

A potato tuber-expressed mRNA with homology to steroid dehydrogenases affects gibberellin levels and plant development

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Summary

Using cDNA-AFLP RNA fingerprinting throughout potato tuber development, we have isolated a transcript-derived fragment (TDF511) with strong homology to plant steroid dehydrogenases. During *in vitro* tuberization, the abundance profile of the TDF shows close correlation to the process of tuber formation. However, when tuberization is inhibited by the addition of gibberellins (GAs) to the growth medium, the appearance of TDF511 in the fingerprint is delayed, then steadily increases in intensity during later stages of development. TDF511 was used to isolate the corresponding cDNA (CB12). The DNA and deduced amino-acid sequences of the cDNA show high homology to a fruit-ripening gene from tomato, a series of steroid dehydrogenases, and the maize *Ts2* gene. A section of the cDNA was cloned in antisense orientation behind a 35S CaMV promoter and transformed into potato. Transgenic plants expressing the antisense gene showed significantly earlier emergence, an increase in height, and longer tuber shape. *In vitro* tuberization experiments reveal extended stolon lengths in comparison to the controls. The analysis of endogenous GA levels showed that the transgenic antisense plants have elevated levels of biologically active GAs and their respective precursors. We propose that this gene plays a role in the metabolism of plant-growth substances important for tuber life cycle and plant development.

Keywords: Tuberisation, gibberellin, cDNA-AFLP, antisense, development.

Introduction

The potato tuber life cycle is composed of tuber formation (tuberization), resource accumulation, dormancy, dormancy breakage and sprouting. These events are likely to be controlled and regulated by a large number of genes differentially expressed throughout the life cycle of the tuber. Tuberization is initiated at the base of the stem by the formation of numerous underground stems, termed stolons, that are characterized by long internodes, nodal bracts and a typical terminal hook structure (Xu *et al.*, 1998b). After a period of rapid longitudinal growth, the stolons swell just behind the apical hook and a burst of cell division occurs, which is accompanied by a re-orientation in the cytoskeleton (Sanz *et al.*, 1996). Morphological changes during the onset of tuberization are accompanied

by the increase of particular proteins, thought to be involved in nitrogen storage (patatin) and prophylactics against pathogen and pest attack (PR-proteins, proteinase inhibitors). In addition, starch accumulation occurs, accompanied by an increase in enzymes involved in starch biosynthesis (sucrose synthase, ADP-glucose pyrophosphorylase, starch synthases, branching enzyme, etc.; Appeldoorn *et al.*, 1999).

It has long been known that tuberization is controlled by factors such as day length, nitrogen supply and levels of phytohormones (Prat *et al.*, 1990). From more recent research it is becoming increasingly clear that one of the key hormones influencing tuber formation is gibberellin (GA) (Carrera *et al.*, 1999; Xu *et al.*, 1998a). The connection

between light perception and GA-regulated development in model plant systems has also been established recently (Kamiya and Garcia-Martinez, 1999). However, GA is likely to be a negative regulator of tuberization while promoting stolon formation. In accordance with this notion, it was demonstrated that GA₁ levels fall sharply in the stolon tip just prior to tuber formation (Xu *et al.*, 1998a). The similarity in general morphology of brassinosteroid biosynthesis mutants to the GA mutants has given rise to research revealing some common signal-transduction pathways (Chory and Li, 1997). A direct interference between the signal perception pathways of these two phytohormones has been shown in *Arabidopsis*. The *Sax1* mutant, thought to be affected in brassinosteroid biosynthesis, shows reduced sensitivity to GA (Ephritikhine *et al.*, 1999). However, the extent and mechanism of GA/brassinosteroid cross-talk remains unclear.

In the search for the genetic basis of tuberization, a number of scientific approaches have been taken. Aerial parts of the plant have been screened for transcripts involved in tuber initiation (Jackson *et al.*, 1993). Alternatively, tubers and allied tissues have been investigated for differentially expressed genes associated with tuber formation (Bachem *et al.*, 1996; Banfalvi *et al.*, 1997; Taylor *et al.*, 1992). These approaches have yielded a number of interesting results regarding important metabolic processes that are active during individual steps of potato organ development. However, no clear indication has arisen from these data as to the key processes that are involved in initiation of tuberization or tuber development.

We have used a synchronized *in vitro* tuberization, dormancy and sprouting system for the systematic analysis of transcripts that are expressed during the potato tuber life cycle. Statistical analysis was carried out on ≈2600 transcript-derived fragments (TDFs) in a total of 18 000 that were visualized using cDNA-AFLP of 24 separate time points during tuber development (Bachem *et al.*, 2000b). The results of this analysis show that a large number of genes expressed early in tuber formation are involved in hormone metabolism (Bachem *et al.*, 2000b).

Here we describe the isolation and characterization of a differential gene expressed during tuber development, whose function appears closely related to the regulation of tuberization and is important for various stages of tuber life cycle and whole-plant development.

Results

TDF511 is tissue-specifically expressed during tuber formation

To analyse genes involved in the physiology and biochemistry of tuber formation, cDNA-AFLP fingerprinting

was carried out on *in vitro*-grown potato stolons and tubers. For this, a cDNA-AFLP template was prepared from a series of time points (daily sampling over 10 days) from potato nodal cuttings cultured on tuber-induction medium (MS medium with 8% sucrose); non-induction medium (MS medium with 1% sucrose); and tuberization suppression medium (MS medium with 8% sucrose and 5 μM GA₄ + GA₇). The axillary buds of the explants tuberize synchronously on day 5–6 when grown on tuber-inducing medium, and develop into shoots under non-induction medium. On growth medium supplemented with GA, however, stolons are formed but tuberization is suppressed over the 10-day period (Xu *et al.*, 1998a). In addition, a template was also prepared from a series of different potato tissues. A screen of 200 cDNA-AFLP primer combinations (using two selective bases) was carried out on these templates (Bachem *et al.*, 2000b). TDFs were isolated when (a) they showed a differential expression pattern at or around the time of tuber formation; (b) their expression was significantly altered in the templates derived from explants grown on medium containing GAs; or (c) the appearance of the TDF showed a high degree of tissue-specific expression.

