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Down regulation of *StGA3ox* genes in potato results in altered GA content and affect plant and tuber growth characteristics

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ABSTRACT

GA biosynthesis and catabolism has been shown to play an important role in regulating tuberization in potato. Active GAs are inactivated in the stolon tips shortly after induction to tuberization. Overexpression of a GA inactivation gene results in an earlier tuberization phenotype, while reducing expression of the same gene results in delayed tuberization. In addition, overexpression of genes involved in GA biosynthesis results in delayed tuberization, while decreased expression of those genes results in earlied tuberization. The final step in GA biosynthesis is catalysed by *StGA3ox1* and *StGA3ox2* activity, that convert inactive forms of GA into active GA_1 and GA_4. In this study we cloned *StGA3ox2* gene in an RNAi construct and used this construct to transform potato plants. The *StGA3ox2* silenced plants were smaller and had shorter internodes. In addition, we assayed the concentrations of various GAs in the transgenic plants and showed an altered GA content. No difference was observed on the time point of tuber initiation. However, the transgenic clones had increased number of tubers with the same yield, resulting in smaller average tuber weight. In addition, we cloned the promoter of *StGA3ox2* to direct expression of the GUS reporter gene to visualize the sites of GA biosynthesis in the potato plant. Finally, we discuss how changes of several GA levels can have an impact on shoot, stolon and tuber development, as well as the possible mechanisms that mediate feed-forward and feed-back regulation loops in the GA biosynthetic pathway in potato.

Introduction

The genes that participate in GA biosynthesis and their effect on plant development have been extensively studied in *Arabidopsis* (Fleet et al., 2003; Pimenta Lange and Lange, 2006; Huang et al., 1998; Schomburg et al., 2003). The biosynthesis of GAs involves a complex network of interacting pathways comprising more than 130 precursors and bioactive GAs that have been identified *in planta* (reviewed in Hedden and Phillips, 2000). GA 20-oxidase and GA 3-oxidase, catalyse the last two steps of active GA biosynthesis, converting inactive forms of GA such as GA_{20} into active forms such as GA_1, while GA2ox catalyzes the breakdown of bioactive GAs. Overproduction of active GAs results in a series of phenotypes, such as longer hypocotyls, increased stem elongation, earlier flowering and decreased seed dormancy (Huang et al., 1998), while plants over-expressing of *GA2ox* genes show a GA dose dependent stem length (Schomburg et al., 2003).

Potato tubers are formed at the tips of diageotropically growing underground shoots, called stolons. The formation of tubers is a result of a signaling cascade involving environmental inputs, hormonal signaling and transcript regulation. GA has been associated with a role in potato tuber formation, in which high concentrations inhibit or delay tuberization while, low levels promote tuber formation (Ewing, 1987; Kumar and Wareing, 1974; Xu et al., 1998). In an *in vitro* tuberization experiment, application of GA results in delayed, reduced and less synchronous tuberization. An estimation of the endogenous GA levels revealed that GA content is lower under tuber inductive conditions (Xu et al., 1998). Moreover, inactivation of the active GA content in the potato stolon tip by induction of the *StGA2ox1* gene occurs soon after induction to tuberization and prior to tuber swelling both *in vitro* and *in vivo* (Kloosterman et al., 2007). Cloning and over-expressing the *StGA2ox1* gene in potato, resulted in a dwarf phenotype reduced stolon growth and earlier *in vitro* tuberization phenotype, while plants with reduced

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expression of *StGA2ox1* showed delayed *in vitro* tuberization and altered tuber morphology, concomitant with altered GA content during the process of tuberization (Kloosterman et al., 2007). Over expression of the *StGA20ox1* gene that is involved in GA biosynthesis, resulted in taller plants with elongated internodes and faster sprout growth while antisense plants were shorter with shorter internodes and an earlier tuberization phenotype (Carrera et al., 2000). In contrast, expression of *StGA3ox2* under a constitutive or leaf specific promoter results in taller plants that tuberize earlier under short-day conditions. Similarly, *phyB* RNAi knock-down transgenic plants are able to produce tubers even under unfavorable long-day conditions and exhibit increased GA content in the stem (Jackson and Prat, 1996), again in contrast with the common notion that higher levels of GAs delay tuberization. However, the site of accumulation of GA is crucial in this respect and a higher level of GA in the stem is often associated with a lower level of bioactive GAs in the stolon most likely due to differential mobility of the various GAs and feedback regulation (Bou-Torrent et al., 2011). Over-expression of StGA3ox2 using a tuber specific promoter had a small effect on plant height and a slightly delayed tuberization phenotype. New evidence on auxin content and auxin related genes during early stages of tuber development support a role for auxin and a crosstalk with GAs after tuber induction to regulate the plane of cell division in the stolons tips to seize longitudinal growth and initiate tuber growth (Roumeliotis et al., 2012).

