Effects of the repression of GIGANTEA gene StGI.04 on the potato leaf transcriptome and the anthocyanin content of tuber skin

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Abstract

Background: GIGANTEA (GI) is a plant-specific, circadian clock-regulated, nuclear protein with pleiotropic functions found in many plant species. This protein is involved in flowering, circadian clock control, chloroplast biogenesis, carbohydrate metabolism, stress responses, and volatile compound synthesis. In potato (Solanum tuberosum L.), its only role appears to be tuber initiation; however, based on findings in other plant species, we hypothesised that the function of GI in potatoes is not restricted only to tuberisation.

Results: To test this hypothesis, the expression of a GI gene in the commercial potato cultivar ‘Désirée’ was repressed, and the effects of repression at morphological and transcriptome level were investigated. Previously, two copies of GI genes in potato were found. A construct to reduce the mRNA levels of one of these genes (StGI.04) was assembled, and the effects of antisense repression were studied in greenhouse-grown plants. The highest level of repression reached around 50%. However, this level did not influence tuber formation and yield but did cause a reduction in tuber colour. Using high-performance liquid chromatography (HPLC), significant reductions in cyanidin 3,5-di-O-glucoside and pelargonidin 3,5-di-O-glucoside contents of tuber peels were detected. Anthocyanins are synthesized through a branch of the phenylpropanoid pathway. The transcriptome analysis indicated down-regulation in the expression of PHENYLALANINE AMMONIA L YASE (PAL), the LEUCOANTHOCYANIDIN OXIDISING enzyme gene LDOX, and the MYB-RELATED PROTEIN Hv1 (MYB-Hv1), a transcription factor coding gene, which is presumably involved in the regulation of flavonoid biosynthesis, in the leaves of a selected StGI.04-repressed line. Furthermore, alterations in expression of genes affecting the circadian clock, flowering, starch synthesis, and stress responses were detected in the leaves of the selected StGI.04-repressed line.

Conclusions: We tested the effects of antisense repression of StGI.04 expression in potatoes and found that as with GI in other plant species, it influences the expression of the key genes of the circadian clock, flowering, starch synthesis, and stress responses. Furthermore, we detected a novel function of a GI gene in influencing the anthocyanin synthesis and potato tuber skin colour.

Keywords: Anthocyanins, Antisense repression, GIGANTEA, Solanum tuberosum, Transcriptome, Tuberisation, Tuber skin colour

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which GI is involved is flowering. It has been shown in several plant species that GI regulates flowering time through the photoperiod-pathway. It is indicated by the gi Arabidopsis mutants that GI acts in the long-day (LD) 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 in [1]). Recently, it has been shown that the GI-FKF1-CDF1-CO module is employed even by mangos in regulating its temperature dependent flowering [2].

Besides flowering time regulation, 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 PHYTOCHROME 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 in [3]).

Another large category of biological functions, in which GI is involved, is the responses of plants to environmental stresses. GI acts by conferring salt and freezing tolerance to Arabidopsis and Brassica nigra. In contrast, in B. rapa and poplar plants, down-regulation of GI leads to enhancement of salt tolerance. GI expression is induced in response to drought stress and in combination with miRNA172, causes suppression of WRKY44, a transcription factor participating in sugar metabolism. GI plays an inhibitory role during oxidative stress by causing down-regulation of the expression of SUPEROXIDE DISMUTASE (SOD) and ASCORBATE PEROXIDASE (APX) (reviewed in [4]). The latest results show that GI influences not only abiotic stress responses but is also involved in response to pathogen defence, and it confers susceptibility to plants during spot blotch attack by regulating the salicylic acid signalling pathway [5].

Brandoli et al. [6] reported a novel role of GI in Petunia hybrida. Flowers of the GI silenced lines emitted 20% less volatile compounds on a fresh weight basis over 24 h 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.

In potato (Solanum tuberosum L.), involvement of GI in initiation of tuberisation was demonstrated by Kloosterman et al. [7]. According to the proposed model based on studying the wild Andean landrace S. tuberosum Group Andigena, a strict short-day (SD) plant for tuberisation, GI influences tuber formation in conjunction with FKF1. As in Arabidopsis, the GI-FKF1 complex can bind CDF1 and target it for degradation by the proteasome. Since CDF1, in an indirect way, increases the transcription of SELF-PRUNING 6A (S6A), a mobile tuberisation signal homologous to the ‘florigen’ FT, GI is an indirect repressor of tuberisation.

Recently, searching for the A. thaliana GI homologue we found two GI transcript variants in potato with 83.73% identity located on chromosomes 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 is partially different. While osmotic stress, cold stress, and heat lead to up-regulation of StGI.04, the same stresses produce down-regulation in the expression of StGI.12. ABA induces StGI.12 but has no effect on StGI.04 [8]. Besides tuberisation, 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. Based on the phenotype of the plants in addition to the effects of StGI.04 repression on the potato leaf transcriptome, a novel role of GI in anthocyanin metabolism was demonstrated and similarities and differences in influencing gene expression in comparison with other plant species were highlighted.

