Gibberellin A$_1$ Metabolism Contributes to the Control of Photoperiod-Mediated Tuberization in Potato

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Abstract

Some potato species require a short-day (SD) photoperiod for tuberization, a process that is negatively affected by gibberellins (GAs). Here we report the isolation of StGA3ox2, a gene encoding a GA 3-oxidase, whose expression is increased in the aerial parts and is repressed in the stolons after transfer of photoperiod-dependent potato plants to SD conditions. Over-expression of StGA3ox2 under control of constitutive or leaf-specific promoters results in taller plants which, in contrast to StGA20ox1 over-expressers previously reported, tuberize earlier under SD conditions than the controls. By contrast, StGA3ox2 over-expressers previously reported, tuberize earlier under SD conditions than the controls. Together, our experiments support that StGA3ox2 expression and gibberellin metabolism significantly contribute to the tuberization time in strictly photoperiod-dependent potato plants.

Introduction

Potato tubers differentiate at the tip of stolons (underground stems), which upon tuber induction stop growing longitudinally and start swelling. They serve as vegetative propagation organs, as well as energy reservoir for the future new plant. Potato tubers are a rich source of carbohydrates widely used in food and industry, being the third crop in economical importance after wheat and rice (faostat.fao.org). Hence, it is of great agronomic interest to unveil signals that control tuber formation.

The control of tuber formation is complex and environmental factors such as photoperiod, temperature, and shortage in nitrogen supply are known to have an important effect [1]. Short days (SD, 8 h light/16 h dark) favor tuberization whereas long days (LD, 16 h light/8 h dark) delay this developmental process. Sensitivity to day length depends on the genetic background; whereas most commercial cultivars of *Solanum tuberosum* were bred to be relatively independent on day length for tuberization, wild potato species like *S. demissum* and several varieties of *S. tuberosum* ssp. *andigena* are strictly dependent on SD for tuber formation [2]. These wild species tuberize under SD conditions, but do not form tubers when grown under LDs.

Current evidence supports the existence of at least a photoperiod- and a GA-dependent pathway in controlling potato tuberization [3,4,5]. Somehow these independent pathways interact and the balance between their positive and negative effects determines tuberization. Therefore, some cross-talk between both pathways is occurring.

Gibberellins (GAs) have also been shown to regulate tuberization. For instance, greater GA content is observed in stolons of plants grown under non-inductive LDs, whereas a decrease in GA activity is found when leaves are exposed to inductive SD [6,7]. Increased GA activity has also been observed in response to high temperatures or continuous nitrate supply, conditions that prevent tuber formation [8,9]. The dwarf *ga* mutant of the photoperiod-dependent *S. tuberosum* ssp. *andigena*, which appears to be blocked in the GA biosynthesis 13-hydroxylation step (Figure 1A), forms tubers after culture for several months under LD conditions [10]. Treatment of potato ssp. *andigena* plants with GA synthesis inhibitors induces tuberization in LD [11]. Altogether, the content of GAs is greater in conditions that inhibit tuberization and conversely a reduction in GA content might induce tuberization even under non-inductive conditions, which led to the generally accepted conclusion that GAs inhibit tuberization.

GA 20-oxidases and GA 3-oxidases catalyze the last two steps of active GA biosynthesis, and GA 2-oxidase their conversion to inactive catabolites (Figure 1A). These three types of enzymes are encoded by small families of *GA20ox*, *GA3ox* and *GA2ox* genes [12,13]. All three enzymatic steps are thought to be main sites of GA biosynthesis regulation (Figure 1A) [14]. *GA20ox* and *GA3ox* expression is under feed-back regulation by GAs, whereas *GA2ox* expression is under feed-forward regulation by bioactive GAs [12]. In addition, *GA2ox* expression is also regulated in response to day length in several plant species [12,15,16,17]. In Arabidopsis and spinach, for example, bolting in response to LD conditions is associated to greater levels of *GA2ox* expression [18,19]. In potato, by contrast, levels of the three *StGA2ox* transcripts were not greater in the leaves of potato plants grown under non-inductive (SD+NB or LD) conditions compared to plants grown under tuber-inducing (SD) conditions. Therefore, it
corresponding transcripts are under negative feed-back regulation, whereas GA20ox mRNA is under positive feed-forward regulation by GA1. (B) Neighbor-joining tree obtained with the alignment of the amino acid sequences: St5ox1 and St5ox2 correspond to potato StGA3ox1 (AF179244) and StGA3ox2 (AF179243); At3ox1 and At3ox2 to arabidopsis AtGA3ox1 (NM101424) and AtGA3ox2 (NM1016683); Ps3ox1 to pea PsGA3ox1 (AF101067), Ls3h1 and Ls3h2 to lettuce LsGA3ox1 (AB012205) and LsGA3ox2 (AB012206); Le3ox1 and Le3ox2 to tomato LsGA3ox1 (AB010991) and LsGA3ox2 (AB010992), tobacco Nty to NtgA3ox1 (AB032198), and Os3ox1 and Os3ox2 are rice genes OsG3ox1 and OsG3ox2 (AB054084 and AB056519).