These selection criteria were fulfilled by a 250 bp TDF designated TDF511 (Figure 1). On medium with 8% sucrose this TDF shows an increase in abundance during the days leading to tuber formation and then a gradual decrease until day 10 (Figure 1a, arrow). When the explants are grown in non-inducing conditions, TDF511 appears only very weakly throughout development (Figure 1b). On medium containing GAs, TDF511 expression is delayed and subsequently increases steadily over the period analysed (Figure 1c). TDF511 is also clearly present in petioles, nodes, and stems (Figure 1d). The results of the cDNA-AFLP fingerprinting (10 days of tuberization and tissue specificity) were further verified by Northern analysis (data not shown). The identity of the TDF511 was confirmed using primers with three selective bases corresponding to the sequence adjacent to the restriction sites in the TDF. The extra selective nucleotides reduced the number of bands in the fingerprint, verifying the identity and the expression pattern of TDF511 (Figure 1e). These results together indicate that the gene corresponding to TDF511 is upregulated on tuber formation, and that the increase in expression is likely to be process-specific.

Isolation of a cDNA corresponding to TDF511

After the isolation and cloning of TDF511, its DNA nucleotide sequence was determined. Homology searching against the NCBI data banks using the BLAST2 program showed a high degree of similarity ($P = 8 \times 10^{-20}$) to a tomato cDNA (Ert10) which is differentially expressed during fruit ripening (Picton *et al.*, 1993). Similarity was

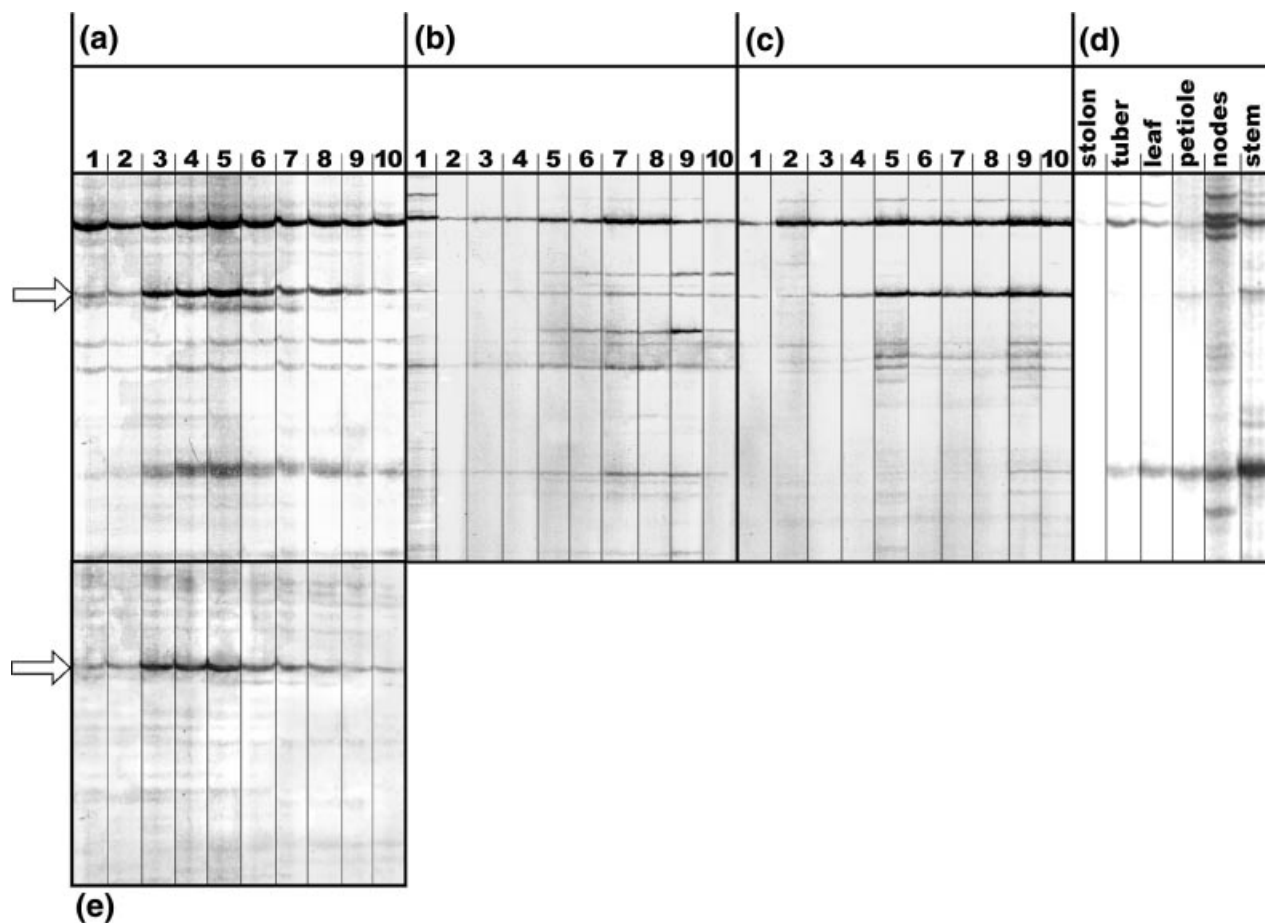


Figure 1. cDNA-AFLP analysis of TDF511.

Individual lanes (1–10) represent fingerprints made from RNA harvested from 10 consecutive days' *in vitro* tuber formation. RNA fingerprints in panels a–d were amplified using the same two selective nucleotides on the PCR primers, while the fingerprints in (e) were amplified using three selective nucleotides.

(a) Fingerprints of RNA from *in vitro* nodal explants grown under tuber-inducing conditions (8% sucrose).

(b) Fingerprints from RNA of explants grown under non-inducing conditions (1% sucrose).

(c) Fingerprints from RNA of explants grown on tuber-suppression medium (8% sucrose + GA).

(d) Fingerprints of RNAs isolated from various potato tissues/organs of greenhouse-grown plants. Fingerprints shown in (a–d) originate from simultaneously run amplifications and gel electrophoresis. The location of TDF511 is indicated by an open arrow.

Fingerprints of the same RNAs shown in panel (a) using an extra selective base in the cDNA-AFLP primers corresponding to the sequence of the *Stgan* gene.

also observed to a series of plant short-chain alcohol dehydrogenases and acyl-carrier proteins.