In this study we cloned the *StGA3ox2* gene and transformed potato plants with a *StGA3ox2* RNAi construct, to modify GA metabolism and study the impact on potato tuber formation and plant growth. In addition, we cloned the promoter of *StGA3ox2* to direct expression of the GUS reporter gene to visualize the sites of GA biosynthesis in the potato plant. We discuss how changes of several GA levels can have an impact on shoot, stolon and tuber development, as well as the possible mechanisms that mediate feed-forward and feed-backward regulation loops in the GA biosynthetic pathway in potato.

Materials and methods

Construction of the StGA3ox2 RNAi and promStGA3ox2::GUS plasmids

Gateway technology (Invitrogen Europe BV, Blijswijk, NL) was used for the construction of the *StGA3ox2* RNAi and the promStGA3ox2::GUS constructs. The *StGA3ox2* gene sequence was retrieved from the NCBI (http://www.ncbi.nlm.nih.gov/) (gene annotation number gb|FJ792643). For the construction of the StGA3ox2 RNAi silencing construct, a 623 bp long fragment was amplified from stolon cDNA library (*S. tuberosum* L. var. *Andigena)* using StGA3ox2 specific primers (5′-caccGTGACGTCATCGAA GAGTAC and 3′-TTAACCTACTTGGACGCCAC) and cloned in an antisense orientation into destination vector pK7GWIWG2 (Karimi et al., 2002) that expresses the antisense under the regulation of the 35S constitutive promoter. The promoter region of *StGA3ox2* (3528 bp) was cloned from *S. tuberosum* group *andigena* using specific primers (5′-GGG-GCA-ACT-TTG-TAC-AAA-AAA-GTT-Gaccaaaaagggaaattctagag and 3′-GGG-GCA-ACT-TTG-TAC-AAC-AAA-GTT-Gtactcttcgatgacgtcactga). Vector pKGFS7 (Karimi et al., 2002), harboring the GUS reporter protein was used for transformation and promoter expression studies. Transgenic plants harboring the *StGA3ox2* RNAi construct and the prom*StGA3ox2*::GUS construct were obtained by Agrobacterium mediated transformation (*AGL0*) of *S. tuberosum* group andigena *in vitro* plantlets as described previously (Visser et al., 1989). Several transgenic clones were obtained for the *StGA3ox2* RNAi. The three clones were selected for further study based on low *StGA3ox2* transcript levels and severity of dwarfism. Similarly, several clones harboring the prom*StGA3ox2*::GUS construct were obtained and selection for the three best clones, named clones 1–3, was based on the performance of the GUS assays. The GUS staining assays was performed as described previously (Stomp, 1992). The incubation of the tissues in GUS substrate X-Gluc was performed overnight at 37 °C. The tissues were washed once with 70% ethanol prior to imaging.

Plant material

Single-node cuttings from short-day-grown potato plants (*Solanum tuberosum* L. var *Andigena*) transformed with the *StGA3ox2* RNAi construct and the prom *StGA3ox2*::GUS as well as the untransformed control were propagated *in vitro*, on standard MS medium (Skoog, 1962) 2% (w/v) sucrose. Potato plantlets were grown for 2 weeks before being transferred to 5 L soil filled pots. Plant height and the number of branches for each plant were monitored weekly. After 10 weeks, plants were transferred to a climate chamber with 8 h light 21 °C and 16 h dark 18 °C in order to induce tuberization. 17 days after plants were placed in the climate chambers, the underground parts of the *StGA3ox2* RNAi transgenic clones plants were harvested, and tuber yield was estimated. For statistical analysis we used the SPSS Statistics v19 (© SPSS, Inc., 2001, Chicago, IL, www.spss.com) software package (*post hoc* comparisons LSD, $a = 0.05$).

Quantitative RT-PCR

Estimation of the level of expression of the *StGA2ox*, *StGA3ox2* and *StGA20ox* genes was performed with quantitative RT-PCR (qRT-PCR). Gene specific primers were used (primer sequences in Supplementary Table 1). RNA was extracted from the samples collected also for GA analysis and estimated using the Qiagen RNaesy Plant mini kit (QIAGEN Benelux B.V. Venlo, NL). One biological sample per clone was investigated. Each biological sample contained tissue from three independent plants from each clone to have a good representation of the transcript levels of all genes in query per clone. The Invitrogen DNAse I Amplification Grade was used to avoid DNA contamination, and the Bio-Rad iScript cDNA synthesis kit was used for cDNA synthesis (Bio-Rad Laboratories B.V., Veenendaal, NL). 1 μg of RNA was used per cDNA synthesis reaction, and the product was diluted 20 times. The Bio-Rad iQ™ SYBR® Green Supermix was used for the qRT-PCR with the Bio-Rad cycler. The reactions were performed in triplicate in a final volume of 10 μl, containing 5 μl of SYBR® Green Supermix, 100 nM of each primer, PCR-grade water and 2 μl of cDNA sample. Reactions were incubated at 95 °C for 5 min to activate the enzyme, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. eIF3e was used as a reference gene (primer sequences in Supplementary Table 1).