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was concluded that changes in GA levels observed during tuber induction do not result from regulated expression of this biosynthetic activity [20].

Light regulation of GA biosynthetic gene expression or activities other than GA 20-oxidases has also been reported [15]. For instance, enhanced synthesis of ent-kaurene under long photoperiods has been observed in the LD plants spinach (Spinacia oleracea) and Agrostemma githago [21]. In Arabidopsis and lettuce (Lactuca sativa) seeds, expression of the GA 3-oxidases AtGA3ox2 (GA4H) and Ls3ox1 genes is induced by seed exposure to red light and this effect is reversed by far-red light treatment. Hence, red light appears to promote GA1 synthesis in these seeds by inducing GA 3-oxidase expression via phytochrome action [22,23]. Transfer of etiolated pea (Pisum sativum) seedlings to light, in its turn, was found to down-regulate expression of Pga3ox1 (Mendel’s Le gene) and up-regulate Pga2ox2, encoding for a deactivating GA 2-oxidase [24,25]. In cowpea (Vigna sinensis), end-of-day far-red treatments, that stimulate epicotyl elongation, resulted in an increase in GA1 levels by inhibiting at least GA 2-oxidase activity in the epicotyl tissues [26]. In potato, Sga2ox1 expression is strongly up-regulated during the early stages of potato tuber development, prior to visible swelling. Characterization of transgenic potato plants with altered levels of Sga2ox1 led to propose a role for this gene in early tuber initiation by reducing GA levels in the subapical stolon region at the onset of tuberization, thereby facilitating normal tuber development and growth [27]. Therefore, the regulated expression of a potato biosynthetic activity different from GA 20-oxidase may modulate reduced GA synthesis under SD conditions and/or contribute to tuberization.

To address the aforementioned possibility, we set out to clone additional GA biosynthetic genes from S. tuberosum ssp. andigena. In this manuscript, we report the functional characterization of Sga3ox2, a gene encoding a GA 3-oxidase from potato, whose expression is differentially regulated in aerial parts and stolons after transfer to SD inductive conditions. Our experiments suggest the importance of this activity in the control of photoperiod-induced potato tuberization.

Materials and Methods

Plant material and growth conditions

Photoperiodic Solanum tuberosum ssp. andigena plants were propagated in vitro in MS media supplemented with 20 g·L⁻¹ sucrose before being transferred to soil, or from tubers. Plant height analysis was performed with plants at the 14-leaf stage, grown in the greenhouse under LD conditions (16 h light/8 h darkness, 22°C). Internode length was measured as reported before [28]. Transformation of potato plants was performed as described elsewhere [29]. About 40 independent transgenic lines were regenerated for each construct, transferred to soil and analyzed at the RNA level. For tuber induction measurements, plants were grown in the greenhouse under LD conditions until

Figure 1. Major reactions in GA biosynthetic pathway and phylogenetic analysis of GA 3-oxidases. (A) The pathway is classified into three groups based on the enzymatic activity and subcellular localization. Plastidial terpene cyclases convert the precursor geranylgeranyl diphosphate (GGDP) into ent-copalyl diphosphate (CDP), and subsequently into ent-kaurene, in a two-step process catalyzed by ent-copalyl diphosphate synthase (CPS) and ent-kaurene synthase (KS). Cytochrome P-450 mono-oxygenases such as ent-kaurene oxidase (KO), ent-kaurenoic acid oxidase (KAO) yield GA12. Cytoplasmic dioxygenases are responsible for the latest steps and conversion to bioactive gibberellins and their inactive catabolites. GA12 in potato is early 13-hydroxylated by GA 13-oxidase (GA13ox). As indicated by curved dotted T-lines or arrows emerging from the bioactive GA1, GA20ox and GA3ox

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they reached a 14-leaf stage and then were transferred to growth chambers under SD conditions (8 h light/16 h dark, 22 °C, SD inducing conditions) or to SD with a light treatment of 30 min or night-break in the middle of the dark period (SD+NB, non-inducing conditions). Light intensity in the growth chambers was about 200 μmol m−2 s−1 provided by high-pressure sodium lamps SON-T AGRO 400 (Philips).

