Dwarf (di)haploid *pito* mutants obtained from a tetraploid potato cultivar (*Solanum tuberosum* subsp. *tuberosum*) via anther culture are defective in gibberellin biosynthesis

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Abstract

Nine dwarf (di)haploid lines (2n = 2 × 24) were obtained from the tetraploid (2n = 4 × 48), long day-adapted potato cultivar ‘Pito’ (*Solanum tuberosum* subsp. *tuberosum*) through anther culture. They grew slowly, had very short internodes, compact and ball-shaped appearance, and dark green leaves. Dwarfism was due to a recessive gene, designated *pito*. Endogenous gibberellin contents were measured in the leaves of dwarf and wild-type lines by gas chromatography linked to mass spectrometry (GC-MS). High amounts of GA19, GA20, GA29, GA1, and GA8 were detected in the wild-type plants, which indicated that the early 13-hydroxylation pathway was predominantly used for GA biosynthesis in *S. t. subsp. tuberosum*. Also GA53, GA15 and GA9 were detected but not quantified. Very low endogenous amounts of all analysed GAs were detected in the *pito* mutants, indicating a block at an early part of the GA biosynthesis pathway. The dwarf lines strongly and quickly responded to the exogenous application of low amounts (79 nM) of bioactive GA (GA3), which restored normal growth and confirmed that the *pito* dwarfs were synthesis mutants and not GA response mutants. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Since the discovery of gibberellin (GA) in the fungus *Gibberella fujikuroi* [1] and the observation that exogenous application of GA can recover the normal growth of the recessive dwarf mutant (*le*) of pea (*Pisum sativum* L.) [2,3], the central role of GAs as growth regulators in plants has been established. While over 100 structures of these diterpenoid compounds are known to date, only few of those containing 19 carbon atoms (C19-GA) are biologically active in plants [4]. The main biological effects are manifested during germination, stem elongation and reproductive development [5,6].

Genetic mutants altered in GA biosynthesis or response to GA have been described in many plant species and families, including Brassicaceae (*Arabidopsis* and *Brassica rapa* L.), Poaceae (barley, *Hordeum vulgare* L.; maize, *Zea mays* L.; and rice, *Oryza sativa* L.), Fabaceae (pea), and Solanaceae (tomato, *Lycopersicon esculentum* Mill.) to mention a few [6–8]. They have been fundamental in resolving the GA biosynthesis pathways in different species. Qualitative and quantitative data on GAs in plants have been significantly increased and improved lately since the highly sensitive detection method, gas chromatography linked to mass spectrometry (GC-MS), has been applied to analysis of endogenous GA contents [4,8]. Current knowledge on the GA biosynthesis pathway permits to divide it into three parts: (i) the synthesis

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of ent-kaurene from geranyl diphosphate by copalyl diphosphate synthase and ent-kaurene synthase; (ii) the conversion of ent-kaurene to GA$_{53}$ by cytochrome P450 enzymes; and (iii) metabolism to bioactive GAs by dioxygenases [8,9].

In the cultivated potatoes (Solanum tuberosum L., Solanaceae), GAs are of special interest because they function as inhibitors of tuberization and, on the other hand, induce sprouting of dormant tubers and promote elongation of stolons [10,11]. However, rather little data is available on the endogenous amounts of GAs in the cultivated potato as compared with the mutants of other major crop species mentioned above. GA$_{20}$ and GA$_{1}$ have been quantified in the sprouts and leaves of potatoes by GC-MS [12–14]. Studies on GAs in stolons suggest that GA$_{1}$ is the main regulator of tuberization [11]. In addition, low amounts of GA$_{4}$ and GA$_{9}$ have been detected in the buds of internodal cuttings and in stolons grown in vitro [11].

Genetic mutants affected in GA metabolism would be helpful in resolving the role of GAs in various important physiological and developmental processes in potato. However, few such mutants have been described to date. A tetraploid clone (2$^n$ = 4 × = 48) of S. tuberosum subsp. andigena Hawkes, the subspecies adapted to short-day conditions and cultivated in the Andean region in South America, contains a recessive gene (ga$^1$) that confers a dwarf phenotype in plants homozygous for this locus [15]. The dwarf plants have smaller and darker green leaves, a compact ball-shaped mass of foliage, flower very sparsely, and are photoperiod-neutral in contrast to the wild type genotypes [14,15]. The normal growth can be restored by foliar application of bioactive GA [15], which, consistent with the reduced amounts of endogenous GAs, indicates that the dwarfs are GA synthesis mutants rather than GA response mutants [5]. Many dwarf di-(haploid) lines resembling the ga$^1$ mutant have been obtained from the potato cultivars Barbara and Titana, of which cultivar Barbara reportedly contains subspecies andigena in the pedigree [16]. However, these dwarf lines have been considered ‘non-vigorous’ for a breeding program [16] and no report on their further characterization is available.