GA measurements

For GA measurements, plants transformed with the *StGA3ox2* RNAi construct and the untransformed control were propagated *in vitro* and placed in the greenhouse as described above. Samples from apex and middle stem were collected from three plants with two biological repeats for two independent clones and the untransformed control. Samples were harvested on two time points. The first harvest took place before the switch to inductive short-day conditions. The second harvest was performed a week after the plants were placed in inductive short-days. Material was immediately frozen in liquid nitrogen and ground into a fine powder. GA measurements were performed as described elsewhere (Lange et al., 2005). One gram of FW of each sample was spiked with 16,

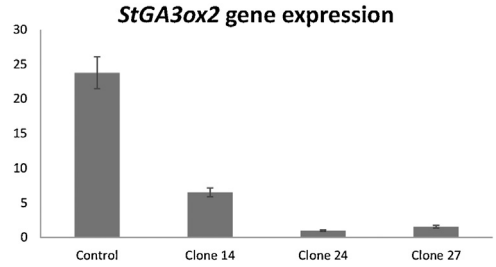

Fig. 1. Expression levels of the *StGA3ox2* gene in the apex in three transgenic clones and the untransformed control grown in the greenhouse. The *StGA3ox2* gene expression for clone 24 is set to one. Error bars represent standard error of the mean using three technical repeats.

17-d2-GA standards (1 ng each; from Prof. L. Mander, Canberra, Australia).

Results

With the aim of examining the role of altered GA content in the stolon tips on tuber development, we cloned a fragment of *StGA3ox2* gene, which is involved in active GAs biosynthesis, in an RNAi construct and we transformed the construct into potato plants.

Expression levels of the StGA2ox2 gene in the RNAi clones

In order to investigate the transcript levels of the *StGA3ox2* in the transgenic clones, RNA was extracted from the apices of independent clones and qRT-PCR was performed with specific primers (primer sequences in Supplementary Table 1). The transcript levels of *StGA30x2* was estimated in several stages and showed several fold decrease in the transgenic clones compared to the untransformed control (data not shown). Transcript levels of *StGA3ox2* in the apexes of the same greenhouse grown plants used for GA content analysis, revealed that the transgenic clones exhibited from 4-fold (clone 14) to more than 20-fold (clone 24) decrease, verifying that the RNAi construct conferred decreased transcript levels for the targeted gene (Fig. 1).

StGA3ox2 RNAi transformed clones have shorter plants with smaller leaves and smaller average tuber weight

Potato plants transformed with the *StGA3ox2* RNAi construct and their untransformed control were placed in the greenhouse and monitored weekly for their height to identify possible differences between the clones and the untransformed controls. After week four, all three transgenic clones exhibited shortened stem length (Fig. 2A) and smaller leaf size (Fig. 2B) compared to the control. All three independent clones exhibited the same phenotypes on stem length and leaves size to different extent. Clone 27 exhibited the most extreme growth retardation phenotype in stem and leaves size compared to all three transgenic clones, even though clone 14 was found to have the lowest levels of *StGA3ox2* transcripts (Fig. 1). Clone 14, had both the highest *StA3ox2* transcript levels and the smallest growth retardation compared to the two other transgenic clones. At week nine, the plants were transferred to climate chambers and grown under short-day conditions in order to induce tuberization. The differences between the stem length of the transgenic plants and the untransformed control continued to increase, resulting in significantly shorter plants for all transformed clones (Fig. 2C). 17 days after the plants were placed in the short-day climate chambers to induce tuberization, tuber fresh weight and number of tubers was scored. Overall the differences in tuber formation were not very large, especially regarding total tuber weight. The control plants had an average of 43 g per plant, while the transgenic clones had 44 g for clones 27 and 24 and 50 g for clone 14. Statistically, only clone 14 showed a significant increase in total fresh weight. On average however, all transgenic plants exhibited either an increased number of tubers (clone 14) or reduced average tuber weight (clone 27) or both (clone 24) (Fig. 3A–C).

Localisation of StGA3ox2 expression

In order to identify the regions of expression of the StGA3ox2 gene, a 3528 kb putative regulatory region of the StGA3ox2 gene was cloned and fused to the GUS reporter gene. Three independent transgenic clones, named clones 1–3, were chosen for further studies, based on their GUS staining performance. All three independent transgenic clones bearing the promStGA30x2::GUS construct exhibited similar GUS staining results. GUS activity was found in the tips of young leaves (Fig. 4A), young flower buds (Fig. 4B), around vascular tissues in a cross section of the stem taken from the region 1 cm directly below the shoot apex (Fig. 4C) and in non-tuberizing stolon tips (Fig. 4D). In the stolon tip, GUS staining is highest in the stolon apex and small leaf scales, and appears to be surrounding the vascular bundles similar to what was found in the shoot stem. No GUS staining was found in the lower stem, roots or in tubers (data not shown). These results indicate that *StGA3ox2* expression appears to be restricted to actively growing tissues.