Results
Selection of StGI.04-repressed lines
A 250-bp fragment of StGI.04 with the less, but still 71.2% identity with the corresponding fragment of StGI.12 (Additional file 1: Fig. S1), was used for generation of StGI.04-repressed ‘Désirée’ (DES) lines (aGI lines). Leaves harvested from plants grown in vitro were tested for the level of repression using reverse-transcription polymerase chain reaction (RT-PCR). Twenty lines were found with lower level of StGI.04 expression than the non-transformed control DES out of which five lines (aGI43, aGI44, aGI52, aGI53, aGI55) with different levels of reduction in StGI.04 transcript level were selected for further studies. Expression of StGI.04 was quantified in the selected five lines with reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The highest repression, 51% of StGI.04 mRNA detected in DES, was
Fig. 1  Level of StGl.04 repression in aGl 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 StGl.04 gene compared to the mean expression values of ACTIN. The means were calculated from three technical replicates in case of (a) and (b) and from 3 biological replicates in case of (c). The standard deviations are indicated by the error bars. Significant differences between the aGl lines and DES were detected by Student’s t-test and labelled by * (p < 0.05) and ** (p < 0.01)
found in aGI52, while only a minimal repression, a 2% reduction, was found in aGI55 (Fig. 1a).

The five selected lines were propagated in vitro, transferred to pots, and grown further under greenhouse conditions in 12 parallel setups. Expression of StGI.04 was re-tested in leaves (Fig. 1b). Four lines possessed significantly ($p < 0.01$) lower StGI.04 expression than found in DES. As in leaves of in vitro plants, a smaller difference compared to DES was found in aGI55, but even this difference was significant at the $p < 0.05$ level. The StGI.04 transcript level was a little bit higher, 63% versus 51%, 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. 1c). In line with the lowest expression in leaves (Fig. 1b), the lowest expression was detected in tubers of the aGI43 plants (Fig. 1c). All lines, except aGI44, showed a significant ($p < 0.01$) level of StGI.04 repression in tubers, including aGI52 with 67% of wild type StGI.04 mRNA level.

**Specificity of repression in aGI52**

The line aGI52 showed a significant ($p < 0.01$) and relatively stable level of reduction in StGI.04 expression based on all three RT-qPCR analyses (Fig. 1). Therefore, this line was selected for further detailed studies.

Although the region of reduced sequence similarity between StGI.04 and StGI.12 was used for generation of aGI lines, the identity of the two regions was still substantial. Thus, the repression of StGI.12 expression by the StGI.04 fragment could not be excluded. To test this possibility, an StGI.12-specific primer pair [8] was used in parallel with the StGI.04-specific primer pair [8] in the RT-qPCR analysis of aGI52 and DES leaves and tubers. Figure 2 shows that the repression in aGI52 was StGI.04-specific and did not extend to StGI.12.

![Fig. 2 Specificity of StGI.04 repression in aGI52. The RT-qPCR analysis was carried out using (a) the GI04spec and (b) the GI12spec primer pair. The leaf RNA was derived from 1-cm-diameter discs of 9 leaves of 9 plants divided into 3 groups. The tuber RNA was the same used in the RT-qPCR analysis presented in Fig. 1c. Y-axis shows mean relative expression values of StGI genes compared to the geometric mean expression values of ACTIN and EF1α. The standard deviations from three biological replicates are indicated by the error bars. DES, non-transformed control ‘Désirée’](image-url)
Phenotypes and tuberisation of aGI plants

Development and morphology of aGI43, aGI44, aGI52, aGI53, and aGI55 plants grown in the greenhouse were visually followed and compared to DES. Plant heights were measured at 7 weeks after transferring them from in vitro into pots. Neither phenotypic changes nor height differences were observed. The earliness of tuberisation was tested also at 7 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. After counting, the plants were replaced in the pots and grown until the end of the vegetation period when the tubers were harvested and measured for weight. No differences in tuber yield were obtained between the aGI lines and DES. The size distribution of aGI tubers was also similar to DES, peaking at 8 to 10 cm in diameter, except aGI44 and aGI55, both of which produced a larger number of small tubers than DES (Additional file 1: Fig. S2).

The molecular model of tuber formation is based on S. andigena, a strict SD plant for tuberisation [7]. Therefore, we wanted to test the effects of StGI.04 repression not only under 12 h light/12 h dark (LD conditions) but also under SD conditions (8 h light/24 h dark). The aGI52 line was compared to DES in this experiment. Even under SD conditions, no difference in canopy phenotype or tuber yield was detected between aGI52 and DES (Additional file 1: Fig. S2).

Anthocyanin content of tuber peels

DES is a red-skinned potato. The skin colour of aGI tubers collected from greenhouse-grown plants was lighter than the skin colour of DES (Additional file 1: Fig. S3a) although, to different extents. The reduction in colour was the most pronounced in aGI52, aGI53 and aGI44. The difference in tuber skin colour was also obvious between aGI52 and DES grown under SD conditions (Fig. 3a). Since anthocyanins determine the skin colour [9], these compounds were extracted from tuber peels and their relative quantity measured. When compared with DES, a 52, 36 and 31% reduction in anthocyanin content was found in greenhouse-grown aGI53, aGI44 and aGI52 tuber peels, respectively (Additional file 1: Fig. S3b). A similar, 43% reduction was observed in the anthocyanin content of aGI52 tubers skins developed under SD conditions (Fig. 3b).

