

Isolation and functional characterization of a stolon specific promoter from potato (*Solanum tuberosum* L.)

Luisa M. Trindade*, Beatrix Horvath, Christian Bachem, Evert Jacobsen, Richard G.F. Visser

Laboratory of Plant Breeding, Department of Plant Sciences, Graduate School Experimental Plant Sciences, Wageningen University, P.O. Box 386, 6700 AJ Wageningen, The Netherlands

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Abstract

In the search for time- and tissue-specific promoters an RNA fingerprinting technique called cDNA-AFLP was used. A transcript derived fragment (TDF511) was isolated which showed high similarity to alcohol dehydrogenases. The gene corresponding to this TDF, named *Stgan*, is likely to be involved in biosynthesis or breakdown of compounds affecting gibberellic acid (GA) levels in the plant [Plant J. 25(6) (2001) 595]. In this article the isolation and characterization of a *Stgan* promoter region is reported. The promoter region of this gene was fused to a reporter gene encoding β -glucuronidase (GUS) and introduced in potato plants. GUS staining was detected uniquely in stolon tips and nodes. RNA in situ hybridization experiments revealed that this gene was specifically expressed in parenchyma cells, in the stolon cortex. Comparison of this promoter sequence with several promoter databases resulted in the identification of several potential binding sites for transcription factors. From the in vitro-culture experiments *Stgan* transcription appears to be induced by long days, sucrose and different hormones such as gibberellic acid, ancymidol, ethylene and cytokinins.

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1. Introduction

Tuberization is a complex developmental process resulting in the differentiation of an underground stolon, a modified stem, into a specialized storage organ, the tuber (Taylor et al., 1992; Visser et al., 1994). The process of tuberization comprises inhibition of the longitudinal growth of the stolon followed by the initiation and growth of the tuber (Ewing and Struik, 1992). During tuberization, major biochemical changes occur, amongst which are the accumulation of starch and formation of storage proteins (Appeldoorn et al., 2002). Potato tubers develop initially from enlargement of existing pith cells in the sub-apical

region of the stolon, followed rapidly by cell divisions in most parenchyma cells, particularly those associated with the perimedulla and inner cortex (Li, 1985). These divisions are thought to cease early in tuber development (once the tuber has reached 30–40 g fresh weight, Li, 1985). The final tuber size is determined by further increase in cell volume of perimedullary and cortical parenchyma tissues (Ewing and Struik, 1992; Taylor et al., 1992).

Although histological studies have identified the changes that occur in cells at the onset and during early stages of tuberization, the molecular mechanisms that determine tuberization are largely unknown. Until now several potato tuber-specific promoters have been isolated such as patatin (Wenzler et al., 1989), starch phosphorylase (St-Pierre and Brisson, 1995), ADP-glucose pyrophosphorylase (Müller-Röber et al., 1994), GBSS (Visser et al., 1991) and sucrose synthase (Fu and Park, 1995). Although they are tissue-specific and therefore adapted to particular requirements, a fine regulation of transgene expression is in most cases not possible even with these promoters. With the increasing demand to express genes that interact and modify pathways

Abbreviations: BAP, benzyl amino purine; CCC, 2-chloroethyltrimethylammonium chloride; epd, eukaryotic promoter database; GA, gibberellic acid; GFP, green fluorescent protein; GUS, β -glucuronidase; JA, jasmonic acid; MS, Murashige and Skoog medium; *Stgan*, *Solanum tuberosum* 'gangly'; TDF, transcript derived fragment; TIS, transcription initiation site.

* Corresponding author. Tel.: +31-31-748-2855; fax: +31-31-748-3457.

E-mail address: luisa.trindade@wur.nl (L.M. Trindade).

in primary and secondary metabolism, it is becoming even more important to be able to fine-tune the expression of genes that are introduced into plants.

In the search for tuber life-cycle related promoters, which are time- and tissue-specific, the screening of differentially expressed genes was performed in tuberization related tissues obtained with a well defined synchronous in vitro tuberization system (Visser et al., 1994). Using an RNA fingerprinting technique called cDNA-AFLP, several hundred transcript derived fragments (TDFs) were identified (Bachem et al., 2000). One of them, TDF511 showed transient expression during the tuberization process and a delayed and increased expression in the presence of gibberellic acid. The related cDNA showed a high level of sequence similarity to a fruit-ripening gene from tomato (*ERT 10*) and several other steroid and alcohol dehydrogenases such as the maize *ts2* gene (Bachem et al., 2001). Potato plants transformed with an antisense *Stgan* construct showed a phenotype with altered tuber morphology, early sprouting, rapid growth during plant development and elongated in vitro stolons termed ‘gangly’ (Bachem et al., 2001). It is thought that *Stgan* is involved indirectly in the biosynthesis or breakdown of compounds that affect GA levels in the plant (Bachem et al., 2001). The involvement of *Stgan* in these metabolic pathways could elucidate specific regulatory elements in the promoter region of this gene, which serve as binding sites for transcription factors directly affecting tuberization.

The aim of this work was to isolate promoters driving genes uniquely expressed in tuberization related tissues. Here, we present the isolation and characterization of a *Stgan* promoter which shows expression exclusively in potato stolons.

2. Materials and methods

2.1. Nucleic acid manipulation

DNA manipulations were conducted using standard procedures as described by Sambrook et al. (1989), unless specified otherwise. DNA was extracted from leaves of potato as described by Rogers and Bendich (1988). *Escherichia coli* DH5 α served as a host for plasmid amplification.

2.2. Plant material

Two different tetraploid varieties of *Solanum tuberosum* were used in these experiments, cv. Karnico and cv. Bintje. Plants of cv. Bintje were used for the cDNA-AFLP experiments and for the Southern blot analysis. Because of the outstanding transformation efficiency cv. Karnico was used for transformation experiments.