A cDNA library derived from swelling stolon tips (Taylor *et al.*, 1992) was screened using TDF511 as a probe. Twelve hybridizing phages were isolated from a plated bank of $\approx 100\,000$ plaques and excised *in vivo*. The inserts of the 12 cDNA clones showed a similarity in restriction sites, so the longest two (both ≈ 1200 bp) were used for sequence analysis. Both clones were shown to have identical 3' regions, and only a slight difference in length at the 5' end. The complete sequence of one clone, CB12, was determined.

CB12 contains an open reading frame of 852 bp coding for a protein with a molecular weight of

30 kDa (Figure 2). In a homology search it was confirmed that the putative protein belongs to the class of short-chain alcohol dehydrogenases. Furthermore, the CB12 cDNA has a high degree of homology to tomato Ert10 cDNA (81%), an *Arabidopsis thaliana* EST (70%), and acyl-carrier reductase proteins. Further similarities were found to hydroxysteroid dehydrogenases (31–37%) and the maize tassel-seed gene *ts2* (18%) (Figure 2). The CB12-derived amino acid sequence also has a consensus for a nucleotide-binding site (Figure 2, region I) and conserved regions for steroid dehydrogenases and short-chain alcohol dehydrogenases (Figure 2, regions II and III, respectively) (Fan and Plapp, 1999; Ghosh *et al.*, 1991). The gene encoded

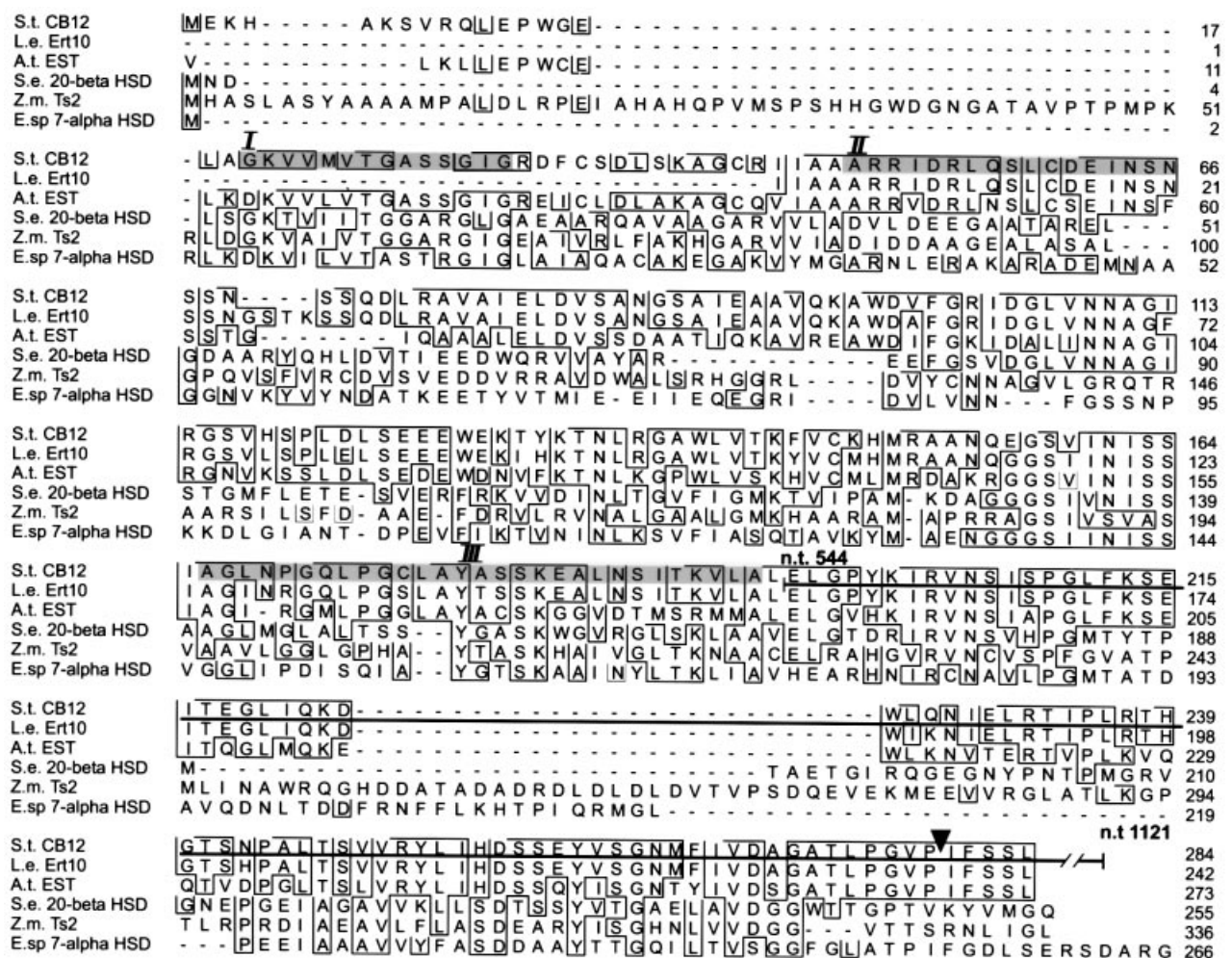


Figure 2. Sequence alignment of the CB12 cDNA-derived amino-acid sequence and various other related sequences.

The derived amino-acid sequences of five related genes were compared to the potato *Stgan* (St CB12) sequence using the CLUSTAL program. Boxed regions show residues identical to the consensus. The sequences are: L.e., Ert10 (tomato ripening-related mRNA), Accession No. X72730; A.t., EST (anonymous *Arabidopsis* sequence), Accession No. CAB75763; S.e., 20-β HSD (*Streptomyces exfolians* 20-β-hydroxysteroid dehydrogenase), Accession No. P19992; Z.m., Ts2 (*Zea mays* alcohol dehydrogenase), Accession No. L20621; E.sp., 7-alpha HSD (*Eubacteria* sp. 7-α-hydroxysteroid dehydrogenase), Accession No. A42468. The three shaded sections represent homologies with functional conservation: I, nucleotide-binding site; II, III, conserved regions of steroid dehydrogenases and short-chain alcohol dehydrogenases. The underlined section of the CB12 sequence represents the region of TDF511 with the nucleotide locations indicated above. Filled triangle, 5' end of the antisense construct.

by the CB12 cDNA has been named *Stgan* (from 'gangly') based on the phenotypes described below.