**Potato GA3ox PCR amplification and plasmid constructs**

Since potato and tomato cDNA collections were very limited at the time this work was initiated, in order to amplify potato cDNAs encoding GA 3-oxidases, degenerated oligonucleotides were designed upon conserved sequences of the AtGA3ox1 (GA4) and PsGA3ox2 (Le) genes from Arabidopsis and pea [30,31,32] and used in PCR. Primers H5 (5′-TGG-GGI-AGT-CCT-CT-CAG-AG(A)-G(T)3′) and H7 (5′-GTA-GTG-TGG-GTC-CTG-AA3′), complementary to the regions 78-WGAFOQ-83 and 227-MGLAAH-233 in AtGA3ox1 respectively yielded a band of expected size. PCR fragments were cloned in pGEM T-easy (Promega) and sequenced. A first strand cDNA obtained from mRNA extracted from leaves of the potato GA deficient mutant ga1 [33] was used as template for PCR amplification. The subcloned B3ox PCR product, which corresponded to a transcript with negative feed-back regulation (Figure S1), was used to screen a cDNA library constructed from leaves of the potato ga1 mutant [20]. Several positives clones were isolated and sequenced (Results S1, Figure S2A), resulting in the isolation of one single full cDNA clone, closely related to the later reported and sequenced (Results S1, Figure S2A), resulting in the isolation of the isolated potato GA 3-oxidase-encoding gene, which was designated as StGA3ox2 [34]. Hence, the isolated potato clone was designated as StGA3ox2 (gb|ACN89834). Specific primers B8 (5′-AGT-CCT-CTA-GTA-ATC-3′) and B9 (5′-GTG-AGT-TGG-CTG-3′), complementary to the tomato SlGA3ox1 [34], were used to amplify a second potato GA 3-oxidase-encoding gene, which was designated as SttGA3ox1 (gb|ACN89835).

To produce StGA3ox2 protein in *E. coli* its coding sequence was amplified using oligonucleotides 3B5 (5′-ATT-ATG-TTG-CCT-ATA-ATG-TGG-TCT-CTG-GCT-GCA-CAA-3′) and 3B3 (5′-GCC-GCT-CGA-GGC-CCTT-GGA-GAC-3′), bearing NdeI and XhoI restrictions sites. The PCR product was conveniently digested and ligated to the pET28 (Novagen) vector site to use for GA extraction and quantification by HPLC and GC-MS as described elsewhere [30].

**GA 3-oxidase recombinant protein and activity assay**

BL21 *E. coli* competent cells were transformed with pET3ox2. Cultures were grown at 30 °C until OD600 = 0.4 and recombinant StGA3ox2 protein accumulation was induced with 1 mM IPTG for 2 h shaking at 30 °C. Aliquots were taken before and after induction, to check the accumulation of the recombinant protein in the soluble fraction. Cell lysates producing StGA3ox2 were prepared by resuspending initial 50 mL pelleted cultures in 2 mL TrisHCl pH = 7.5, 4 mM DTT and 1 mg/mL lysozyme. After 15 min incubation at room temperature and sonication, extracts were ultracentrifuged at 35,000 rpm for 30 min at 4 °C. Supernatants were frozen for activity assays. 85 μL aliquots of these cell lysates were incubated at 30°C for 2 h with either [3H]-GA20 and [3D]-GA3p or with [3H]-GA20 in a total volume of 100 μL containing 4 mM 2-oxoglutarate, 4 mM ascorbate, 0.5 mM ferrous sulphate, 4 mM DTT and 2 mg/mL BSA, as described in [39]. The reaction mixture was analyzed by HPLC, detecting the radioactivity of each sample in a Beckman scintillation counter. Fractions at elution times corresponding to GA1 and GA4 were analyzed by GC-MS for confirmation of the reaction products.

**Results**

**Potato GA 3-oxidase is encoded by at least two genes**

When this work was initiated, potato and tomato cDNA collections were very limited and no information was available on GA 3-oxidase-encoding genes from these species. Therefore cDNA prepared from leaves of the *S. tuberosum* ssp *andigena* ga1 mutant was used as template to amplify StGA3ox genes, because this mutant is supposed to accumulate high levels of the GA 3-oxidase-encoding transcripts due to negative feed-back regulation. As a result (check Methods for detailed information), we identified a full length clone with a higher percentage of homology to the pea *PcGA3ox1* (Le) and the Arabidopsis AtGA3ox2 (GA4) genes [30,31,32] than to any other 2-oxoglutarate dependent dioxygenase sequence available at that moment, thus suggesting that the encoded potato GA 3-oxidase. The sequences from tomato *(Solanum lycopersicum)*, StGA3ox1 and StGA3ox2, were later reported [34], and because the potato full length clone shared 92% identity with StGA3ox2, it was designated as StGA3ox2. Based on the nucleotide sequence of the StGA3ox1 gene and using RT-PCR and specific primers (B8 and B9) on potato RNA as a template, a new cDNA was cloned, named as SttGA3ox1, which included the complete coding region corresponding to this gene (Figure S2B). From these results we conclude that potato, like tomato, has at least two independent genes encoding GA 3-oxidase enzymes as shown in the phylogenetic tree of Figure 1B.