2.3. Isolation of *Stgan* promoter by screening a genomic library

A genomic library made from *S. tuberosum*, variety Desiree (kindly supplied by Professor Uwe Sonewald, IPK, Germany) was used. The fragments were partially digested with the restriction enzyme *Sau3A* and inserted into the *Bam*HI site of lambda ZAP Express vector system (Stratagene). The titre of the genomic library was estimated to be 10⁸ pfu/ml. In total two library screens were performed. In each library screening the two first screen rounds were performed in the phage form and after in vivo excision a third screen was done. The cDNA clone of *Stgan*, named CB12 (Bachem et al., 2001), was used as a probe for the selection of the genomic clones.

In the first library screen 1 million phage plaques were plated resulting in the isolation of two positive clones after the three screening rounds. Both clones enclosed a region downstream of the first codon. A second screen was considered necessary in order to isolate *Stgan* clones containing a fragment upstream of the translation initiation site. For the second library screening, 20 million phage plaques were plated from which 17 independent colonies were isolated after in vivo excision. In order to identify which clones contained a region upstream of the ATG, a primer homologous to the *Stgan* 5' coding region (GTAAC-CATCACCACCTTTTCC) was used in combination with two vector primers T3 and T7.

2.4. Molecular analysis

For Southern blot analysis, DNA was digested with restriction enzymes, the products were resolved by electrophoresis through 1% agarose gel (4 μ g/lane) and transferred onto a nylon membrane (Hybond N⁺, Amersham). The membrane was pre-hybridized for 3 h at 65 °C in modified Church buffer. Hybridization was carried out for 16 h at 65 °C in modified Church buffer with an 1802 bp fragment containing *Stgan* promoter region (mentioned in the Section 3) labelled with [α -³²P]dCTP by random primed labelling. The blot was washed at 65 °C for 15 min each time, the two first times in 2 $\times$ SSC, 0.1% SDS and finally in 1 $\times$ SSC, 0.1% SDS. The radioactively labelled blots were incubated with a X OMAT S and AR films (Kodak) and after 2 days they were developed.

2.5. Isolation of *Stgan* promoter II with the Universal GenomeWalker kit

Isolation of a second *Stgan* promoter was performed with the Universal GenomeWalker kit (Clontech) according to manufacturer's instructions. DNA was extracted from potato leaves var. Bintje as described by Rogers and Bendich (1988). The DNA was digested for 16 h with five blunt-end restriction enzymes: *Dra*I, *Eco*RV, *Pvu*II, *Sca*I and *Stu*I. After purification of DNA with phenol/chloroform

GenomeWalker adaptors were ligated to the ends left by each restriction enzyme. Two rounds of PCR were performed using the Advantage Genomic Polymerase mix (Clontech) in a Perkin Elmer (PE) 2400 thermocycler. The primary PCR was performed using an adaptor-related primer (5'-GTAATACGACTCACTATAGGGC-3') and a gene-specific primer (5'-¹⁹³¹CTCATCGCATAGAGATTTCAATCGATC-3'), followed by a second PCR with a nested adaptor-related primer (5'-ACTATAGGGCACGCGTGGT-3') and a nested gene-specific primer (5'-¹⁹⁰⁴GAGCGGCGGCGATGATTCTACAACCAG-3'). The parameters used during the primary PCR reactions were as follows: 7 cycles: 94 °C, 2 s; 72 °C, 3 min; 37 cycles: 94 °C, 2 s; 67 °C, 3 min, in the last cycle an additional 7 min at 67 °C. For the nested PCR the same profiles were used and just the number of cycles was different: 5 cycles with the first profile and 25 cycles with the second profile. For the PCR reactions the Advantage Genomic polymerase mix (Clontech) was used. This mix includes a *Tth* DNA polymerase as primary polymerase, a proofreading *Vent_R* DNA polymerase and TthStart antibody to provide 'hot-start' PCR; in this way the errors made by *Tth* DNA polymerase are reduced 6.9-fold (Barnes, 1994).

The PCR fragments were analysed on a 1% agarose/EtBr gel and cloned into pGEM-T Easy vector system I (Promega) according to manufacturer's recommendations.

2.6. Analysis of the *Stgan* promoter I sequence

The *Stgan* promoter I sequence was compared with the GenBank eukaryotic promoter database (epd) using the BLASTn sequence alignment program (Altschul et al., 1997).

The search for plant specific boxes was performed using a computer program called MatInspector professional (Quandt et al., 1995) in the TRANSFAC (transcription factors) database.

For the localization of the putative TATA box two different programs were used: PC gene and Gene Runner (version 3.05) (Hastings software).

2.7. Identification of *Stgan* transcription initiation site (TIS)

Total RNA was isolated from potato stolons cv. Bintje with the GenElute Mammalian total RNA Miniprep kit (Sigma–Aldrich) according to the manufacturer's instructions. Messenger RNA was extracted from 5 µg total RNA with paramagnetic streptavidin coated beads as described in Bachem et al. (2000). About 200 ng of mRNA were used to proceed with the GeneRacer Kit (Invitrogen, Life Technologies) according to manufacturer's recommendations. The mRNA was reverse-transcribed with Super-Script II RT (Invitrogen, Life Technologies). PCR reactions were performed in the Perkin Elmer (PE) 9700 thermocycler with the Advantage Genomic Polymerase mix (Clontech) and the following primers: an anchor-related primer (5'-

CGACTGGAGCACGAGGACACTGA-3') and the gene-specific primer (5'-²⁰⁸⁴ACGTCCAAAGGCGTTCCAAGCTTTCTG-3') for the primary PCR and for the nested PCR the anchor-related primer (5'-GGACACTGACATGGACTGAAGGAGTA-3') and the gene-specific primer (5'-²⁰⁰⁸GTACGGCTCGAAGCTCCTGACTTGAA-3').