High-performance liquid chromatography (HPLC) was used to specify the anthocyanins extracted from tuber peels. Three anthocyanins, cyanidin 3,5-di-O-glucoside, pelargonidin 3,5-di-O-glucoside and delphinidin 3,5-di-glycoside were detected with pelargonidin 3,5-di-O-glucoside being present in the largest amount. A significant reduction in cyanidin 3,5-di-O-glucoside and pelargonidin 3,5-di-O-glucoside content of aGI52 peels compared to DES peels was demonstrated (Fig. 3c). An attempt was also made to detect malvidin 3-galactoside; however, it was not present in a detectable amount.

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**Fig. 3** Anthocyanin content of aGI52 and DES tubers grown under SD conditions. a Morphology of mature tubers of three aGI52 and three non-transformed, control DES plants. b The relative anthocyanin content of tuber skins determined spectrophotometrically. c The anthocyanin content of tuber skins analysed using HPLC. Abbreviations: Cya, cyanidin 3,5-di-O-glucoside; Pel, pelargonidin 3,5-di-O-glucoside; Del, delphinidin 3,5-di-glycoside. 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. The standard deviations are indicated by the error bars. Significant differences between aGI52 and DES were detected by Student’s t-test and labelled by * (p < 0.05) and ** (p < 0.01). Fw, fresh weight
Transcriptome analysis of aGI52 leaves

To explore the effects of StGl0.4 repression on the global transcription profile, the same leaf RNA of greenhouse-grown aGI52 and DES plants tested for the specificity of repression (Fig. 2) was used for RNA-seq analysis in three biological replicates. Parameters presented in Additional file Table S1, Fig. S4 and S5 indicate that the analysis had a good quality.

To assess the alterations in unigene expression we analysed the differentially expressed unigenes (log2 (Fold Change)] > 1 and padj < 0.05) in the comparison of aGI52 and DES. We found 454 and 247 uniquely expressed genes in DES and aGI52, respectively (Fig. 4a). We obtained 488 differentially expressed genes (DEGs): (1) 289 were up- and (2) 199 were down-regulated in aGI52 (Fig. 4b and Additional file 1: Fig. S6). Gene ontology (GO) enrichment analysis revealed that mainly those genes were up-regulated, which are related to photosynthesis, while peptidase regulators and inhibitors were down-regulated in aGI52 (Fig. 4c, d and Additional file 1: Fig. S7). The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that the glyoxylate and dicarboxylate metabolism, the carbon metabolism and the peroxisomal pathways were activated, while the metabolisms of some amino acids were suppressed (Fig. 4e). Nevertheless, none of these pathways were significantly altered (corrected p > 0.05).

Transcription factors (TFs) are key regulators of plant development and stress responses. Thus, first we focused on differentially expressed TFs. Fourteen up-regulated and 11 down-regulated TFs were identified in aGI52 (Fig. 5a). The up-regulated category included the gene for bHLH63/CIB1, a positive regulator of flower development [11], NF-YB-3/HAP3C, which can interact with CO when it replaces HAP2 in the ternary HAP complex and promotes flowering in Arabidopsis [12], IBH1 that negatively regulates cell and organ elongation in response to gibberellin and brassinosteroid signalling [13], an ABI3-like gene similar to the major mediator of ABA repression of growth and floral transition in Arabidopsis [14, 15] and RVE1, a clock output affecting plant development [16]. Furthermore, TF genes involved in plant defence responses, ERF1B, ZAT10, WRKY11, MYB1R1, and TGA2.1, were also activated [17–21] in addition to ASR3 that acts in an opposite and negative manner to regulate immune gene expression in Arabidopsis [22]. Interestingly, unlike NF-YB-3/HAP3C, NF-YA-1/HAP2A, encoding the DNA-binding subunit of the HAP complex, is down-regulated. RAP2–7 encoding an ethylene responsive TF with an APETALA2 (AP2) domain is also down-regulated. AP2-like target genes act as floral repressors [23]. Furthermore, GATA2.1, a repressor of the gibberellin signalling pathway that also represses flowering [24], and a CONSTANS-LIKE gene, COL13, is expressed at lower levels in aGI52 than in DES. Figure 5b demonstrates that the expression of the majority of TFs listed in Fig. 5c are highly synchronised. The exceptions are ZAT10, ERF1B, and bHLH72, whose expression pattern is similar only to that of TGA2.1, MYB1R-1, and ASR3.

The KEGG analysis highlighted some DEGs involved in glyoxylate and dicarboxylate metabolism, carbon metabolism, and peroxisomal pathways (Additional file 1: Fig. S8–10). These included the genes encoding the key enzymes of starch synthesis, ADP-GLUCOSE SYNTHASE (AGS), STARCH SYNTHASE (SS), and STARCH PHOSPHORYLASE (SP) in addition to TREHALOSE-6-PHOSPHATE SYNTHASE (TPS), a sugar messenger connecting metabolism and development, and the first line defence antioxidants, SUPEROXIDE DISMUTASE (SOD) and CATALASE (CAT) as shown in Fig. 6a. Nevertheless, while SOD was up-regulated, CAT was down-regulated (Fig. 6b). In contrast, down-regulation of starch synthesis genes was highly coordinated (Fig. 6c).

