence of pCP60::aBB in kanamycin resistant colonies was tested by isolating plasmid DNA using the same miniprep method as for *E. coli* with the following exceptions: [1] The cells were washed with 1 ml of 0.1 M NaCl before adding the DNA extraction buffer I [2] After precipitation the proteins with the extraction buffer III, the supernatant was further purified with phenol-chloroform extraction. Identity and orientation of the inserted fragment in pCP60 was verified by Sanger sequencing (BIOMI Ltd., Gödöllő, Hungary).

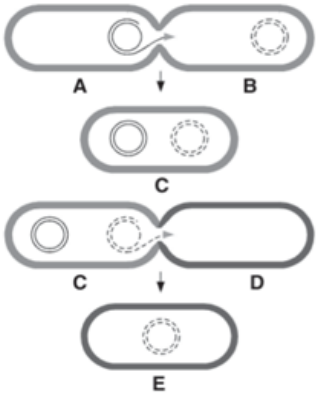


Figure 3. Diagram depicting triparental mating. The *E. coli* helper and donor strains have been mixed together with the *Agrobacterium* recipient strain. (A) The *E. coli* helper strain transfers the self-transmissible plasmid pRK2013 (solid circles) to *E. coli* donor strain. (B) The *E. coli* donor strain carries an engineered plasmid that is mobilizable but not self-transmissible (dotted circles). (C) The donor strain acquires pRK2013 from the helper strain and now carries both plasmids. (D) Using transfer functions supplied by pRK2013, the donor strain transfers the engineered plasmid to the *Agrobacterium* recipient. (E) The engineered plasmid replicates and becomes established in the *Agrobacterium* cells (copied from WISE et al. 2006).

Potato transformation was carried out using the method of DIETZE et al. (1995). Middle leaves of potato plants grown in MS medium for 3-4 weeks were used for transformation as an explant. After removing the petioles, leaves were cut and placed in abaxial position into Petri dishes containing 30-40 ml of liquid MS medium. Then 100 µl of *A. tumefaciens* (pCP::aBB) suspension (OD_{600}=0.6) was added into Petri dishes containing the leaf explants and incubated in darkness at 24°C After 2 days of incubation leaves were transferred onto the surface of solid CIM medium supplemented with 250 mg/l claforan, 50 mg/l kanamycin and incubated in a culture room at 24°C under a photoperiod of 16 h/8 h day/night cycle at a light intensity of 75 µmol m^{-2} s^{-1} for 1 week. Leaves were transferred from CIM to selective solid SIM medium and subcultured weekly in the same medium until the shoots were regenerated. After the shoot regeneration, the shoots were sub-cultured in selective RM medium for rooting.

4.2.5. RNA isolation and cDNA synthesis

The protocol of STIEKEMA et al. (1988) was used for RNA isolation. 100 mg of grinded tissue powder were solved in RNA isolation buffer (660 µl 3 M sodium acetate, pH=5.2, 200 µl 0.5 M EDTA pH=8.0, 1 ml 10% SDS, 8.14 ml distilled water) and homogenized quickly by using a vortex. Centrifugation was carried out at 4°C with 13,000 rpm for 10 min. The supernatant was taken out and transferred into a new tube containing phenol/chloroform (1:1) mixture. After

vortexing, the mixture was centrifuged at the same condition as in the previous step. In the following step, the phenol/chloroform extraction was repeated. 125 µl of 10 M LiCl was added to ~500 µl supernatant and kept at 0°C for 1 hour. After precipitation, the pellet was collected by centrifugation. The supernatant was removed, and the pellet was washed with 1 ml of 2.5 M LiCl and centrifuged. After removing the supernatant, the pellet was washed with 1 ml of 70% ethanol twice and dried under a laminar flow. The dried RNA was solved in 30 µl of distilled water and the concentration measured at OD_{260} using a nanodrop spectrophotometer.

Complementary DNA (cDNA) was synthesised using the Maxima H minus First Strand cDNA Synthesis Kit with dsDNAse according to the manufacturer's instruction.

4.2.6. Gene expression analysis

cDNA quality was checked by reverse transcription PCR (RT-PCR) in a T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) using the ACTIN primer pair (primer sequences are listed in Table 4). *ACTIN* is a commonly used reference gene for reverse transcription quantitative PCR (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. Data were analysed with the Light Cycler-96 Software version 1.1 (Roche Diagnostics GmbH, Mannheim, Germany). Expression analysis of the genes was carried out using the primer pairs listed in Table 4. Parallel reactions to amplify *ACTIN* and *EF1α*, the commonly applied reference genes (NICOT et al. 2005), were used to normalize the amount of template. Relative expression was calculated based on the geometric mean of the expressions of the two 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.

4.2.7. *In silico* DNA sequence analysis

Three thousand-bp sequences of *S. tuberosum* Group Phureja located upstream from the translational start site of the *StBB* transcript variant XM_006356317.2 were retrieved from the Potato Genomic Resource Spud DB (http://solanaceae.plantbiology.msu.edu/) and the binding sites of transcription factors were predicted by the online platform The Plant Transcriptional Regulatory Map (http://plantregmap.gao-lab.org/).

4.2.8. Stress and ABA treatments

Leaves of potato plants grown in pots for 6-8 weeks were subjected to different abiotic stress treatments. For the salt and drought treatment, composite leaves with petioles were placed into a beaker filled with distilled water (control), 200 mM NaCl or 20% PEG 6000 solution and incubated at room temperature for 6 h. For ABA treatment composite leaves with petioles were incubated in 0.1 mM ABA solution for 24 h at room temperature. For cold and heat treatments, composite leaves with petioles in distilled water were incubated at 4°C and 42°C, respectively, for 6 h. After treatments, discs of 1 cm in diameter were cut out from leaves, frozen in liquid nitrogen and stored at -70°C until utilization. Stress treatments were validated by testing the *Δ1-PYRROLINE-5-CARBOXYLATE SYNTHETASE* (*P5CS;* LIU et al. 2019), α-*GLUCAN, WATER DIKINASE* (*GWD;* ORZECHOWSKI et al. 2021) and *HEAT SOCK PROTEIN 20-44* (*HSP20-44*; ZHAO et al. 2018) at mRNA levels. DNA sequences of primers are listed in Table 4.

4.2.9. Determination of anthocyanin pigment content

Freshly harvested matured potato tubers grown in pots were peeled. A simplified method of TOGURI et al. (1993) was used for anthocyanin extraction from 1 g skin with 10 ml 1% HCl in methanol overnight at 4°C. Relative concentrations of the chloride forms of the anthocyanin pigments were determined spectrophotometrically by measuring the absorbance at 540 nm.

4.2.10. Statistical analysis

Quantitative phytochemical analysis was performed by principal component analysis (PCA) and variable importance projection (VIP) plots by partial least squares discriminant analysis (PLS-DA) of the first principal components (MetaboAnalyst 4.0, https://www.metaboanalyst.ca). A value of 1.0 was selected as the cut-off for the VIP values. As pre-treatment, sample normalization was carried out based on median values, and the data were log transformed. Significance of differences ($p < 0.05$ and $p < 0.01$) between two groups of data was detected by Student's *t*-test. Significance of differences ($p < 0.05$ and $p < 0.01$) between multiple groups of data was detected by One-way ANOVA (Analysis of Variance) with post-hoc Tukey HSD (Honestly Significant Difference) (https://www.astatsa.com).

5. RESULTS AND DISCUSSION

5.1. Metabolite analysis of tubers and leaves of two potato cultivars and their grafts

5.1.1. Effect of grafting on growth and tuberization

Formerly, grafting experiments were successfully used to identify signals of initiation of potato tuber development (EWING and STRUIK 1992; DUTT et al. 2017). However, tuberization and metabolite composition of leaves and tubers of grafted potato cultivars has not been studied in detail.

To unravel the influence of vegetative organs on the primary polar metabolite content of potato tubers and the effect of tuberization on the metabolite content of leaves, grafting experiments were carried out testing two potato cultivars 'Hópehely' (HP) and 'White Lady' (WL), which have different tuber morphologies (Fig. 4a and 4b). A previous study (URI et al. 2014) demonstrated that the metabolic composition of tubers of these two cultivars grown under screen-house conditions is different.

Plantlets were grown *in vitro* and transferred into pots and grown further under greenhouse conditions. After two weeks of acclimatisation, homo- and hetero-grafts were prepared and grown further, together with non-grafted control plants, in a greenhouse. Two weeks after grafting, plants with well-developed root and shoot systems (Fig. 4c and 4d) were counted. Three consecutive experiments were carried out, with a grafting success of 64–85%. Six weeks after grafting, the number of tubers was tested by tipping the plants out of the pots. The canopy development of the plants was also estimated at this time by measuring the area of the five largest leaves of the plants. A positive correlation ($R^2 = 0.6947$) between the leaf growth rate and the number of tubers formed on plants was detected (Fig. 5). The leaf area as well as the number of tubers was low in the case of HP and homo-grafted HP/HP plants, while these parameters were high in the case of WL and WL/WL plants. The hetero-grafts, either WL (HP/WL) or HP (WL/HP) of the rootstock, possessed intermediate values, indicating that canopy development and tuber initiation are influenced by grafting.

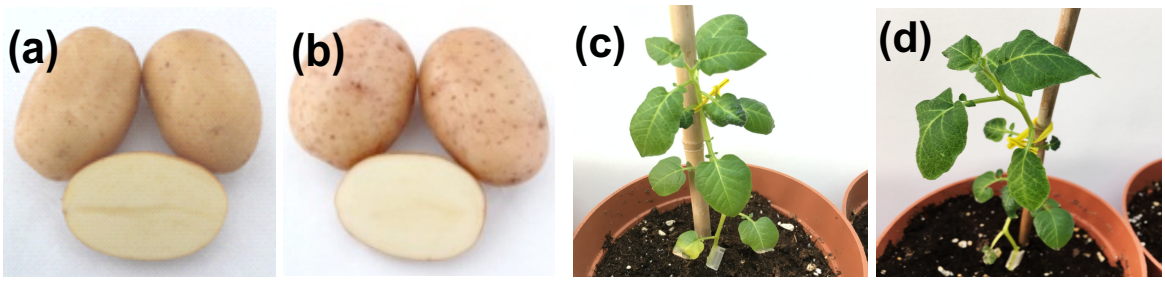


Figure 4. Tuber and canopy morphology of two Hungarian potato cultivars, 'White Lady' (a and c) and 'Hópehely' (b and d). Tubers were grown in field conditions (photography by Z. Polgár).

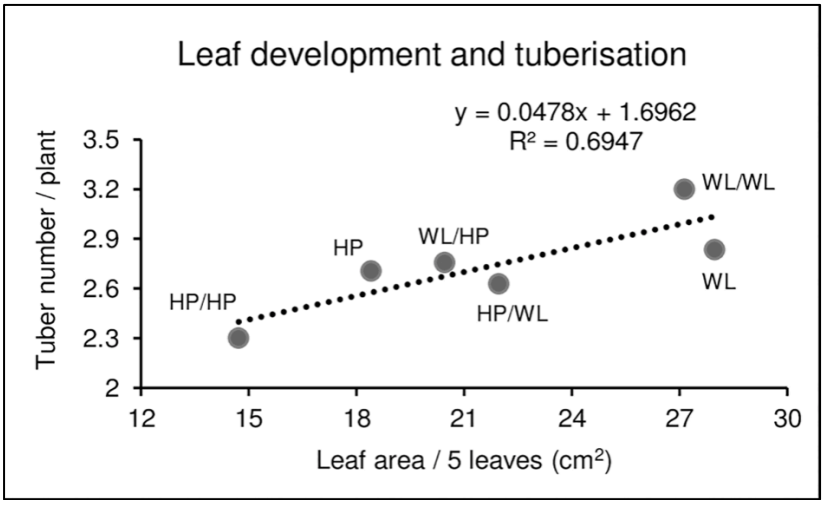


Figure 5. Correlation between leaf size and tuberization six weeks after grafting. WL and HP, non-grafted controls; WL/WL homo-grafted WL; HP/HP, homo-grafted HP; WL/HP, hetero-graft: WL scion/HP rootstock; HP/WL, hetero-graft: HP scion/WL rootstock.

At the end of the vegetation period, the mature tubers were collected and visually characterised, the number of tubers per plant was counted and the mass of the tubers was measured. The HP tubers were roundish with yellow to pinkish skin colour (Fig. 6a), while the WL tubers were short-oval and white yellowish (Fig. 6b). The grafting did not change the colour of the tubers (Fig. 6c-6f). In contrast, the shape of several tubers collected from hetero-grafted plants resembled that of the scion tubers (Fig. 6a,b,e and f).

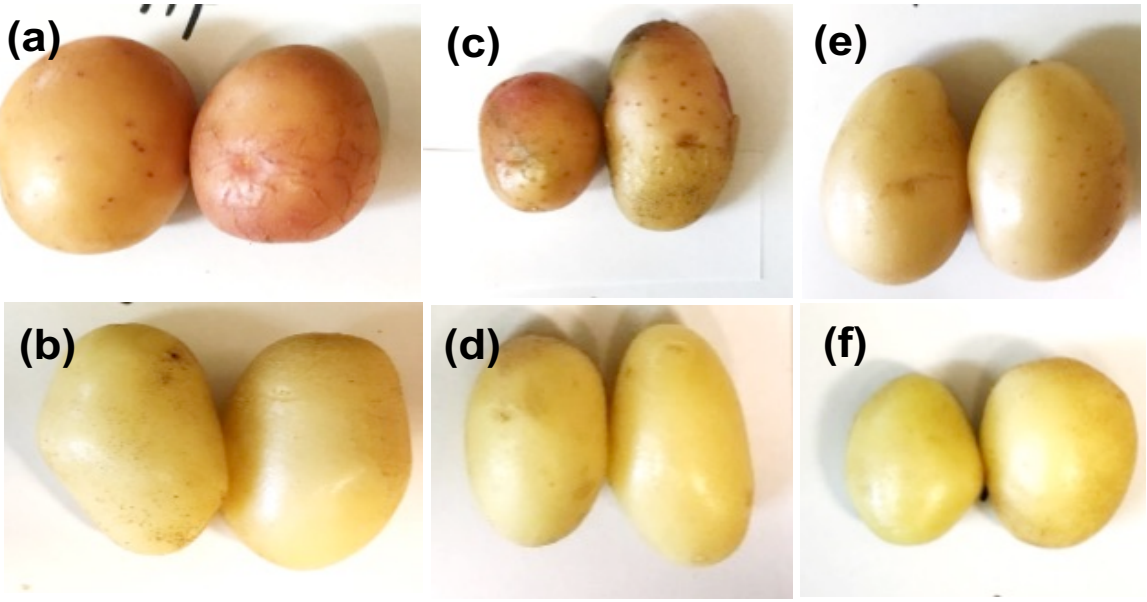
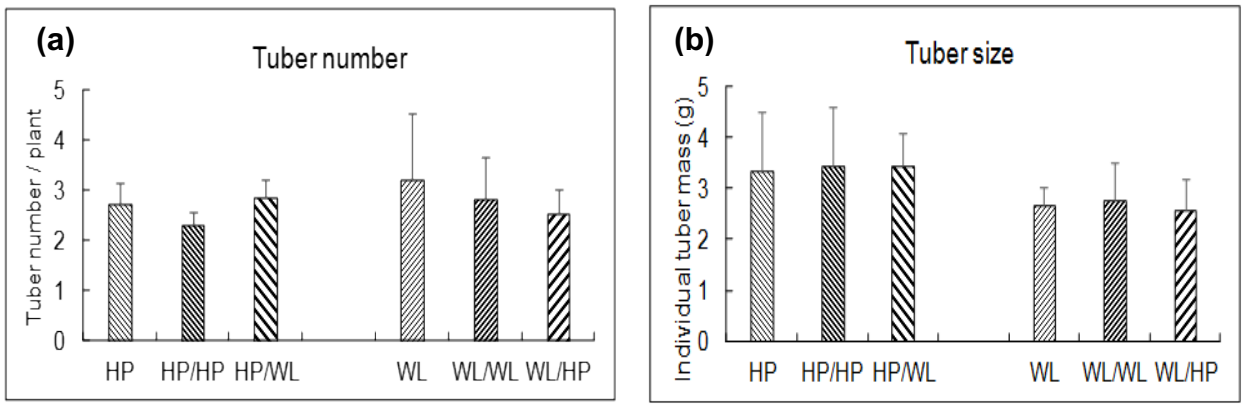


Figure 6. Morphology of mature tubers collected from the non-grafted and grafted potato plants. (a) non-grafted HP, (b) non-grafted WL, (c) homo-grafted HP/HP (d) homo-grafted WL/WL, (e) hetero-graft WL/HP, HP is the rootstock (f) hetero-graft HP/WL, WL is the rootstock

The average number of tubers per pot ranged from 2.3 to 3.2 (Fig. 7a), with a mass of 2.6 to 3.6 g/tuber (Fig. 7b). The tuber yield was between 7.2 and 10.2 g/plant (Fig. 7c). There was no significant difference in these parameters between the HP and WL plants, and the values were not changed by grafting.



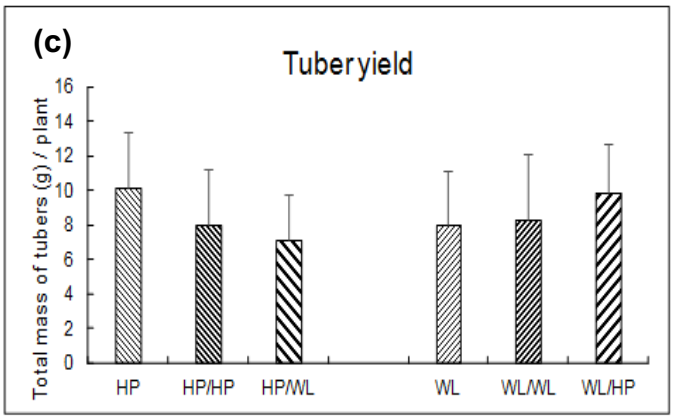


Figure 7. Tuber number (a), size (b) and yield per plant (c) at the end of the vegetation period. The means are obtained from three consecutive experiments. The standard deviations are indicated by error bars. No significant differences were detected between the means at $p \leq 0.05$ by one-way ANOVA with post-hoc Tukey HSD test.