2.8. Transformation and regeneration of transgenic plants

For the analysis of *Stgan* expression in transgenic plants two constructs were made containing the 1.8 and 0.9 kb 5' region of clone L22. Both promoter fragments were obtained by PCR using the primers LC1 (5'-GGAT¹²CCAGGTGTC CCAATTTTCTC-3') and LC3 (5'-GGA¹⁸¹⁰TCCGGC-GAGTTCTCCC-3') for the 1.8 kb fragment, and LC1 plus LC2 (5'-GGATCC⁸⁷³ATTGCCTCTCTGTTCTTTT-3') for the 0.9 kb fragment. These fragments were introduced into the *Bam* HI site of a promoterless bifunctional reporter vector pMP2490 (Quaedvlieg et al., 1998) which encodes β-glucuronidase (GUS::intron) and green fluorescent protein (GFP), and transferred into Karnico potato plants using *Agrobacterium tumefaciens*. Potato transformation with *A. tumefaciens* was carried out as described by Visser (1991). Karnico stem segments were immersed for 10 min in *A. tumefaciens* and drained in a filter paper for the excess of fluid. The explants were transferred to MS medium supplemented with 20 g/l sucrose, 8 g/l agar, 200 mg/l claforan, 200 mg/l vancomycin, 100 mg/l kanamycin and 1 mg/l zeatin, and incubated at 24 °C and 16 h light. Every 3 weeks the segments were placed on fresh media. Around 25 independent transformants were obtained from each of the two constructs.

To determine the GUS activity of the transformants, 3–5 clones were produced from each of the primary transformants and grown on MS medium supplemented with 10 g sucrose (MS10) and kanamycin (50 ng/ml) with 16 h light. When the plantlets had 6–7 leaves the top was cut off and regrown on MS10, and when these plantlets were 5–6 cm long they were transferred into soil. Several tissues of the in vivo and in vitro plants were analysed for GUS expression including roots, stolons (young and swelling stolons), tubers (young and old), stems (nodes and internodes), leaves (young and old), flowers, and tuber sprouts. The tissues detached from in vivo plants were collected at three different time points: 10, 20 and 35 weeks after planting in soil. At the first time point the plants had stolons and small tubers but no flowers, in the second stage plants were flowering with small and big tubers and almost no stolons, and in the third stage dormant and sprouting tubers were analysed.

Untransformed Karnico plants were used as negative control, while plants transformed with plasmid pMP2482 (Quaedvlieg et al., 1998), containing a double enhanced CaMV 35S promoter, served as positive control.

2.9. Determination of GUS activity

Tissues were cut in 1–1.5-cm sections and placed in P-buffer (0.05 M Na₂HPO₄ and 0.05 M KH₂PO₄), vacuum infiltrated for 15 min, then incubated at 37 °C in 0.05 M NaHPO₄, 0.05 M KH₂PO₄, 10 mM EDTA, 5 mM K-ferricyanide and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronidase until it turned blue (usually between 16 and 38 h). Tissues without GUS staining were kept in the same buffer for 1 week.

For the detection of GFP protein, experiments were performed but no signal could be detected. These results were already expected due to the low and very specific signal obtained with GUS gene expression. To be able to detect GUS gene expression more than 100 molecules need to be present in the cell; however, for the GFP signal more than 10,000 molecules are needed (Köhler, 1998). For the same reason promoter deletions were not performed.

2.10. In situ mRNA hybridization

In situ mRNA hybridizations were carried out essentially as described by Appeldoorn et al. (2002). RNA probes were labelled with dioxigenin-11-rUTP using the Boehringer nucleic acid labelling kit, according to manufacturer's recommendations. Primary and secondary stolons were fixed in 10 mM sodium phosphate buffer (PBS), pH 6.8, containing 4% paraformaldehyde, 0.25% glutaraldehyde and 100 mM NaCl for 1 h under vacuum at room temperature. The samples were swirled and incubated for another 20 min under vacuum. The fixative solution was replaced and the samples incubated for 16 h at room temperature without vacuum. The fixed samples were rinsed in PBS, dehydrated in ethanol:xylene series, and embedded in paraffin (Para Clean, Klinipath, Duiven, NL). Seven-micrometre thick longitudinal sections were cut and attached to slides coated with 2% (v/v) 3-aminopropyl-triethoxy-silane (Sigma, Deisenhofen, Germany). After removal of the paraffin, hybridization took place in the dark in a humid atmosphere for 16 h at 42 °C. The tissues were washed four times with 4× SSC (0.6 M NaCl and 0.06 M sodium citrate) at room temperature. RNase treatment was performed to remove non-hybridized RNA with a solution containing 50 µg RNase A/ml in 500 mM NaCl, 1 mM EDTA, 10 mM Tris–HCl (pH 7.5) for 50 min and finally washed in 2 × SSC at room temperature for 30 min.

Antibody binding was allowed for 1 h at room temperature after which excess antibody was removed by washing three times for 15 min in 100 mM Tris–HCl (pH 7.5). The sections were subsequently incubated in a reaction medium of 0.45 mg/ml nitro-blue tetrazolium chloride (NBT) and 0.175 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 100 mM Tris–HCl (pH 9.5), containing 100 mM NaCl and 5 mM MgCl₂. The reaction was performed for 18 h in the dark at room temperature and positive signal appeared as dark purple staining. The reaction was stopped

by washing the sections two times with 10 mM Tris–HCl (pH 8.0), 1 mM EDTA for 5 min at room temperature. The slides were subsequently washed twice with distilled water for 5 min and mounted in glycergel (DAKO Glycergel, Mounting Medium, Uithoorn, NL).