The Stgan gene is represented at low copy number in the potato genome

In order to study the genomic organization of the gene(s) corresponding to the CB12 cDNA, total DNA was isolated from potato leaves and digested with the restriction enzymes *EcoRI*, *BamHI*, *EcoRV*, *HindIII*, *PstI* and *XbaI*. The products were immobilized on a nylon membrane and hybridized with a 732 bp probe comprising the 5' end of a genomic clone. The autoradiogram (Figure 3) shows two

major hybridizing bands for the *BamHI*, *HindIII* and *XbaI* digests, which have no sites in the region used as a probe, and more bands for the other enzymes. These results indicate that the *Stgan* gene is present at low copy number in the potato genome.

Antisense expression of Stgan leads to developmentally altered plants

To gain more understanding about the function of the *Stgan* gene product, a construct was made in a binary *Agrobacterium tumefaciens* vector (a pBI121 derivative) that expresses a section of the *Stgan* gene in reverse

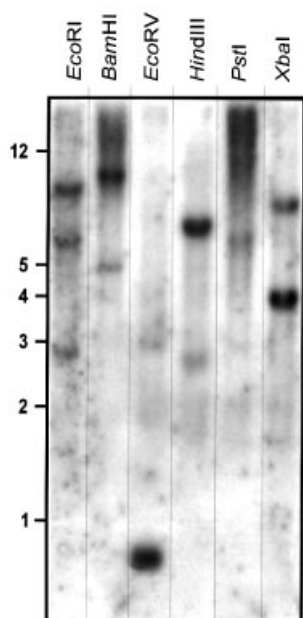


Figure 3. Genomic organization of the of *Stgan* genes by Southern analysis.

Genomic DNA of wild-type potato (Karnico) was digested with restriction enzymes *EcoRI*, *BamHI*, *EcoRV*, *HindIII*, *PstI* and *XbaI*. After electrophoretic separation and blotting, the immobilized fragments were hybridized with a probe from a section of the *Stgan* gene.

orientation from a 35S CaMV promoter. The construct was transformed into the potato variety Karnico via *A. tumefaciens* co-cultivation. Forty putative transformed lines were potted and grown in the greenhouse in five repeats. Transformation was confirmed in all lines using Southern hybridization (data not shown). Initial studies of the transgenic plants containing the antisense construct (designated AS-*Stgan*) showed that the phenotype was largely normal with respect to general morphology and yield. However, several transgenic lines clearly showed a taller growth habit (*GANGLY* phenotype) in comparison with the non-transformed controls. To validate this observation, tubers from seven lines including a control (Karnico) were harvested and planted in pots (with six repeats) in a growth chamber, under highly controlled conditions with regard to light, temperature and humidity. Under these conditions, the transgenic tubers sprouted earlier and the plants were taller at the end of the growth period in comparison to the controls. Figure 4 shows the growth curves (a) and the phenotype (c) of *GANGLY* plants in comparison with the Karnico control. The growth curves show that the *GANGLY* plants emerged earlier and maintained their initial growth advantage during further plant development. The tubers of *GANGLY* had an altered length-to-width ratio (Figure 4b) resulting in longer, narrower tubers, as can be seen in Figure 4d (left panel). This morphology is most pronounced in the lines CB12-1,

CB12-4, CB12-9 and CB12-10. The wild-type Karnico potatoes break dormancy ≈ 17 weeks after detachment from the haulm (harvest). At this time point the *GANGLY* plants were ≈ 2 weeks into sprouting (Figure 4d, left panel). One week after planting and subsequent growth in the dark, the *GANGLY* tuber have sprouts up to 15 cm while the Karnico plants have sprouts of 1 cm or less (Figure 4d, right panel).

RNA was isolated from etiolated sprouts of the six transgenic lines used in the growth experiment and the non-transformed control, and probed with the CB12 cDNA section used in the antisense constructs. Figure 4(e) shows the autoradiogram of the hybridization experiment. The arrow indicates the *Stgan* transcript, which is only present in the Karnico control and not in the other transgenic lines, indicating a high level of antisense suppression.

Plants harbouring the AS-Stgan construct have altered tuberization phenotypes in vitro

In potato nodal sections, tuberization can be induced *in vitro* by high sucrose (Bourque *et al.*, 1987). The addition of GAs to the tuber-induction medium, however, inhibits or delays tuber formation. Gibberellin-treated explants generally show elongated, stolon-like structures with a single terminal tuber after 2 weeks in culture. In order to analyse the structures produced by *GANGLY* plants, nodal sections were taken from plants grown under the same conditions as described (Hendriks *et al.*, 1991) and, after surface sterilization, were cultured on tuber-inducing medium. Since the *in vitro* tuber formation is not synchronous in the potato variety Karnico, assessing the time point of tuberization of the *GANGLY* plants and the controls was not possible. However, the *GANGLY* plants showed clearly elongated, stolon-like structures when compared to the control plants, which showed very short or no stolons (Figure 5).

GANGLY plants have high levels of active GA_s levels and their respective precursors

The similarity between the *in vitro* tuberization structures of explants grown on GA and those produced from *GANGLY* explants, together with *in vivo* phenotypes of the *GANGLY* plants, raises the possibility that the *Stgan* gene plays a direct or indirect role in the metabolism of one or more phytohormones. In order to assess this possibility, GA concentrations were determined in tissues with strong longitudinal growth. Etiolated sprouts were harvested at ≈ 30 cm in length and GA fractions were extracted (Kappers *et al.*, 1997). The concentrations of two active GA species (GA₁, GA₄) and their respective precursors (GA₂₀, GA₉) were determined in the three tallest-