StGA3ox2 RNAi construct alters the levels of the various GAs in the apex

In order to investigate the effect of the *StGA3ox2* RNAi construct on the GA content in the potato plant, we measured the concentration of five major GA precursors, the two bioactive GA species as well as four inactivation products. These GAs were measured in the apex, middle stem and tuber in two independent transgenic clones and an untransformed control under long-day conditions to achieve a spatial and temporal overview of GA metabolism (Fig. 5 and Table 1).

The transgenic clone 14 had higher levels of GA_9 and GA_{20}, in comparison to the control plants, both substrates for StGA3ox2 activity (Fig. 5). In addition, GA_{44}, a precursor of GA_{20} two steps up in the biosynthesis pathway, had higher concentration in the transgenic clones compared to the untransformed control. GA_9 and GA_{20} are also substrates for the StGA2ox enzyme that converts these two GAs into GA_{51} and GA_{29}, respectively. In clone 14, both products are increased. Contrary to expectations, active GA_4 and GA_1, products of the StGA3ox2 step, were not lower compared to the untransformed control. Instead, GA_4 levels were even higher in clone 14 compared to the control. GA_4 and GA_1 are substrates for StGA2ox enzymes to produce GA_{34} and GA_8. Only GA_{34} levels were lower in clone 14.

Gene expression analysis on the same samples as scored for GA content, revealed that in clone 14, three out of four identified *StGA20ox* genes had a two to three fold increase in expression levels compared to the untransformed control (Fig. 5). In addition, out of five identified *StGA2ox* genes, only two had a 3-fold (*StGA2ox1*) or a mild 0.5 fold (*StGA2ox5*) increase (expression levels of genes coding for GA biosynthesis enzymes are shown in Supplementary Figure 1).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2013.04.003.

In clone 27, down-regulation of *StGA3ox2* was also observed. In addition, only GA_{20} but not GA_9 content was higher. Moreover, as noticed for clone 14, concentrations of GA_{44}, GA_{51} and GA_{29} were also higher. On the other hand, in contrast to clone 14, increase in GA_9 and GA_4 content was not observed. Furthermore, no increase in the expression levels of the *StGA2ox* genes was noticed, while