Although the anthocyanin content in leaves was so low that only cyanidin 3,5-di-O-glucoside could be detected the difference in the concentration of this compound between aGI52 (22.4 ±0.2 ng/g Fw) and DES (24.1±0.2 ng/g Fw) was significant (p<0.01). This prompted us to make a manual search for DEGs involved in anthocyanin metabolism. Anthocyanins are a class of flavonoids synthesized through a branch of the phenylpropanoid pathway. The beginning genes include PHENYLALANINE AMMONIA LYASE (PAL), CINNAMATE 4-HYDROXYLASE (C4H), and 4-COLIMAROL COA LIGASE (4CL). The next steps are divided into early and late enzymatic steps. The early biosynthetic genes include CHALCONE SYNTHASE (CHS), CHALCONE ISOMERASE (CHI), FLAVANONE 3-HYDROXYLASE (F3H), and FLAVANONE 3’-HYDROXYLASE (F3’H). The late biosynthesis genes include DIHYDROFLAVONOL 4-REDUCTASE (DFR), ANTHOCYANIDIN SYNTHASE (ANS), and UDP-GLUCOSE:FLAVONOID 3-O-GLUCOSYL
Fig. 4 (See legend on previous page.)
Identification of AtMYBL2 as the protein with the highest protein sequence was carried out. This search resulted in a search for the most similar Arabidopsis thaliana proteins since the function of these proteins has not been known. Transcriptome data are highly reliable. RT-qPCR and RNA-seq were similar, indicating that the proteins of Nicotiana tomentosiformis and Solanum lycopersicum Hv1 has the highest relationship with the MYB3-like domain. The phylogenetic analysis showed that StMYB-Hv1 is one of the largest TF families in plants which contain 1–4 tandem incomplete repeats (Rs) at the N-terminal region. According to the number of Rs the MYB family is divided into four categories (reviewed in [25]). It was found that StMYB-Hv1 is made up of 122 amino acids and belongs to the R3 MYB TFs carrying a single R3 type domain. The phylogenetic analysis showed that StMYB-Hv1 has the highest relationship with the MYB3-like proteins of Nicotiana tomentosiformis and Solanum lycopersicum (Fig. 8a and Additional file 1: Fig. S11). However, since the function of these proteins has not been known thus far a search for the most similar Arabidopsis thaliana protein sequence was carried out. This search resulted in identification of AtMYBL2 as the protein with the highest similarity to StMYB-Hv1 (Fig. 8b).

**Discussion**

Despite detection of the pleiotropic function of GI in several plant species, only its role in tuber initiation was studied in potato. Based on the function of GI in other plant species, we supposed that it is not restricted to tuberisation in potato. To test this hypothesis, we repressed the expression of a GI gene in the commercial potato cultivar ‘Désirée’ and investigated the effect of repression at morphological and transcriptome level.

Recently, we have identified two GI genes in potato, designated StGI.04 and St.GI.12, with different promoter sequences, different organ-specific expression, and stress responses [8]. Thus, we presumed that the two genes possess different functions at least in part. In this study, StGI.04 was targeted and its mRNA level reduced by constitutive expression of its less homologous region to St.GI.12 in antisense orientation. Nevertheless, even this region showed 71.2% identity with St.GI.12 cDNA sequence. Although, no reduction in St.GI.12 transcript level was detected by RT-qPCR due to the high level of homology existing between the two GI copies, the influence of St.GI.12 on the results obtained cannot be excluded.

Reduction of StGI.04 mRNA level in leaves and tubers of antisense aGI plants ranged between 2 and 51%. The most stable and relatively high level of repression (33–49%) was detected in aGI52. Thus, this line was studied in more detail than the others in our experiments.

Morphological characterisation of five aGI lines was carried out under greenhouse conditions. No differences in phenotype, growth rate, and tuber yield of the aGI plants compared to the control were detected. Although there was one-on-one line that showed differences in earliness of tuberisation or size distribution of tubers it was not in correlation with the level of StGI.04 repression. Growing aGI52 plants under SD condition did not influence the tuberisation either. Earlier, we identified two POTH20 TF binding sites in the promoter region of StGI.04 [8]. It was shown previously that overexpression of POTH1, a KNOTTED-like homeobox gene with 73% identity to POTH20, caused in vitro enhancement in tuberisation under both SD and LD photoperiods in several potato lines [27]. Therefore, we thought that the repression of StGI.04 would result in alterations of tuber formation. However, this situation did not occur. Thus, we concluded that the level of reduction in the StGI.04 transcript level that we could achieve might not be high enough for influencing tuberisation in the commercial potato cultivar ‘Désirée’.

**Validation of RNA-seq data by real-time quantitative PCR**

To validate the results of RNA-seq data, we selected three significantly up-, and five down-regulated DEGs in aGI52. These genes included four TFs, a diagnostic indicator of sulphur nutritional status, an F-box/LRR-repeat protein, a transmembrane transporter, and a heat shock protein (Fig. 7). The expression trends of each selected gene using RT-qPCR and RNA-seq were similar, indicating that the transcriptome data are highly reliable.