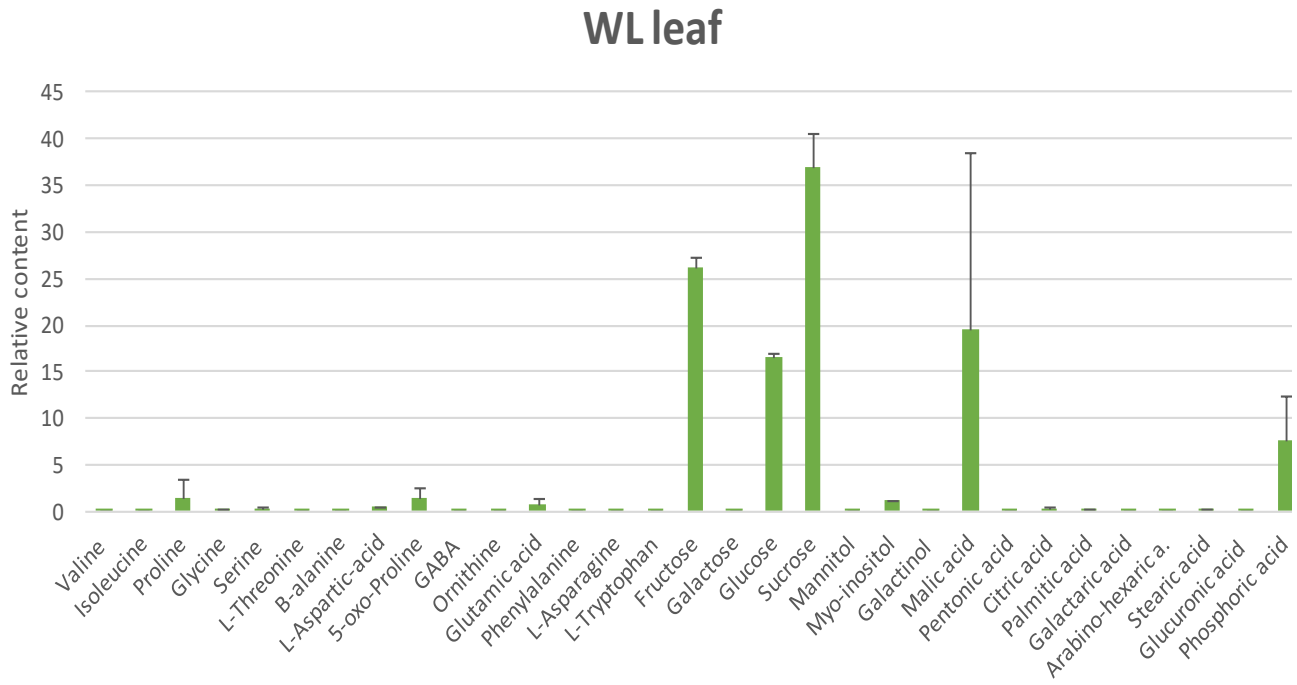
Generally, hetero-grafting potato and Solanaceae plants have been used as an efficient method to improve potato hybridization. Phenotypically, tomato/potato hetero-grafting showed decreased amount of stolon number and stolon length which indicate that tomato scion is less effective at producing substances or signals to induce tuberization but promotes stolon development into aerial stems and sprouting (ZHANG et al. 2019). Also, two *Solanaceae* plants, *Datura stramonium* and *S. tuberosum* cv Qingshu 9 were hetero-grafted and hetero-grafted potatoes had larger number of flower than self-grafted potatoes (ZHANG et al. 2022). In our study, no phenotypical changes were found when two potato cultivars were hetero-grafted, but the time of tuber initiation was altered. A positive correlation between the growth rate of the leaves and the time of tuber initiation was detected supporting the previous finding (KLOOSTERMAN et al. 2013) that tuberization is triggered by source-derived mobile signals. Nevertheless, further experiments involving a large number of genotypes are necessary to see how general this correlation is in different potato cultivars.

5.1.2 Metabolic profiling of leaves

To investigate the influence of grafting on the metabolite composition of leaves, samples were collected six weeks after grafting in two consecutive experiments, and metabolite profiling was carried out using GC-MS. A total of 31 polar metabolites were identified in the leaf extracts, including 15 different amino acids, 4 sugars, 3 sugar alcohols, 8 organic acids and 1 inorganic acid (Fig. 8). The major compounds in the leaves were sugars and malic acid. The average concentration

of malic acid was very much the same in the two cultivars. However, it fluctuated substantially in the individual groups of samples. Among the amino acids, proline, glutamine (measured as oxo-proline; FIEHN 2006), glutamic acid and aspartic acid had the highest peaks. The main fatty acids were palmitic and stearic acid.

The concentrations of sugars in the leaves were quantified by comparing the peak sizes of the leaf samples to those of authentic standards. Glucose, fructose and sucrose were present in the highest amounts at concentrations of 1.13±0.26, 1.10±0.27 and 3.52±0.38 mg/g fresh weight (FW) in the HP leaves, while the concentrations of the same compounds were 2.15±0.05, 2.57±0.10 and 6.82±0.70 mg/g FW in the WL leaves, respectively. Despite the differences detected in the fructose, glucose and sucrose concentrations in the leaves of the two cultivars, no significant differences were found in the sugar content of the leaves of the hetero-grafted plants compared to their homo-grafted counterparts (Fig. 9), indicating that the sugar content of scions is not influenced by rootstocks.



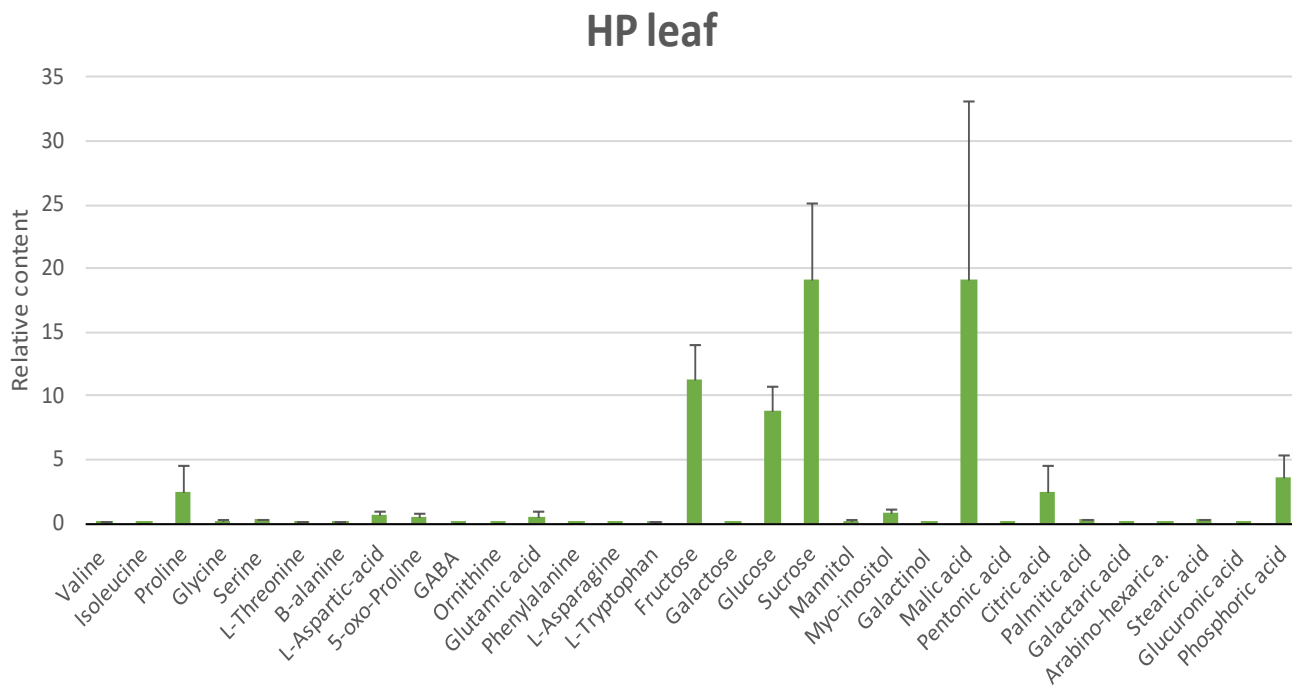


Figure 8. Metabolite composition of leaves. The relative data shown on the *Y*-axis are derived from a comparison of the peak sizes of the samples and the internal standard, ribitol (0.2mg/l). The means were obtained from two consecutive experiments from three-four biological repeats.

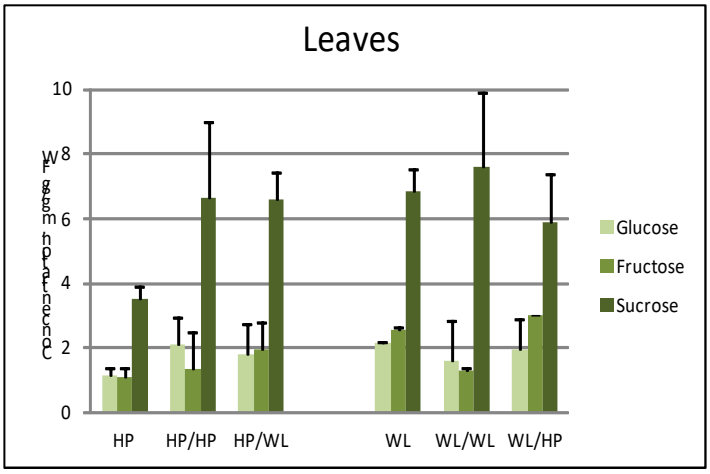


Figure 9. Sugar concentrations in leaves. The concentrations were calculated by comparing the peak sizes to those of authentic standards.

PCA of the HP and WL leaf metabolite data revealed distinct profiles causing samples to cluster based on genotype. The first and second principal components, representing 68.7% and 12.7% of the total sample variance, respectively, clearly separated the HP and WL samples in the loading plots (Fig. 10a).

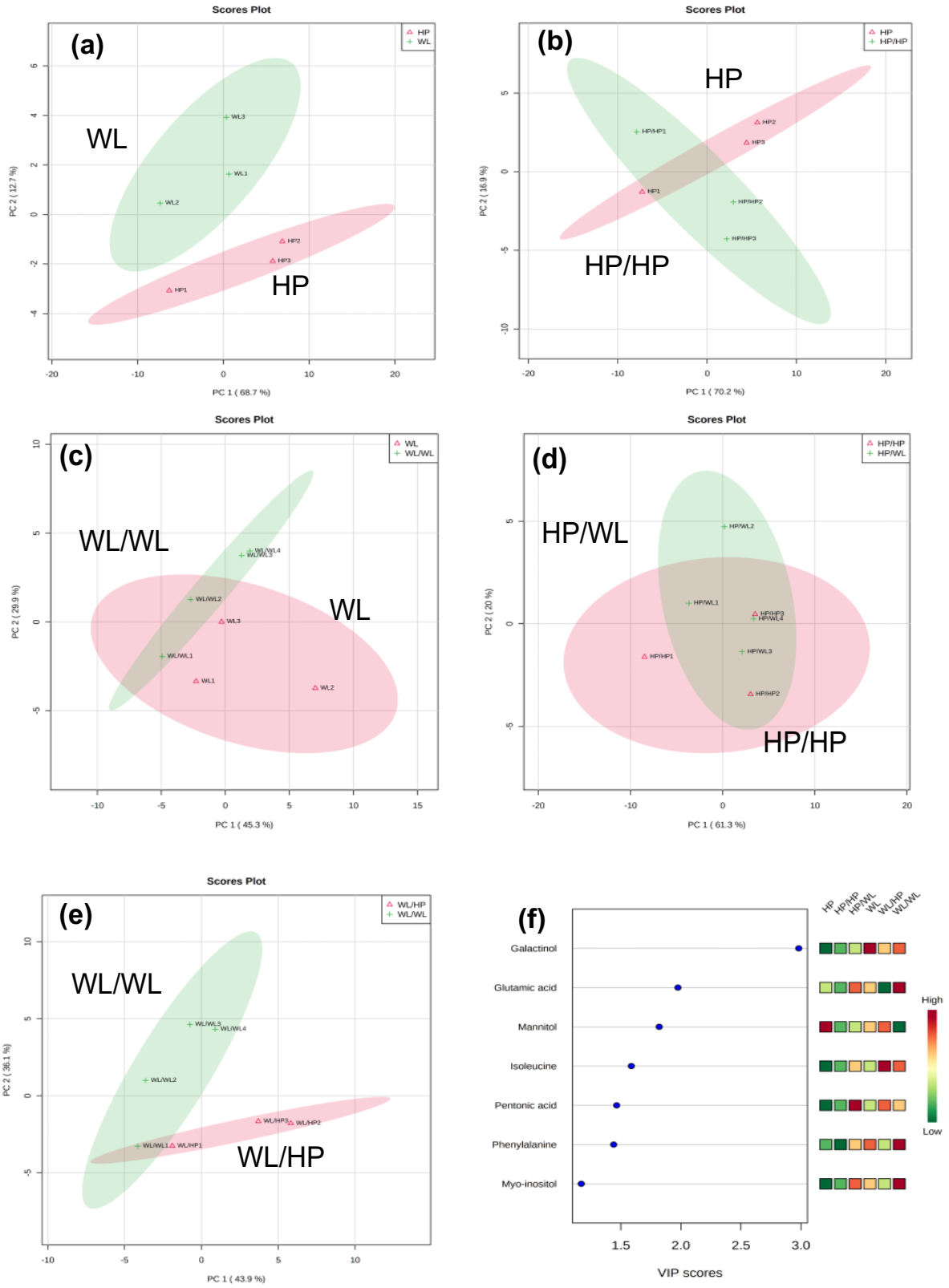


Figure 10. PCA and VIP plots calculated from PLS-DA showing the metabolite differences in the source leaves of non-grafted (a), homo-grafted (b and c) and hetero-grafted (d and e) plants. The VIP plots (f) indicate the major differences between each category.

Metabolite analysis of the homo-grafted plants, carried out in the same way as for the non-grafted controls, showed that although there were some differences compared to the non-grafted controls, interruption of plant development by cutting and healing did not substantially influence the constitution of the leaf metabolome as a whole. The hetero-grafting did not substantially change the metabolome of the leaves (Fig. 10d and e). Nevertheless, compared to the leaves of the homo-grafted plants, the hetero-rootstocks evoked some differences in the leaves, especially in the concentrations of glutamic acid, citric acid, proline, β-alanine and ornithine. To determine which compounds caused the major differences between the leaves of the six sets of plants, variable importance projection (VIP) plots by partial least squares-discriminant analysis (PLS-DA) were utilized. Seven compounds had a VIP score higher than 1.0, out of which four compounds (galactinol, glutamic acid, mannitol and isoleucine) possessed values above 1.5 (Fig. 10f).

5.1.3. Metabolic profiling of tubers

To analyse the metabolite composition of the tubers, three freshly collected mature tubers approximately 1.5–2.0 cm in diameter were grouped together. For tubers derived from each category of plants, four to five groups of tubers were prepared, and the levels of the same 31 metabolites detected in the leaves were tested in the tubers using GC-MS. In the tubers, as in the leaves, sugars were the most abundant metabolites. However, while in the leaves, the sucrose concentration was approximately the same as the concentrations of fructose and glucose (Fig. 9), the sucrose concentration in the tubers was much higher than the concentrations of the other two sugars (Fig. 11).

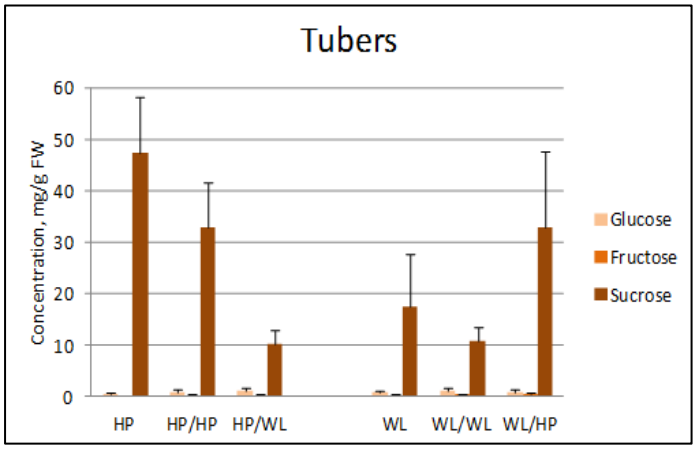


Figure 11. Sugar concentrations in tubers. The concentrations were calculated by comparing the peak sizes to those of authentic standards.

Unlike in the leaves, in which malic acid was the most abundant organic acid, citric acid was the dominant organic acid in the tubers. In terms of amino acids, asparagine was present in the highest amount in the tubers. The fatty acids palmitic and stearic acid were identified in low amounts similar to those detected in the leaves (Fig. 12).

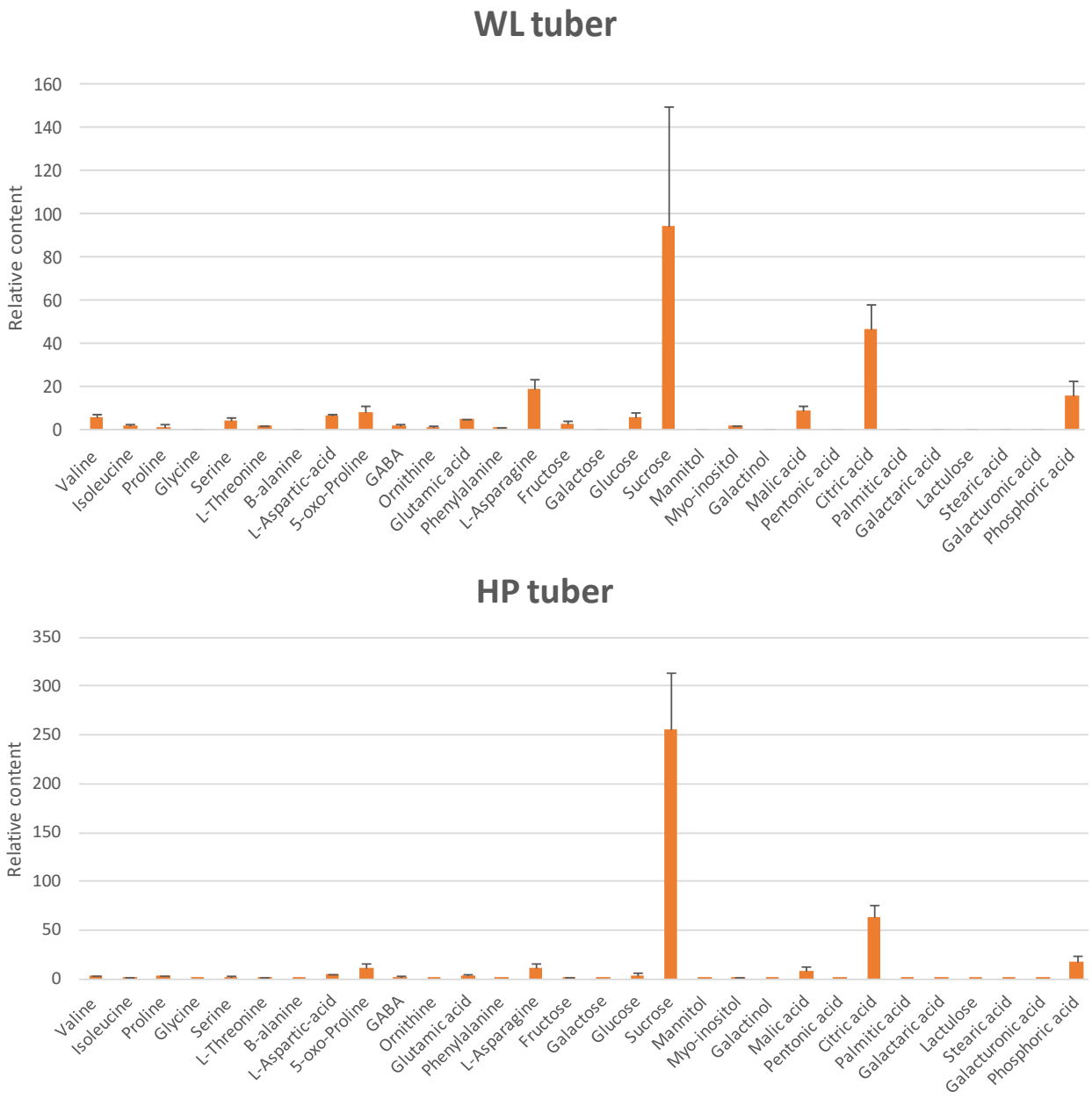
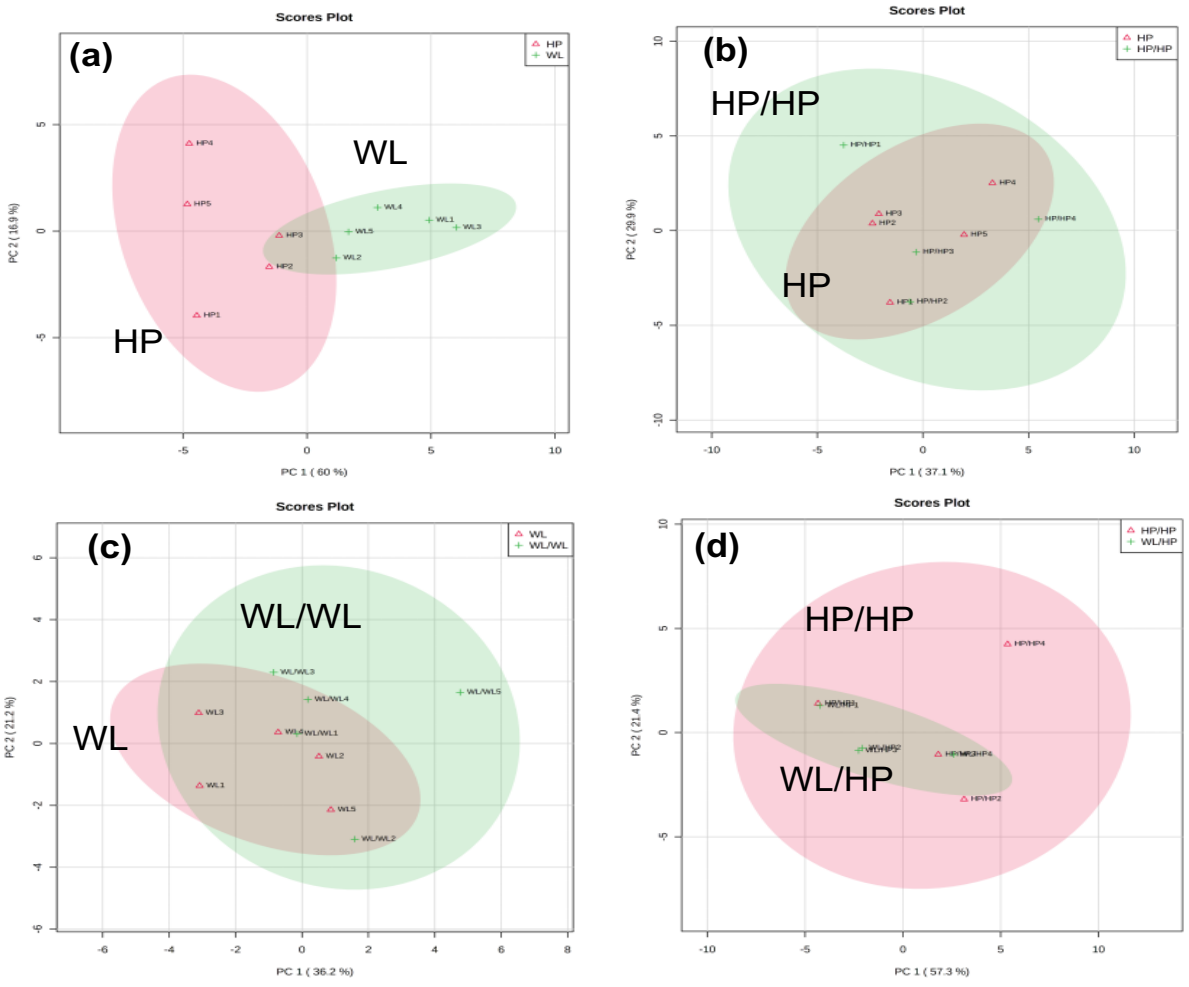