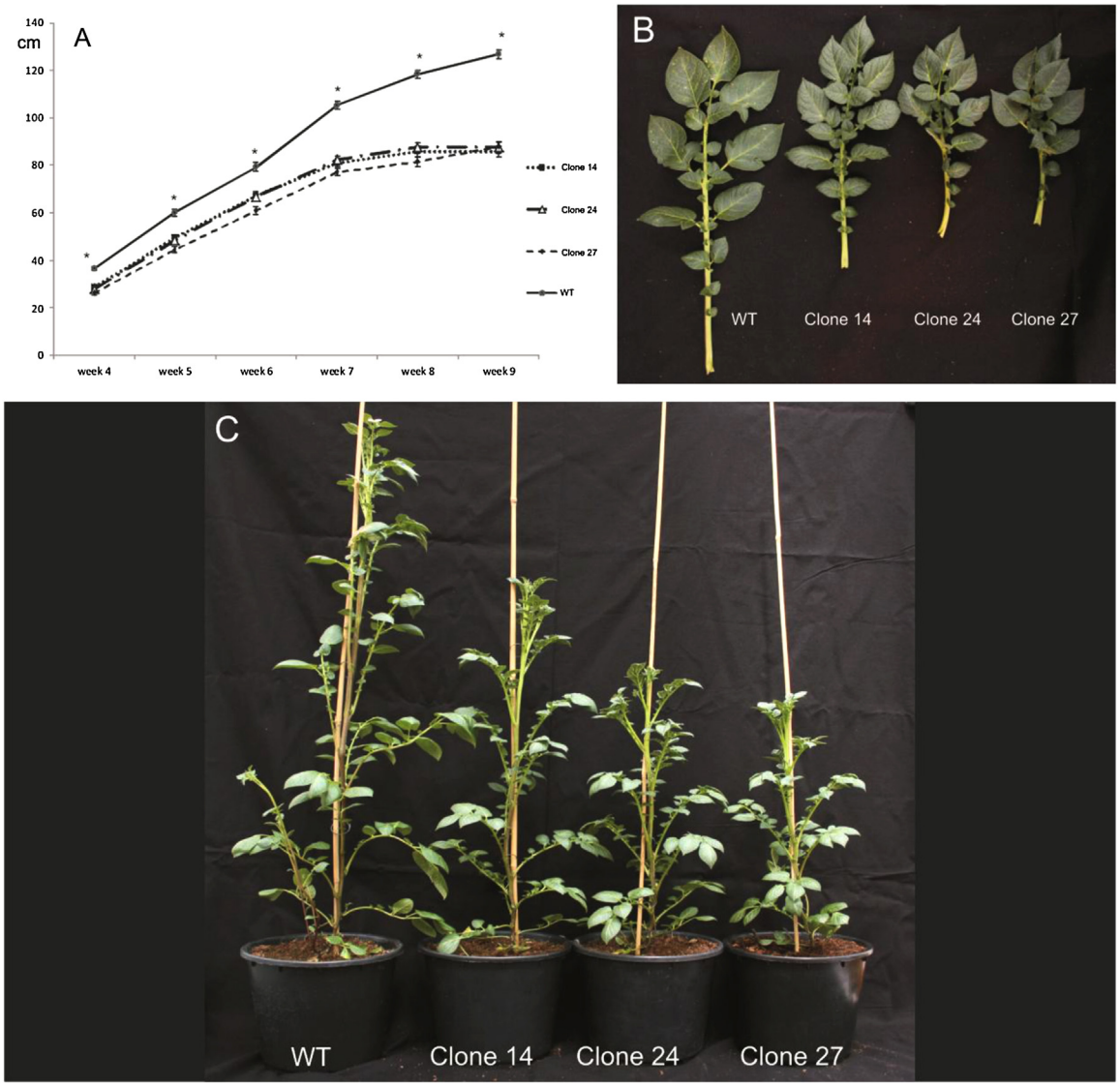

Fig. 2. (A) Scoring of plant height (cm) of the wild type (WT) *S. tuberosum* (var. *Andigena*) and three transgenic clones 14, 24 and 27. Error bars represent standard error for nine repeats per clone and six repeats for the wild type control. Asterisks (*) represent statistical significant difference between control and transgenic clone (*post hoc* comparisons LSD, $a = 0.05$). (B) Comparison between leaves of the wild type *S. tuberosum* (var. *Andigena*) and transgenic clones 14, 24 and 27. (C) Comparison between control and transgenic clones 14, 24 and 27.

Table 1
Concentrations of the GAs scored in the apices of two transgenic clones and the wild type untransformed control under long days and after 1 week in tuber inductive short-day conditions and the standard error. The values are averages of two independent biological repeats.

ng/g FW	Apex – long days						Apex – short days					
	Clone 14		Clone 27		WT control		Clone 14		Clone 27		WT control	
	Average	SE	Average	SE	Average	SE	Average	SE	Average	SE	Average	SE
GA_9	1.45	0.15	0.7	0.3	0.05	0.05	0.2	0	0.35	0.05	0	0
GA_4	1.65	0.25	0.6	0.4	0.35	0.05	0	0	0.15	0.05	0.3	0.2
GA_{34}	9.6	0.1	6.05	1.95	14.15	1.85	0.4	0.1	0.6	0.3	4.95	0.35
GA_{53}	1.25	0.55	1.1	0.3	0.65	0.15	0.05	0.05	0.05	0.05	0	0
GA_{44}	2.45	0.25	1.95	0.45	0.9	0.3	0.9	0.2	0.55	0.25	0.9	0.2
GA_{19}	5.65	0.35	6.4	1.2	5.65	0.95	2.75	0.65	2.85	0.15	2.95	0.25
GA_{20}	>20	n/a	>20	n/a	10.2	1.6	13.5	0.4	9.1	7.2	8.6	0.7
GA_1	1.15	0.05	0.65	0.25	0.95	0.05	0.35	0.15	0.3	0.1	0.3	0.1
GA_8	13.35	1.35	14.75	2.05	>20	n/a	4.7	0	5.3	1.4	14.75	2.35
GA_{51}	4.15	0.45	3	0.8	0.6	0.2	2.6	0.2	2.35	0.35	0.95	0.15
GA_{29}	>20	n/a	>20	n/a	9.05	0.65	>20	n/a	>20	n/a	17.55	0.35

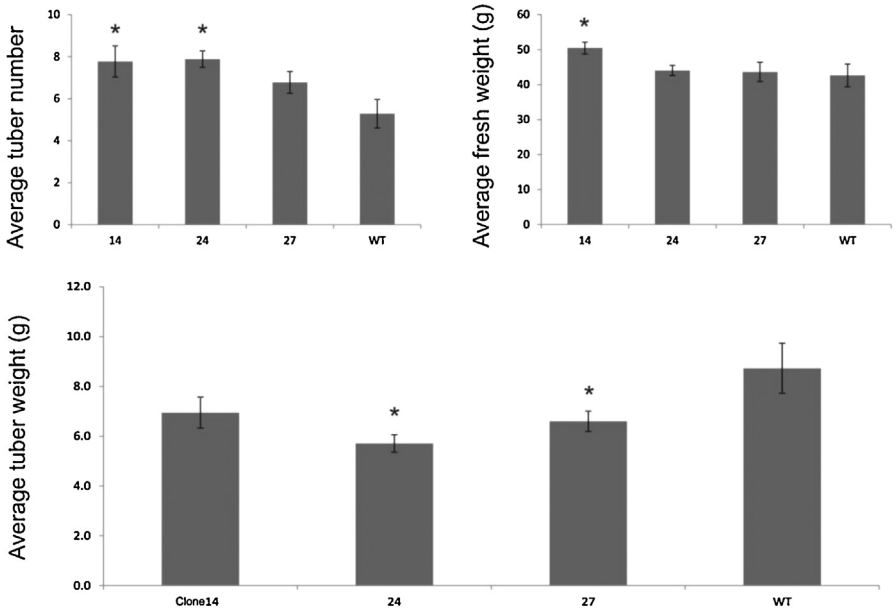

Fig. 3. (A) Scoring of the number of tubers, (B) the fresh weight and (C) the average tuber weight of the wild type (WT) and the transgenic clones 14, 24 and 27. Error bars represent standard error for nine repeats per clone and six repeats for the wild type control. Stars represent statistical significant differences (*post hoc* comparisons LSD, $a = 0.05$).