We have recently found several dwarf genotypes among the (di)haploid (2$^n$ = 2 × = 24) lines produced from the tetraploid potato cultivar Pito, a genotype representing the long day-adapted subspecies tuberosum of the cultivated potato predominantly grown outside South America [17–19]. The (di)haploids are called haploids in the following, consistent with the use of the term haploid for sporophytes with gametic chromosome number [20]. The aim of this study was to measure the endogenous amounts of GA in the leaves of the long day-adapted potato by GC-MS and to characterize the dwarf mutants for GA biosynthesis and response to exogenous application of bioactive GA.

2. Materials and methods

2.1. Plant material and culture

Haploid lines of cultivar Pito were produced through anther culture and regenerated as previously described [18]. Regenerated plants were multiplied by internodal cuttings in vitro.

In vitro plantlets of the haploids and cultivar Pito were transferred to soil and grown in a greenhouse or in a growth chamber. In the greenhouse, cultivation was under natural daylight supplemented with illumination from sodium halide lamps (photoperiod 18 h; 200 μmol/s/m$^2$, measured at crop height). The daily minimum and maximum temperatures were 19°C and 24–25°C, respectively. In the growth chamber, (8.8 m$^2$; Weiss Umwelttechnik, Germany), photoperiod was 18 h (250 μmol/s/m$^2$), temperature was 19/17°C (day/night), and relative humidity was 40%. Plants were watered daily and fertilized weekly with 0.5% N:P:K fertilizer (5:7:6) (Kukkien Y-lannos, Kemira OY, Finland).

2.2. Determination of ploidy levels

Ploidy levels were determined by measuring the DNA content of nuclei (2C value). Nuclei were extracted from leaves, chicken red blood cells (2C = 2.33 pg [21]) added as internal standards, the mixture stained with propidium iodide (PI), and analysed using a FACSort flow cytometer (Becton Dickinson, USA) as previously described [22].
2.3. Determination of endogenous gibberellin (GA) content

Leaf samples (1.0 g fresh weight, f.w.) were extracted over night in 20 ml of 80% methanol. Deuterated GAs (17,17-[2H2]GA) obtained from Professor L. Mander, Canberra, Australia, were added as internal standards prior to extraction. After extraction, the sample was reduced to aqueous phase in vacuo and diluted with 20 ml of H2O. The aqueous phase was adjusted to pH 2.8 with 1 M HCl and partitioned three times with equal volumes of ethyl acetate. The ethyl acetate extracts were combined, the H2O freeze out, and thereafter reduced to dryness in vacuo. The residue was dissolved in 2 ml of H2O, pH adjusted to 8.0 with 1 M KOH, and applied to a pre-equilibrated QAE Sephadex (Pharmacia-LKB, Uppsala, Sweden) anion-exchange column (30 mm × 10 mm i.d.). The column was washed with 15 ml of H2O (pH 8.0) prior to elution with 25 ml 0.2 M formic acid which was run directly onto a pre-equilibrated 500-mg C18 ISOLUTE cartridge (Sorbent AB, V. Frölunda, Sweden) which was then eluted with 4 ml of methanol. The methanol eluate was dried and thereafter subjected to reversed-phase HPLC.

The HPLC system consisted of a Waters model 600 pump (Waters Associates, Milford, MA, USA) connected via a Waters 717-autosampler to a 4-μm Nova–Pak C18 column, 150 mm × 3.9 mm i.d. (Waters Associates). The mobile phase was a 20-min linear gradient of 20–100% methanol in 1% aqueous acetic acid at a flow rate of 1 ml/min. Five fractions corresponding to the GAs of interest were dried, methylated with ethereal diazomethane, and after evaporation trimethylsilylated in 20 ml of dry pyridine/BSTFA/trimethylchlorosilane (50:50:1, v/v) at 70°C for 30 min. The derivatization mixture was then reduced to dryness and dissolved in dichloromethane. Samples were injected in the splitless mode into an HP 5890 gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) fitted with a fused silica glass capillary column (30 m long, 0.25 mm i.d.) with a chemically bonded 0.25 μm DB-5MS stationary phase (J&W Scientific, Folsom, CA, USA). The injector temperature was 270°C. The column temperature program varied depending on which GA was observed. The column effluent was introduced into the ion source of a JMS–SX/SX102A mass spectrometer (JEOL, Tokyo, Japan). The interface temperature was 280°C and the ion source temperature was 250°C. The acceleration voltage was 10 kV. Ions were generated with 70 eV at an emission current of 500 μA. For quantification, samples were analysed in either high resolution selected ion monitoring (HR-SIM) mode or in selected reaction monitoring mode (SRM) [23]. For all the GA analyses, calibration curves were recorded from 0.5 to 20 pg of GA with 20 pg of [2H2]GA as the internal standard. All data were processed by a JEOL MS–MP7010 data system.