StGA3ox1 and StGA3ox2 show a differential spatial pattern of expression

The expression of the *SttGA3ox1* and *SttGA3ox2* genes was analyzed by Northern blot using RNAs from different organs of *S. tuberosum ssp andigena* plants (leaf-14 stage) grown under non-inducing LD conditions (Figure 2A). Because at this developmental stage plants had very few or no stolons, these organs were not harvested in our analyses. Different patterns of expression were observed for the two *SttGA3ox* genes. Relatively high levels of the *SttGA3ox2* transcript were found in the apex, internodes and stem nodes, lower levels in flowers and leaves and almost no expression in roots. *SttGA3ox1* transcripts were detected mainly in flowers and,
to a lower extent, in apex, nodes, internodes and roots. Almost no expression was detected in leaves (Figure 2A). Thus, whereas StGA3ox2 was expressed in most vegetative tissues, StGA3ox1 transcripts seemed to be more abundant in floral organs. Consequently, we focused on the study of StGA3ox2. Consistent with our results, data on the expression levels of the two StGA3ox genes in non-tuberizing stolon tips detected expression of only StGA3ox2 [27].

StGA3ox2 encodes a functional potato GA 3-oxidase

In order to assess the GA 3-oxidase activity of the StGA3ox2 protein, we obtained pET3ox2 clone, containing the coding sequence of StGA3ox2 in frame in a pET28 (Novagen) expression vector. E. coli BL21 strain was transformed with the pET3ox2 clone and grown at 30°C. The recombinant protein was allowed to accumulate by induction with IPTG for 2 h (Figure 3A). Crude extracts of the expression cultures were incubated for 2 h at 30°C in the presence of the radiolabeled substrates, either [3H]-GA20 or [14C]-GA9. HPLC analysis of the reaction products resulted with radioactivity peaks at the same elution time as 3H-GA1 and 14C-GA4 in each reaction (Figure 3B). These fractions were further identified by GC-MS as GA1 and GA4 (data not shown), thus confirming that StGA3ox2 encodes a functional GA 3-oxidase.

Expression of StGA3ox2 in the stolons is strongly repressed under tuber-inducing (SD) conditions

In previous studies no major changes were detected in the levels of expression of the StGA20ox genes between potato plants induced (SD) and non-induced (SD+NB or LD) to tuberize [20]. Therefore, we have investigated whether photoperiod regulated the expression of StGA3ox2 in different organs of 14-leaf plants grown for 3 additional weeks under inductive (SD) and non-inductive (SD+NB or LD) conditions (Figure 2B). In plants grown under non-inductive conditions (SD+NB or LD) levels of StGA3ox2 transcript were similar but not identical (compare Figs. 2A and 2B). Remarkably high levels of StGA3ox2 mRNA were detected in the underground stolons. By contrast, in plants grown under inductive SD conditions, StGA3ox2 expression was clearly up-regulated in apex, leaves and nodes. The most conspicuous change in gene expression however was found in the stolons, where StGA3ox2 expression was completely repressed, with no transcript detected either in stolons of plants induced to tuberize or tubers already formed (Figure 2B). Since exposure of potato plants to SD tuber-inducing conditions results in a large decrease in stolon GA levels [7], our correlative data led us to hypothesize that the repression of this GA 3-oxidase activity participates in the reduction of stolon GA content associated with tuber induction.

Expression of StGA3ox2 in the aerial part of the plant is diurnally regulated and is increased under tuber-inducing (SD) conditions

The levels of StGA3ox2 transcript in leaves were found to be dependent on the time of the day at which leaves were sampled. To analyze whether this was due to a diurnal rhythm of expression, we studied the accumulation of StGA3ox2 messenger in potato leaves of plants grown under inducing (SD) and non-inducing (SD+NB) conditions, over a period of 24 h. Similar patterns of transcript abundance were observed in plants entrained to SD or SD+NB conditions within the 24 h period (Figure 4). The expression of StGA3ox2 fluctuated during the day, with transcript levels reaching a peak around 3 h after starting the light period (lights were switched on at 11:00 h and maximal levels of transcript were detected at 14:30 h) and a valley around 2 h after lights switch-off, after which they gradually recovered during the night period (Figure 4). Although the pattern of StGA3ox2 transcript accumulation was similar in leaves of plants entrained...
to SD or SD+NB conditions, levels of StGA3ox2 mRNA were higher in SD- than SD+NB-grown plants over the entire 24 h period. This agrees with the results shown in Figure 2B and suggests that, in contrast with that observed in the underground stolons, increased levels of StGA3ox2 mRNA occur in leaves and other aerial part of the shoot of plants entrained to SD conditions compared to plants grown under SD+NB non-inducing conditions.

