Tuber formation in the wild potato species *Solanum demissum* Lindl.

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Tuber formation in the wild potato species *Solanum demissum* Lindl.

Proefschrift

ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus
dr. C.M. Karssen
in het openbaar te verdedigen
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des namiddags te vier uur in de Aula
van de Landbouwuniversiteit te Wageningen

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The drawing of *Solanum demissum* Lindl. on the cover is adapted from an article written by Professor J. Lindley (1799-1865) in the fourth volume of Professor C. Lemaire's book 'Jardin Fleuriste, contenant l'histoire, la description, la figure et la culture des plantes les plus rares et plus méritants nouvellement introduites en Europe' (1854, pp. 20-27).
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STELLINGEN

1. Bij *Solanum demissum* Lindl. leidt blootstelling aan lange-nachtcondities en gematigde nachttemperaturen, (5-20)°C, niet tot de aanmaak van knolinducerende substanties in de bladeren maar tot het transportabel maken en het transport van deze stoffen.

   Dit proefschrift

2. Het is onjuist dat 12-hydroxy-jasmijnzuur wordt aangeduid als 'tuberonic acid' (Yoshihara *et alii* 1989), het is hoogstens 'a tuberonic acid'.

   T. Yoshihara *et alii* 1989. Agric. Biol. Chem. 53: 2835-2837.
   Dit proefschrift

3. Bij de aardappelplant zijn veranderingen in de fructoseconcentratie in stoloonuiteinden indicatief voor veranderingen in de wijze van saccharose-ontlading in die stoloonuiteinden.

   Dit proefschrift

4. De celwandgebonden zure-invertase-activiteit, die wordt aangetroffen in zich ontwikkelende knollen van de aardappelplant, is een relict uit de tijd dat deze knollen nog ongezwollen, longitudinaal groeiende stoloonuiteinden waren.

   Dit proefschrift

5. De idee, dat bepaalde bladeren van de aardappelplant exclusief in verbinding zouden staan met bepaalde stoloonuiteinden (Chapman 1956), was al in 1918 ondergraven door Artschwager.

   E.F. Artschwager. 1918. J. Agric. Res. 14: 221-252.
   H.W. Chapman. 1958. Physiol. Plant. 11: 215-224.

6. Bij de bestudering van genexpressie in met *Globodera rostochiensis* geïnfecteerde aardappelwortels duiden Gurr *et alii* de aardappelkloon pPMR1 aan als 'syncytium specifiek'. Het is echter uiterst onwaarschijnlijk dat een (aardappel)plant 'pathogeen-specifieke' genen zou bezitten.

Gurr *et alii* 1991. Mol. Gen Genet. 226: 361-366.

7. Als men een traject van een bepaalde grootheid wil aanduiden is het logisch de randwaarden door haakjes te omgeven en de gebruikte eenheid buiten de haakjes te plaatsen, bijvoorbeeld (5-20)°C in plaats van 5-20°C. Het is onterecht en van weinig exactheid getuigend dat dit door sommige wetenschappelijke tijdschriften geweigerd wordt.

Helder *et alii* 1993. Physiol. Plant. 88: 647.

8. In wetenschappelijke verhandelingen wordt *et alii* afgekort tot *et al.*, dit levert een besparing op van één teken. Het is daarmee de minst efficiënte afkorting in de Engelse taal en het ware beter voluit *et alii* te schrijven of bijvoorbeeld *e.a.*

9. Voor personen die werken in een chemisch laboratorium verdient het sterkere aanbeveling de handen te wassen voor een toiletbezoek dan daarna.

10. In 'Lauda per la natività del Signore' van Ottorino Respighi wordt voor een deel van het laatste tutti een tempo aangegeven van 108 halve noten per minuut. Dit deel komt beter tot zijn recht als een tempo van 95 tot 100 halve noten per minuut wordt aangehouden.

11. De in de vergetelheid geraakte verkeersregel, die gebiedt te stoppen voor voetgangers die een weg oversteken via een zebraoversteekplaats of daartoe aanstalten maken, dient in ere te worden hersteld.

12. Verwondering is een uitstekende drijfveer voor het doen van wetenschappelijk onderzoek.

Stellingen behorend bij het proefschrift, getiteld 'Tuber formation in the wild potato species *Solanum demissum* Lindl.' door Johannes Helder.

Wageningen, 9 februari 1994.

Ter herinnering aan mijn opa,
Johannes Breugem

CONTENTS

VOORWOORD

Laat ik beginnen te vertellen dat ik een plezierige tijd heb gehad op de vakgroep Plantenfysiologie. Voor het doen van onderzoek dient aan enige randvoorwaarden in de koudbloedige sfeer (denkend aan bijvoorbeeld analyse-apparatuur, plantmateriaal en klimaatruimten) te zijn voldaan. Dat is vrijwel nooit een probleem geweest. De warmbloedige factoren zijn echter zeker zo belangrijk: technisch gezien omdat je een proefschrift onmogelijk zonder hulp van anderen kunt schrijven, maar vooral sociaal: het zijn deze factoren die je aanmoedigen, een hart onder de riem steken en waarmee je (on)benullige gesprekken voert. Deze factoren bepalen of je je ergens thuis voelt en als dat het geval is dan komt dat de gewenste productiviteit beslist ten goede.