2.4. Treatment of plants with GA

Dwarf haploid lines were grown for 3 months in the greenhouse or growth chamber (two experiments in each) before GA treatment. The whole plant was sprayed, until moist, with 79 nM (27.5 ppm) GA3 (Sigma, St. Louis, MO, USA) dissolved in distilled water. The treatment was repeated four times at 2-day intervals.

3. Results

3.1. Ploidy of the anther culture-derived dwarf regenerants

Eight dwarf genotypes were found among the 67 androgenetic genotypes obtained through anther culture of the tetraploid cultivar Pito (2n = 48). Nuclear DNA content of the dwarf lines (1.73 ± 0.03 pg) and the two normally growing haploid lines (1.76 ± 0.05 pg) indicated that they were at the diploid level (2n = 24). In the tetraploid cultivar Pito (2n = 4 × 48), nuclear DNA content was 3.31 pg. The dwarf lines were phenotypically uniform and indistinguishable. Because cultivar Pito and other haploids grew normally, it was concluded that dwarfism was a recessive trait and attributable to homozygous condition of a recessive mutant gene allele tentatively designated as pito. Such gametes [i.e. anther culture-derived (di)haploids] can be obtained assuming that the tetraploid cultivar Pito is heterozygous for the corresponding locus. Further genetic analysis was not done in this study.
3.2. GA content

The amounts of GAs were determined by GC-MS in the leaves of three *pito* dwarfs picked by random (lines 52, 103 and 145), one normally growing haploid (line 142), and Pito (Table 1). The analysed GAs were GA19 (substrate for GA 20-oxidase), GA20 (metabolite of GA19 and end-product of GA 20-oxidase), GA29 (inactive 2β-hydroxylation product of GA20), GA1 (bioactive 3β-hydroxylation product of GA20) and GA8 (inactive 2β-hydroxylation product of GA1) [6,7]. They define the ‘early 13-hydroxylation pathway’ [6,8] utilized for synthesis of bioactive GAs from GA12 that is the common precursor of all GAs [4,6]. GA29, GA19 and GA8 have not been quantified in potatoes previously.

The amounts of all GAs (Table 1) were greatly reduced in the dwarfs as compared to the normally growing haploid line 142 and Pito, the differences ranging from ca. 6-fold (GA1) to ca. 40-fold (GA19). The low amounts of the bioactive GA1 were quite similar (100–114 pg/g f.w.) in the three *pito* dwarfs and, on the other hand, the amounts of GA1 were similarly high (653 and 685 pg/g f.w., respectively) in the haploid line 142 and the tetraploid ‘Pito’ that grew normally. Also, low amounts of GA53 (13-hydroxylated GA12; the first precursor of the early 13-hydroxylation pathway) were detected. Due to the very low amounts and some unknown interfering substances quantification of GA53 was considered inaccurate and is not presented here. For the same reason, the amounts of GA15 and GA9 produced via the non-hydroxylation pathway [6,7] could not be accurately determined, but their existence showed that some GA was synthesized also via the alternative pathway.

Table 1

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<thead>
<tr>
<th>Genotype</th>
<th>GA contenta</th>
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<td></td>
<td>GA19</td>
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<td>Dwarf lines</td>
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<tr>
<td>Line 52</td>
<td>&lt;40</td>
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<td>Line 103</td>
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<tr>
<td>Line 145</td>
<td>&lt;40</td>
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<tr>
<td>Normally-growing controls</td>
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<tr>
<td>Line 142</td>
<td>1635 (79)</td>
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<tr>
<td>Pito</td>
<td>1434 (85)</td>
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a pg/g fresh weight (f.w.). S.D. is in parentheses (n = 3). Molecular weights: GA19, 362.4; GA20, 332.4; GA29, 348.4; GA1, 348.4; and GA8, 364.4. The GAs are listed from left to right in their respective order of synthesis via the early 13-hydroxylation pathway.

3.3. Phenotypic responses to treatment with GA

Experiments in the greenhouse and growth chamber produced similar results. The *pito* mutants grew very slowly and were 9–10 cm high after 3 months. They had very short internodes, compact and ball-shaped appearance, and dark green leaves (Fig. 1). In a few *pito* mutants, small flower buds developed but were soon aborted and no flowers developed, whereas the wild-type haploids initiated flowering after ca. 8 weeks of growth. The *pito* mutants and wild-type genotypes both formed tubers during the 3 months of observation.