2.11. In vitro plants grown in different media

In vitro potato plants var. Bintje were grown for 9 days under 19 different conditions (Table 1). These 19 different hormones, sugars and light conditions were chosen based on their influence (inducing or inhibiting) in gene expression during the potato life cycle (Ewing and Struik, 1992 and references therein; Li, 1985). RNA was isolated from 16 whole plantlets grown in each of 19 different media and cDNA-AFLP template was synthesized as described by Bachem et al. (2000). The mRNA was extracted from whole plantlets, as in any of the media supplemented with different hormones and sugars no stolons could be obtained.

3. Results

3.1. Isolation of *Stgan* promoter I by screening a genomic library

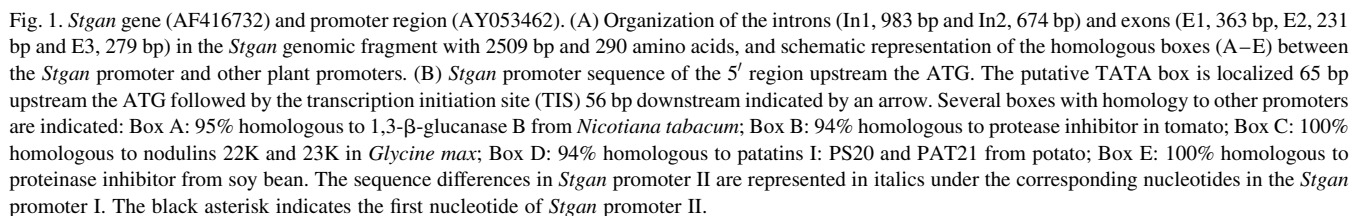
In order to isolate the promoter region of the *Stgan* gene, two library screenings were performed and two relevant clones (LT22 and LT71) were identified. Clone LT71 contained a 2.5 kb fragment including almost the entire coding region of *Stgan*, from 69 bp downstream of the START codon till the STOP codon, while the other clone (LT22) consisted of a 2.7 kb fragment. Using a *Stgan* specific primer based on the 5' end of the coding region, it was demonstrated that this clone included 2.2 kb upstream of the putative translation initiation codon and 0.5 kb of the 5' translated region.

Comparison of the 2509 bp long fragment with the cDNA showed that the *Stgan* gene comprises three exons and two introns whose size and organization are depicted in Fig. 1A. The splice junctions of both introns obey the GT/AG boundary rule and conform to the consensus sequences for splicing junctions in other plant genes (Brown and

Table 1
Different conditions in which 2-week-old plants were grown for 9 days

1. MS10 (16 h light)	11. MS10 + 50 µM Dicamba
2. MS10 (8 h light)	12. MS10 + 50 µM 2,4D
3. MS10 (0 h light)	13. MS10 + 7 mM ethephon
4. MS + 234 mM sucrose	14. MS10 + 50 µM Kinetine
5. MS + 300 mM sorbitol	15. MS10 + 100 µM ABA
6. MS + 300 mM fructose	16. MS10 + 200 µM methyl jasmonate
7. MS + 300 mM glucose	17. MS10 + 1.75 mg/ml CCC
8. MS + 300 mM mannitol	18. MS10 + 50 µM GA ₄₊₇
9. MS + 300 mM sucrose	19. MS10 + 5 mg/l ancymidol
10. MS10 + 25 mg/l BAP	

MS10 is MS medium supplemented with 10 g sucrose per litre of media.



The sequence of the upstream region of the *Stgan* gene was analysed with two different computer programs, PC gene and GeneRunner, and a putative TATA box was identified 65 bp upstream of the first in-frame ATG codon. The transcription initiation site was determined experimentally with GeneRunner kit and it is localized 9 bp upstream the first ATG (Fig. 1).

For the identification of putative *cis*-elements in *Stgan* promoter different databases and computer programs were used. Firstly, the *Stgan* promoter was compared with other

plant promoters present in the eukaryotic promoters database (epd) from the National Center for Biotechnology Information (NCBI) databases (Altschul et al., 1997), and secondly compared with the TRANSFAC database which contains several known *cis*-acting elements specific for plants. The 5' untranslated region of the *Stgan* gene did not show a long stretch of sequence homology to any other sequences in the NCBI databases, but contained several small regions that are conserved among promoters of patatin I and II (Box D, potato), proteinase inhibitor (Box B, tomato; Box E, soybean) and nodulin 22K, 23K (Box C, soybean). These results are shown in Fig. 1A and B. Searching in the TRANSFAC database with the program MatInspector Professional (Quandt et al., 1995), several putative transcription factor binding sites were identified (Table 2). Very high similarity was found between the *Stgan* promoter and the binding sites of the four Dof (zinc finger) transcription factors: Dof1, Dof2, Dof2 and PBF, which are unique to plants (Yanagisawa and Schmidt, 1999). The *Stgan* promoter shared high homology in three different locations to the binding site of a GA-regulated myb transcription factor from barley (Gubler et al., 1999). Furthermore, a 21 bp-long sequence (1579–1590) showed similarity to the binding site of the RAV1 protein which interacts with the AP2 domain, conserved among ethylene response elements in tobacco (Kagaya et al., 1999).

Comparison of the putative transcription factor binding sites (Table 2) and the homologies between the *Stgan* promoter to other plant promoters (Fig. 1) resulted in the identification of at least one common putative *cis*-element, corresponding to the Box B (Fig. 1) and the Dof2 transcription factor (Table 2).