**Phylogenetic and similarity analysis of StMYB-Hv1**

MYB TFs are one of the largest TF families in plants which contain 1–4 tandem incomplete repeats (Rs) at the N-terminal region. According to the number of Rs the MYB family is divided into four categories (reviewed in [26]). It was found that StMYB-Hv1 is made up of 122 amino acids and belongs to the R3 MYB TFs carrying a single R3 type domain. The phylogenetic analysis showed that StMYB-Hv1 has the highest relationship with the MYB3-like proteins of Nicotiana tomentosiformis and Solanum lycopersicum (Fig. 8a and Additional file 1: Fig. S11). However, since the function of these proteins has not been known thus far a search for the most similar Arabidopsis thaliana protein sequence was carried out. This search resulted in identification of AtMYBL2 as the protein with the highest similarity to StMYB-Hv1 (Fig. 8b).
Fig. 5 (See legend on previous page.)

(c) Table:

| Name          | Gene ID         | Function                                                                 |
|---------------|-----------------|--------------------------------------------------------------------------|
| ANAC083       | Soltu.DM.01G007500 | Integrates ABA-mediated abiotic stress signals into leaf aging; involved in xylem development |
| bZIP44        | Soltu.DM.01G049720 | Seed germination                                                         |
| ANAC100       | Soltu.DM.03G029980 | Chlorophyll catabolic processes                                           |
| NF-YB-1       | Soltu.DM.10G028300 | Transition from vegetative to reproductive phase; seed development; embryo development ending in seed dormancy |
| GATA21        | Soltu.DM.12G025800 | Repressor of the GA signaling pathway that represses flowering and modulates greening, in a SOC1-dependent manner; chloroplast development |
| RAP2-7        | Soltu.DM.11G025890 | Regulation of flowering and innate immunity; interacts with CRV2 to regulate CO and FT |
| ATHB-5        | Soltu.DM.11G003380 | Regulation of ABA-activated signaling pathway                            |
| TC4           | Soltu.DM.12G029960 | Cotyledon, leaf, embryo development, leaf senescence                     |
| WRKY20        | Soltu.DM.07G026960 | Response to 1-aminocyclopropane-1-carboxylic acid and wounding           |
| CRF2          | Soltu.DM.08G028690 | Responses to ETH and CK                                                  |
| COL13         | Soltu.DM.09G022940 | CONSTANS-LIKE 1                                                         |
| bhUH72        | Soltu.DM.03G029660 | De-etiolation; red, far-red light phototransduction; response to light stimulus |
| ERF18         | Soltu.DM.11G001310 | ETH and JA mediated signaling pathways; pathogen defense response       |
| ZAT10         | Soltu.DM.12G006170 | Both a positive and a negative regulator of plant defenses              |
| IBH1          | Soltu.DM.09G016010 | BR and GA mediated signaling pathways; regulation of growth              |
| WRKY11        | Soltu.DM.08G003290 | Defense response to bacterium                                            |
| GATA24        | Soltu.DM.10G013010 | Zinc protein binding                                                    |
| MYB-Hv1       | Soltu.DM.05G004710 | May be involved in the regulation of flavonoid biosynthesis in barley    |
| bhUH63        | Soltu.DM.03G004150 | Positive regulation of flower development                                |
| NF-YB-3       | Soltu.DM.07G027340 | Flowering time regulation                                                |
| RVE1          | Soltu.DM.03G010030 | Morning-phased 1F integrating the circadian clock and auxin pathways    |
| ABIS-like     | Soltu.DM.04G033590 | ABA and glucose mediated signaling pathways                              |
| ASR3          | Soltu.DM.03G033480 | Negative regulation of immune response                                   |
| MYB18-1       | Soltu.DM.07G010910 | Activation of drought-regulated genes                                    |
| TGAC1         | Soltu.DM.10G026520 | Enhanced tolerance to Xanthomonas                                       |
The role of GI in photoperiodic flowering and circadian clock has been extensively investigated in several plant species, and it seems that flowering time regulation and circadian clock control are general GI functions. In line with this conclusion, binding sites for TFs involved in the regulation of flower development, including SOC1 and ABI5, were predicted, and expression of StGI.04 in floral organs was demonstrated via RT-qPCR [8]. This study showed that StGI.04 repression influences the expression of several TFs involved in regulation of flowering; the positive regulators of flowering bHLH63/CIB1 and ABI5-like are up-regulated, while the repressors RAP2–7 and GATA21 are down-regulated in aGI52 leaves. The CO-LIKE gene, COL13, however, appears to be out of line because it is expressed at a lower level in aGI52 versus DES, while CO is an activator of flowering in Arabidopsis [28]. Although, we note that the function of COL13 has not been studied yet, and it can differ from that of CO. Repression of StGI.04 produced up-regulation in the expression of REVEILLE1 (RVE1), a Myb-like, clock-regulated TF that links the clock and auxin networks by positive regulation of the expression of the auxin biosynthetic gene YUCCA8 (YUC8), thereby facilitating plant growth [16]. These results indicate that as in other plant species, GI is involved in circadian clock control.