Spraying with GA3 induced rapid stem elongation, of which the first signs were clearly visible only 1 day after the first GA treatment. On average, dwarf plants elongated 17.3 ± 3.3 cm (31 plants measured in two experiments) in 10 days after the first GA treatment (Fig. 1). Leaves of the GA-treated plants were slightly more pale green than the leaves of the non-treated dwarfs, and similar to the wild-type haploids. The dwarf genotypes initiated flowering 18–21 days after treatment with GA. No differences were observed in the response to GA treatment among the dwarf haploids.

4. Discussion

Detection of GA3 and the high amounts of GA19 in the haploid line 142 and cultivar Pito indicated that the long day-adapted cultivated potatoes of subspecies *tuberosum* utilize primarily the early 13-hydroxylation pathway for synthesis of bioactive GAs. All GAs in plants are synthe-
Fig. 1. Two plants of the haploid pito mutant (line no. 10) grown in the greenhouse for 3 months. The plant to the right was sprayed with 79 nM GA3 and 3 days before photography, whereas the plant to the left was sprayed with distilled water. Height of the GA-treated plant is ca. 30 cm.

sized from the tetracyclic hydrocarbon ent-kaur-ene by a series of oxidative reactions catalysed by monoxygenases or 2-oxoglutarate dependent dioxygenases [6,7]. The first reactions are catalysed by monoxygenases, resulting in the formation of GA12 and GA53. Further oxidation steps resulting in, e.g. GA19, GA20, GA29 or GA1, and GA8, respectively, are carried out by dioxygenases. GA1 is the main bioactive GA in most species, causing the major effects such as stem elongation [5], whereas GA29 and GA8 are inactive 2β-hydroxylation products from GA20 and GA1, respectively [7].

The early 13-hydroxylation pathway is believed to be the most common one for synthesis of bioactive GAs in plants [6,7]. For example, it is the only active pathway detected in maize [24]. Our study showed that this pathway is predomin-
The very low endogenous amounts of GA in the pito mutants indicated that GA biosynthesis was blocked. The extremely low amounts of GA$_{19}$ and detection of some GA$_{53}$ suggested that the gene pito attributable to the defective GA synthesis is probably involved in the early (monooxygenase-catalysed) part of the pathway [4,6,8]. The strong and quick response to the exogenous application of low amounts (79 nM) of GA$_3$ confirmed that the dwarf haploids were, indeed, synthesis mutants and not GA response mutants; the latter show reduced or no response to the bioactive GAs [5]. GA$_1$ is considered to be the major bioactive GA in plants [5], while GA$_{25}$, a more persistent 1,2-dehydro analogue of GA$_1$ originally described from the fungus Gibberella fujikori, is synthesized and highly bioactive also in plants [24,27].

The phenotype and the reduced amounts of bioactive GA$_1$ in the haploid pito dwarfs closely resembled the previously described recessive ga1 mutant detected in an accession of the short day-adapted, tetraploid subspecies andigena [15]. The pito dwarfs contain ca. 6.5-fold lower amounts of GA$_1$ than the wild type genotypes, whereas ca. 25-fold reduction has been observed in the ga1 dwarfs [14]. The dwarfs of subspecies andigena tuberize under long and short days, i.e. are day-length neutral, in contrast to the wild-type genotypes that tuberize only under short days [14,15]. This is consistent with the role of GA as an inhibitor of tuber initiation [10,11]. Because subspecies tuberosum (e.g. cultivar Pito) is day-length neutral and tuberizes under long days, no effect due to reduced GA biosynthesis was expected and studied in this respect in the pito dwarfs. The block of GA biosynthesis in the ga1 mutant is reported to occur between GA$_{12}$ and GA$_{33}$ [13], and the dwarf phenotype can be reversed and flowering capacity recovered by foliar application of bioactive GA [15]. Thus, the pito and ga1 mutants share many similarities, even though they originate in different subspecies of S. tuberosum based on the pedigree data available [15,17].

It is assumed that because the media used for anther culture, haploid induction and regeneration of shoots contained no GA in our study [18], many dwarf genotypes defective in GA biosynthesis probably regenerated and grew slowly and were not transferred to rooting medium. However, detection of the recessive pito mutant by haploid induction from a tetraploid cultivar demonstrated that this method, which avoids interference by genes inherited from other genotypes in crosses, is powerful in revealing recessive gene mutations carried in potato cultivars. The ga1 and pito mutants originate in two different subspecies of S. tuberosum and are the only GA synthesis mutants reported in the large and economically important genus Solanum to date. Therefore, genetic localization, isolation and characterization of the ga1 and pito genes will be an interesting subject for future studies. These efforts should benefit from working on pito at the diploid level using the haploids described in this study.

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