only one out of four *StGA20ox* genes had higher expression levels compared to the untransformed control (Supplementary Figure 1). Our measurements verified that in the apex during long days the GA_{20} content is higher in the partial silenced *StGA3ox2* transgenic clones, compared to the wild type control. In the middle stem the GA content was significantly lower for measured GAs but similar to the shoot apex. GA_{20} concentration tends to be higher in the transgenic clones. GA_8 and GA_{34}, products of the GA_1 and GA_4 catabolism by StGA2ox, show no significant difference between the untransformed plants and the transgenic clones in the middle stem. In addition, the concentration of the active form GA_1 did not differ in the wild type and the transgenic plants (data not shown).

GA content was also measured after switch to tuber inductive short-day conditions. The GA content in tuber inductive short days is much lower for all GAs measured in our research compared to the non-inductive long days. In the apex, the difference in the content of the precursor GA_{20} noticed in long days is decreased and the GA_1 content does not differ between the *StGA30x2* RNAi clones and the control (Table 1). Significant differences are visible only in the content of GA_8 and GA_{34} that is lower in the knock-down

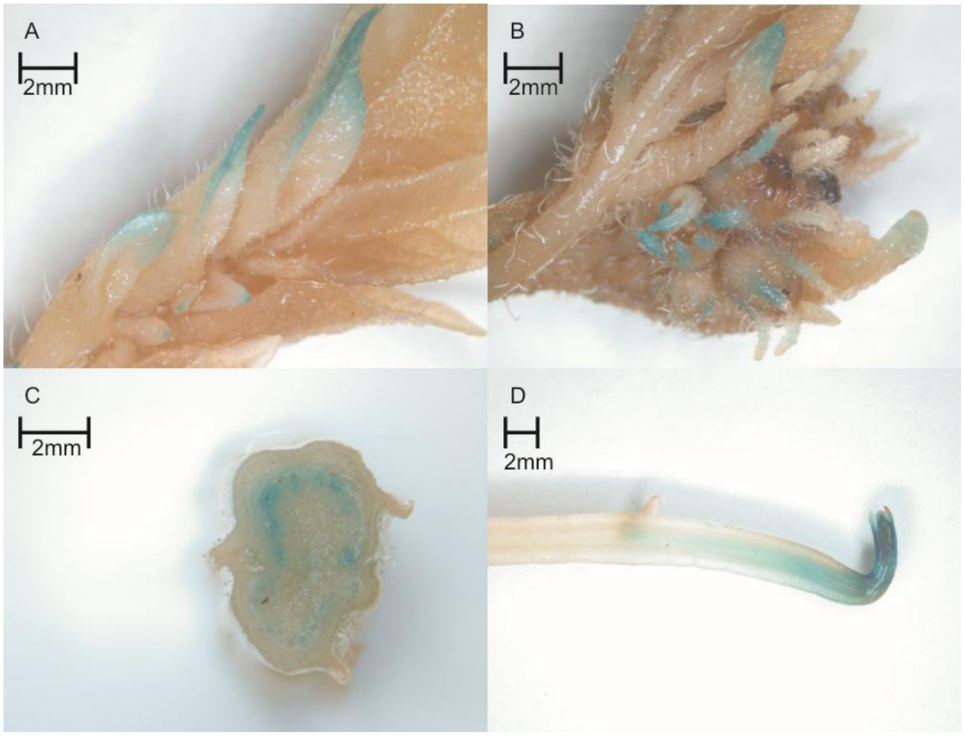

Fig. 4. prom*StGA3ox2* directed GUS activity staining in the tips of leaf primordia in the apex (A) of prom*StGA3ox2*::GUS transgenic clone 1, in the flower buds in the apex (B) of transgenic clone 2, in the vascular bundles in the shoot close to the apex (C) of transgenic clone 1 and in the stolon tips (D) of the transgenic clone 3.