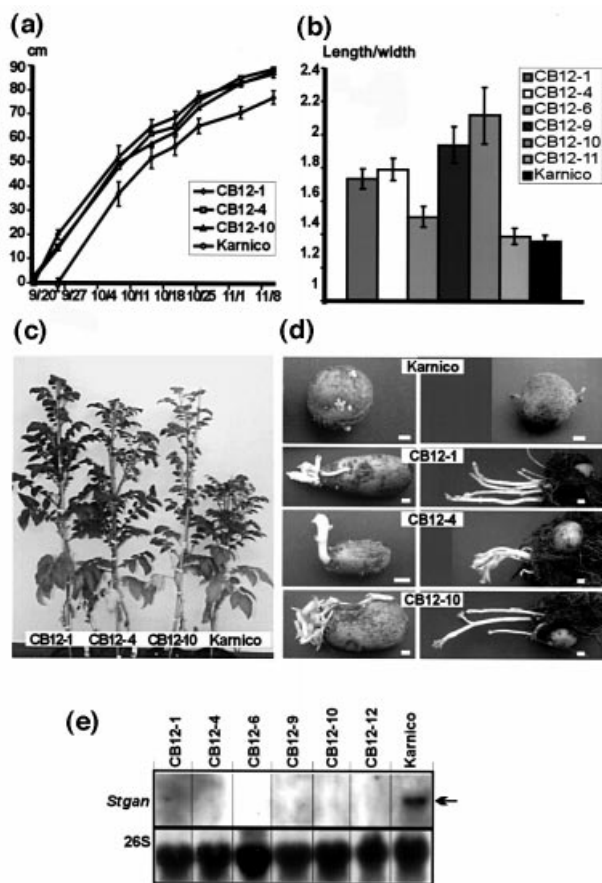


Figure 4. Molecular and phenotypic analysis of transgenic AS-*Stgan* lines. (a) Mean growth curves of selected *GANGLY* lines (CB12-1, CB12-4, CB12-10) in comparison with untransformed control (Karnico) during the growing period. Error bars show SEM. (b) Length/width ratios of the lines tested (CB12-1, CB12-4, CB12-6, CB12-9, CB12-10, CB12-11). Error bars show SEM. (c) Height difference of selected AS-*Stgan* lines (CB12-1, CB12-4, CB12-10) compared to control. (d) Phenotype of *GANGLY* and Karnico tubers at the time point of dormancy breaking in control (left), and the same lines 1 week after sprouting in the dark (right). White bars represent a 1 cm length standard. (e) Northern analysis of the *Stgan* transcript (arrow) in the six lines used in the growth experiment. The same RNA blot probed with a 26S ribosomal gene (shown below) as a control for RNA concentrations.

growing *GANGLY* lines and in Karnico controls. The results show (Figure 6) that in all cases the *GANGLY* plants had higher levels of GA than the controls. In particular, the two active GAs (GA_1 , GA_4) were over sevenfold higher for the transgenic line CB12-1 (Figure 6). In addition, the increase in GA_1 in the three lines tested were broadly in line with the phenotypic differences in growth habit *in vivo* (Figure 4c) and *in vitro* (Figure 5). This result indicates that the reduction of *Stgan* gene expression in the antisense lines is concomitant with increases in active GA levels (in particular GA_1) and their precursors.



Figure 5. *In vitro* stolon growth of *GANGLY* lines compared to non-transgenic Karnico controls. Nodes from pot-grown potato plants were harvested and grown *in vitro* on tuber-induction agar medium (MS + 8% sucrose). Transgenic lines containing the AS-*Stgan* construct, CB12-10, CB12-4 and CB12-1 are labelled as 1, 2 and 3, respectively, and the three Karnico controls are shown as 4, 5 and 6.

Furthermore, the levels of GA agree well with the other observed phenotypic changes in the *GANGLY* plants.

Discussion

Here we present the isolation and characterization of a gene that is expressed in the early stages of tuber formation. The phenotype of *GANGLY* potato plants shows altered tuber morphology, early sprouting, rapid early growth during plant development, and elongated stolons *in vitro*. Although the phenotype of the transgenic *GANGLY* plants can be explained by the increase in gibberellins, the difference in concentrations of active GAs in etiolated sprouts is not proportional to the observed phenotypes in fully grown plants. This may be due to the fact that *Stgan* is primarily expressed during

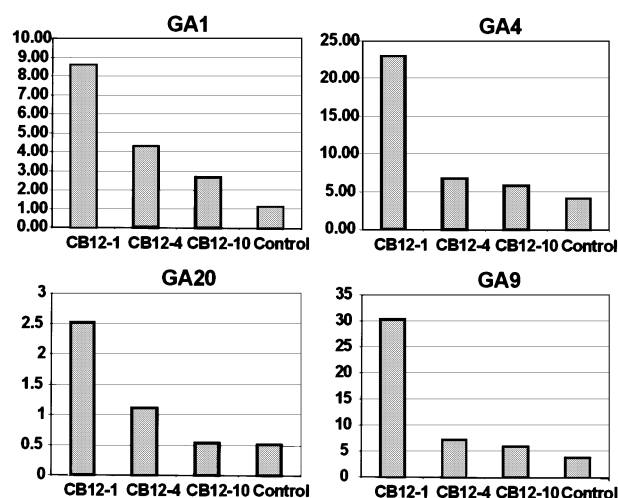


Figure 6. Gibberellin content of *GANGLY* plants and non-transformed controls. GA levels, represented as bars, for each potato line (CB12-1, CB12-4, CB12-10) in ng g⁻¹ FW.

early stages of plant development, leading to a reduced impact on the phenotype in fully grown plants. However, the changes in plant architecture produced by a suppression of *Stgan* gene expression indicate that *Stgan* genes are active throughout the plant, not only in tubers. Unlike other genes that show expression at early stages of tuber development, such as the *stsnap* gene (Bachem *et al.*, 2000a) and the SAMDC from potato (Taylor *et al.*, 1992), the *Stgan* gene codes for a catalytic protein with no known biochemical activity in plants. From the analysis of the nucleotide and derived amino-acid sequence, it is clear that *Stgan* has a striking resemblance to short-chain alcohol dehydrogenases. This large family of proteins is involved in a very wide array of biochemical pathways and acts on a variety of substrates, including steroids, sugars and acetyl-CoA (Persson *et al.*, 1991).