3.2. Analysis of *Stgan*-GUS expression in different tissues

The 5' region of the *Stgan* gene was cloned into a vector

containing a bi-functional translational fusion of reporter genes amongst which the β -glucuronidase gene (GUS::intron) (Quaedvlieg et al., 1998). Two different constructs were made; one carried an 1802 bp fragment while the other one included 900 bp region upstream of the ATG from the LT22 genomic clone. Twenty and 25 transformants were regenerated with each construct, respectively. Plants were grown in vitro and different tissues from all lines were analysed for GUS expression: leaves (young and mature), stems, roots and tubers (no stolons, flowers or fruits were obtained under in vitro conditions). Under in vitro conditions no GUS expression could be detected. Therefore ten randomly chosen lines from each of the two constructs in 3–5 fold repeats were transferred to soil. Different tissues of the greenhouse-grown plants were analysed for GUS expression such as leaves (young and mature), nodes, internodes, roots, flowers, stolons (young, swelling, primary and secondary), tubers (young, mature) and tuber sprouts (no fruits were obtained under in vivo conditions). *Stgan* promoter activity was detected uniquely in stolons by means of GUS assays. A large number of stolons in different developmental stages showed GUS activity. Some stolons showed expression in the main stem nodes (Fig. 2A3,A4, A5), while others had blue staining in the tip as well. In contrast, stolons were also found with no coloration at all. No other tissue showed staining even after 1 week of incubation in GUS assay buffer, indicating that this promoter is extremely organ-specific. Additionally, no blue staining could be observed in the non-transgenic control plants even after 36 h in the GUS staining media.

3.3. In situ hybridization

In order to confirm the expression pattern of the *GUS::intron* gene under the control of the *Stgan* promoter and to analyse in which cell type *Stgan* gene is expressed,

Table 2
Homology between *Stgan* promoter and several transcription factors (TF) binding sites

Name of family/matrix	Further information	Position	Strand	Sequence	Reference
P\$ADOF/PBF_01	PBF (MPBF)	1741–1751	(+)	acaaAAAGgga	Yanagisawa and Schmidt (1999)
P\$AHBP/ATHB1_01	<i>A. thaliana</i> homeobox protein 1	867–880	(–)	gagcaATTAttttt	Sessa et al. (1993)
		1195–1208	(+)	aaataATTAttttag	
P\$MYBL/GAMYB_01	GA-regulated myb gene from barley	539–546	(+)	ggtgGTTG	Gubler et al. (1999)
		1147–1154	(+)	gacgGTTG	
		1479–1486	(+)	gttcGTTG	
P\$OPAQ/O2_02	Opaque-2	1523–1532	(+)	ttcACGTaca	Izawa et al. (1993)
P\$RAV5/RAV1_01	Interacts with AP2 domain	1579–1590	(–)	acccAACAtaat	Kagaya et al. (1999)
P\$ADOF/DOF1_01	Zinc finger transcription factor	623–633	(+)	atgtAAAGaac	Yanagisawa and Schmidt (1999)
		793–803	(–)	aaatAAAGaaa	
P\$ADOF/DOF2_01	Zinc finger transcription factor	439–449	(–)	taataAAGcat	Yanagisawa and Schmidt (1999)
		1061–1071	(–)	ttttAAAGcta	
P\$ADOF/DOF3_01	Zinc finger transcription factor	635–645	(+)	gagaAAAGgag	Yanagisawa and Schmidt (1999)

These results were obtained by searching the TRANSFAC database (Wingender et al., 2001) with the computer programme MatInspector Professional (Quandt et al., 1995). In the third column the position of the putative TF binding site in *Stgan* promoter is indicated. In the fourth column it is specified the *Stgan* strand (+ or –) in which the box was found, and in the fifth the sequence of *Stgan* homologous to the TF binding site. The core sequence of the TF binding site is represented in capital letters.

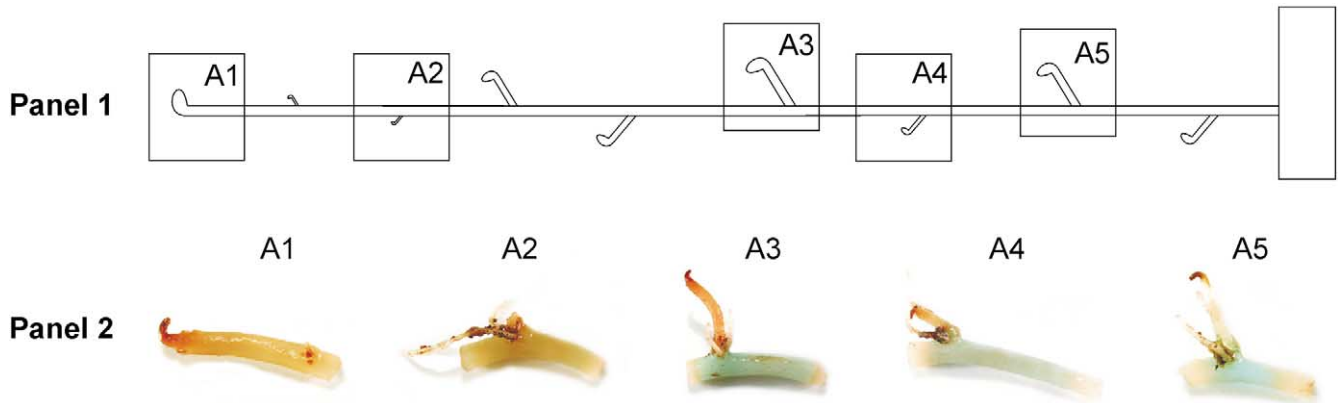


Fig. 2. GUS activity in transgenic potato plants containing GUS–*Stgan* promoter fusion. Panel 1 shows a drawing of the complete stolon from which 1–1.5-cm sections have been cut. Panel 2 shows some of the individual sections after incubation for 16 h in GUS detection buffer.