Constitutive over-expression of StGA3ox2 in the aerial parts results in elongated plants and early tuberization under SD conditions

The putative regulatory role of GA biosynthesis catalyzed by StGA3ox2 led us to investigate its function in the photoperiod-dependent tuberization. Plants over-expressing the StGA3ox2 transcript under the control of the constitutive 35S promoter (35S:3ox2 lines) or the green tissue specific StLS1 promoter (LS1:3ox2 lines) were obtained. The StLS1 promoter drives expression to green tissues of the leaves and stem [36]. Several transformants were regenerated that accumulated higher levels of StGA3ox2 transcript than the controls (Figure 5A). These lines exhibited an elongated phenotype as compared to the wild-type controls (Figure 5B and 6), with taller stems caused by an increase in internode length and not by an elevated number of internodes (see Figure 5C). GA content was quantified in shoots (leaves and stems) of four different StGA3ox2 over-expressing lines. Higher levels of GA1 (ranging from 1.3 to 2.2 fold-increase), and much lower levels of its 13-hydroxylated precursor GA20 and of GA29 (a GA20 catabolite) were found in both types of over-expressing lines (35S:3ox2 and LS1:3ox2) compared to wild-type plants (Table 1). Transformants with a more elongated phenotype also exhibited the highest levels of GA1, thus evidencing a positive correlation between increased levels of bioactive GAs and internode growth. No apparent differences in GA53, GA44 and GA19 (GA1 precursors) were detected (Table 1).

Figure 3. GA 3-oxidase activity assay in recombinant E.coli lysates expressing StGA3ox2 protein. (A) Recombinant StGA3ox2 accumulated in BL21 E.coli growing at 30°C. After 2 h induction with IPTG, we detected a band with a molecular weight of 43 kDa corresponding to StGA3ox2. (B) HPLC chromatogram after 2 h incubation of E.coli lysates expressing recombinant StGA3ox2 feeded with the radiolabeled putative substrates 3H-GA20 and 14C-GA9. The corresponding products peaked at the 3H-GA1 and 14C-GA4 corresponding elution times. doi:10.1371/journal.pone.0024458.g003

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None of the StGA3ox2 over-expressing lines formed tubers under LD conditions but, when transferred to inducing SD conditions, they tuberized earlier than the controls (Figure 5D). The early tuberization response was significantly observed in both types of over-expressing lines (35S:3ox2 and LS1:3ox2) compared to wild-type plants (Table S1). Consistently, the yield in mass of tubers per plant was significantly higher in the over-expressing lines than in the controls (Table 2).

Reduced levels of StGA3ox2 in the aerial parts results in shorter plants and delayed tuberization under SD conditions

An exception in our analyses was the LS1:3ox2–16 line that was significantly shorter than the controls and in which reduced levels of StGA3ox2 transcript were detected (Figure 5A–C), likely due to a co-suppression effect. Interestingly, the LS1:3ox2–16 plants tuberized significantly later than wild-type controls (Figure 5D; Table S1B). In addition, the co-suppressed LS1:3ox2–16 line produced smaller tubers and a lower tuber yield per plant (Table 2).
The GA 3-oxidase activity plays a role in controlling both early and late responses after transfer to SD inductive conditions. We report the isolation of StGA3ox2 and StGA3ox1, two potato cDNA clones encoding GA 3-oxidases (Figure 1A). The recombinant product of StGA3ox2 is a functional enzyme catalyzing the conversion of both 3H-GA20 and 14C-GA9 into 3H-GA1 and 14C-GA1, respectively (Figure 3B). This agrees with pea PgGA3ox1 and Arabidopsis AtGA3ox2 genes encoding GA 3-oxidase proteins catalyzing the synthesis of active GAs both in the early- and non-C13-hydroxylation pathways [30,31,32]. The higher StGA3ox2 expression in the shoots of the 3S:3ox2 and 3S:3ox2 transgenic plants resulted in lower levels of GA20 (the substrate) and higher levels of GA1 (the product) than in control plants (Table 1), demonstrating that StGA3ox2 encodes an enzyme with GA 3-oxidase activity also in planta. In addition, GA 3-oxidase activity might have a role in the control of bioactive GA synthesis during tuber induction, as we detected a strong down-regulated expression of StGA3ox2 in stolons induced to tuberize, possibly contributing to the drop in GA1 levels observed in the stolons of plants exposed to SD tuber-inducing conditions (Figure 2B) [27].