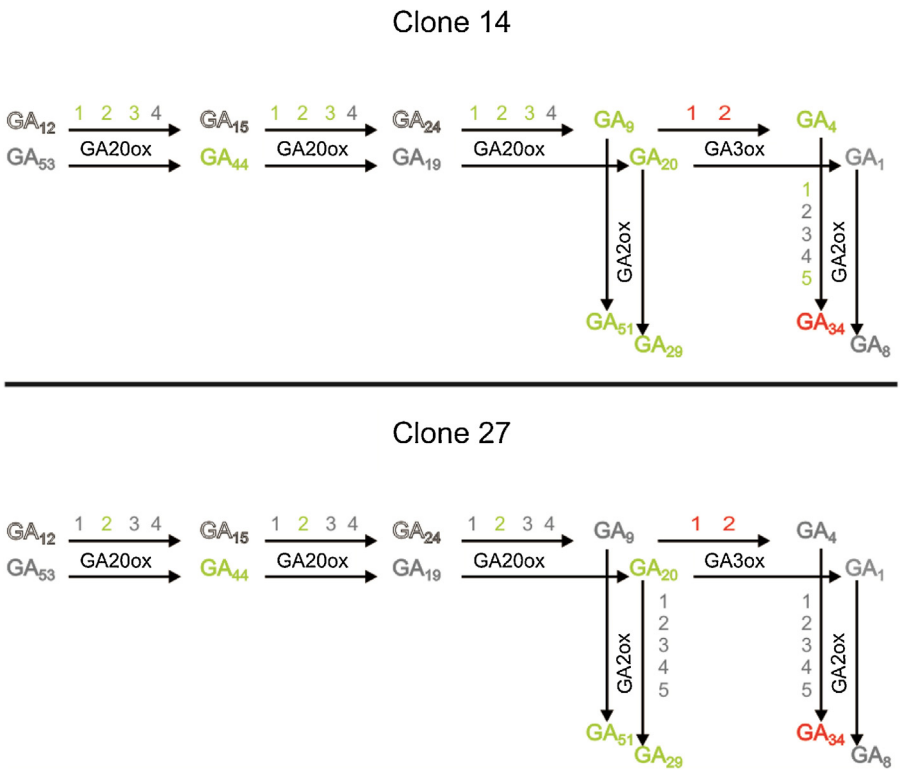

Fig. 5. Changes in the gibberellins biosynthetic pathway for clone 14 (top) and clone 27 (bottom) in comparison to control plants under long-day conditions. GA_{12}, GA_{15} and GA_{24} were not scored (white letters with black outline). GAs indicated in green had higher concentration, GAs indicated with red had lower concentrations and GAs indicated with grey had no statistical changes compared to the untransformed control. The gene encoding the enzyme catalyzing each biosynthetic step is written next to corresponding step, and numbers represent the several members of the same gene family. The gene family members that were down-regulated are indicated with red colour, the family members that were up-regulated are indicated with green colour and the family members that did not show differences in expression are indicated with grey. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

clones compared to the untransformed control (Table 1). GA levels in potato tuber samples were also measured under tuber inductive short-day conditions in several tissues including the potato tubers, but virtually no GA was detected in any of the samples (data not shown). Therefore, no concrete conclusions can be drawn from this set of results.

Discussion

Recently, the cloning and expression of a *StGA3ox2* under the control of 3 different promoters was described (Bou-Torrent et al., 2011). CaMV-35S regulated expression of this gene and the expression by the leaf specific promoter resulted in taller plants with altered GA content under tuber inductive short day conditions. A leaf specific expression resulted in shorter plants and the authors suggest that this was the result of PTGS co-suppression. In our study, the RNAi mediated reduction in expression of *StGA3ox2* resulted in shorter plants with shorter internodes, a similar phenotype to the *StGA20ox1* antisense clones (Carrera et al., 2000) and *StGA2ox1* overexpression clones (Kloosterman et al., 2007). The *StGA20ox1* and *StGA3ox2* RNAi clones seem to have reduced capacity to produce GA_1 and GA_4 while the *StGA2ox1* overexpression results in increased conversion rate of GA_1 and GA_4 to GA_8 and GA_{34}. These results imply that the shoot and internode length of the potato is strongly correlated with the ability of the potato plant to produce and catabolize the bioactive GA_1 and GA_4.

The concentration of several GAs was examined in two independent *StGA3ox2* RNAi clones. GA_{20}, the substrate of StGA3ox2 was found to be higher in both clones and GA_9 also substrate for StGA3ox2 was found to be higher in clone 14, likely due to the reduced *StGA3ox2* expression levels. GA_{20} and GA_9 are also a substrate for StGA2ox in a catabolic step that produces GA_{51} and GA_{29}. In both clones, GA_{51} and GA_{29} were found to have higher concentrations suggesting that this metabolic pathway is working in higher rates in the transgenic clones compared to the untransformed control.

Additional evidence that the metabolic flux of active GAs is altered is provided by levels of GA_8 and GA_{34} in short-day conditions (Table 1). Switch to tuber inductive short-day conditions are known to induce *StGA2ox1* gene expression that regulates catabolism of the active GA_1 and GA_4 into GA_8 and GA_{34} (Kloosterman et al., 2007). Interestingly, levels of GA_1 were not altered in the transgenic clones however its breakdown product, GA_8, is reduced, indicating a reduction of GA_1 turnover as a result of lowered GA_1 biosynthesis through GA3ox2. In addition, GA_{51} and GA_{29}, products of GA_9 and GA_{20} turnover are more abundant in the transgenic clones compared to the untransformed control. These results indicate that the GA_1 and GA_4 pathway exhibits reduced activity in favor of the GA_{20} and possibly the GA_9 pathway. Therefore, it could be suggested that the observed phenotypes might be attributed to the differentiated GA content in the transgenic clones.