Although both NF-YA-1/HAP2A and NF-YB-3/HAP3C are components of the NF-Y/HAP heterotrimer TF complex, NF-YA-1/HAP2A was down-regulated, while NF-YB-3/HAP3C was up-regulated in aGI52 leaves. In Arabidopsis, the NF-Y/HAP complex was shown to stimulate the transcription of various genes by recognizing and binding to a CCAAT motif in promoters. NF-Y complexes, among others, have been found to be involved in the control of flowering, embryogenesis, seed maturation, drought resistance, and ABA perception. The Arabidopsis genome encodes 10 distinct NF-YA, NF-YB, and NF-YC proteins that allow an enormous combinatorial and functional diversity for the complex (reviewed in [29]). The number of NF-Y genes may be even higher in the tetraploid potato than in Arabidopsis, and the down-regulated NF-YA-1/HAP2A and the up-regulated NF-YB-3/HAP3C may be the subunits of different NF-Y/HAP complexes with different regulation pathways and functions.

Besides flowering, the role of GI in responses of plants to environmental stresses is also well-known. We found StGI.04 to be induced by osmotic-, cold- and
Fig. 7 (See legend on previous page.)
Fig. 8 Phylogenetic tree (a) and alignment of StMYB-Hv1 and the *Arabidopsis* MYBL2 proteins. b Sequences producing significant alignments with StMYB-Hv1 (PGSC0003DMG400030548) were retrieved from iTAK and Phylogeny.fr was used to generate the tree. An NCBI protein-protein blast search was carried out and AtMYBL2 (NP_001321410.1) identified as the *Arabidopsis* protein with the highest similarity to StMYB-Hv1. Identities and similarities of sequences are presented as a result of a Clustal Omega multiple sequence alignment. St, *Solanum tuberosum*; Nt, *Nicotiana tomentosiformis*; Sl, *Solanum lycopersicum*; Vv, *Vitis vinifera*; JC, *Jatropha curcas*; Gm, *Glycine max*; Cc, *Cajanus cajan*; Gh, *Gossypium hirsutum*; Gr, *Gossypium raimondii*.
heat stresses [8] and identified five TF genes involved in plant defence responses (ERF1B, ZAT10, WRKY11, MYB1R1, TGA2.1), which were up-regulated in aGI52. ERF1 and WRKY11 led to an increase in tolerance to Bacillus species in Arabidopsis and tomato [17, 19], while the activity of TGA2.1 caused retardation in rice plants in defence against Xanthomonas oryzae [21]. A similar duality was observed in the case of ZAT10. Mittler et al. [18] found that both overexpression and knockout zat10 mutants of Arabidopsis were enhanced in tolerance to salinity, heat, and osmotic stresses. In contrast, MYB1R-1 caused an unambiguous increase in drought tolerance in potato [20]. After considering these literature data and our results, it is suggested that StGI.04 has an ambivalent role in terms of both the biotic and abiotic stress responses in potato.

Superoxide dismutase and catalase (SOD and CAT, respectively) are antioxidant enzymes and integral parts of plants’ defence mechanisms to avoid damage caused by active oxygen species. Interestingly, however, while SOD was up-regulated, CAT was down-regulated in aGI52 leaves. GI is a repressor of SOD also in Arabidopsis as it is indicated by enhanced tolerance of the gi-3 mutant to oxidative stress, which is associated, at least in part, with constitutive activation of SOD and APX genes [30]. However, unlike in potato, the expression levels of CAT genes in Arabidopsis were 1.5–2-fold higher in the gi mutants than in the wild type [31].

We found that the key genes of starch synthesis, AGS1, AGS2, SS, and SP were suppressed, while TPS, a sugar messenger, was activated in aGI52. It has already been known that the Arabidopsis circadian system is sensitive to sucrose, the GI protein is stabilised by sucrose in the night and the gi mutants have increased starch contents [32–34]. Rice plants carrying a null mutation in GI showed a significantly increased sucrose and starch content in the leaves [35]. Our transcriptome result suggests that potato may be a third type of plants, in which repression of StGI.04 decreases the starch content in leaves. A recent study identified TREHALOSE-6-PHOPHATE SYNTHASE 8 (TPS8) as a direct interactor of GI in Arabidopsis [36] and we found TPS7 among the up-regulated genes. Our result supports the previous conclusion that TPS is involved in the mediation of GI effects.

Repression of StGI.04 expression caused a reduction in tuber colour and the anthocyanin pigmentaion of tuber skins. Significant reductions were detected in cyanidin 3,5-di-O-glucoside and pelargonidin 3,5-di-O-glucoside contents in tuber peels. Nevertheless, no significant alterations were found in the biosynthetic pathway of these compounds at the transcriptome level in leaves. The exceptions were PAL, the beginning gene of the pathway and LDOX, encoding a leucoanthocyanidin oxidising enzyme, which were down-regulated. The lack of transcriptional changes in leaves might be due to the general inactivity of the anthocyanin biosynthesis pathway, which is reflected by the very low amount of anthocyanin pigments detected in leaves. While the anthocyanin concentration was in a range from 30 to 430 μg/g Fw in tuber skin it was only 22–24 ng/g Fw in leaf samples and the only anthocyanin which could be detected in leaves was cyanidin 3,5-di-O-glucoside. However, its concentration in aGI52 leaves was slightly, but significantly lower than in DES leaves.