One of the earlier events associated with the onset of tuberization is a change in the plane of cell division, from longitudinal to transversal, of the cells at the sub-apical region of the stolon. As a consequence of this switch, stolon growth is arrested and lateral tuber expansion begins [40,41]. Polarized cell expansion in plant cells is controlled by cortical microtubules localized below the plasma membrane [42]. Orientation of these microtubules has been shown to be regulated by GAs, which promote a perpendicular distribution of the microtubules to the growing axis of the cell, thereby directing cell elongation along the longitudinal axis [43]. In induced stolons, decrease in GA1 levels partially caused by down-regulated expression of the StGA3ox2 gene expression is thus likely to contribute to tuber formation, in concert with changes in the expression of other GA metabolism genes [27]. A complex regulatory mechanism at the transcriptional level has been proposed for such coordinated induction of SGA2ox1, SGA20ox1, and SGA20ox3 expression and repression of StGA3ox2 expression, after the switch from LD to SD conditions, possibly mediated by a common mechanism [27].

Transfer of plants to SD conditions induces at the same time a drop in the StGA3ox2 mRNA levels in the stolons and a rise in the levels of transcript in the aerial part of the plant (shoot apex, leaves and the nodes; Figure 2B). These changes in gene expression are accompanied by an increase in stem elongation as a result of the reduced number of hours of light that the plants receive per day. While stem elongation is already observed after 1 week of transfer the plants to SD conditions, tuber formation requires at least 2 weeks under inductive conditions and, thereby, stem elongation.
was considered to be a short-term adaptive response independent of tuberization [3]. Our results suggest that up-regulated expression of StGA3ox2 in the leaves might mediate this short-term elongation response. Alternatively, it might reflect the positive feed-back of a general reduction of bioactive GA levels in plants that have already stopped growing and started to senesce [3].

Over-expression of StGA3ox2 in the leaves results in elongated plants and early tuberization

We have investigated the function of a GA 3-oxidase in the regulation of tuber induction by generating transgenic potato plants that over-expressed StGA3ox2 under the control of the constitutive 35S CaMV (35S:3ox2) or the potato green tissue-specific StLS1 (LS1:3ox2) promoters. As expected, transformants accumulating high levels of the transgene exhibited a taller phenotype due to longer internodes (Figure 5). Consistently, the co-suppressed line LS1:3ox2–16, with reduced StGA3ox2 mRNA levels, showed reduced plant height with shorter internodes. Increase of plant height was similar in both constitutive (35S:3ox2) and leaf-specific (LS1:3ox2) over-expressers but always smaller than that observed in transformants over-expressing StGA20ox1 [28]. This suggests that GA 20-oxidase activity may be limiting in potato plants (hence, the GA20 precursor) and/or that negative feed-back regulation of the genes encoding this enzyme is tighter than that of genes encoding GA 3-oxidase activity.

Over-expression of StGA3ox2 induced early tuberization under SD inductive conditions, with a higher yield in mass of tubers per plant in the transgenic lines as compared to the wild-type controls (Table 2). Consistently, the co-suppressed line LS1:3ox2–16 tuberized later and formed smaller tubers than the controls. Thus, a direct correlation between increased StGA3ox2 expression in the leaves and early tuberization in SD was found in these lines, with those accumulating the highest levels of StGA3ox2 mRNA (lines 35S:3ox2–10 or LS1:3ox2–18) also exhibiting the earliest tuberization onset. This observation is in apparent contradiction with the widely accepted notion that GAs inhibit tuberization. However, it is important to note that levels of GA1 in these transgenics were only determined in the shoots (Table 1) and we have been unable to quantify them in the stolons. To bypass this technical problem, we obtained transgenic lines that over-expressed StGA3ox2 in tubers (Tub1:3ox2 lines; Figure 7). These lines should accumulate higher levels of GA1 in tubers. Indeed, lines exhibiting increased levels of the StGA3ox2 transcript in tubers showed a slight delay in the tuberization onset under SD conditions (Figure 7C). The patatin B33 promoter is a marker for tuber formation assumed to become active once the stolon starts to differentiate into a tuber [29]. Hence, the Tub1:3ox2 lines should begin to accumulate GA1 in the stolons during their early transition into tubers, with this late increase in GA1 content having a mild impact on the tuberization onset. The Tub1:3ox2 lines, however, formed a higher number of tubers per plant than the controls, but with reduced weight and