Figure 12. Metabolite composition of tubers. The relative data shown on the *Y*-axis are derived from a comparison of the peak sizes of the samples and the internal standard, ribitol. The means were obtained from two consecutive experiments from four-five biological repeats. The standard deviations are indicated by error bars.

The PCA plot showed that the metabolite compositions of the HP and WL tubers were quite different; however, they were not separated completely by the first and second components, which represented 57.3% and 21.4% of the total sample variance, respectively (Fig. 13a). The major differences between the two cultivars were in the sugar, phenylalanine and ornithine contents. The highest disparity was detected in the sucrose concentration, which was 47.3±11.0 in the HP tubers and 17.6±10.3 mg/g FW in the WP tubers. Although the PCA biplots showed some differences the grafting, in general, did not severely change the metabolite profile of the tubers. Among the six different categories of tubers, a VIP score higher than 1.0 was found for 10 compounds, of which sucrose, galactinol, pentonic acid, galacturonic acid and lactulose had values higher than 1.5 (Fig. 13f).



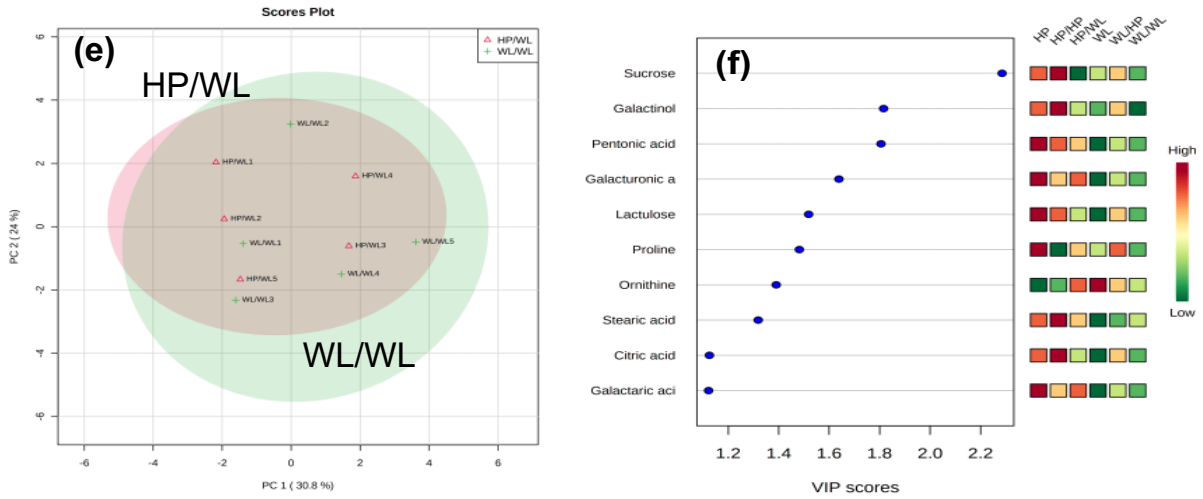


Figure 13. PCA and VIP plots from PLS-DA showing the metabolite differences in freshly harvested mature tubers of non-grafted (a), homo-grafted (b and c) and hetero-grafted (d and e) plants. The VIP plots (f) indicate the major differences between each category.

In a previous study, URI et al. (2014) found a large difference in the metabolite composition of HP and WL tubers grown from first generation seed tubers under open air screen-house conditions. In this study, plants were propagated *in vitro*, potted and grown further under greenhouse conditions. Although the majority of the detected compounds were identical in the previous and current study, the PCA plots of the tubers grown in a screen-house had a higher separation rate than those grown in a greenhouse. This difference might be explained by the better controlled environmental conditions in a greenhouse than in a screen-house. An alternative explanation may be based on the genetic diversity of the starting material, which could be different in the case of seed tubers and *in vitro* propagated plants.

5.1.4. Testing the effect of grafting on specialized metabolites

To gain insight into the effect of grafting on the concentrations of the metabolites highlighted by the VIP plots as the most important features separating the different plant categories (i.e., those compounds with values >1.5), bar graphs were prepared for each compound, and ANOVA with post hoc Tukey HSD tests were applied to identify the significant differences. In the leaves, four compounds had a value >1.5 (Fig. 10f); however, only galactinol, the metabolite with the highest VIP score, showed a tendency of grafting-dependent changes. The galactinol concentration was 8-fold lower in the HP leaves than in the WL leaves. The grafting, especially hetero-grafting, increased the amount of galactinol in the HP leaves and decreased it in the WL

leaves. Although, none of these changes were significant at the $p < 0.05$ level, there was a clear tendency of changes (Fig. 14a) suggesting that the concentration of this compound is influenced by the hetero-rootstock. Galactinol is formed from UDP-galactose and myo-inositol. Galactosyl-sucrose oligosaccharides, as for example raffinose and stachyose, are synthesised from galactinol and sucrose and from galactinol and raffinose, respectively, producing myo-inositol as a by-product (SENGUPTA et al. 2015). The level of myo-inositol was similar in the leaves of all plants, while the amounts of raffinose and stachyose were under the detection level. Thus, we suppose that the different galactinol concentrations in different plant categories are rather related to differences in the rate of catabolism or phloem transport from source to sink organ than to galactinol or galactinol-derived oligosaccharide synthesis.

In the case of the tubers, five compounds had a VIP score >1.5 (Fig. 13f); of those compounds only the concentration of the most important compound, sucrose, was significantly ($p < 0.01$–0.05) different between the tubers of the homo- and hetero-grafted plants. Fig. 14b shows that there was a 2.7-fold difference between the sucrose content of the HP and WL tubers and a 3.0-fold difference between the sucrose content of the HP/HP and WL/WL tubers, and these differences were not changed significantly by hetero-grafting (Fig. 14b).

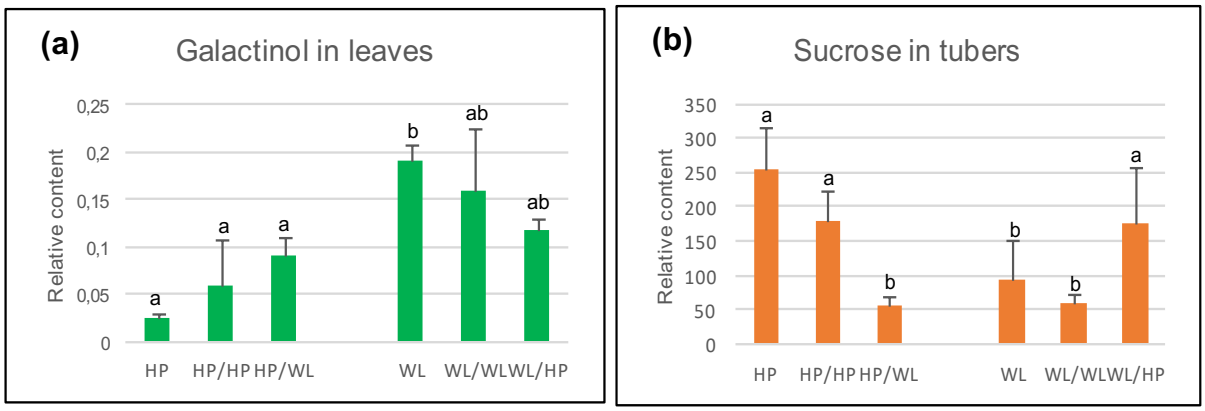


Figure 14. Differences in the galactinol and sucrose contents of leaves and tubers, respectively, detected by GC-MS; the relative data shown on the *Y*-axis are derived from a comparison of the peak sizes of the samples and the internal standard, ribitol.

Sugar transport between source and sink tissues has been excessively studied and found that it is mainly facilitated by the translocation of sucrose molecules (FERNIE et al. 2002). Several experiments showed that by manipulating the expression level of enzymes involved in starch synthesis the sucrose and glucose contents of tubers can be strongly influenced (MÜLLER-

RÖBER et al. 1992; TAUBERGER et al. 2000; TRETHEWEY et al. 2001; FERNIE et al. 2002; HAJIREZAEI et al. 2003; JUNKER et al. 2006). In those experiments, however, expression of target genes was manipulated by expression of a suitable construct driven by the *CaMV35S*, *PATATIN* or *rolC* promoter in the whole plant or in a tuber- or vascular tissue specific manner, respectively. In contrast, in our experiments, only natural signals derived from scion could influence gene expression and/or metabolite pathway regulation in rootstock and vice versa. Since no significant alterations in sucrose concentrations of tubers developed on hetero-grafted compared to homo-grafted plants were detected it was concluded that the sucrose content of HP and WL tubers is genetically determined.

Other traits than sucrose or galactinol content of tubers, however, can be influenced by grafting. ZHANG and GUO (2019) demonstrated that the starch content and vitamin C level in the potato tubers were significantly decreased after grafting with tomato, but the reducing sugar content increased. The tuber yield was decreased and several tubers sprouted at harvest that might be the reason of reduced starch- and elevated reducing sugar concentrations of tubers. Tomato/potato hetero-grafting changed the expression of almost three-thousand genes (ZHANG et al. 2019).

5.2. Characterisation of the *GIGANTEA (GI)* genes in potato

5.2.1. Organ-specific expression of *GI* genes in *S. tuberosum* cv. 'Désirée'

Two transcript variants of *GIGANTEA* gene were found in *S. tuberosum* Group Phureja. One of them, represented by XM_006358978.2, located on chromosome 4 (*StGI.04*), while the other one, represented by XM_006361554.2, located on chromosome 12 (*StGI.12*). Their promoter sequence was analysed *in silico* using The Plant Regulation Data and Analysis Platform (PlantRegMap) and it was concluded that the regulation of the two *StGI* genes is not identical. Although some *cis*-elements were identified in the promoter region of both genes the locations of these elements and several other *cis*-acting regulatory elements (CAREs) were different (KARSAI-REKTENWALD et al. 2022).

To test the expression of *StGI.04* and *StGI.12* in different organs of potato gene-specific primers were designed (Table 4) and used in RT-qPCR analysis. *StGI.04* mRNA was detected in each tested organ with the highest levels in root, stolon and sepal, lowest levels in tuber and petal and medium levels in stem, petiole and source- and sink leaves. In the case of *StGI.12*, little or no

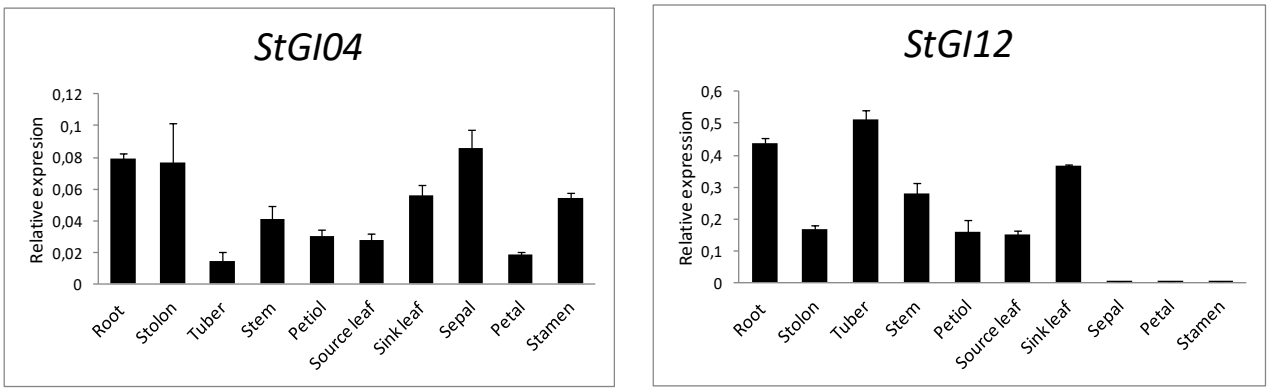


Figure 15. Organ-specific expression of *StGI.04* and *StGI.12* genes in *S. tuberosum* cv. 'Désirée'. *Y*-axis shows mean relative expression values of *StGI* genes compared to the geometric mean of Ct values of *ACTIN* and *EF1α* ± standard deviation, from three technical replicates.

expression was detected in flower organs whereas it was expressed at relatively high levels in root, tuber and sink leaves and at moderate levels in stolon, stem, petiole and source leaves. In general, the level of *StGI.12* expression was higher than that of *StGI.04*. In root, for example, the *StGI.12* mRNA level was 5-fold, whereas in tubers, it was 30-fold higher than that of *StGI.04* (Fig. 15). These results indicate that the expression pattern of the two *GI* genes is unique and organ-specific.

Organ-specific expression of *GI* gene was previously reported in *Arabidopsis,* soybean and sweet potato. In *Arabidopsis*, *AtGI* had high expression level in the inflorescence apices, young flowers, and young siliques (FOWLER et al. 1999) and higher mRNA level in shoots than in roots (LEE and SEO 2018). In soybean (*Glycine max*), three *GI* homologues (*GmGI*) were identified. Under LD conditions, *GmGI* transcripts had the highest level in the 2nd trifoliolates and floral buds at flowering. Under SD conditions, *GmGI1* showed the highest expression levels in roots at unifoliolate opening and in leaves at flowering. However, *GmGI2* and *GmGI3* always had the highest mRNA level in roots (LI et al. 2013). TANG et al. (2017) reported that in sweet potato (*Ipomea batata*), the *IbGI* expression was stronger in leaves and roots than in stems. In our study, *StGI.04* showed the highest transcript levels in root, stolon and sepal, while *StGI.12* in root, tuber and sink leaves. Thus, the organ specificity of *GI* expression appears to be species- and allele-specific.

Expression level of *StGI.12* in root and shoot organs was approximately five times and in tubers thirty times higher than the expression level of *StGI.04*. Regulation of transcription is a complex process, which depends on the availability and activity of TFs and the type, number,

position and combination of regulatory elements present in and around the promoter (reviewed by HERNANDEZ-GARCIA and FINER 2014). Thus, considering the results of a previous work (KARSAI-REKTENWALD et al. 2022) we speculate that the higher activity of *StGI.12* may be explained by the higher proximity of CAREs in the *StGI.12* promoter with the core promoter region than in the *StGI.04* promoter.

5.2.2. Effect of stress treatments on the expression of *GI* genes in *S. tuberosum* cv. 'Désirée'

JOSE and BÁNFALVI reviewed (2019) that *GI* is involved in abiotic stress regulation and in a few plant species it was shown that the expression of *GI* in leaves is influenced by stresses. Moreover, *in silico* analysis of *GI* promoter regions resulted in prediction of binding sites for TFs responding to ABA and abiotic stresses, such as salt, water deprivation, cold and heat (KARSAI-REKTENWALD et al. 2022). To test the effect of the predicted factors on the transcription of *StGI* genes detached source leaves of plants grown in a greenhouse were subjected to the various treatments and analysed by RT-qPCR. The stress inducible genes *P5CS*, *GWD* and *HSP20-44* were used to test the efficiency of treatments. PEG, cold and heat up-regulated *StGI.04*, but down-regulated *StGI.12* and while ABA induced *StGI.12* expression it had no effect on *StGI.04* (Fig. 16). The salt stress repressed *StGI.12*, but did not influence the *StGI.04* mRNA level. Thus, one can conclude that the two *StGI* genes respond to stresses and ABA in a different way.