A number of plant genes with significant homology to *Stgan* have been characterized. The homologies between these genes split into four major phylogenetic groups (Figure 7), the most similar to the *Stgan* sequence being the tomato-ripening gene Ert10 (Picton *et al.*, 1993) and an *A. thaliana* gene (At EST; Figure 7). However, no function has yet been ascribed to either of these genes. Another related set of genes includes the *gad3* gene from tomato (Jacobsen and Olszewski, 1996), which was shown to be downregulated on GA application, and the *sadA* gene from pea, which is induced under UV light stress (Brosche and Strid, 1999) and was suggested to be involved in phyto steroid metabolism. Another member of this group, the *Ts2* gene from maize, is responsible for the activation of the male developmental programme of the tassel (DeLong *et al.*,

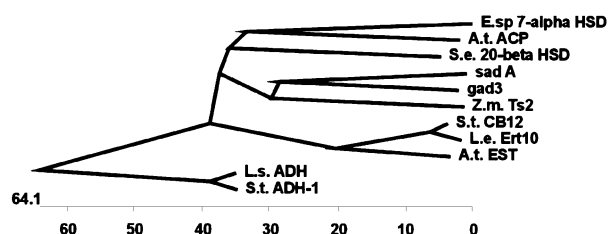


Figure 7. Phylogenetic tree of various genes related to *StGAN*. The phylogenetic tree was generated using the DNA-STAR program MEGALIGN and displays the same derived amino-acid sequences related to the *Stgan* gene. The same sequences were used as shown in the multiple alignment from Figure 2, with additional sequences as follows: A.t., ACP (*Arabidopsis thaliana* acyl-carrier protein), Accession No. CAB75765; *gad3*, tomato *gad3* gene, Accession No. U21801; *sadA*, pea *sadA* gene, Accession No. AF053638; S.t., ADH-1 (potato alcohol dehydrogenase 1), Accession No. M25154; L.s., ADH (lettuce alcohol dehydrogenase), Accession No. JC4320.

1993). The *Ts2* gene product, as the putative *StGAN*-protein, resembles steroid dehydrogenases, and it has been suggested that the gene may have an effect on phytohormone metabolism to reduce the levels of this phytohormone as a prerequisite for masculinization. More recently, it has been reported that the *Ts2* gene product is directly involved in programmed cell death, regulating the elimination of pistils during early development in the maize male inflorescence. Furthermore, it is suggested that this process may be the result of the elimination or biosynthesis of an unknown signalling molecule possibly related to steroids (Calderon-Urrea and Dellaporta, 1999).

In *GANGLY* plants we find a significant increase in the levels of active GA molecules and their precursors. Unlike an ADH-type gene from lettuce, which also shows homology to *Stgan* (Toyomasu *et al.*, 1995), *Stgan* does not appear to be directly induced by GA, since gene expression, revealed by cDNA-AFLP, does not rise directly after culture on GA-containing medium. However, gene expression is higher in the GA-treated explants than under a non-inducing regime without GA (Figure 1). The treatment of potato plants with an inhibitor of GA increases levels of TDF511, indicating that *Stgan* gene regulation is likely to be complex (unpublished results).

The connection between catalytic activity of the *Stgan* gene product and increased phytohormone content in the antisense plants remains elusive. Recently reported experiments on a potato GA biosynthetic enzyme, namely a GA-20 oxidase, shows a significant effect on tuberization and plant development (Carrera *et al.*, 2000). The similarity between the phenotype of *GANGLY* plants and those of the *StGA20ox1* over-expression plants obtained by Carrera *et al.* (2000) could lead to the speculation that *StGAN* is involved

in a biochemical route for the breakdown of GA. However, it seems unlikely that a short-chain alcohol dehydrogenase is directly involved in the catabolism of GA, as the molecular biology of biochemical pathways of GA breakdown is well elucidated (Hedden and Proebsting, 1999). Thus, an indirect route for GA depletion via a second regulatory compound, the biosynthesis of which is catalysed by the StGAN gene product, appears more likely. From the homology to *Ts2* and other bacterial steroid dehydrogenases, the StGAN enzyme is likely to be involved in the biosynthesis or breakdown of a steroid-like compound, which in turn affects GA levels in the plant, leading to the phenotypic effects observed in transgenic *GANGLY* plants. Further experiments with isolated StGAN protein will help elucidate its biochemical activity, and provide new clues as to its biological role in tuber formation and plant development.

Experimental procedures

Plant transformation, plasmid construction and plant material

For synchronized *in vitro* tuberization experiments, plants of *Solanum tuberosum* cv. Bintje were used. After day-length preconditioning (Hendriks *et al.*, 1991), nodal stem sections without leaves were harvested from potato plants and surface sterilized. These explants were placed on agar medium, and the axillary buds or their products were harvested at various times after treatment, as described in the text. Transformations with *Agrobacterium tumefaciens* were carried out on the potato cv. Karnico. Internodal sections of 3–5 mm were taken for transformation by *A. tumefaciens* using co-cultivation (Visser, 1991). A section of the CB12 cDNA (Accession No. AF349916), including 50 bp of the vector at the 5' end of the construct, was cloned into a binary vector described below using PCR-aided cloning (Bachem *et al.*, 1996). The primers used to amplify the region to be cloned were: 5'-primer, [TCTAGACTCCACCGGTTGGCGGCC] bp 826 in AF349916; 3'-primer, [TCTAGAGGGTAAAGTAGCTCCTGCAT] complementary to vector sequence adjacent to base pair 67 bp in AF349916. The resulting 849 bp PCR product contains terminal *Xba*I sites (Figure 2, italic), and a section of coding region up to the black arrow indicated in Figure 2. This PCR fragment was cloned directly into the vector pGEM-T (Promega-Benelux, Leiden, The Netherlands). After DNA restriction-enzyme analysis the insert was excised with *Xba*I restriction enzyme and re-cloned into an *Xba*I site of the pBI121ΔGUS binary vector (a pBIN121 derivative; Clontech, Palo Alto, CA, USA). Total DNA was isolated from putative transgenic potato lines (Jones and Sutton, 1997), which rooted on kanamycin and the integration of the AS-*Stgan* construct verified by Southern hybridization.

RNA for cDNA-AFLP template production was isolated from axillary buds grown *in vitro* over a 10-day period, as described previously (Bachem *et al.*, 1998). Other plant tissues were isolated from greenhouse-grown potato plants, and these include stolons (non-tuberizing); tubers (young sink tubers); leaves (fully expanded source-leaf lamina); petioles; nodes (with axillary buds and without petioles); and stems (with nodes and internodes). All amplification reactions for RNA-fingerprinting and re-amplifica-

tion of isolated fragments were carried out on a PE-9600 thermocycler using Taq DNA polymerase (PE Biosystems, Foster City, CA, USA). All oligonucleotides used in the cDNA-AFLP procedure were obtained from Eurogentec (Seraing, Belgium). A detailed protocol for cDNA-AFLP is available at <http://www.spg.wau.nl/aflp.htm>.