It has been known for a long time that three loci, D (developer), R (red), and P (purple) determines tuber colour. Jung et al. [37] 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 [38] and shown that an R2R3-MYB is a direct target of the small RNA regulation [39]. However, we did not find these MYB genes among the aGI52 DEGs. Instead, we found an R3-type MYB-coding gene up-regulated in aGI52 leaves, the MYB-RELATED PROTEIN Hv1 (MYB-Hv1), similar to MYBL2 in Arabidopsis. AtMYBL2 is a transcriptional repressor that negatively regulates anthocyanin biosynthesis [40]. We presume a similar function for MYB-Hv1 in potato and are going to study it in tuber skin.

The biological processes, namely circadian clock regulation, flowering, stress responses, starch synthesis and anthocyanin metabolism and major genes influenced by StGI.04 at transcript level in potato leaves are presented in Fig. 9.

Conclusions

GI GIANTEA was discovered almost 60 years ago as a “supervital” Arabidopsis mutant with a late flowering phenotype [41]. Since that time, it has been detected in many plant species, and its pleiotropic effect extending from flowering through metabolism to stress responses has been demonstrated (reviewed in [3]). In most of the species, GI is a single copy gene; however, it is duplicated in petunia and potato [8, 42]. In this study, functional analysis of one of the potato GI genes, designated StGI.04, was performed. 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 (Fig. 9). In addition, however, StGI.04 has a new function not detected earlier in other plant species, namely promotion of the synthesis of anthocyanins in tuber skin. The function of the other potato GI gene, StGI.12, is still unknown. However, based on the differences in regulation of StGI.04
and StGI.12 [8], functional differences between the two potato GI genes are expected.

**Methods**

**Plant growth conditions**

The commercial potato (Solanum tuberosum L.) cultivar ‘Désirée’ was used in all experiments. It was obtained from Fritz Lange KG (Bad Schwartau, Germany), cultivated under axenic conditions for tissue culture at the Max Planck Institute of Molecular Plant Physiology (Golm, Germany) as described by Dietze et al. [43], and transferred to our laboratory. The plants were propagated in vitro from stem segments in the rooting medium RM (MS without vitamins [44]) containing 2% (w/v) sucrose and 0.8% agar in 40-ml tubes closed with paper plugs at 24°C under a photoperiod cycle of 16 h/8 h day/night at a light intensity of 75 μmol m⁻² s⁻¹.

For morphological studies, 4-week-old plantlets obtained by tissue culture in tubes were transferred into pots containing Tabaksubstrat sterile soil A200 (Stender GmbH, Schermbeck, Germany) and grown further under greenhouse conditions from January to April at a temperature regime of 18 to 24°C and soil humidity up to approximately 80% provided by regular watering. The photoperiod was set at 12 h light and 12 h dark. In winter, the ambient light conditions were supplemented with artificial lightening by sodium lamps. Pesticides and fungicides were regularly applied for pest and fungal pathogen control.

For the short day (SD) tuberisation experiment, plantlets transferred from in vitro into pots were grown in the greenhouse for a month and then moved into a growth chamber and grown further until the end of the vegetation period under well-controlled SD conditions (8 h light/16 h dark, 23°C, 100 μmol m⁻² s⁻¹ light intensity, 72% humidity).

**Generation of aGI-repressed ‘Désirée’ lines**

Antisense GIGANTEA (aGI) plants were generated by amplifying a 250-bp fragment of StGI.04 using the primer pair GI250 (Additional file 2: Table S2) from ‘Désirée’ genomic DNA isolated according to Shure et al. [45]. The PCR fragment was inserted into pGEM-T Easy (Promega, Madison, WI, USA) and re-cloned in antisense orientation into the binary vector pCP60 as a KpnI-EcoRI-fragment behind the constitutive CaMV35S promoter. pCP60 (Additional file 1: Fig. S12) is a derivative of pBIN19 [46] constructed and was generously provided by P. Ratet, CNRS, Paris, France.
The recombinant plasmid DNA was transformed into *Escherichia coli* DH5α and introduced into *Agrobacterium tumefaciens* LBA4404 by tri-parental mating [47]. Identity and orientation of the 250-bp *StG1.04* fragment in pCP60 was verified by Sanger sequencing (BIOMI Ltd., Gödöllő, Hungary).

‘Désirée’ leaves propagated in vitro in 500-ml jars in MS medium containing 2% (w/v) sucrose and solidified with 0.8% agar (5 plants/jars) were used for transformation, and shoots were regenerated as described by Dietze et al. [43]. Kanamycin in a concentration of 25 μg ml⁻¹ was used for selection of transgenic lines.

**Analysis of StG1 gene expression**

Total RNA was extracted from leaves and tubers using the method described previously by Stiekema et al. [48]. RNA concentration and quality were tested using a NanoDrop spectrophotometer and reverse transcribed into first-strand cDNA using the Maxima H minus First Strand cDNA Synthesis Kit with dsDNase (Thermo Scientific Molecular Biology, Waltham, MA, USA). Semi-quantitative reverse transcription PCR (RT-PCR) analysis of the cDNAs was carried out using the ACTIN, *G1.04spec* and *G1.12spec* primer pairs (Additional file 2: Table S2) and visualising the PCR products on agarose gels. For these PCR amplifications, DreamTaq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) was used.

RT-qPCR assays were performed using a Light Cycler-96 thermal cycler (Roche Diagnostics GmbH, Mannheim, Germany) and a Luminaris Color HiGreen Flourescein qPCR Master Mix (Thermo Scientific Molecular Biology, Waltham, MA, USA). *ACTIN* and *EF1α* served as reference genes [49]. Primers are listed in Additional file 2: Table S2.