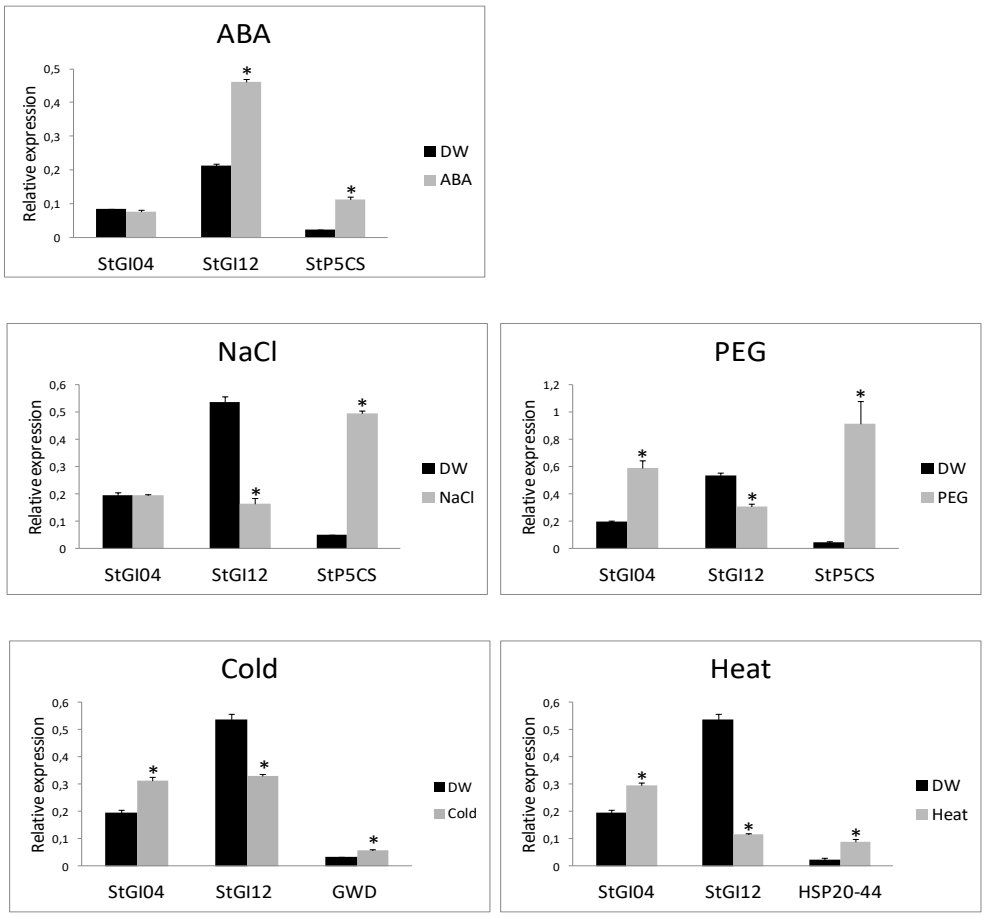


Figure 16. Expression level of *StGI* genes determined by RT-qPCR using total RNA from stress-treated leaves. *Y*-axis shows mean relative expression values of *StGI* genes compared to the Ct values of *ACTIN.* The treatments were carried out with 3 source leaves of 6-week-old greenhouse-grown plants. Efficiency of treatments was tested by the up-regulation of *Δ1-PYRROLINE-5-CARBOXYLATE SYNTHETASE (StP5CS), α-GLUCAN, WATER DIKINASE (GWD)* and *HEAT SOCK PROTEIN 20-44 (HSP20-44).* DW- distilled water, non-treated leaves.

HAN et al. (2013) showed that the peak level of *AtGI* mRNA, for example, is up-regulated under drought stress. Also, PALTIEL et al. (2006) demonstrated a strong increase in *GI* expression to elevation of temperature in both *Arabidopsis* and *Medicago truncatula*. In *Ipomea batata*, *GI* expression is up-regulated by high temperature, drought, and salt stress but down-regulated by cold stress (TANG et al. 2017). Here we showed that the expression of *StGI.04* is induced by cold, heat and osmotic stresses. In contrast, *StGI.12* expression is repressed by the same stresses and also by salt stress, which has no effect on *StGI.04*. It has been known for a long time that ABA rapidly accumulates in plants in response to environmental stress and plays a pivotal role in the reaction to various stimuli (reviewed by SIRKO et al. 2021). ABA induced *StGI.12* but not *StGI.04* expression. The reason of all these differences may be the presence of MYB TF binding sites in

StGI.04, which is lacking from *StGI.12* promoter. The MYB TFs present in the *StGI.04* promoter respond mainly to salicylic- and jasmonic acid suggesting that different signal transduction pathways lead to up- and down-regulation of the two *StGI* genes as a reaction to different abiotic stresses. Nevertheless, the core sequence ACGTG for binding sites of ABA-responsive TFs are present in both *StGI* promoters (KARSAI-REKTENWALD et al. 2022).

5.2.3. Selection of *StGI.04*-repressed lines

Besides tuberization, no detailed characterisation of the *GI* genes has been reported thus far in potato; therefore, it was decided to study their function by antisense repression of gene expression starting with *StGI.04*. To reach this goal JOSE (2019) cloned a 250-bp fragment of *St.GI04* in an antisense orientation into the plant transformation vector pCP60 between the constitutive *CaMV35S* promoter and the *nos* terminator and generated 56 transgenic 'Désirée' (DES) lines.

Primary selection of *StGI.04*-repressed lines (aGI lines) out of the 56 transgenic lines was based on RT-PCR of leaves harvested from plants grown *in vitro* using a *StGI.04*-specific primer pair tested earlier by F. Karsai-Rektenwald. Twenty lines were found with lower level of *StGI.04* expression than the non-transformed control DES by visual observation of the intensity of bands in ethidium bromide-stained agarose gels. Five lines (aGI43, aGI44, aGI52, aGI53, aGI55) with different level of reduction in *StGI.04* transcript level were selected for further studies. Expression of *StGI.04* was quantified in the selected five lines with RT-qPCR. The highest repression, 49% of *StGI.04* mRNA amount detected in DES, existed in aGI52, while only a minimal, 2% reduction was found in aGI55 (Fig. 17a).

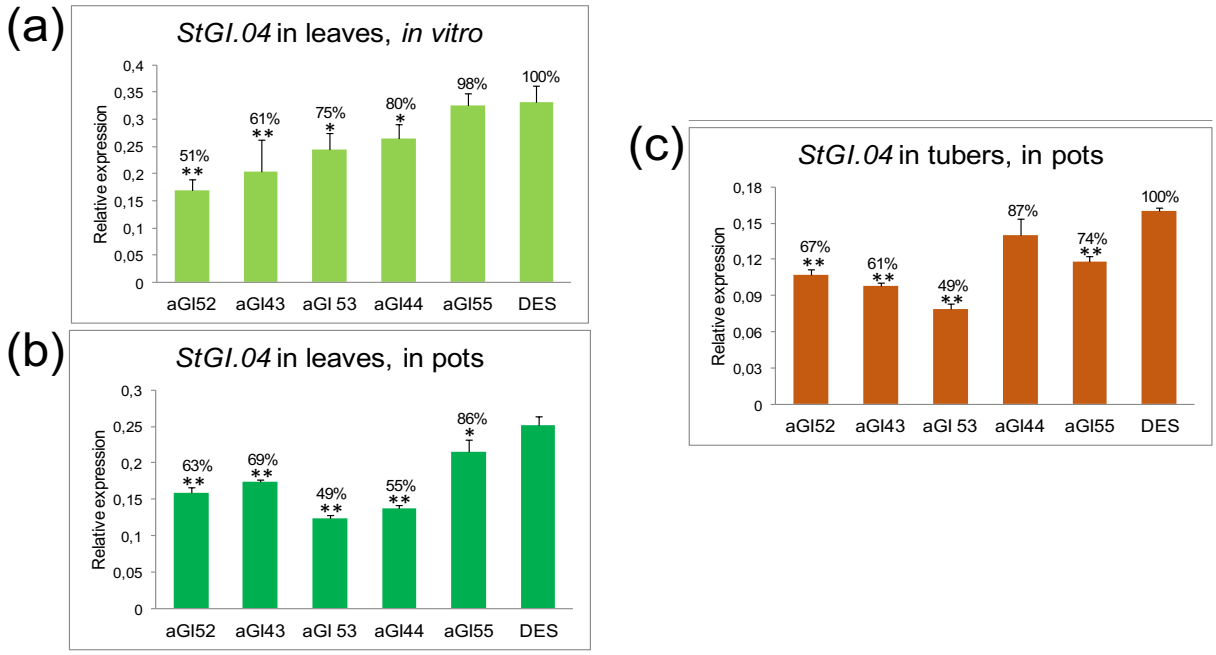


Figure 17. Level of *StGI.04* repression in aGI lines compared to the non-transformed control 'Désirée' (DES). RNA was isolated from (a) middle leaves of *in vitro* plants; 3 leaves/line harvested from 3 plants, (b) source leaves of 8-week-old plants grown in pots in a greenhouse; 9 leaf discs of 1 cm in diameter/line harvested from 9 plants, (c) mature tubers harvested at the end of the vegetation period; 3 sets/line, 3 tubers/set composed from the largest tubers of each line distributed into approximately equal groups. *Y*-axis shows mean relative expression values of *StGI.04* gene compared to the mean expression values of *ACTIN*.

The five selected lines were propagated *in vitro*, transferred to pots and grown further under greenhouse conditions in 12 parallels. Expression of *StGI.04* was re-tested in leaves (Fig. 17b). Four lines possessed significantly ($p < 0.01$) lower *StGI.04* expression than DES. Like in leaves of *in vitro* plants, the less difference compared to DES was found in aGI55, but even this difference was significant at $p < 0.05$ level. The *StGI.04* transcript level was a little bit higher, 63% versus 49%, in aGI52 leaves of greenhouse-grown plants versus leaves of *in vitro*-grown plants.

At the end of the vegetation period, the tubers were harvested and the level of *StGI.04* expression was tested in tubers (Fig. 17c). In line with the lowest expression in leaves (Fig. 17b), the lowest expression was detected in tubers of the aGI43 plants (Fig. 17c). All lines except aGI44 showed a significant ($p < 0.01$) level of *StGI.04* repression in tubers including aGI52 with 43% of wild-type *StGI.04* mRNA level.

The line aGI52 showed significant ($p < 0.01$) and relatively stable level of reduction in *StGI.04* expression in all three RT-qPCR analyses (Fig. 17). Therefore, this line was used to test

the specificity of antisense repression. Although the less homologous region of *StGI.04* to *StGI.12* was used for generation of aGI lines the identity of the two regions was still 71.2%. Thus, the repression of *StGI.12* expression by the *StGI.04* fragment could not be excluded. To test this possibility a *StGI.12*-specific primer pair (Table 4) was used in parallel with the *StGI.04*-specific primer pair (Table 4) in the RT-qPCR analysis of aGI52 and DES leaves and tubers. Fig. 18 shows that the repression in aGI52 was *StGI.04*-specific and did not extend to *StGI.12*.

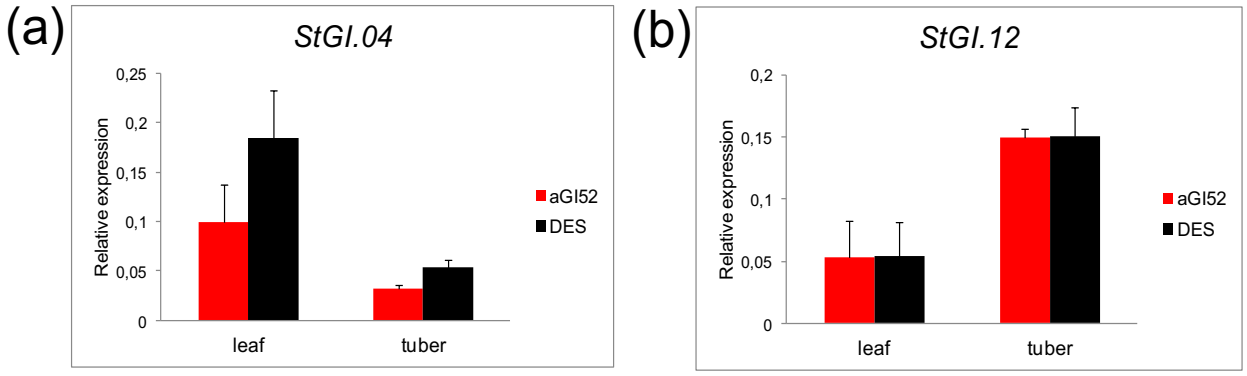


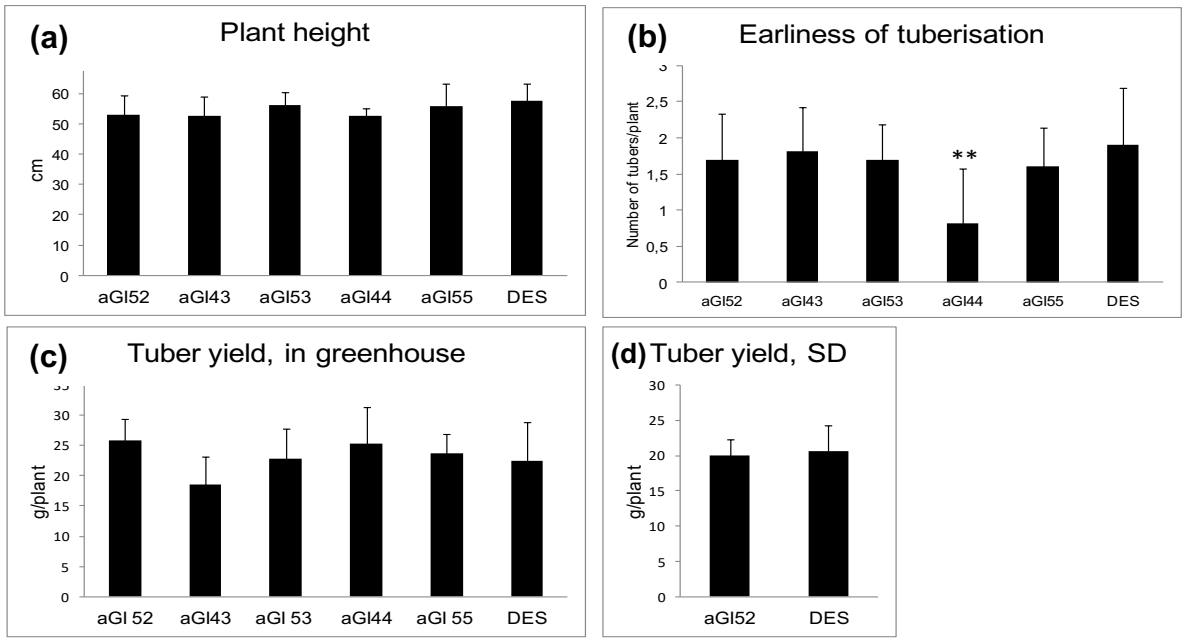
Figure 18. Specificity of *StGI.04* repression in aGI52. The RT-qPCR analysis was carried out using (a) the GI04spec and (b) the GI12spec primer pair. *Y*-axis shows mean relative expression values of *StGI* genes compared to the mean expression value of A*CTIN*.

5.2.4. Phenotypes and tuberization of aGI plants

Development and morphology of aGI43, aGI44, aGI52, aGI53, aGI55 plants grown in the greenhouse was visually followed and compared to DES. Height of the plants was measured at seven weeks after transferring them from *in vitro* into pots. Neither phenotypic changes nor height differences were observed (Fig. 19a). The earliness of tuberization was tested also at seven weeks after planting by counting the number of tubers after carefully tipping the plants out of the pots. Significant delay in tuber initiation was detected only in the line aGI44 (Fig. 19b). After counting, the plants were replaced into the pots and grown until the end of vegetation period when the tubers were harvested and measured for weight. No difference in tuber yield was obtained between the aGI lines and DES (Fig. 19c). The size distribution of tubers was also similar to DES peaking at 8-10 cm in diameter except aGI44 and aGI55, which produced larger number of small tubers than DES (Fig. 19e-j).

The molecular model of tuber formation is based on *S. andigena*, a strict SD plant for tuberization (KLOOSTERMAN et al. 2013). Therefore, we wanted to test the effect of *StGI.04* repression not only under LD (12h light/12h dark) but also under SD conditions (8h light/16h dark). The line aGI52 was compared to DES in this experiment and the plants cared by J. Jose at the Centre for Agricultural Research in Martonvásár. Even under SD conditions, no difference in canopy phenotype or tuber yield was detected between aGI52 and DES (Fig. 19d).

Earlier, two POTH20 TF binding sites were identified in the promoter region of *StGI.04* (KARSAI-REKTENWALD et al. 2022). It was shown earlier that overexpression of POTH1, a KNOTTED-like homeobox gene with 73% identity to POTH20, enhanced *in vitro* tuberization under both SD and LD photoperiods in several potato lines (ROSIN et al. 2003). Therefore, we had the idea that the repression of *StGI.04* would result in alteration of tuber formation. However, it was not the case. Thus, it was concluded that the level of reduction in *StGI.04* transcript level that we could achieve might not be high enough for influencing the tuberization in the commercial potato cultivar 'Désirée'.



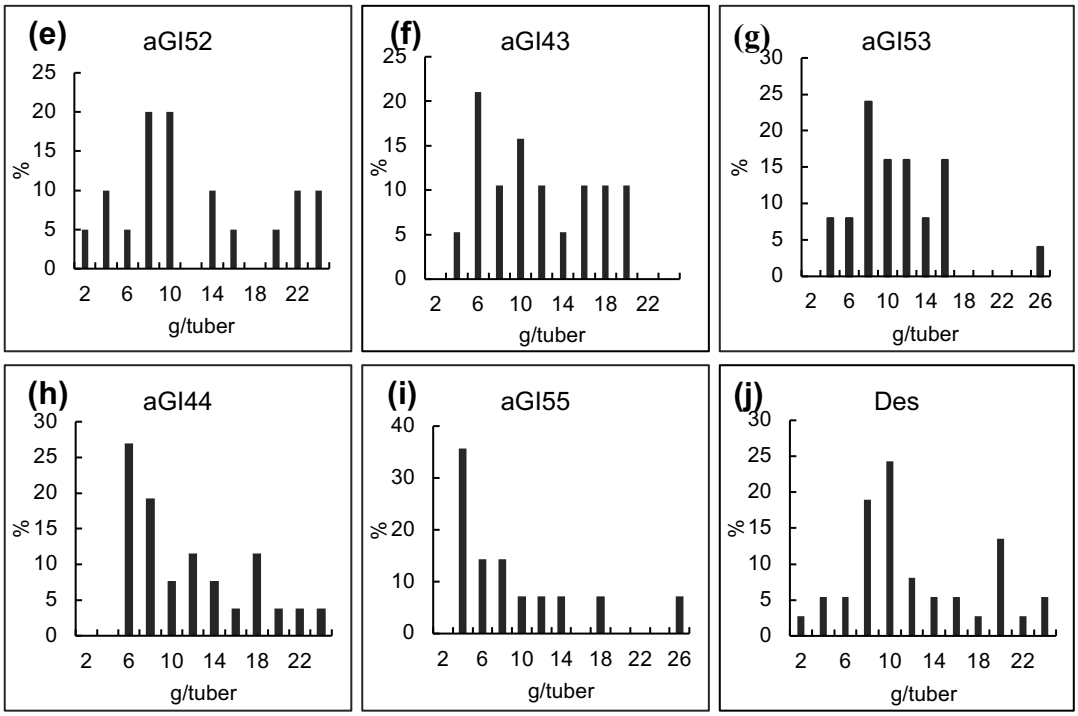


Figure 19. Growth and tuberization parameters of aGI lines compared to the non-transformed DES control. (a) Height of plants and (b) Earliness of tuberization tested 7 weeks after transferring the plants from *in vitro* culture into pots. (c) Tuber yield under greenhouse conditions at the end of the vegetation period. (d) Tuber yield under SD conditions at the end of the vegetation period. (e-j) Distribution of tuber sizes in percentage at the end of the vegetation period.

5.2.5. Anthocyanin content of tuber peels

DES is a red-skinned potato. The skin colour of aGI tubers, although with different extent, but was lighter than the skin colour of DES (Fig. 20). The reduction in colour was more or less in accordance with the level of *StGI.04* repression in tubers being the most pronounced in aGI52 and aGI53. The difference in tuber skin colour was also obvious between aGI52 and DES grown under SD conditions (Fig. 21a). Since anthocyanins determine the skin colour (LEWIS 1997) these compounds were extracted from tuber peels and their relative quantity measured in aGI52 and DES. In comparison with DES, 43% reduction in anthocyanin content was found in aGI52 tuber peels (Fig. 21b).