RNA in situ hybridization experiments were performed in potato stolons. In situ hybridization experiments revealed that *Stgan* gene is indeed expressed in potato stolon (Fig. 3). After 18 h exposure the difference in signal between the *Stgan* antisense and *Stgan* sense probes was evident. The dark purple precipitate was found in the tip of the stolon (Fig. 3A,C) and in the main stem of the stolon (Fig. 3A,B).

In both stolon tips and main stem the expression was stronger in parenchyma (-like) cells of the cortex region.

3.4. Molecular analysis

As the *Stgan* promoter I driven GUS expression is unique to stolons, whereas plants with silenced *Stgan* gene showed

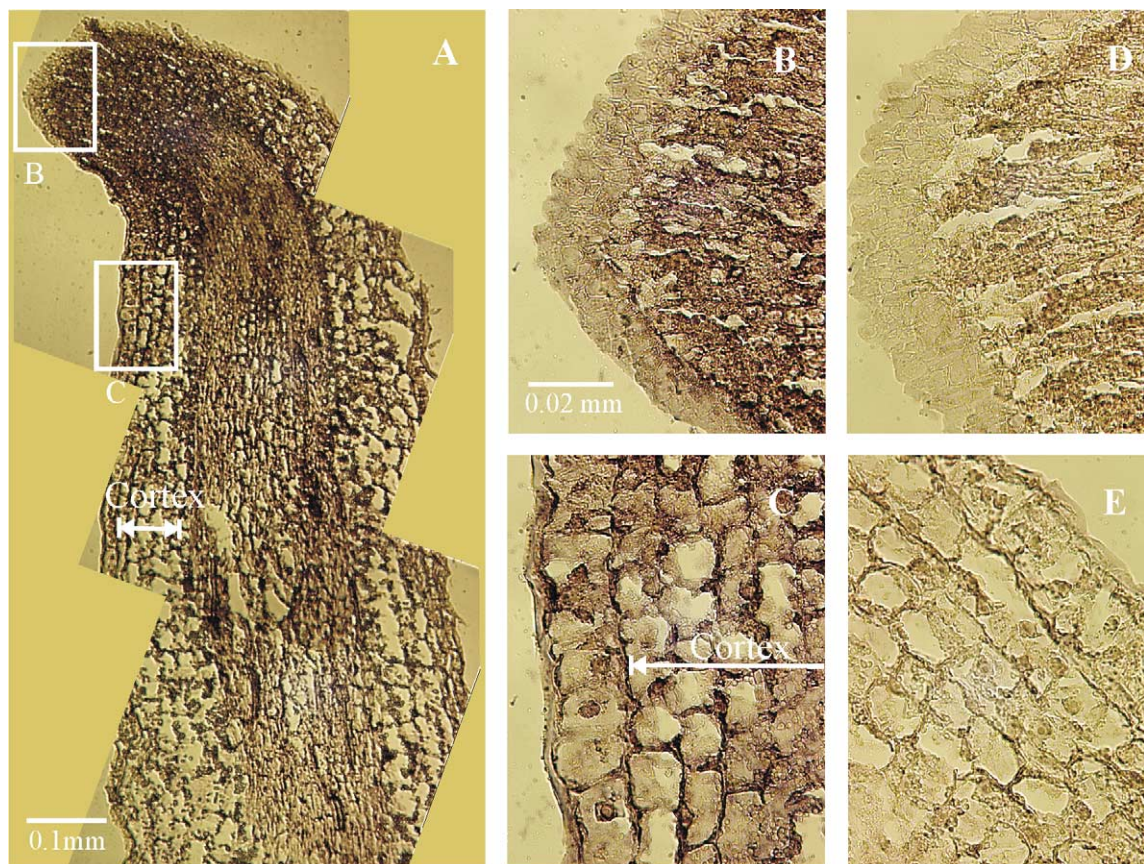


Fig. 3. Localization of *Stgan* mRNA in longitudinal sections of potato stolons by in situ hybridization with digoxigenin-labelled probes. The stolons were viewed under light-field illumination and the signal is dark purple. (A–C) were hybridized with the *Stgan* antisense probe, (D,E) were hybridized with the *Stgan* sense probe, which was used as negative control. (B–E) were captured with the same light conditions and with the same magnification, which is five times higher than (A). (B) and (D) show a detail of the tip of the stolon, and (C) and (E) the body of the stolon. The arrows in (A) and (C) indicate the stolon cortex and the dark purple precipitate can be observed in the cytoplasm of the parenchyma (-like) cells.

an altered phenotype also in stems and sprouts (Bachem et al., 2001), we suspected the presence of an *Stgan* gene with two different regulation and expression patterns. Therefore a detailed analysis of the genetic arrangement of *Stgan* promoters was carried out by Southern blot hybridization. Genomic DNA was digested with six different restriction enzymes: *Bam*HI, *Hinc*II, *Hind*III, *Eco*RV, *Eco*RI and *Dra*I (Fig. 4). Hybridization with the 1.8 kb *Stgan* promoter fragment yielded two and three bands with *Eco*RI and *Eco*RV, respectively. Four bands could be detected in the DNA digested with *Hind*III, which has a site in the region corresponding to the probe. *Dra*I and *Hinc*II produced more than four bands and each of them cut seven and two times, respectively, in the probe. These results suggested the presence of two (or three) copies of the *Stgan* promoter in the tetraploid potato variety Bintje.

3.5. Isolation of *Stgan* promoter II with the Universal GenomeWalker kit

Since the Southern blot analysis revealed the presence of more than one copy of the *Stgan* promoter region, experiments were performed in order to isolate the other promoter(s).

The second *Stgan* promoter (II) was isolated using the Universal GenomeWalker kit. A 1160 bp fragment was obtained from which 1009 bp were upstream of the translation initiation site. The 151 bp corresponding to the coding region of the *Stgan* gene were 100% homologous to the clone obtained from the library screening. However, the

sequences corresponding to the promoter region were just 95.5% identical. The differences in sequence between the promoters (I and II) are shown in Fig. 1. In both promoters Box B and Box C (Fig. 1) contained some differences in their sequence.