In *Arabidopsis*, the presence of a possible feed-forward regulation of *GA2ox* genes through GA_1 and GA_4 to stabilize GA concentrations has been described (Thomas et al., 1999). In clone 14, higher concentration of GA_4 is observed. Expression analysis of five *StGA2ox* genes in clone 14 revealed that two *StGA2ox* genes had higher expression levels. In clone 27 no differences in GA_1 and GA_4 content was observed and *StGA2ox* gene expression was not altered. Thus, at least for clone 14 there seems to be a positive correlation between the GA_4 levels and the *StGA2ox* gene expression.

Studies in several species, including *Arabidopsis*, wheat and maize, indicate the existence of a negative feedback mechanism

that regulates *GA20ox* gene expression (gibberellin metabolism and regulatory loops reviewed in Thomas et al., 2005). Application of GA_1 negatively affects *StGA20ox* and *StGA3ox* gene expression. In transgenic clone 14, two out of three *StGA20ox* genes investigated are up-regulated and in transgenic clone 27, one *StGA20ox* gene is up-regulated. These findings could be a result of the reduced capacity of the transgenic clones to produce GA_1 and GA_4. Without high capacity to produce GA_1 and GA_4 the lack of negative feedback regulation would promote *StGA20ox* gene expression, resulting in higher levels of GA_9 and GA_{20} due to the inability of GA3ox to catabolize these pre-cursors at a sufficient rate.

As shown in Fig. 5, both *StGA3ox* genes exhibit a down-regulated profile in the transgenic clones. It is likely that the RNAi construct, despite being specifically designed to target *StGA3ox2*, also affects transcript levels of *StGA3ox1*, due to sequence similarity. However, the steady state levels of GA_1 are not significantly different. This could also be attributed to the higher levels of the substrates for the StGA3ox enzyme, which may be able to sustain this metabolic step despite the lower *StGA3ox* gene expression levels.

Leaf specific expression of *StGA3ox2* resulted in earlier tuberization, while a leaf specific co-suppression line for *StGA3ox2* gene resulted in delayed tuberization under tuber inductive short-day conditions (Bou-Torrent et al., 2011). These results can largely be explained by the different modes of transport of the various GAs. Different GAs have been found to be differentially transported in pea (Proebsting et al., 1992). Leaf specific expression of a *StGA3ox* gene would result in increased GA_1 biosynthesis in the stem, using a large part of the available and mobile GA_{20} substrate pool, resulting in reduced availability of the GA_{20} in the stolon. A reduction of GA_{20} in the stolon can result in decreased capacity to produce the less mobile GA_1, promoting earlier tuberization (Bou-Torrent et al., 2011). Consistent with that, the sink effect of the above-ground parts is not occurring in the tuber specific promoter driven *StGA3ox* gene expression, which results in local increase of GA_1 in the stolon tips, delayed tuberization and altered number of tubers produced by each plant.

In the shoot apex, bioactive GAs show little or no changes in observed concentrations. Based on the physiological similarity between the shoot apex and stolon apex, a similar steady-state level of bioactive GAs could be expected to occur in the stolon top. The fact that we could not observe changes in the time point of potato tuber initiation may be explained by this assumption.

Scoring the number of tubers and tuber yield produced by the transgenic clones revealed that two out of three of the *StGA3ox2* RNAi clones had a smaller average tuber weight (Fig. 3C), due to increased number of tubers (Fig. 3A) rather than reduced yield (Fig. 3B). Tuber specific promoter driven *StGA3ox2* expression also resulted in increased number of tubers but not altered yield (Bou-Torrent et al., 2011). In our transgenic clones, *StGA3ox2* gene expression is down regulated, while two *StGA2ox* genes are up regulated (Fig. 5).

In pea, decreased auxin content through shoot decapitation results in increased *PsGA2ox1* and decrease *PsGA3ox1* gene expression resulting in reduced GA_1 content (Ross et al., 2000). Auxin is known to be one of the major factors controlling branching (Hayward et al., 2009; Ongaro and Leyser, 2008) and the mechanisms that control branching in stolons are similar to the mechanisms that control branching in shoots (Roumeliotis et al., 2012). Altered GA:Auxin ratio within the stolon due to reduction of GA3ox2 expression may therefore result in increased stolon branching and tuber formation. This might provide a possible explanation for the increased tuber numbers observed on the stolons for some of the transgenic clones (Fig. 3A).

In this study we investigated the effects of the *StGA3ox2* RNAi transformation on the plant development, tuber formation and GA content in potato plants. In agreement with previous studies, potato clones transformed with the *StGA3ox2* RNAi construct were shorter with reduced internode length, and had altered content for various GAs, but did not alter the total yield produced or the time point of tuber initiation.

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