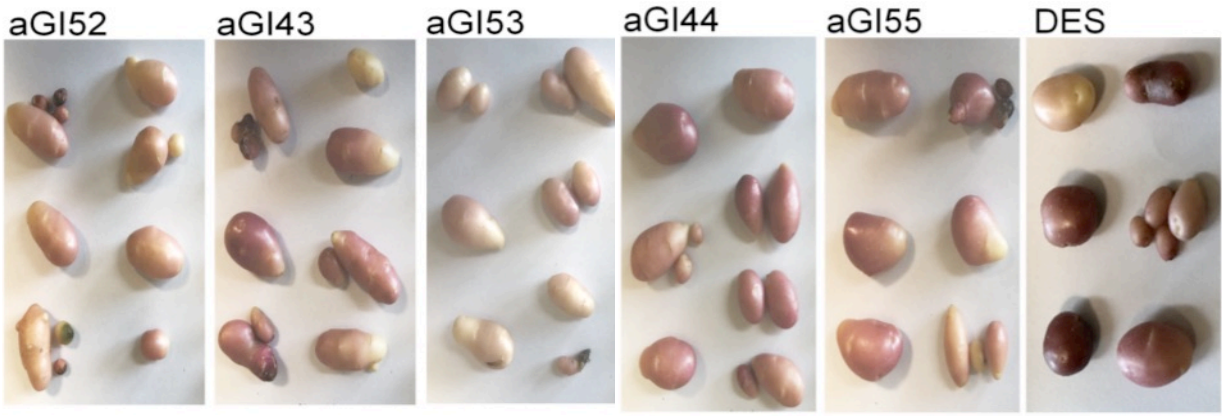


Figure 20. Morphology of mature tubers collected from 6-7 plants/line grown in a greenhouse in pots.

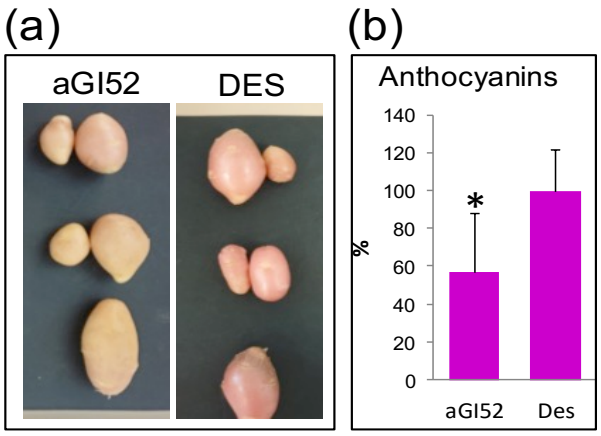


Figure 21. Morphology of mature tubers of three aGI52 and three non-transformed, control DES plants grown under SD conditions (a) and the anthocyanin pigment content of the aGI52 tuber peels compared to DES tuber peels in percentage (b) Anthocyanins were measured spectrophotometrically. Tuber skins were peeled from 3 sets of largest tubers per line; each set contained 3 tubers. The extraction was from equal amounts of skins.

It has been known for a long time that three loci, *D* (developer), *R* (red), and *P* (purple) determines tuber colour. JUNG et al. (2009) demonstrated that the *D* locus encodes an R2R3 MYB TF, a part of the MYB-bHLH-WD40 complex, regulating anthocyanin synthesis. Several alleles of R2R3 MYBs as StMYBA1 and StMYB113 were identified in cultivated tetraploid potatoes (JUNG et al. 2009) and shown that an *R2R3-MYB* is a direct target of the small RNA regulation (BONAR et al. 2018). Thus, we presume that the repression of *StGI.04*, directly or indirectly, influences the activity of these transcription factors and thereby regulates the synthesis of

anthocyanins in potato. To or knowledge, this function has not been reported for *GI* in any other plant species investigated thus far.

5.3. Characterisation of the *BIG BROTHER* (*BB*) gene in potato

5.3.1. Identification of the *BB* gene in potato

As it is shown in Figure 4, we found a positive correlation between the canopy development and the time of tuber initiation in two commercial potato cultivars and their grafts. It has been already known at that time that the *BB* gene restricts the leaf development in *Arabidopsis* (VERCRUYSSE et al. 2020). Assuming a similar function of *BB* gene in potato we thought that down-regulation of *BB* gene expression might result in a higher leaf expansion rate and earlier tuberization in potato. In order to identify the *BB* gene in potato a search for the *A. thaliana* RING/U-box superfamily protein (*AtBB*) NM_148885.3 homologue was carried out using the nucleotide BLAST tool available at NCBI. Two transcript variants XM_006356317.2 and XM_006356318.2 both located on chromosome 11 were found. However, it turned out that they were 100% identical at transcript level and predicted to be the *S. tuberosum* E3 ubiquitin ligase *BIG BROTHER-like* (*StBB*) gene with 70% identity to *AtBB* (Fig. 22).

```
AtBB      -CGACGTCGTTTTGTCTCCTTCCACACACTCTTTCCTCTCTCTTTCTTCTCTCTTTCTTT      59
StBB      GAGAGAGAGAAAAAAGTGAAAAAAAACTCAATTTG-C--------AACTCTGTCTTCATCT      52
            **    *       *      * ** *  ***            *** ****  * *

AtBB      TCTCTCTCTCTCCTCTGCTCCTCCGTCTCT--CGTCTACAGTGCCC----TCCGCATCAC      113
StBB      TCTCTCTCTCATGCCATTTTCACACACAAAGGAGAAAAAAGAAGAAGAAGAGAGAACAAC      112
          **********    *   * * *   *      *   * **           * *  **

AtBB      CTTTTTCCTTGTCCTAT-GAATTTGGT-----CGAAATGCCCTTCTCCTCCTCCTCCTTC      167
StBB      TTTGATGAACTGCATAAGGCATTCAATAAAGCAAACAAACTAATGATTGCCAAATCTGAA      172
           **  *      * **  * ***   *       * *  *   *     **   **

AtBB      CACTAATCTCAAATATATCCTTCGAGACTCTCCCTTGCCGTCTCCAATTG----CCACTC      223
StBB      GAGGATCC--ACATTTATTATCTTGGAATCAAAGTTTCATAAGTTGATTGTTCATCTCTG      230
           *  *  *  * ** ***  *    ** **    ** *        ****     * **

AtBB      ACCGCTCCAACTCTCTTCGAATTAGCTGAAA--------T-GAATGGAGATAATAGACCA      274
StBB      TGCTCTCCAGAAGAATTAGTGTGTGCTGCAGAATAGTAATTCACCATGAACTGGAATCAG      290
            * *****      ** *  *  **** *         *  *      *    *  *

AtBB      GTGGAAGATGCTCATTACACGGAGACAGGTTTCCCTTATGCTGCTACTGGAAGTTACATG      334
StBB      CAAACGGAAATTTATTACACAAATGGTGCTGTGCCTTATAATTCAATTGGAAGTTTTATG      350
                **   * *******  *    * * * ******  * * * ********  ***

AtBB      GACTTTTATGGTGGTGCGGCTCAGGGGCCTCTTAACTACGATCATGCCGCAACTATGCAT      394
StBB      GATTTCTTTGGAGGTGTTACATATGACCATGTTAATTATATATTTGCCGATCCTCCCTAT      410
          ** ** * *** ****   *  * *  * * **** **      *****   **    **

AtBB      CCTCAGGACAATCTGTACTGGACCATGAATACCAATGCATACAAGTTTGGGTTTTCAGGA      454
StBB      GCTCAGGAGAGTTTATATCCATCCATCAGCACCAATCCATACAAATTTGGTTATTCTGAA      470
           ******* * * * **     **** *  ****** ******* ***** * *** * *

AtBB      TCAGATAATGCTTCTTTCTATGGTTCATAT---------GACATGAACGATCATTTATCG      505
StBB      GCAGGTAGTTTCTCGTATTACGATTATGATCATGAATATGTGGTGAATGATCATGTATCT      530
           *** ** *   ** *  ** * **   **         *   **** ****** ****

AtBB      AGGATGTCCATAGGGAGAACAAATTGGGACTATCATCCCATGGTGAACGTTGCTGATGAT      565
StBB      GGAATCGAGGAGCATGATAGACATTTAGAAAACCCTTCAACTGCCAC---------TGTA      581
           * **             * * ***  **  * * * * *  *  *          **

AtBB      CCTGAAAACACAGTTGCACGTTCCGTCCAAATCGGAGACACAGATGAGCACTCTGAAGCT      625
StBB      AATGTAGCTGCAAATGTGCATAGAGAGGAAATTTCAGGCTCCAATTCACTCACTAATTCT      641
            ** *    **  **  * *   *   ****   ** * *  **   * * ** *  **

AtBB      GAAGAATGCATTGCAAATGAGCATGATCCCGACAGTCCTCAGGTATCCTGGCAAGATGAC      685
StBB      GTGGAATGTCCCAGGGGTCAAATTAATACTCGTGACAGTGAGGTTGTTTGGCACGATAGT      701
          *  *****         * *   * ** *         * ****    ***** ***

AtBB      ATTGATCCTGATACAATGACCTATGAGGAATTAGTAGAGCTGGGGGAAGCAGTAGGAACA      745
StBB      ATCGACCCTGACAACATGACCTATGAGGAATTACTTGAGTTGGGGGAGGCTGTTGGAACT      761
          ** ** ***** *  ****************** * *** ******* ** ** *****

AtBB      GAAAGCAGGGGGTTGTCTCAGGAACTCATAGAAACGCTTCCCACTAAAAAGTATAAGTTT      805
StBB      CAAAGCAGAGGCCTTTCCCAAGATCAAATCTCCTCGCTTCCAGTCACAAAGTTTAAGTGT      821
           ******* **  * ** ** ** * *  **     *******    * ***** ***** *

AtBB      GGGAGCATCTTCTCCAGGAAAAGAGCTGGGGAGAGGTGTGTGATATGCCAGCTCAAGTAC      865
StBB      GGCTTTTTCTCAAGAAAGAAATCAAGAAAGGAAAGGTGTGTGATCTGCCAGATGGAATAC      881
          **     ***     * ****  *     *** *********** ****** *  * ***

AtBB      AAGATAGGGGAGAGGCAAATGAATCTGCCGTGCAAGCATGTGTATCATTCTGAATGCATT      925
StBB      AAACGAAAGGATCGGCAGGTCACGCTTCCTTGCAAACATGTCTATCATTCTGGTTGTGGA      941
          **   *  ***  ****  * *  ** ** ***** ***** **********  **
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AtBB      TCCAAATGGCTAAGCATCAACAAGGTTTGCCCGGTGTGTAACAGCGAGGTCTTTGGGGAG      985
StBB      AGCAGATGGCTAAGTATCAACAAAGCTTGCCCAATTTGCTACTCAGAAGTGGTGATCAAT      1001
            ** ********* ******** * ******  * **  **   ** **  *     *

AtBB      CCCAGCATTCATTGATCGGCACAAGGGGC------------TCCTCCTCTTCTTTTCTTT      1033
StBB      ACATCAAAGCGGTGAAGCAAATAATAAGCACCGATGGGGACTGAAATCATATTTTTCTTC      1061
           *    *  *  ***     * **   **            *       *  *******

AtBB      TTG--------------GCTTTTTATAT------------CGAGGCTCATCAAGTAATTG      1067
StBB      TTGGTCAAAGCATTTTCCCTTTCTATTTTCGTTATAGAATCTTATATTTCCTGTTCCTTG      1121
          ***               **** *** *            *     *   *   *  ***

AtBB      TTTTAGTGTAGTGAAAACCCCAAAAAATAGTCTAAAAGATGTC--CACACTATACTCTCT      1125
StBB      TGTTACATTAGAGAAATAGTTTAGTGGTAAATAAGAACATAAAGAAACACCACATTCGTT      1181
          * ***   *** ****      *    **    * ** **      **** * * **  *

AtBB      CATGTTCAGTCCTTCTCTGTACATGTAATTTTTCTTCTAGTTCCATTTTCGCTTGTGTGT      1185
StBB      AACGATGGAGCTTT-GTACCACGTGTTCTGTTTTACATAT-------TTAGCCTTGATCT      1233
           * * *    * **      ** ***  * ***    **        ** ** *   * *

AtBB      GCTTTAAGTTTAACAGTCACTCGTATTGTATACTAAATGCTAAGTCAAAAACGCTGAATC      1245
StBB      --CAAAACTTTGTCACAGAAAGGATTTCTAGTTTTACTTTTTA-----------------      1274
               ** ***  **   *   *  ** **   * * *  * *

AtBB      CATAT    1250
StBB      -----    1274
```

Figure 22. Nucleotide sequences of *AtBB* and *StBB* aligned by Clustal Omega multiple sequence alignment tool.

5.3.2. *In silico* prediction of transcription factors binding to the *StBB* promoter

The Plant Transcriptional Regulatory Map Platform (PlantRegMap) was used to predict transcriptional binding sites in the 3000-bp region upstream from the *StBB* translation start site (chromosome 11, from 37009829 bp to 37012829 bp, reverse complement) in the *S. tuberosum* Group Phureja genome sequence. The search resulted in identification of 48 binding sites for 29 TFs in the *StBB* promoter region at a threshold *p*-value $\leq 1e^{-5}$. 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 7). Location of the TF binding sites with the indication of TF families is presented in the top of Figure 23.

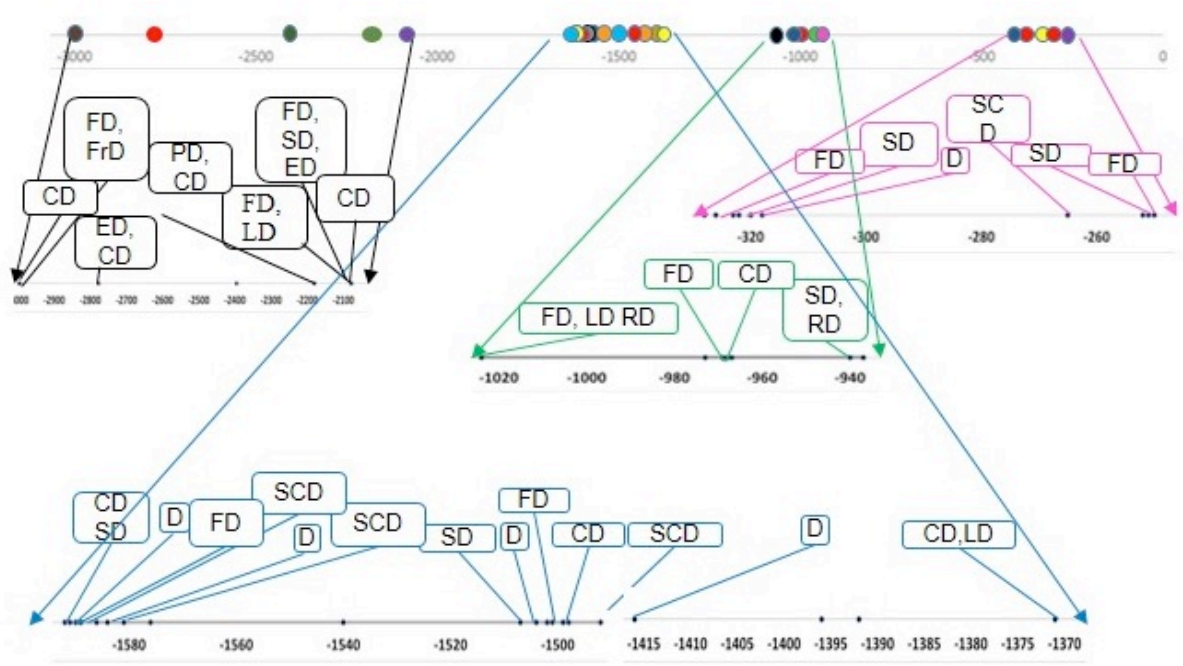

Figure 23. Predicted binding sites of TFs in the promoter region of the *S. tuberosum* Group Phureja *StBB* gene. Thin line represents the promoter region from the translation start site to -3000 bp. Round shapes in different colour represent predicted TF families: red, DOF; light blue, BBR-BPC; yellow, 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.

The predicted TFs are involved in a wide range of biological processes and respond to different internal and external stimuli (Table 7). Nevertheless, we found that 20 out of the 24 TFs with known functions are related to developmental processes as for example, the flower-, leaf-, stem- and root development or cell cycle regulation (Fig. 23) and only a few of them are involved in stress responses.

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. (2008), 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 and KEBEDE 2021).

5.3.3. Identification of the main CAREs in the *StBB* promoter

The binding sequences and locations of TFs with known functions are listed in Table 7. 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 family 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 (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 and 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 (BORNER et al. 2000; THEIßEN et al. 2016). 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 (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).

Table 7. Transcription factors with known functions binding to the *StBB* promoter

Family	Name	Position	Matched sequence	Function*
BBR-BPC	BPC6	-1589	ACTTTTTTCTCTCTCTCTCTC	Response to ET
		-1540	TTCATCTTCTCTCTCTCATGC	
	BPC1	-1591	AGGAGAGAGAGAGAGAAAAAAGTG	Response to ET, regulation of development
		-1416	AAGAAAGGAAGAATAATAAAGAGA	
		-1502	GAGAAAAAAGAAGAAGAAGAG	
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	AAAAGGGAAAAGCAAAGAAAA	Response to chitin
		-1581	GAGAGAAAAAAGTGAAAAAAA	
		-973	CTGAACAAAAAGGAAAACAAA	
		-1504	AGGAGAAAAAAGAAGAAGAAG	
	DOF5.9	-320	AACAAAAGGGAAAAGCAAAGA	Phloem or xylem histogenesis
		-1507	CAAAGGAGAAAAAAGAAGAAG	
		-251	AACCAAGCCAAAAAGGAAAAT	
	OBP3	-1586	TTCACTTTTTTCTCTCTCTCT	Photomorphogenesis
		-318	TTTGCTTTTCCCTTTTGTTAA	
	OBP1	-1498	TTCTCTCTTCTTCTTCTTTTT	Response to AUX and SA, cell wall modification, positive regulation of cell cycle
		-967	ATTCCCTTTGTTTTCCTTTTT	
GRAS	GAI	-1589	GAGAGAGAGAGAGAAAAAAG	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
		-1492	AAGAAGAAGAGAGAACAACT	
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	CTTTTTTCTCTCTCTCTCTCC	Translocation, response to cold and GA, positive
		-1501	TCTCTTCTTCTTCTTTTTTCT	

		-969	TCCCTTTGTTTTCCTTTTTGT	regulation of flower development
		-326	TTTTTTTCTTTGCTTTTCCCT	
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

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 7).

Hence analysis of promoters and CAREs is important for genetic engineering of crops, additional experiments are needed to decide whether the predicted CAREs are functional or not in the *StBB* promoter.

5.3.4. 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 24. 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.