The potato cultivar Karnico was used for all transformation experiments and non-transformed, regenerated Karnico lines were used as controls in all cases. Transformation events were verified using Southern blot analysis, and ploidy levels were checked in transformed lines by flow cytometry. Growth phenotypes of the transformed lines CB12-1, CB12-4, CB12-6, CB12-9, CB12-10 and CB12-12 were assessed in a growth chamber with wide-spectrum light sources (mercury vapour lamps, electric discharge tubes and tungsten bulbs) with a light/dark regime as described. Temperatures were set at 20 and 18°C for day and night, respectively, and the relative humidity was adjusted to 70%. The plants were potted in six repeats and arranged in a randomized complete block formation. The statistical significance of differences was calculated using Student's *t*-test with $P = 0.01$. The length/width ratios were measured individually; the width measurement was taken as the narrowest dimension across the tuber, and the length as the distance between haulm attachment site and apical eye (bud).

Other molecular techniques

Basic DNA manipulations and molecular techniques were as described (Sambrook *et al.*, 1989). Restriction enzymes were from Pharmacia (Uppsala, Sweden) with the exception of *Asel* (NE-Biolabs, New Brunswick Scientific, NJ, USA).

Sequence analysis

Sequence determination of the transcript-derived fragment was carried out on plasmid using an automated ABI-sequencer (ABI-Perkin Elmer BV, The Netherlands). All sequences were analysed for homology to data banks using the BLAST 2.0 program (Altschul *et al.*, 1997). Other analyses and manipulations were carried out using the DNA-STAR programs.

Determination of gibberellins

Non-dormant tubers from both the transgenic *GANGLY* lines (CB12-1, CB12-4 and CB12-10) and the non-transformed Karnico controls (in triplicate) were planted in moist compost and allowed to sprout in the dark. Sprouts were harvested at ≈30 cm long. During harvest, adventitious roots and larger, etiolated leaves were removed, and the sprouts were cut into 10 cm sections before freezing in liquid nitrogen. Sprouts from control plants were harvested ≈1 week later than those of the antisense lines, due to the more rapid growth of the latter. Frozen sprout tissues (10 g FW) were ground to a fine powder under liquid nitrogen. For the extraction of GAs, ice-cold methanol containing 0.1% ascorbic acid was added [MeOH : tissue = 4 : 1 (v/w)]. For quantification of GAs, [17-²H₂]GA₁, [17-²H₂]GA₄, [17-²H₂]GA₉ and [17-²H₂]GA₂₀ (1 × 10⁻⁸ g of each) were added as internal tracers. After a 4 h extraction (stirring at 4°C) the homogenate was centrifuged (10 min, 4°C, 5000 r.p.m.) and the pellet was re-extracted with 80% methanol (+0.1% ascorbic acid) by stirring overnight at 4°C. After centrifugation, combined extracts were evaporated under reduced pressure until methanol-free. For determination of GAs

in the extracts, the procedure described by Kappers *et al.* (1997) was followed after reversed-phase HPLC using a ChromSpher 5 C₁₈ column (Chrompack, Bergen op Zoom, The Netherlands; 250 × 10 mm). Four fractions containing the GAs to be determined were collected, based on the retention times of standards, and evaporated to dryness. Prior to GC-MS analysis, the samples were methylated with excess ethereal diazomethane and taken to dryness under an N₂ stream. Subsequently, methylated GA₁, GA₄ and GA₂₀ fractions were trimethylsilylated. Derivatized samples were GC-MS analysed using a Hewlett-Packard 5890 GC coupled to a Hewlett-Packard 5972 mass selective detector (Hewlett-Packard, Wilmington, DE, USA). Derivatized samples were injected splitless into a fused silica capillary column (HP5ms; 30 m × 0.25 mm × 0.25 µm) and separated by a temperature gradient. The ion pairs monitored by GC-SIM (selected ion monitoring) were 506/508 (GA₁), 284/286 (GA₄), 298/300 (GA₉), and 418/420 (GA₂₀). Quantification of GAs was achieved using calibration curves constructed by mixing protonated and deuterated GAs in various amounts, including corrections made for naturally occurring isotopes.