3.6. Regulation of *Stgan* expression

Expression of the *Stgan* gene(s) was studied using the cDNA-AFLP fingerprinting technique in potato plants grown for 9 days under 19 different conditions. Several hormones, sugars and light conditions were used in order to analyse factors that influence *Stgan* expression. The *Stgan* transcript was most abundant in plants grown with 16 h light (long days) supplemented with sucrose (8% (w/v) and 10% (w/v)), gibberellic acid (GA), ancymidol (an anti-GA), ethefon or BAP (benzyl amino purine) (Fig. 5). No *Stgan* expression could be detected in the plants grown in media supplemented with fructose, glucose, sorbitol or mannitol. Hormones such as auxins, kinetin, abscisic acid, jasmonic acid and CCC (2-chloroethyltrimethylammonium chloride) did not have a positive effect on *Stgan* expression.

4. Discussion

The analysis of the potato tuber life cycle by cDNA-AFLP resulted in the isolation of TDF511. The corresponding gene is very similar to the tomato fruit ripening gene *ERT10* and by homology it belongs to the family of the short-chain alcohol dehydrogenases (Bachem et al., 2001). This gene appears to be up-regulated during tuberization, and addition of gibberellic acid, an inhibitor of tuber formation, leads to a delayed but also higher expression (Bachem et al., 2001). Our previous results (Bachem et al., 2001) suggested that *Stgan* may be indirectly involved in a biochemical route for the breakdown of GA, affecting tuber-life cycle and plant development. In this article we describe the analysis of a *Stgan* promoter that regulates gene expression in a tissue- and process-specific manner.

The expression pattern of the *Stgan* promoter in stolons shown by GUS assays described above is likely to represent a continuum between no expression and a relatively high expression. We propose that *Stgan* expression in stolons is correlated with the time point and location of tuberization onset. Several results support this hypothesis, starting with the fact that *Stgan* is transiently expressed during tuberization. During the 10 days in vitro tuberization system, where tubers are formed synchronously around day 5, *Stgan* expression increases until day 5 and declines thereafter. This expression profile closely resembles the pattern visualized in the GUS assays described here, where neither small (young) stolons nor tuber-bearing (older) stolons show GUS expression and only stolons presumed to be just prior to tuberization show a higher level of GUS staining.

It is well known that when gibberellic acid is added to

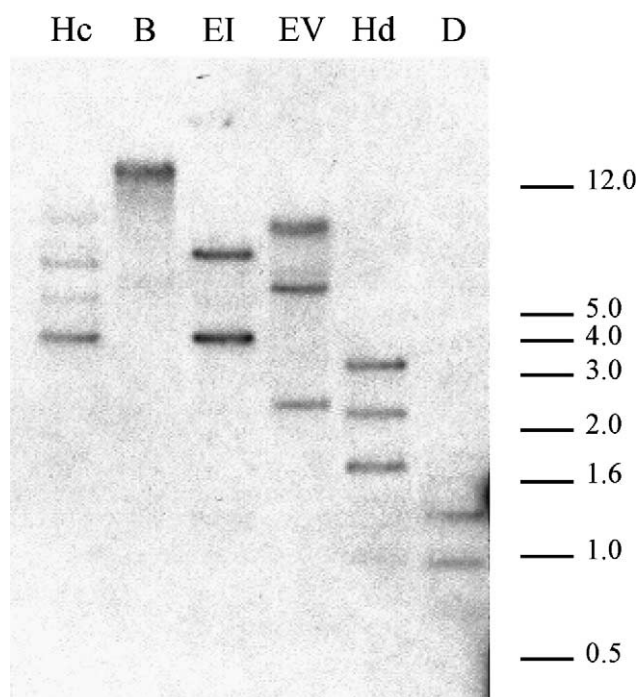


Fig. 4. Results of Southern blot analysis of *Stgan* promoter region. Genomic DNA was digested with six different enzymes: *Bam*HI (B), *Dra*I (D), *Eco*RI (EI), *Eco*RV (EV), *Hinc*II (Hc), *Hind*III (Hd) and *Sca*I (S).