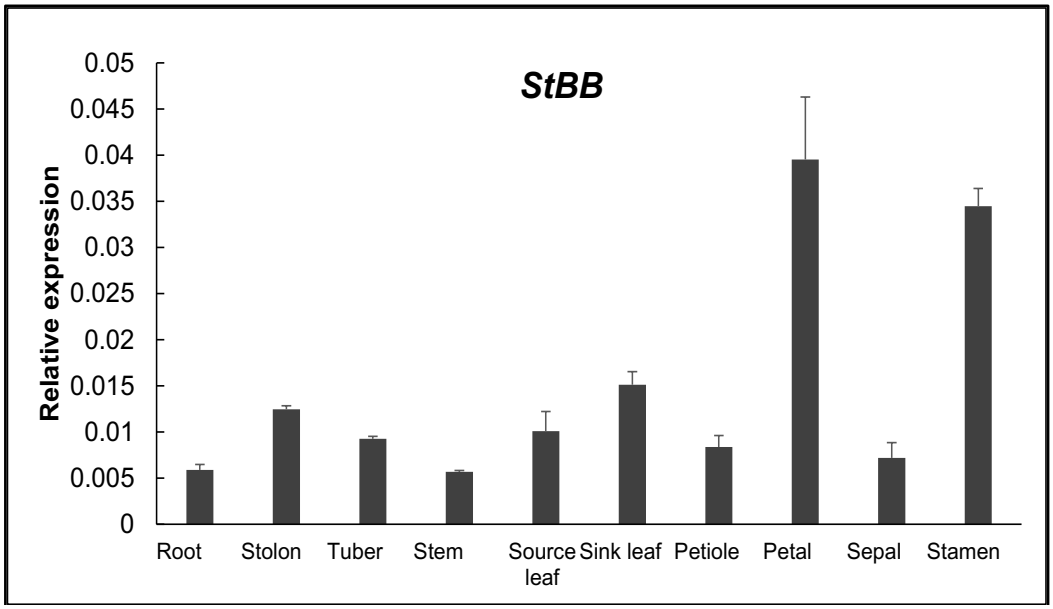

Figure 24. Expression profile of the *StBB* gene in different organs of *S. tuberosum* cv. 'Désirée' determined by RT-qPCR. Bars indicate mean relative expression values of *StBB* gene compared to the mean expression values of *ACTIN* and *EF1α* + SE (n = 3 technical replicates). Samples were collected from organs of 3-5 plants.

As shown in the section 4.3.3, 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.

5.3.5. Stress response of *StBB* gene in *S. tuberosum* cv. 'Désirée'

The *in silico* analysis of *StBB* promoter predicted some binding sites for TFs responding to salt, drought and cold stress and the stress-mediator, ABA (Table 7). To test the effect of stresses and ABA on the transcription of *StBB* gene detached source leaves of greenhouse-grown potato plants were subjected to different treatments and analysed by RT-qPCR. The stress-responsible genes *P5CS*, *GWD* and *HSP20-44* were used as control genes in the treatments. Figure 25 shows that under the NaCl and PEG treatments the leaves lost water and started to collapse, while the cold- and heat stress and ABA did not influence the phenotype of leaves. Data analysis revealed that the transcript level of *StBB* was slightly increased by the salt stress, however, statistically significant increase was observed only after 6 h of PEG treatment. No changes in expression were detected under cold and heat stresses or after ABA treatment (Fig. 26).

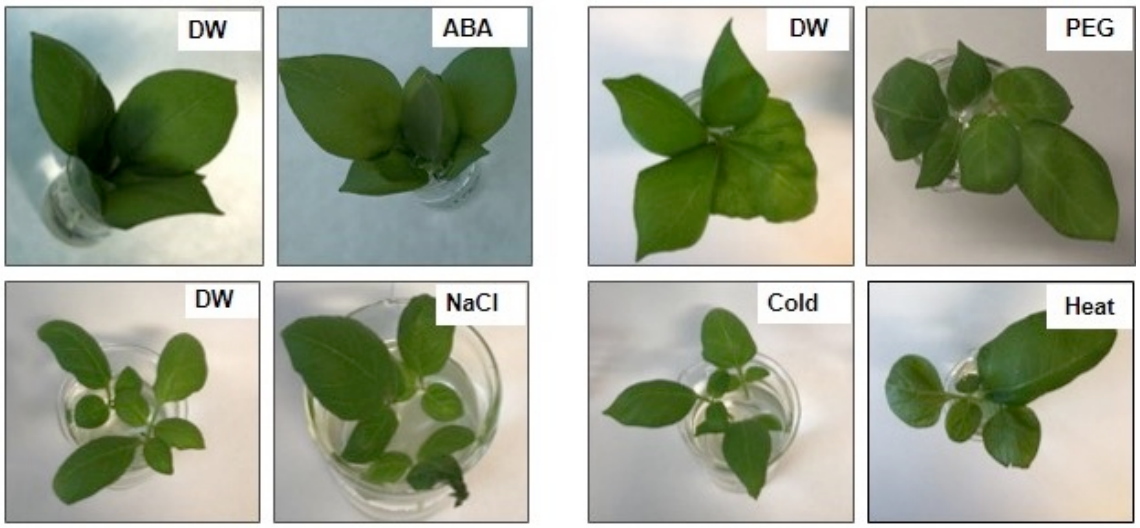

Figure 25. Effects of ABA treatment and abiotic stresses on the leaf phenotype of *S. tuberosum* cv. 'Désirée'

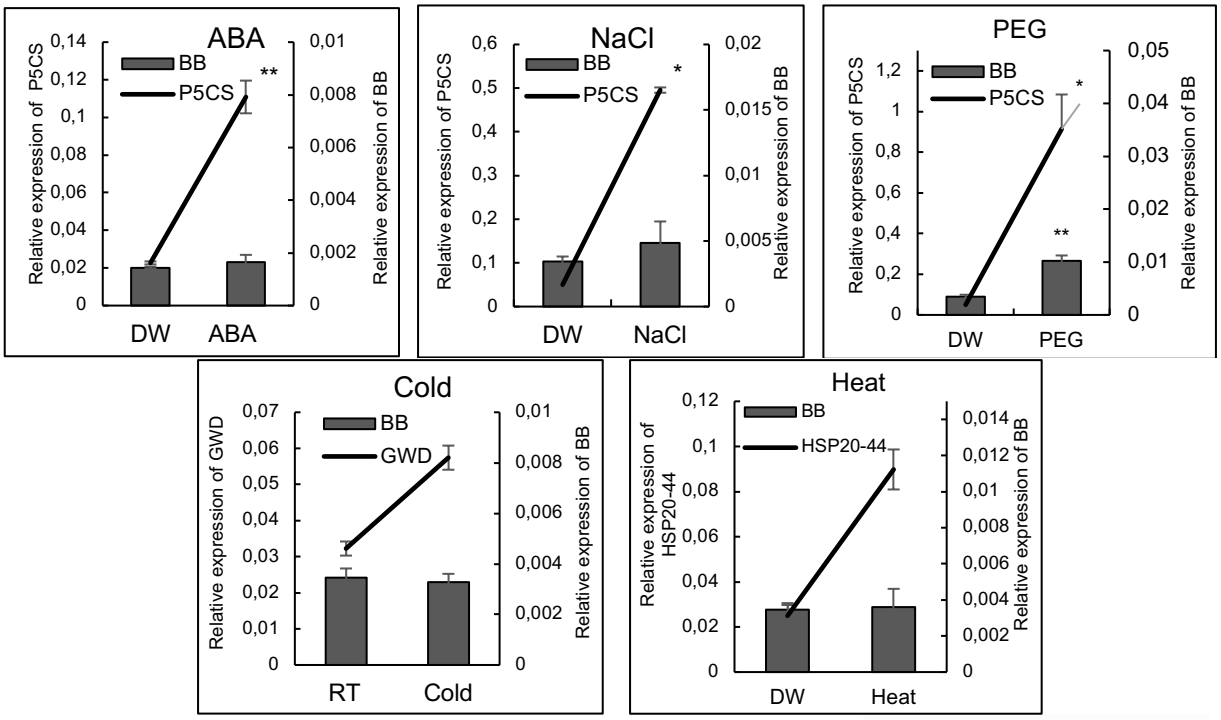

Figure 26. Relative expression level of *StBB* gene determined by RT-qPCR using total RNA from stress-treated leaves. The treatments were carried out with 3 composite source leaves of 6-8-week-old greenhouse-grown plants. Efficiency of treatments was tested by the up-regulation of *Δ1-PYRROLINE-5-CARBOXYLATE SYNTHETASE* (*StP5CS*), *α-GLUCAN, WATER DIKINASE* (*GWD*) and *HEAT SOCK PROTEIN 20-44* (*HSP20-44*). *Y*-axis shows mean relative expression values of genes compared to the mean expression values of *ACTIN* and *EF1α* + SE ($n = 3$ technical replicates). Statistical significance of the measurements was determined by the Student's *t*-test ($p \leq 0.01$) and labelled by an asterisk. DW, distilled water; RT, room temperature.

It is known that salt and drought stresses negatively affect the plant dry mass. For instance, after the drought stress treatment on pot-grown potato, the average yield of shoot mass is reduced by approximately 30% (BOGUSZEWSKA-MAŃKOWSKA et al. 2020). Furthermore, HAYASHI et al. (2011) demonstrated that plant photosynthesis is influenced by salinity and osmosis because inappropriate concentration of salinity disturbs nutritional balance and thereafter changes cellular structure. Another study showed that potato plant height, stem diameter, plant weight, root length and the number of lateral roots as well as stomatal apertures were reduced when the plantlets were cultured in a medium containing PEG, mannitol or NaCl (ZHU et al. 2020). In contrast, the short-term (3-6 h) cold stress did not significantly affect the relative water content and chlorophyll content of leaves of potato (ORZECHOWSKI et al. 2021). LIU et al. (2021) treated the potato cv. 'Hezuo 88' with a short period of heat and found that most stomata were opened after 6 h, however, no phenotypical changes on leaves were observed, the later which is in line with our observation.

Our results showed that the transcript level of *StBB* was slightly increased by the salt stress, however, statistically significant increase was obtained only after PEG treatment. QI et al. (2020) reported that the expression of one of the RING-finger ubiquitin ligase E3 genes, *StRFP2* (potato *RING-FINGER PROTEIN 2*), is up-regulated by the PEG-elicited osmotic stress, thereby enhancing the drought tolerance of potato. Hence *StBB* and *StRFP2* are both contain a RING-finger domain and *BB* is a functional E3 ubiquitin ligase (DISCH et al. 2006) we suppose that *StBB*, like *StRFP2,* enhances the drought tolerance of potato by limiting the plant growth under drought stress condition.

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. Nevertheless, based on the presumed function of StBB under drought condition the drought tolerance of the *StBB*-repressed plants may be reduced. 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 under optimal and sub-optimal growth conditions.

5.3.6. Selection of *StBB*-repressed lines *in vitro*

Functional analysis of the *StBB* gene was carried out as that of *StGI.04*; antisense repressed DES lines were generated and tested for phenotype and tuberization. Approximately 55-60 DES leaves in six Petri-dishes were transformed with the antisense construct using an *Agrobacterium*-mediated transformation protocol. After regeneration, shoots were isolated from individual leaves or distant positions of a leaf, out of which 10 rooted in antibiotic-containing medium (Fig. 27).

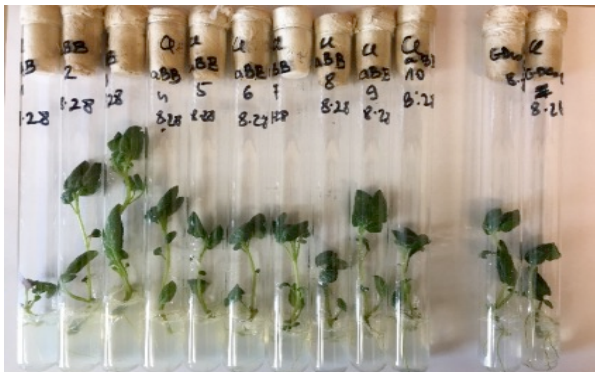

Figure 27. Four-week-old *in vitro* plantlets of aBB lines and the non-transformed control plant 'Désirée' (DES).

Primary selection of *StBB*-repressed lines (aBB lines) was based on RT-PCR of leaves harvested from plants grown *in vitro* using the StBB primer pair (Table 4). In a separate reaction, equal quality and quantity of cDNAs was checked with PCR using the *ACTIN* primers. Three lines were found with lower level of *StBB* expression than the non-transformed control DES by visual observation of the intensity of bands in ethidium bromide-stained agarose gels.

Expression of *StBB* was quantified with RT-qPCR in the three lines (aBB2, aBB3, aBB9) with lower expression than DES. Compared to the non-transformed control 26-33% reductions in *StBB* mRNA levels were detected. However, these reductions were statistically ($p < 0.05$) not significant (Fig. 28).

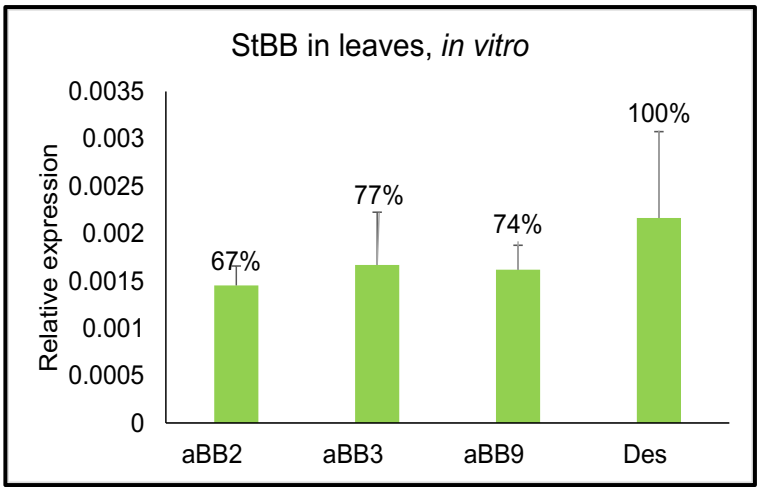

Figure 28. Level of *StBB* repression in aBB lines compared to the non-transformed control 'Désirée' (DES). RNA was isolated from the middle leaves of *in vitro* plants; 3 leaves/line harvested from 3 plants. *Y*-axis shows mean relative expression values of *StBB* gene compared to the mean expression values of *ACTIN*.

5.3.7. Phenotypes and *StBB* expression in aBB plants grown in pots

Despite the insignificance of reduction in *StBB* expression level in *in vitro* grown plants, we were interested in testing the plants also under greenhouse conditions. The three selected lines were propagated *in vitro*, transferred to pots and grown further under greenhouse conditions in 6 parallels with the control in 12 parallels. The height of the plants was measured at six weeks after transferring them from *in vitro* into pots. Only one line, aBB3, differed in height from DES as it was higher (61.0±15.1 cm) than the non-transformed control (47.1±6.8 cm) at significant $p < 0.05$ level (Fig. 29).

Expression of *StBB* was re-tested in leaves of plants grown in pots. Reduction in expression was detected only in aBB2. However, this was a minimal (4%), non-significant ($p < 0.05$) reduction compared to DES (Fig. 30).

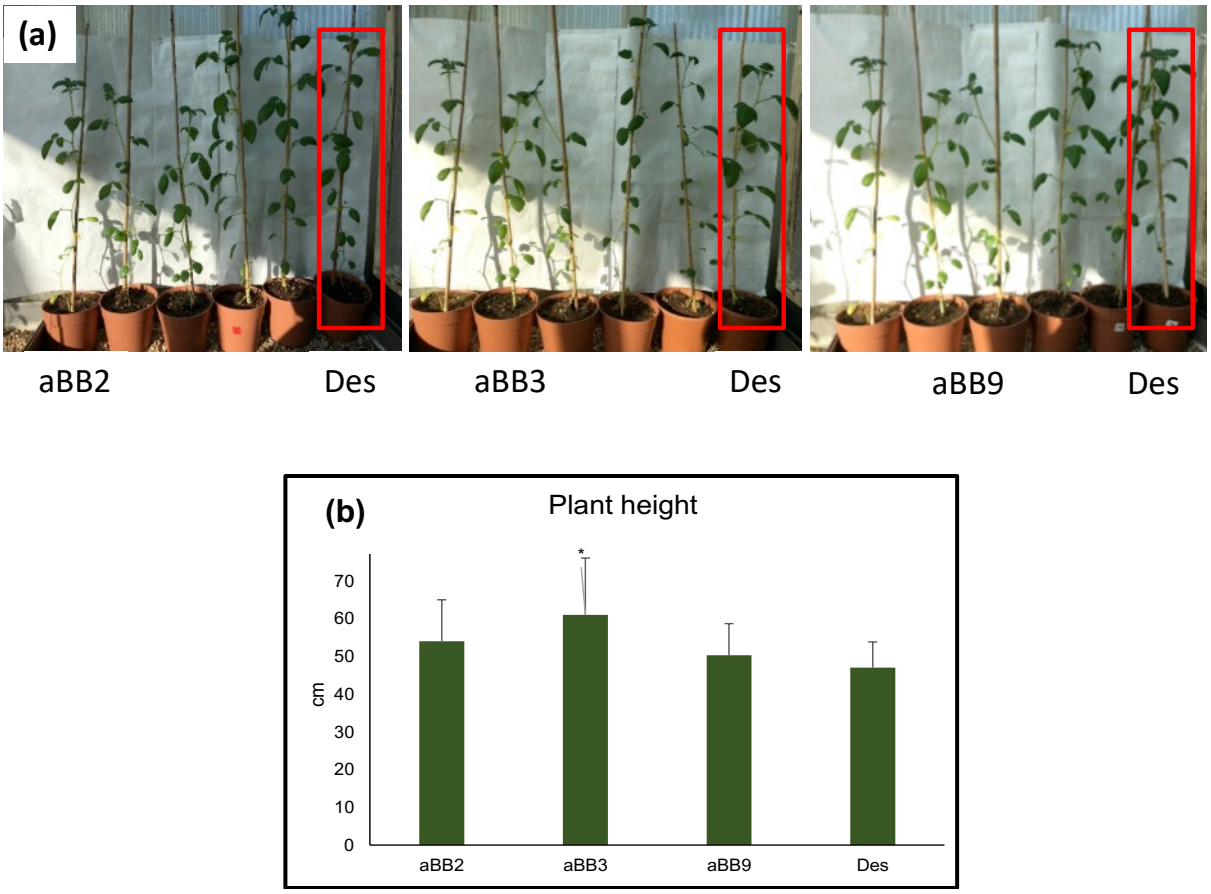

Figure 29. Morphology of aBB lines compared to the non-transformed DES control 6 weeks after transferring the plants from *in vitro* culture into pots. (a) 5 plants/aBB lines placed next to DES. The control plant is in a red box. (b) Plant height. *Y*-axis shows plant height in cm.

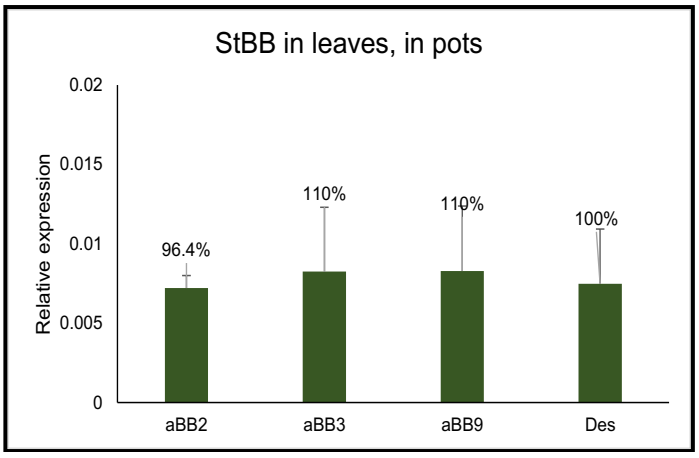

Figure 30. Level of *StBB* expression in aBB lines compared to the non-transformed control DES. RNA was isolated from middle leaves of 6- week-old pot-grown plants. *Y*-axis shows mean relative expression values of *StBB* gene compared to the mean expression value of *ACTIN*.

Tubers were harvested at the end of the vegetation period and measured for weight. The average weight of tuber per line of aBB2, aBB3, aBB9 and DES was 4.7±2.7 g, 4.8±3.8 g, 3.8±2.2 g and 3.2±1.5 g, respectively. However, because of the high deviation, the differences in tuber yield between aBB lines and DES were not significant (Fig. 31).