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References

- Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl. Acids Res.* **25**, 3389–3402.
- Appeldoorn, N.J.G., De Bruijn, S.M., Koot Gronsvelt, E.A.M., Visser, R.G.F., Vreugdenhil, D. and van der Plas, L.H.W. (1999) Developmental changes in enzymes involved in the conversion of hexose phosphate and its subsequent metabolites during early tuberization of potato. *Plant Cell Env.* **22**, 1085–1096.
- Bachem, C.W.B., van der Hoeven, R.S., de Bruijn, S.M., Vreugdenhil, D., Zabeau, M. and Visser, R. (1996) Visualisation of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development. *Plant J.* **9**, 745–753.
- Bachem, C.W.B., Oomen, R.J.F.J. and Visser, R.G.F. (1998) Transcript imaging with cDNA-AFLP: a step-by-step protocol. *Plant Mol. Biol. Rep.* **16**, 157–173.
- Bachem, C.W.B., Oomen, R.J.F.J., Kuyt, S., Horvath, B.M., Claassens, M.M.J., Vreugdenhil, D. and Visser, R.G.F. (2000a) Antisense suppression of a potato alpha-SNAP homologue leads to alterations in cellular development and assimilate distribution. *Plant Mol. Biol.* **443**, 473–482.
- Bachem, C.W.B., van der Hoeven, R.S., Lucker, J., Oomen, R.J.F.J., Casarini, E., Jacobsen, E. and Visser, R.G.F. (2000b) Functional genomic analysis of potato tuber life-cycle. *Potato Res.* **43**, 297–312.
- Banfalvi, Z., Molnar, A., Kostyal, Z., Lakatos, L. and Molnar, G. (1997) Comparative studies on potato tuber development using an *in vitro* tuber induction system. *Acta Biol. Hung.* **48**, 77–86.
- Bourque, J.E., Miller, J.C. and Park, W.D. (1987) Use of an *in vitro* tuberization system to study tuber protein gene expression. *In Vitro Cell Dev. Biol.* **23**, 381–386.
- Brosche, M. and Strid, A. (1999) Cloning, expression, and molecular characterization of a small pea gene family regulated by low levels of ultraviolet B radiation and other stresses. *Plant Physiol.* **121**, 479–487.
- Calderon-Urrea, A. and Dellaporta, S.L. (1999) Cell death and cell protection genes determine the fate of pistils in maize. *Development*, **126**, 435–441.
- Carrera, E., Bou, J., Garcia-Martinez, J.L. and Prat, S. (2000) Changes in GA 20-oxidase gene expression strongly affect stem length, tuber induction and tuber yield of potato plants. *Plant J.* **22**, 247–256.
- Carrera, E., Jackson, S.D. and Prat, S. (1999) Feedback control and diurnal regulation of gibberellin 20-oxidase transcript levels in potato. *Plant Physiol.* **119**, 765–774.
- Chory, J. and Li, J. (1997) Gibberellins, brassinosteroids and light-regulated development. *Plant Cell Envir.* **20**, 801–806.
- DeLong, A., Calderon-Urrea, A. and Dellaporta, S.L. (1993) Sex determination gene *TASELSEED2* of maize encodes a short-chain alcohol dehydrogenase required for stage-specific floral organ abortion. *Cell*, **74**, 757–768.
- Ephritikhine, G., Fellner, M., Vannini, C., Lalous, D. and Barbier-Brygoo, H. (1999) The *sax1* dwarf mutant of *Arabidopsis thaliana* shows altered sensitivity of growth responses to abscisic acid, auxin, gibberellins and ethylene and is partially rescued by exogenous brassinosteroid. *Plant J.* **18**, 303–314.
- Fan, F. and Plapp, B.V. (1999) Probing the affinity and specificity of yeast alcohol dehydrogenase I for coenzymes. *Arch. Biochem. Biophys.* **367**, 240–249.
- Ghosh, G., Weeks, C.M., Grochulski, P., Duax, W.L., Erman, M., Rimsay, R.L. and Orr, J.C. (1991) Three-dimensional structure of holo 3, 20 β-hydroxysteroid dehydrogenase: a member of a short-chain dehydrogenase family. *Proc. Natl Acad. Sci. USA*, **88**, 10064–10068.
- Hedden, P. and Proebsting, W.M. (1999) Genetic analysis of gibberellin biosynthesis. *Plant Physiol.* **119**, 365–370.
- Hendriks, T., Vreugdenhil, D. and Stiekema, W.J. (1991) Patatin and four serine proteinase inhibitor genes are differentially expressed during potato tuber development. *Plant Mol. Biol.* **17**, 385–394.
- Jackson, S.D., Sonnewald, U. and Willmitzer, L. (1993) Characterization of a gene that is expressed in leaves at higher levels upon tuberisation in potato and upon flowering in tobacco. *Planta*, **189**, 593–596.
- Jacobsen, S.E. and Olszewski, N.E. (1996) Gibberellins regulate the abundance of RNAs with sequence similarity to proteinase inhibitors, deoxygenases and dehydrogenases. *Planta*, **198**, 78–86.
- Jones, P.D. and Sutton, J.M. (1997) Protocol 4a: genomic DNA extraction. In *Plant Molecular Biology, Essential Techniques* (Jones, P.D. and Sutton, J.M., eds). Chichester, UK: John Wiley & Sons, pp. 24–25.
- Kamiya, Y. and Garcia-Martinez, J.L. (1999) Regulation of gibberellin biosynthesis by light. *Curr. Opin. Plant Biol.* **2**, 398–403.
- Kappers, I.F., Jordi, W., Maas, F.M. and van der Plas, L.H.W. (1997) Gibberellins in leaves of *Alstroemeria hybrida*: identification and quantification in relation to leaf age. *J. Plant Growth Reg.* **16**, 219–225.
- Persson, B., Krook, M. and Jornvall, H. (1991) Characteristics of short-chain alcohol dehydrogenases and related enzymes. *Eur. J. Biochem.* **200**, 537–543.

- Picton, S., Gray, J., Barton, S., AbuBakar, U., Lowe, A. and Grierson, D.** (1993) cDNA cloning and characterisation of novel ripening-related mRNAs with altered patterns of accumulation in the ripening inhibitor (*rin*) tomato ripening mutant. *Plant Mol. Biol.* **23**, 193–207.
- Prat, S., Frommer, W.B., Hofgen, R. et al.** (1990) Gene expression during tuber development in potato plants. *FEBS Lett.* **268**, 334–338.
- Sambrook, S., Fritsch, E.F. and Maniatis, T.** (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
- Sanz, M.J., Mingo Castel, A., Van Lammeren, A.A.M. and Vreugdenhil, D.** (1996) Changes in the microtubular cytoskeleton precede *in vitro* tuber formation in potato. *Protoplasma*, **191**, 46–54.
- Taylor, M.A., Arif, S.A., Kumar, A., Davies, H.V., Scobie, L.A., Pearce, S.R. and Flavell, A.J.** (1992) Expression and sequence analysis of cDNAs induced during the early stages of tuberisation in different organs of the potato plant (*Solanum tuberosum* L.). *Plant Mol. Biol.* **20**, 641–651.
- Toyomasu, T., Yamauchi, T., Yamane, H., Murofushi, N. and Inoue, Y.** (1995) cDNA cloning and characterization of gibberellin-responsive genes in photoblastic lettuce seeds. *Biosci. Biotechnol. Biochem.* **59**, 1846–1849.
- Visser, R.** (1991) Regeneration and transformation of potato by *Agrobacterium tumefaciens*. In *Plant Tissue Culture Manual: Fundamentals and Applications*, Vol. **B5** (Lindsey, K., ed.). Dordrecht: Kluwer Academic Publishers, pp. 1–9.
- Xu, X., Van Lammeren, A.A.M., Vermeer, E. and Vreugdenhil, D.** (1998a) The role of gibberellin, abscisic acid, and sucrose in the regulation of potato tuber formation *in vitro*. *Plant Physiol.* **117**, 575–584.
- Xu, X., Vreugdenhil, D. and Van Lammeren, A.A.M.** (1998b) Cell division and cell enlargement during potato tuber formation. *J. Exp. Bot.* **49**, 573–582.