**RNA-seq**

RNA was isolated according to Stiekema et al. [48] from a sample set of three source leaves harvested from three 8-week-old plants grown in pots in the greenhouse. Three sample sets were prepared from aGI52, and three sets were prepared from the non-transformed control ‘Désirée’. A total of 5 to 7.5 μg of RNA per sample set was transported to the Novogene (UK) Company Ltd. (Cambridge, UK) and used for the generation of sequencing libraries after quality control. A paired-end 150 bp sequencing strategy was used to sequence the samples and the resulting data was also checked for its quality by the company. The bioinformatic analysis including mapping of quality reads to the *S. tuberosum* group Phureja DM1–3 v.6.1 reference genome (http://solanaceae.plantbiology.msu.edu/dm_v6_1_download.shtml), gene expression level analysis (FPKM distribution, Pearson correlation between samples, coexpression Venn diagram), differential gene expression (log2 fold change of DEGs, Vulcano plot) and functional analyses (GO and KEGG enrichment analyses) was carried out also by the company Novogene.

**Spectrophotometric analysis of anthocyanins**

Freshly harvested, mature potato tubers grown in pots were peeled. A simplified method of Toguri et al. [50] 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.

**HPLC analysis of anthocyanins**

The anthocyanin profile of potato tuber skin was analysed by the modified method on the basis of our previous studies [51–53]. The anthocyanin extract of tuber skin was centrifuged in Eppendorf tubes in a Hettich Mikro 22R centrifuge (15,000 rpm min⁻¹ for 2 min). The supernatant of centrifuged extract was filtered on a 0.45 μm MILLEX®-HV Syringe Driven Filter Unit (SLHV 013 NL, PVDF Durapore), purchased from Millipore Co. (Bedford, MA, USA), and injected into the HPLC system. The WATERS® High Performance Liquid Chromatograph (Waters Co., 34 Maple Street, Milford, MA, USA) was equipped with an absorbance detector (2487 Dual λ), a binary HPLC pump (1525), and in-line degasser, a column thermostat and an autosampler (717 plus) (set at 5°C) and was controlled using EMPOWER™ 2 software. A SYMMETRY C18 5 μm 4.6 × 150 mm (pore size 100 Å) column was installed. The isocratic flow rate of mobile phase (2.5% glacial acetic acid containing Milli-Q® water, MeOH and ACN = 35:35:10) was set to 1 cm³ min⁻¹ the pressure in the column was 1600 ± 15 psi (cca 11 ± 0.1 MPa) at a column temperature of 40°C. The running time was 10 min. The injected volumes of standards and samples were 20 μl. Detection started at 0.80 min to eliminate the peak of the extraction mixture, especially MeOH. The sampling rate was 10 pt. sec⁻¹, and the anthocyanins were monitored at a wavelength of 530 nm. The retention times of the standards were cyanidin 3,5-di-O-glucoside chloride (cyanin chloride) (CAS number: 2611–67–8) 1.6 min, pelargonidin 3,5-di-O-glucoside chloride (pelargonin chloride) (CAS number: 17334–58–6) 3.3 min, delphinidin 3,5-di-glycoside chloride (delphin) (CAS number: 17670–06-3) 4.3 min, and malvidin 3-galactoside chloride (primulin) (CAS number: 30113–37–2) 5.8 min.
Phylogenetic and sequence similarity analysis

DNA and protein sequences were aligned with the NCBI nucleotide and protein blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) or with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Plant Transcription Factor and Protein Kinase Identifier and Classifier (iTAK; http://itak.feilab.net/cgi-bin/itak/index.cgi) was used to search for proteins with significant similarity. The phylogenetic tree was constructed using Phylogeny.fr (https://www.phylogeny.fr/). All analyses were carried out with default parameters.

Statistical analysis

Heatmaps were generated by the Metaboanalyst 5.0 (https://www.metaboanalyst.ca). Statistical significance of the measurements was determined by Student’s t-test.

Abbreviations

DEG: Differentially expressed genes; DES: Désirée; GIGANTEA; KEGG: Kyoto encyclopedia of genes and genomes; LD: Long day; SD: Short day; St: Solanum tuberosum; RT‑qPCR: Reverse transcription‑quantitative polymerase chain reaction; TF: Transcription factor.

Supplementary Information

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Authors’ contributions

KO executed the plant tests under LD conditions and performed the RT‑qPCR analysis, JJ created the agI lines and grew the plants under SD conditions, FK‑R participated in RNA‑seq data analysis, GF evaluated the result of HPLC analysis, GS prepared the samples for HPLC analysis, GV performed the HPLC analysis, ZB conceived, designed and supervised the study and wrote the manuscript with the help of GV in description of the HPLC analysis. All authors have read and approved the final version of the manuscript.

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Availability of data and materials

The raw RNA‑seq data has been submitted to GEO (https://www.ncbi.nlm.nih.gov/geo/info/linking.html) under the Series record: GSE189956. All other data generated or analysed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate

All methods were performed in accordance with the relevant guidelines and regulations.

Consent for publication

NA

Competing interests

The authors declare that they have no competing interest.

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