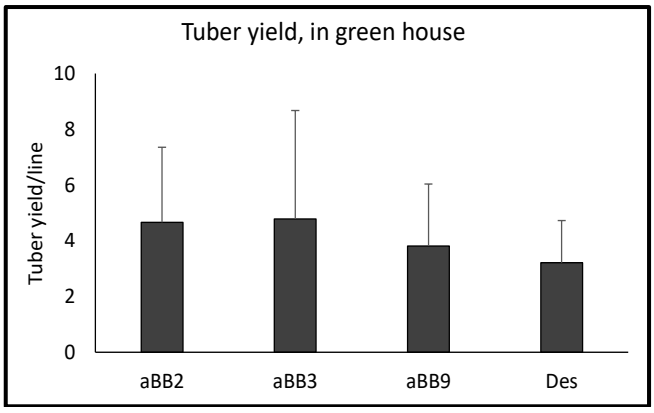

Figure 31. Tuber yield of aBB2, aBB3 and aBB9 compared to the non-transformed control DES from two independent experiments. Tuber yield was tested at the end of vegetation period namely 12-14 weeks after transferring the plants from in vitro culture into pots. *Y*-axis shows tuber yield in g.

It has been demonstrated that BB has a role in regulation of organ size in *Arabidopsis, Saltugilia*, *Nicotiana tabacum*, etc. (VANHAEREN et al. 2017; LANDIS et al. 2017; 2020). We attempted to get information on the function of *BB* gene in potato by antisense repression, however, we could not reach a significant level of reduction in any of the lines tested. A possible explanation might be related to the very low level of *StBB* expression even in control leaves (0.06-fold of the expression of *ACTIN*), which could result in a high error rate in RT-qPCR. Thus, other methods, as for example gene-editing, should be used in the future to get information about the function of *BB* gene in potato.

6. CONCLUSIONS AND RECOMMENDATIONS

The first part of the study provided an understanding of the effects of intercultivar grafting in potato and summarized the morphological and metabolic outcomes. We identified the major polar metabolites in leaves and tubers of two commercial potato cultivars (*Solanum tuberosum* L. cv. 'White Lady' and *Solanum tuberosum* L. cv. 'Hópehely') and found characteristic differences in the metabolite compositions in both organs of the two cultivars. In comparison to non-grafted and homo-grafted controls no major effect of hetero-scions and rootstocks on the metabolite concentrations were detected suggesting that the level of major metabolites is under genetic control. The only exception was galactinol, the concentration of which was slightly influenced by hetero-grafting in leaves. Furthermore, the grafting experiments resulted in detection of a positive correlation between the rate of leaf growth and the time of tuber initiation which supports the idea that tuberization is triggered by source-derived mobile signals. To the best of our knowledge, no information at molecular level is available regarding the responses of two potato cultivars grafted on each other. Although the grafting did not have considerable influences on metabolite composition in potato leaves and tubers under greenhouse conditions similar experiments can be performed under field conditions with involvement of more potato genotypes to get more information on transported metabolites under natural environmental conditions. Moreover, hence the signal transports largely depend on vascular formation (KUROTANI and NOTAGUCHI 2021), it is important to focus on the mechanism of vascular development.

To unravel the influence of some candidate genes to tuberization we turned to the study of *GIGANTEA* (*GI*) and *BIG BROTHER (BB)* because these genes are ubiquitous in the plant kingdom and are involved in diverse processes from flowering to stress responses and from leaf sizes to tuberization. The two genes were studied in several plant species including *Arabidopsis*, however, no details on the regulation of *GI* and *BB* gene expression in potato have been reported thus far. Thus, one of the aims of our work was the characterization of *GI* and *BB* in *Solanum tuberosum* L. cv. 'Désirée'.

In this study, expression analysis of the two potato *GI* genes homologous to *Arabidopsis*, designated *StGI.04* and *StGI.12*, was performed and found that the responses of the two genes to abiotic stresses and ABA as well as their organ-specific expression is different. Thus, we presume that the function of *StGI.04* and *StGI.12* are at least partially different. This study laid foundation for further investigation of the roles of *GI* genes in potato.

Analysis of a *StGI.04*-repressed line demonstrated that StGI.04, as with *GI* in other plant species, influences the circadian clock, flowering, stress responses, and starch synthesis via the alteration of expression of key genes of these processes in leaves of potato plants (KARSAI-REKTENWALD et al. 2022). Expression of *StGI.04* was quantified in *StGI.04*-repressed lines and found that the highest repression achieved was around 50% compared to the non-transformed control plant 'Désirée'. However, no differences either in phenotype including plant height or tuber yield were observed. Thus, we concluded that the repression of *StGI.04* may not result in alteration of tuber formation in 'Désirée' or the level of reduction in *StGI.04* transcript level that we could achieve was not high enough for influencing the tuberization in this potato cultivar. Unexpectedly, however, we identified a novel function for StGI.04 not detected earlier in other plant species, namely, promotion of the synthesis of anthocyanins in tuber skin. In collaboration with MATE Institute of Horticulture and Eszterházy Károly Catholic University it was found that the level of anthocyanins: cyanidin 3,5-di-O-glucoside and pelargonidin 3,5-di-glycoside, were reduced in tuber peels of *StGI.04*-repressed lines. In order to study how StGI.04 influences the anthocyanin level, the next step could be focused on the transcriptome analysis of tuber peels of 'Désirée' and the *StGI.04*-repressed lines. Moreover, presuming a similar trend of phenolic levels with anthocyanin contents, detecting the major phenolic compounds in the tuber skin of *StGI.04*-repressed lines in comparison to 'Désirée' is suggested.

The function of the other potato *GI* gene, *StGI.12*, is still unknown. However, as it was concluded above, based on the different expression pattern of *StGI.04* and *StGI.12* we suppose that their function is at least partially different and *StGI.12* may have higher influence on tuberization than *StGI.04*. To investigate this hypothesis, the further study could be focused on investigating how *StGI.12* gene repression influences tuberization and gene expression at transcriptome level under stress conditions in different organs. In addition, testing the involvement of *StGI.12* gene in anthocyanin metabolism is recommended. These could be the essential studies to understand the role of GIs in commercial potato cultivars.

The *S. tuberosum* E3 ubiquitin ligase coding *BIG BROTHER-like* (*StBB*) gene is 70% identical with the *Arabidopsis BB* gene *(AtBB).* Supposing that the *BB* gene, like in *Arabidopsis*, has an important role in the development of potato, a 3.0-kb promoter sequence of the potato *BB* gene was analysed *in silico.* 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. Twenty 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. The level of *StBB* expression was studied in different organs and under different stress conditions. The *StBB* mRNA was detected in each organ tested with the largest amounts in petal and stamen. Also, statistically significant increase in *StBB* transcript level was detected after the osmotic stress. These results suggest that the function of *StBB* is similar to that of *AtBB*. We have attempted to test this by antisense repression of *StBB* expression. However, no lines with statistically different *StBB* mRNA level could be obtained. Thus, further experiments are required to understand how the level of *StBB* expression is determined and how *StBB* influences organ growth at the molecular level under optimal and sub-optimal growth conditions.

7. NEW SCIENTIFIC RESULTS

- Grafting experiments between the commercial potato cultivars ‘Hópehely’ and ‘White Lady’ indicated that the sucrose concentration of tubers is genetically determined, whereas the galactinol concentration in the leaves can be influenced by the developing tuber.
- A positive correlation between the growth rate of the leaves and the time of tuber initiation was detected.
- A putative *BIG BROTHER (StBB)* gene homolog has been identified in *Solanum tuberosum*.
- It was found that *StGI.04, StGI.12* and *StBB* genes are differentially expressed in response to different abiotic stress treatments and in different organs.
- A new function for *GIGANTEA* in potato was discovered by demonstrating that repression of *StGI.04* expression leads to the reduction of anthocyanin content of tuber skin.

8. SUMMARY

Potato is the fourth most consumed crop in the world in accordance with their nutritional status. However, many commercial cultivars suffer from a number of production and quality issues caused by unfavorable environmental conditions. Thus, productivity is still a key issue for breeders. Early tuberizing potato cultivars are more profitable for the growers and are candidates for stress escapers. Therefore, there is a high interest in breeding early bulking potatoes.

The aim of our research work was double: [1] to get information about the influence of vegetative organs on the primary polar metabolite composition of potato tubers and vice versa, to get information about the effect of tuberization on the primary polar metabolite composition of leaves; [2] to extend our knowledge on those genes, which may influence potato organ development and earliness of tuberization.

To achieve our first goal grafting experiments were carried out between two commercial potato cultivars, 'Hópehely' and 'White Lady'. This experiment resulted in detection of a positive correlation between the rate of leaf growth and the time of tuber initiation. The grafting did not influence substantially the metabolite composition of leaves and tubers except the galactinol level in leaves. Although there was a big difference in the sucrose concentration of tubers between the two cultivars, this was not altered by grafting. Thus, we concluded that the sucrose concentration of tubers is genetically determined.

To achieve our second goal two genes *GIGANTEA* (*GI*) and *BIG BROTHER* (*BB*) were characterized in the *Solanum tuberosum* L. cv. 'Désirée'. Based on the previous studies in the model plant *Arabidopsis* and other plant species we hypothesised that these genes influence tuberization.

To test this hypothesis expression analysis of the two potato *GI* genes, *StGI.04* and *StGI.12* was carried out. We found that *StGI.04* is expressed in each organ, whereas *StGI.12* is not expressed in flower. The osmotic stress, cold and heat up-regulated *StGI.04* but down-regulated *StGI.12*, and while ABA induced *StGI.12* expression it had no effect on *StGI.04*. Thus, we presumed that the function of *StGI.04* and *StGI.12* are at least partially different.

Function of *StGI.04* was investigated by antisense repression of *StGI.04* in 'Désirée'. A maximum of approximately 50% reduction in *StGI.04* expression was achieved, however, it did not result in alteration of tuber formation in 'Désirée'. Thus, we concluded that *StGI.04* may not be involved in tuber development or the level of reduction in *StGI.04* transcript level was not high

enough for influencing the tuberization in this potato cultivar. At the same time, however, we identified a novel function for *StGI.04*, namely, the promotion of anthocyanin synthesis in tuber skin. As the function of *StGI.12* is still unknown the next study will focus on this gene. It will be interesting to know whether this gene also influences the anthocyanin content of potato or not.

Similar analysis as with *StGI* genes were performed with the *StBB* gene. *StBB* mRNA was detected in each organ tested, with the highest amounts in floral organs. Out of the abiotic stresses (osmotic stress, heat, cold) and ABA treatment, only the osmotic stress up-regulated the *StBB* expression. *In silico* promoter analysis of *StBB* resulted in prediction of binding sites for transcription factors involved in developmental processes such as for example, the flower-, leaf-, stem- and root development or cell cycle regulation. For functional analysis of the gene generation of antisense *StBB*-repressed 'Désirée' lines was attempted, however, none of the lines obtained showed a reduced *StBB* transcript level under greenhouse conditions. Thus, further experiments are required to determine the role of the *StBB* gene in potato organ development.

9. APPENDICES

A1: REFERENCES

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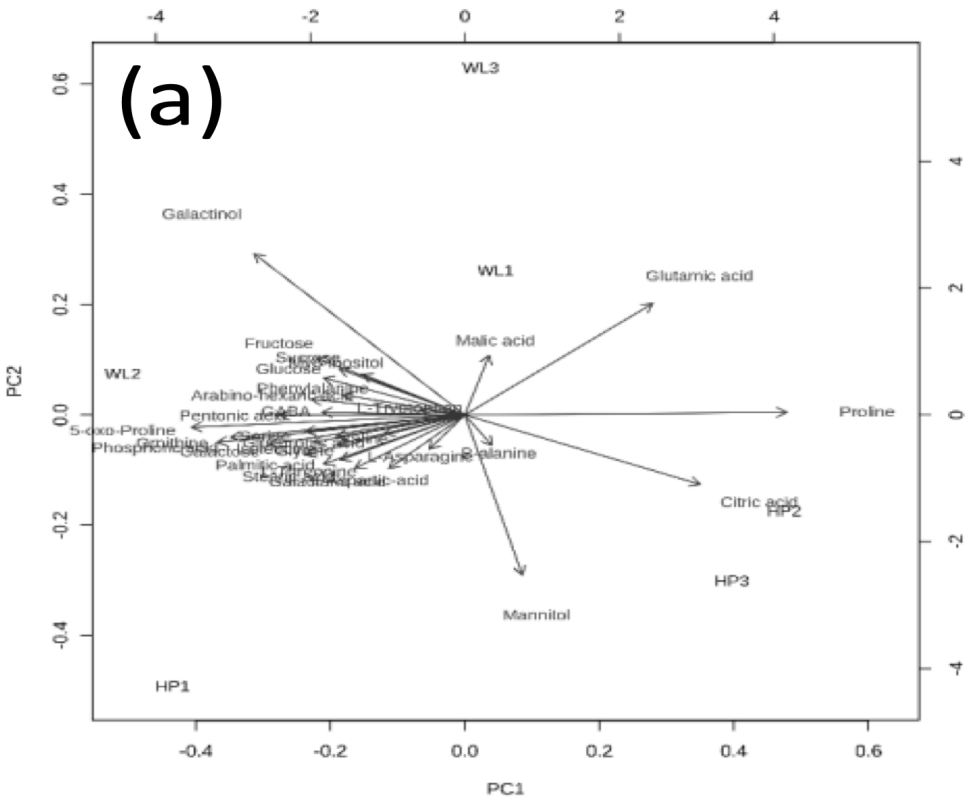
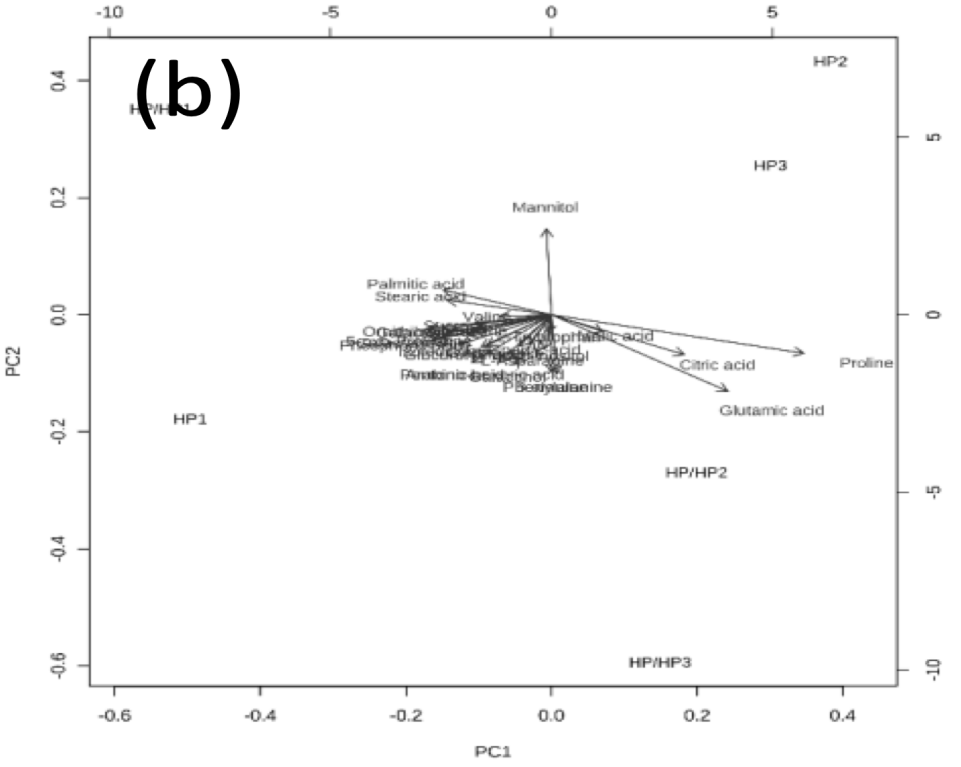
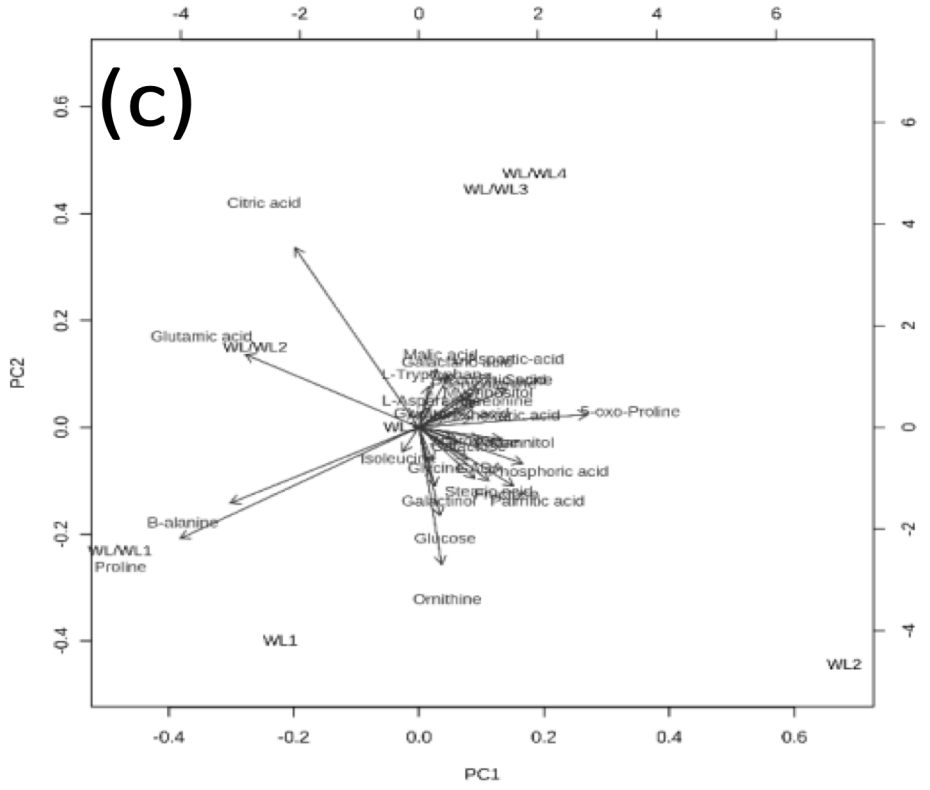
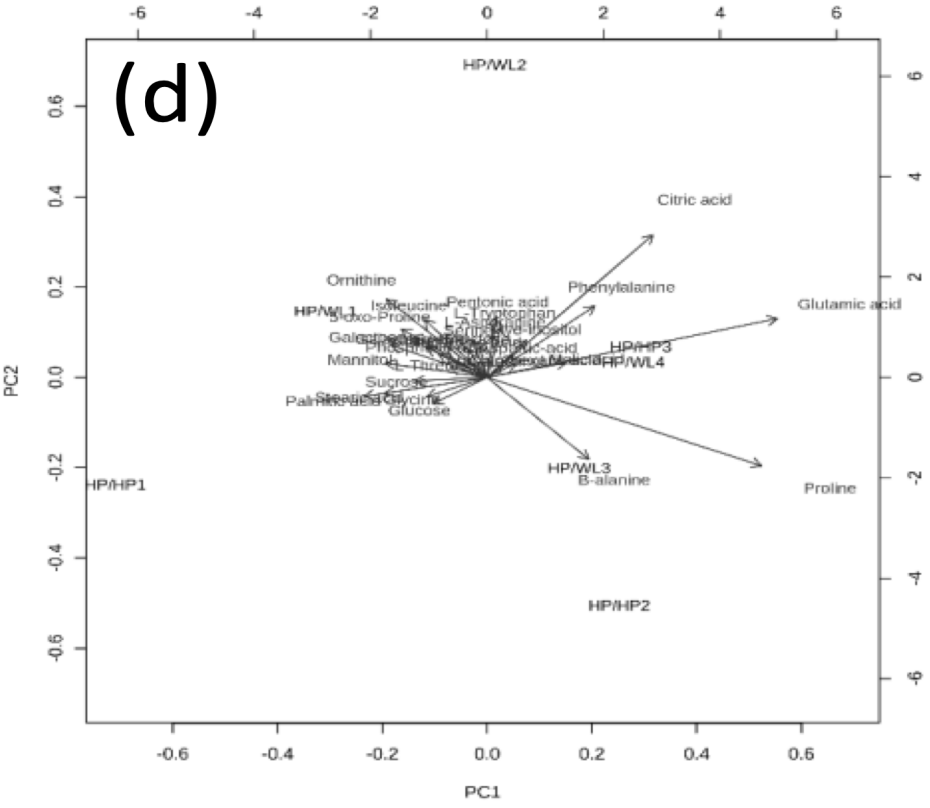
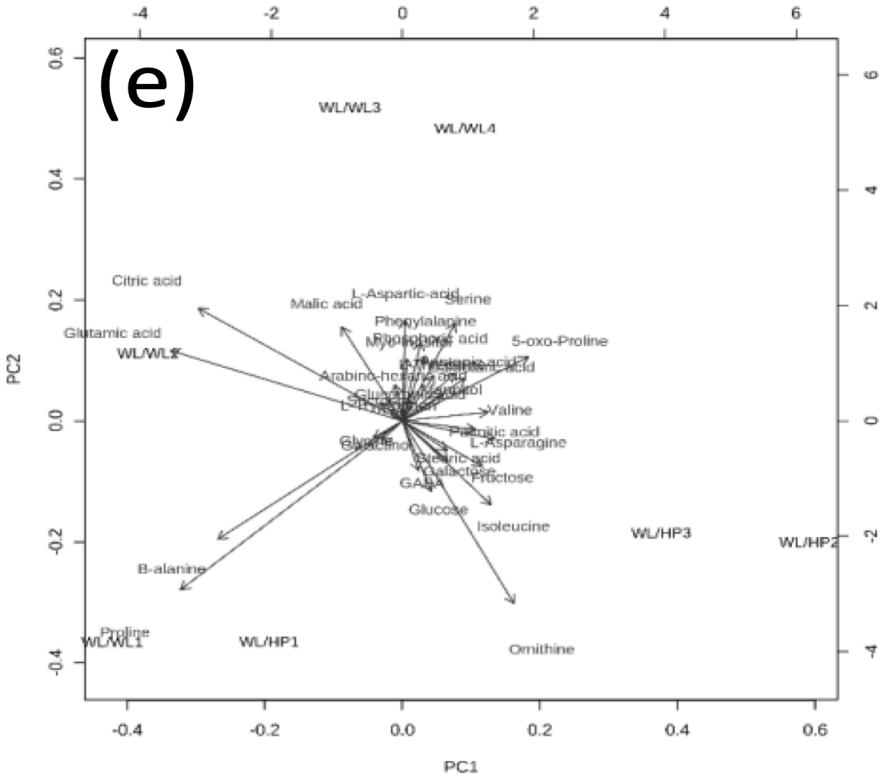
A2: SUPPLEMENTARY FIGURES