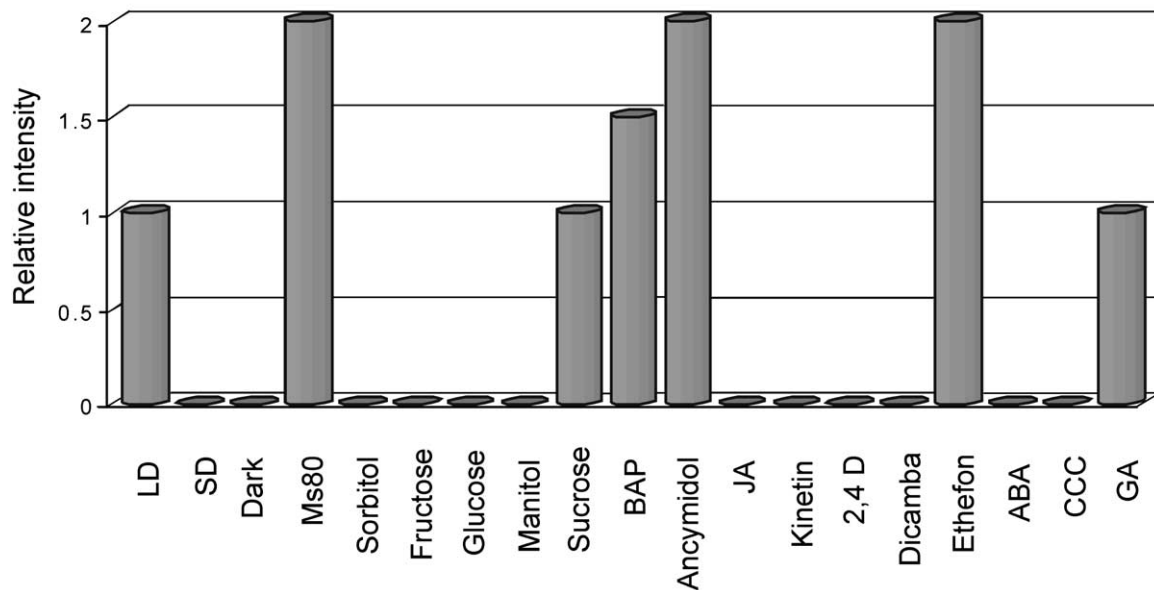


Fig. 5. cDNA AFLP analysis of TDF 511 expression in Karnico in vitro plants grown for 9 days in 19 different conditions. LD, long days (16 h light); SD, short days (8 h light); dark (0 h light); MS80, MS plus 234 mM sucrose; JA, jasmonic acid; GA, gibberellic acid. More details about the different media are indicated in Table 1.

tuberization media, explants produce longer stolons and the formation of tubers is delayed. This is consistent with the altered expression of *Stgan* when GA was added to the medium as visualized by cDNA-AFLP (Bachem et al., 2001). Furthermore, the levels of GA decrease in the stolons just prior to tuberization (Ewing and Struik, 1992), and as previously suggested *Stgan* may be involved in GA breakdown pathway which affects tuber life-cycle progression and plant development (Bachem et al., 2001). Alcohol dehydrogenases have been previously reported to be related with GA metabolism (Jacobsen and Olszewski, 1996), although the exact mechanism of GA regulation remains unclear. Furthermore, *Stgan* is not expressed in every stolon and it is known that tuberization is not induced in all stolons at the same time (Ewing and Struik, 1992). Finally, *Stgan* is expressed in parenchyma cells, and as shown by Taylor et al., 1992, potato tubers develop from a rapid cell division in the parenchyma cells.

Previous results in our laboratory (Bachem et al., 2001) demonstrated that *Stgan* genes express in stolons, petioles and stem nodes. However, in potato plants transformed with the *Stgan* promoter I::GUS construct, GUS expression was restricted to stolons. This indicates that the expression pattern displayed by cDNA-AFLP is a result of the activity of promoter I as well as other *Stgan* homologues. Differential gene expression in members of gene families were shown in other potato systems such as in non-specific lipid transfer proteins (nsLTPs) (Horvath et al., 2002) and for the MADS-box genes (Kang and Hannapel, 1996). In the other hand, it cannot be excluded that the level of expression observed with cDNA-AFLP is not sufficient to give a detectable GUS signal. With the cDNA-AFLP method a single transcript can be detected (Bachem et al., 2000),

while with GUS assays, 100 molecules per cell have been estimated to be required (Köhler, 1998). These results also illustrate the care necessary when generalizing on the expression of multicopy genes. The expression observed for a certain gene may be the sum of the expression of the different copies.

The localization of the most common *cis*-acting elements in the *Stgan* promoter region are in agreement with the results previously obtained by Joshi (1987) for other plant promoters. Joshi (1987) describes 9–200 bp as the most common distances between the transcription initiation site (TIS) and the translation-starting site this distance is 9 bp in *Stgan* promoter. The first translated codon is ATG as in 92% of other plant genes. The distance between the putative TATA box and the TIS is 56 nucleotides, slightly longer than the one determined statistically by Joshi (1987) as being 32 ± 7 bp.

Comparing the GUS staining obtained in plants transformed with the two *Stgan* promoter I constructs (900 bp *Stgan*-promoter::GUS and 1800 bp *Stgan*-promoter::GUS), we could conclude that Boxes E and D (Fig. 1) are not responsible for tissue specificity since both showed specific expression in stolons. However, these boxes might respond to compounds such as sugars or hormones.

The genes coding for patatin and PIN (proteinase inhibitor) respond to sugars and particularly to sucrose. Therefore the homologous boxes between these promoters and the *Stgan* promoter may be related to sugar response. However, this box has a different sequence from the SURE (sucrose responsive element, Grierson et al., 1994).

Root nodules of leguminous plants are sites of high metabolic activity with net import of assimilates and can thus be regarded as sink organs. Equally, stolons developing

into tubers represent strong sink organs. Similarities between potential sites for transcription-factor binding in regulatory regions of genes expressed in these organs may come from such functional relationships.

Experiments with different hormones, sugars and light conditions revealed that the *Stgan* transcript was most abundant in plants grown with 16 h light (long days), supplemented with either sucrose (8% (w/v) and 10% (w/v)), gibberellic acid (GA), ancymidol (an anti-GA), ethefon or BAP (benzyl amino purine). Whether these hormones and sugars act directly or indirectly to induce and/or up-regulate *Stgan* gene activity is unknown. The induction by GA is consistent with the promoter sequences showing high similarity to the 8 bp transcription factor binding site from a GA-regulated gene from barley (Table 2) in the *Stgan* 5' upstream region. Ethefon is a compound that releases ethylene (Jacobsen and Olszewski, 1996) and inhibits tuberization. The higher expression of *Stgan* in the presence of ethylene is consistent with the putative binding site for the transcription factor RAV1 found in *Stgan* promoter region. The RAV1 protein interacts with the AP2 domain that is conserved among a family of tobacco ethylene response elements (Kagaya et al., 1999).

The *Stgan* promoter will be an important tool for the comparison with other sequences of promoters that are also cell type- and tissue-specific, aiding the identification of consensus sequences that confer spatial and temporal expression specificity.

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