Figure 5. Over-expression of StGA3ox2 under constitutive (35S:3ox2) and leaf-specific (LS1:3ox2) promoters. (A) RNA blot analysis from LD-grown shoots, 30 μg of total RNA from each sample was electrophoresed and equal loading on the gel was assessed by ethidium bromide staining, a representative picture is shown out of at least two independent Northern analysis. (B) Stem height and (C) Internode length of overexpressing lines, wt and cosupressed line grown under LD conditions. Mean value obtained from 10–15 individual plants from each line. Errors bars indicate SE, asterisks (*) highly significant differences (P<0.01) and circles () significant differences (P<0.05) compared to wt and for the 5th internode in C. (D) Tuber induction data, represent the average of 10–15 independent replicates for each line from a representative experiment out of three with similar results. doi:10.1371/journal.pone.0024458.g005
with several small tubers of elongated shape attached to each stolon (Table 2). This phenotype indicates that the StGA3ox2 product accumulated in the tubers of these lines, probably leads to increased GA1 content affecting tuber size and shape, as well as tuber apical dominance, with growth of secondary tubers observed in these lines. Although there are reports indicating that the patatin promoter is somewhat leaky and some reporter expression can be observed in “pre-tuberizing” stolons [44], the subtle tuberization time phenotypes observed in Tub1:3ox2 lines are likely to relate to the effect of altered GA levels on tuber development, whereas highly specific stolon promoters should be used to assay StGA3ox2 photoperiodic control of tuber induction. Altogether, our results seem to suggest that increased levels of GA1 in stolons induced to tuberize have an inhibitory effect on tuber formation, in agreement with the accepted negative role of GAs in this process. Recent studies have suggested that GAs can move inside the plant, for instance a basipetal transport was observed in Populus and from leaves to the shoot apical meristem in Arabidopsis [45,46], whereas upwards (acropetal) transport was described in pea stems [47].

Despite the subtle but significant phenotypes of our StGA3ox2 over-expression lines, we performed grafting experiments between the wt and 35S:3ox2–5 transgenic line (data not shown). We could not detect any significant difference between the grafted specimens, possibly due to technical problems, such as earlier tuberization time in all grafts induced in older plants or by a tight regulation of GA1 metabolism and degradation possibly mediated by StGA2ox1 [27].

Contrasting effects on tuber induction time in StGA3ox2 and StGA20ox1 over-expressers: the importance of the different mobility of GA20 and GA1 in the plant and the balance between shoots and tubers in metabolizing GA20

Both StGA20ox1 and StGA3ox2 over-expressers display elongated stems and high levels of the bioactive GA1 in the shoots (Table 1; [28]). A relevant difference between these transgenic lines is the different accumulation in the shoots of GA20, the immediate precursor of the bioactive GA1; it was high in StGA20ox1 over-expressers and it was low in StGA3ox2 over-expressers. In addition, the early tuberization observed in the 35S:3ox2 and LS1:3ox2 lines (Figure 5) is in clear contrast with previous results showing that over-expression of StGA20ox1 results in delayed tuber formation under SD inductive conditions [28].

A possible explanation for this paradox is that GA20 and GA1 have different mobility within the potato plants, as occurs in pea. In this species, using le (blocked in GA 3-oxidase) and na (blocked prior conversion to GA12-aldehyde) mutants it was found that while GA1 application induces elongation of le lines, the stature of le is not altered when grafted to wild-type rootstock, indicating that GA1 is not transported in sufficient amount from the wild-type rootstock to the le scion. By contrast, stem elongation of the na line increased after GA application or by grafting to either wt or le rootstocks, indicating that GA20 or earlier precursors are readily transported across the graft.

Table 2. Tuber yield of transgenic lines.

<table>
<thead>
<tr>
<th>Line</th>
<th>n’ tubers/plant</th>
<th>g of tubers/plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>1.7 ± 0.3</td>
<td>6.0 ± 0.6</td>
</tr>
<tr>
<td>35S:3ox2–10</td>
<td>2.3 ± 0.3</td>
<td>11.4 ± 0.7 *</td>
</tr>
<tr>
<td>35S:3ox2–5</td>
<td>2.1 ± 0.2</td>
<td>10.0 ± 0.4 *</td>
</tr>
<tr>
<td>LS1:3ox2–18</td>
<td>1.5 ± 0.3</td>
<td>7.5 ± 0.3 *</td>
</tr>
<tr>
<td>LS1:3ox2–3</td>
<td>1.6 ± 0.3</td>
<td>9.6 ± 0.3 *</td>
</tr>
<tr>
<td>LS1:3ox2–16</td>
<td>2.5 ± 0.4</td>
<td>3.2 ± 0.2 *</td>
</tr>
<tr>
<td>Tub1:3ox2–30</td>
<td>3.6 ± 0.1 *</td>
<td>5.6 ± 0.4</td>
</tr>
<tr>
<td>Tub1:3ox2–37</td>
<td>3.2 ± 0.4 *</td>
<td>6.5 ± 0.3</td>
</tr>
</tbody>
</table>

Plants were grown for 4 weeks under SD inducing conditions before tubers were harvested. Data of number of tubers per plant and g of tubers are the average of 15 independent replicates for each transgenic line and 9 independent replicates for wild-type (± values indicate SE). Significant differences relative to wild-type are indicated with * (P<0.01). doi:10.1371/journal.pone.0024458.002

Table 1. Levels of endogenous GAs in transgenic lines.