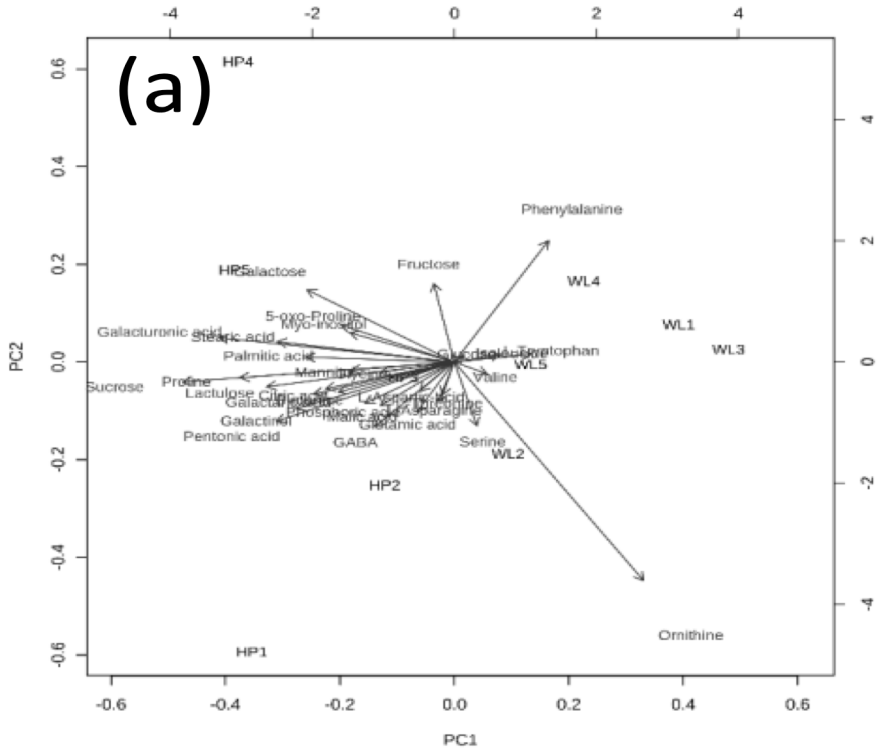



(c)
WL/WL4
WL/WL3
Citric acid
Glutamic acid
WL/WL2
Malic acid
5-oxo-Proline
Isoleucine
Glycine
Phosphoric acid
Palmitic acid
B-alanine
Glucose
WL/WL1
Proline
Ornithine
WL1
WL2
PC1
PC2


(d)
HP/WL2
Citric acid
Ornithine
Phenylalanine
Glutamic acid
Pelgonic acid
HP/WL1
Isoleucine
5-oxo-Proline
HP/HP3
HP/WL4
Mannitol
Sucrose
Glycine
Glucose
HP/WL3
B-alanine
Proline
HP/HP1
HP/HP2
PC1
PC2



Supplementary figure 1. PCA biplot analysis of the leaf metabolites. (a) HP-WL, (b) HP-HP/HP, (c) WL-WL/WL, (d) HP/HP-HP/WL, (e) WL/WL-WL/HP


(a)
HP4
Phenylalanine
Fructose
HP5
Galactose
WL4
5-oxo-Proline
WL1
Galacturonic acid
Stearic acid
WL3
Palmitic acid
WL5
Sucrose
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GABA
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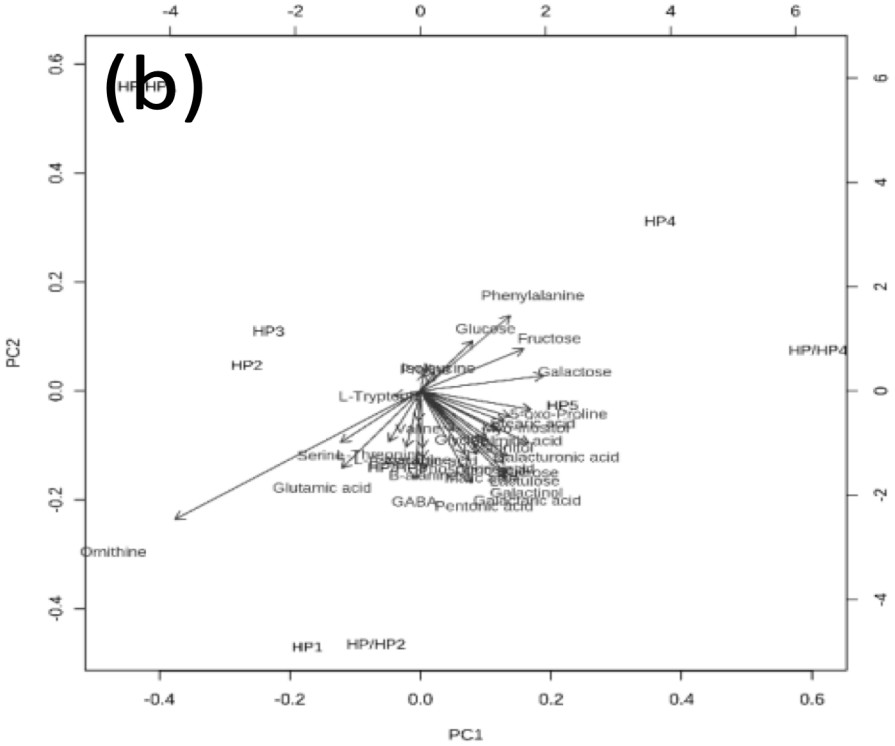
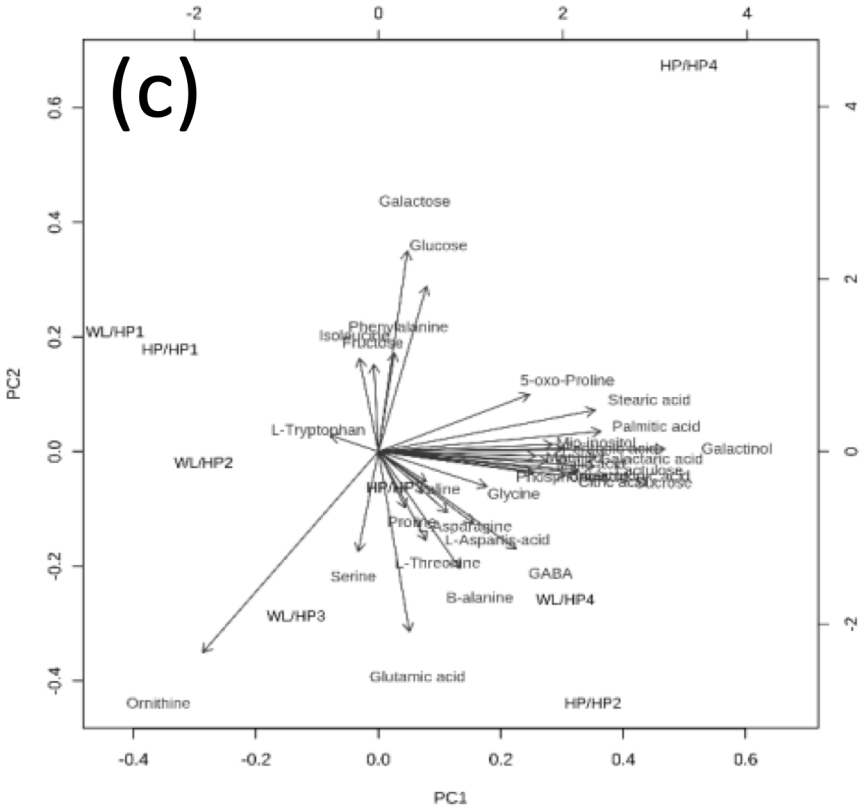
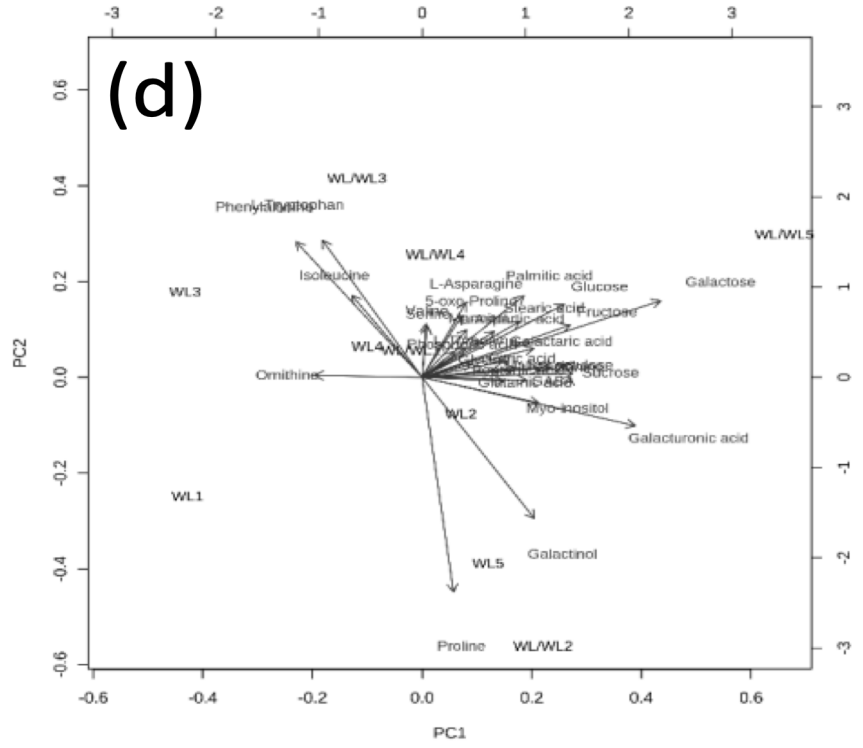
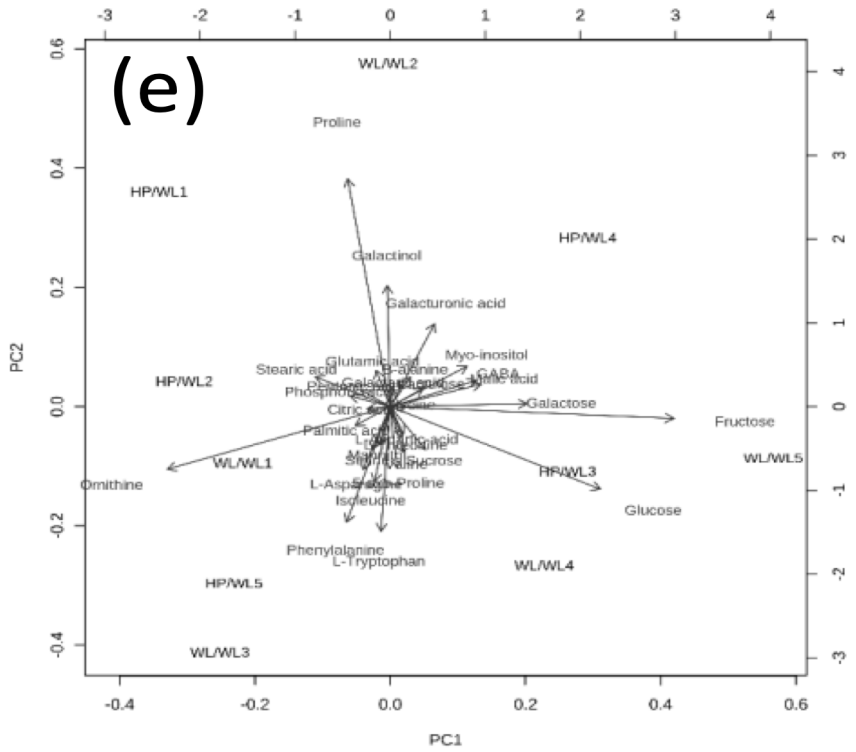
Serine
WL2
HP2
Ornithine
HP1
PC1
PC2


(b)
HP4
Phenylalanine
HP3
Glucose
Fructose
HP2
HP/HP4
Galactose
HP5
5-oxo-Proline
Galacturonic acid
Glutamic acid
GABA
Pentonic acid
Ornithine
HP1
HP/HP2
PC1
PC2


(c)
HP/HP4
Galactose
Glucose
WL/HP1
HP/HP1
Phenylalanine
Isoleucine
Fructose
5-oxo-Proline
Stearic acid
Palmitic acid
L-Tryptophan
Galactinol
WL/HP2
Glycine
Asparagine
L-Asparagine
L-Threonine
Serine
GABA
B-alanine
WL/HP4
WL/HP3
Glutamic acid
Ornithine
HP/HP2
PC1
PC2


(d)
WL/WL3
WL/WL4
WL/WL5
Isoleucine
L-Asparagine
Palmitic acid
Glucose
Galactose
WL3
5-oxo-Proline
Stearic acid
Fructose
WL4
Galactaric acid
Ornithine
Sucrose
Myo-inositol
WL2
Galacturonic acid
WL1
Galactinol
WL5
Proline
WL/WL2
PC1
PC2



Supplementary figure 2. PCA biplot analysis of the tuber metabolites. (a) HP-WL, (b) HP-HP/HP, (c) WL-WL/WL, (d) HP/HP-WL/HP, (e) WL/WL-HP/WL

A3: LIST OF TABLES AND FIGURES

A4: LIST OF SCIENTIFIC ACTIVITIES

Articles published in peer reviewed scientific journals

Karsai-Rektenwald F., **Odgerel K.,** Jose J., Bánfalvi Z. (2022): *In silico* characterization and expression analysis of *GIGANTEA* genes in potato. *Biochemical Genetics.* (IF:1.89) https://doi.org/10.1007/s10528-022-10214-7

Odgerel K., Bánfalvi Z. (2021): Metabolite analysis of tubers and leaves of two potato cultivars and their grafts. *PLoS One.* 16:e0250858. (IF:3.24) https://doi.org/10.1371/journal.pone.0250858

Articles submitted in peer reviewed scientific journals

Odgerel K., Bánfalvi Z. (2022): Promoter and expression analysis of the *BIG BROTHER* gene in potato. *Columella – Journal of Agricultural and Environmental Sciences*. (submitted)

Odgerel K., Jose J., Karsai-Rektenwald F., Ficzek G., Gergely Simon G., Végvári G., Bánfalvi Z. (2022): Effects of the repression of *GIGANTEA* gene *StGI.04* on the potato leaf transcriptome and the anthocyanin content of tuber skin. *BMC Plant Biology*. (resubmitted)

Articles and abstracts published in conference books

Villányi V., **Odgerel K.**, Karsai-Rektenwald F., Bánfalvi Zs. (2021): Az oltvány hatása a burgonya koraiságára és a gumók metabolit összetételére. XXVII. Növénynemesítési Tudományos Napok. Március. Összefoglaló kötet, 37.

Odgerel K. (2021): The promoter analysis and gene expression of potato *BIG BROTHER* gene. Proceeding of the "Scientific Conference of PhD. Students - 2021". Nitra, Slovakia. Nov. pp.16

Karsai-Rektenwald F., **OdgereL K.,** Jose J. (2021): Regulation of *GIGANTEA* genes in potato. Proceeding of the "Scientific Conference of PhD. Students - 2021". Nitra, Slovakia. Nov. pp.14

Odgerel K., Bánfalvi Z. (2020): The use of grafting to study the influence of canopy growth rate on earliness of tuber initiation and metabolite composition of potato tubers. Proceeding of "The 8th Plant Genomics & Gene Editing Congress". Rotterdam, the Netherlands. Mar. pp. 19

Odgerel K., Kondrák M., Bánfalvi Z. (2019): Studying leaf-derived signalling on tuberization and tuber metabolome by grafting experiments in potato. The 2nd international conference on Plant and Molecular Biology. Amsterdam, the Netherlands. Oct. pp. 30

Bánfalvi Z., **Odgerel K.,** Csákvári E., Jose J., Kondrák M. (2019): Studying the relationship between tuber bulking and canopy growth in potato. Proceedings of the XXV. Plant Breeding Scientific Day. Budapest, Hungary. Mar. pp. 149-153

Oral and poster presentations

Odgerel K. (2021): The promoter analysis and gene expression of potato *BIG BROTHER* gene. Scientific Conference of PhD. Students – 2021. Nitra, Slovakia. Nov. 10.

Odgerel K., Bánfalvi Z. (2020): The use of grafting to study the influence of canopy growth rate on earliness of tuber initiation and metabolite composition of potato tubers. The 8th Plant Genomics & Gene Editing Congress. Rotterdam, the Netherlands. Mar. 4-5.

Odgerel K., Kondrák M., Bánfalvi Z. (2019): Effect of leaf mobile signals on potato tuber induction and metabolite composition. The MBK Days - Scientific meeting. Gödöllő, Hungary. Nov. 29.

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