<table>
<thead>
<tr>
<th>Line</th>
<th>GA44</th>
<th>GA19</th>
<th>GA29</th>
<th>GA29</th>
<th>GA1</th>
<th>GA8</th>
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<tr>
<td>wild-type</td>
<td>0.58</td>
<td>0.60</td>
<td>2.5</td>
<td>15.1</td>
<td>0.30</td>
<td>19.4</td>
</tr>
<tr>
<td>35S:3ox2–10</td>
<td>nd</td>
<td>0.58</td>
<td>0.13</td>
<td>3.2</td>
<td>0.63</td>
<td>27.1</td>
</tr>
<tr>
<td>35S:3ox2–5</td>
<td>0.56</td>
<td>0.73</td>
<td>0.1</td>
<td>nd</td>
<td>0.50</td>
<td>nd</td>
</tr>
<tr>
<td>LS1:3ox2–18</td>
<td>nd</td>
<td>nd</td>
<td>0.62</td>
<td>5.5</td>
<td>0.67</td>
<td>nd</td>
</tr>
<tr>
<td>LS1:3ox2–3</td>
<td>0.69</td>
<td>0.38</td>
<td>0.43</td>
<td>nd</td>
<td>0.40</td>
<td>11.7</td>
</tr>
</tbody>
</table>

Levels of endogenous GAs (ng g⁻¹ FW) in the shoot apices of the 35S:3ox2 and LS1:3ox2 lines. GAs were quantified by GC-MS using internal standards.

doi:10.1371/journal.pone.0024458.0001

Figure 6. Phenotype of representative GA 3-oxidase and GA 20-oxidase transgenic plants. Plant height of the constitutive 35S:20ox1 and the tuber-specific Tub1:3ox2–30 (Tub1:3ox2) over-expressers is shown in comparison to that of lines over-expressing the GA 20-oxidase activity (35S:20ox1) or wild-type (wt) plants. The number of nodes was the same in all plants.

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Table 1. Levels of endogenous GAs in transgenic lines.
from the wt or le rootstocks to the na scions, where they can be metabolized to GA$_1$ [47]. In our transgenic Tub1:3ox2 lines (Figure 7), StGA3ox2 is over-expressed in tubers, thus likely accumulating higher local levels of GA$_1$ (as deduced from the elongated tubers phenotype) and delaying tuberization; they however show slightly longer stem lengths or very similar to wt elongated internodes (high levels of GA$_1$ in shoots) and pale green leaves, co-existed with a strong induction of tuber formation [3,48]. Conversely, reduced rates of stem elongation may occur concomitantly with an inhibition of tuberization, as it was observed in potato plants over-expressing Arabidopsis CONSTANS (CO) [4]. Both PHB and CO are components of the photoperiod pathway that seem to interact with GA metabolism. However, since StGA3ox2 over-expressing or co-suppressing lines still require SD photoperiods to produce tubers, this indicates that impairment of this transcript levels does not constitutively block and/or activate the photoperiod pathway, but modulates it.

In conclusion, we have shown that differential pattern of StGA3ox2 expression in stolons plays a role in tuberization induction, unveiling a possible mechanism by which photoperiod- and GA-dependent pathways cross-talk in controlling potato tuberization. Our study also points out that the role of GAs in the regulation of photoperiod-controlled tuberization results from the combination of the enzymatic activities involved in their local production and metabolism, together with the differential distribution of GA$_1$ and its precursors within the plant. It also highlights the regulatory role played by the integration of local levels of GAs with the photoperiod-dependent signal that induces tuberization.

**Supporting Information**

**Results S1** Isolation of potato GA 3-oxidase clones. (PDF)

**Figure S1** Negative feed-back regulation of the corresponding mRNA hybridizing to PCR product B3ox. (PDF)

**Figure S2** Structure of the StGA3ox2 clones and amino acid sequence comparison to other GA 3-oxidases. (PDF)

**Table S1** Statistical analysis on the tuberization time in transgenic lines over-expressing StGA3ox2. (PDF)

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**Author Contributions**

Conceived and designed the experiments: SP. Performed the experiments: JB-T. Analyzed the data: JB-T SP JFM-G JLG-M. Contributed reagents/materials/analysis tools: JLG-M JFM-G. Wrote the paper: JB-T SP. Manuscript corrections: JLG-M JFM-G. Financial help: SP JFM-G.
References