Een aantal mensen wil ik graag noemen. Op de eerste plaats wil ik mijn promotoren en co-promotor bedanken. Prof.dr. J. Bruinsma heeft met name in het begin en aan het einde van dit onderzoeksproject een belangrijke rol gespeeld: in het begin omdat U kennelijk vertrouwen had in de kandidaat J. Helder (dat geldt uiteraard ook voor de andere (co-) promotoren), die U, behalve in de collegezaal, nog nooit eerder had ontmoet, in de latere fasen van de wording van dit proefschrift omdat U met grote zorg de ontwerpteksten heeft doorgenomen en verbeterd. Het bespreken hiervan ging op een plezierige wijze en ik heb er veel van geleerd. Prof.dr.ir. P.C. Struik (beste Paul), ik wil niet onvermeld laten de keren dat ik rond half zes of later in de middag onaangekondigd bij je langs kwam en dat je, ondanks een zeer volle agenda, tijd had om mee te denken over het onderzoek en de daarbij behorende problemen. Tevens wil ik graag de snelheid en de accuratesse noemen waarmee je de verschillende ontwerpteksten hebt doorgewerkt, die waren respectievelijk hoog en groot. Veel dank hiervoor.

Met mijn co-promotor, dr. D. Vreugdenhil, heb ik het meest frequent contact gehad; bijna dagelijks. Dick, ik heb jouw manier van begeleiden (vrijwel loslaten en toch sturen) enorm gewaardeerd. Je hebt weleens gezegd dat je dat in dit geval het beste leek en ik denk dat dat klopt. Ik wil je graag bedanken voor de tijd en de energie die je in mijn begeleiding hebt gestoken, waarbij ik niet onvermeld wil laten dat jij degene bent geweest die het initiatief genomen heeft tot dit project. Naast dit alles heb je ermee ingestemd dat Elly Koot-Gronsveld gedurende meer dan twee jaar aan dit onderzoek heeft meegewerkt en dat is geen sinecure. Elly, ik durf gerust te stellen dat dit proefschrift door jouw

bijdrage dikker en substantiëler is geworden. Jij hebt veel inzicht in plantenfysiologische onderwerpen die biochemisch getint zijn en ik heb de wijze waarop wij samengewerkt hebben de afgelopen jaren plezierig gevonden.

Prof. dr. Cees M. Karssen, ik zal niet onder stoelen of banken steken dat ik mij gevleid voelde toen je opperde om de rector magnificus dit keer niet te vervangen door een waarnemer. Bedankt. Hiernaast is er een groot aantal collega's die op één of andere wijze hebben bijgedragen aan de totstandkoming van dit proefschrift. Een aantal ervan wil ik graag noemen: Steef de Bruijn, Folkert Hoekstra en Frans Tetteroo. Steef, wij zijn cohort-genoten die praktisch in fase zijn gebleven. Ik wil hier graag je enorme hulpvaardigheid noemen met name op het PC-vlak maar ook op andere terreinen. Onze levenshoudingen lopen nu niet direct parallel, maar ik wil graag zeggen dat ik, hoewel mij een groot aantal dingen ontgaan, veel waardering heb voor de consequente wijze waarop jij aan je geloof gestalte geeft. Tsja, Folkert, ik chargeer als ik zeg dat ik niet zo gauw zou weten waar wij het de afgelopen jaren niet over gehad hebben. Onder andere het kabinetsbeleid, de herkomst van het water op aarde en de voor- en nadelen van promiscuïteit (in evolutionair perspectief) passeerden de revue en ik moet zeggen dat dit niet de minst interessante uren waren die ik op de vakgroep heb doorgebracht. Met Frans heb ik tientallen uren achter de Dionex doorgebracht, een zeer gevoelig apparaat voor het doen van suikerbepalingen waarmee wij beiden een haat/liefde verhouding hebben/hadden. Verbaal dollen is iets waarmee wij ons met enige regelmaat bezighielden, en dat behoort tot de dingen die je op de rails houden.

Een aantal studenten hebben hun bijdrage geleverd aan de totstandkoming van dit proefschrift en dat zijn, in volgorde van binnenkomst, Jeanine Geelhoed, Bjorn Dirks, Connie Eijkelboom, Iris Kappers, Arie van der Maarl en Jolanda Schrauwen.

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Dit geschrift was nog niet af toen ik als post-doc begon bij de vakgroep Nematologie. Ik wil graag de zeer coulante houding noemen van dr.ir. Fred Gommers en dr.ir. Jaap Bakker. Zij boden mij de ruimte om dit proefschrift, gedeeltelijk binnen werktijd, te voltooien.

I would like to thank Dr. Otto Miersch and Prof.dr. G. Sembdner from the Institute of Plant Biochemistry Halle (Halle, Germany). I would like to put it rather clear: without

your help and your expertise in the field of (hydroxylated) jasmonic acids Chapter 2 would have been far less substantial than it is now. Thank you very much.

Hierbij bedank ik de stichting 'Fonds landbouw export bureau 1916/1918' voor de financiële ondersteuning die ik gekregen heb ten behoeve van mijn deelname aan een congres in Bar Harbor (Maine, USA).

Heel graag wil ik mijn ouders, mijn broer Onno, Marion en Lotte bedanken voor hun belangstelling en meeleven.

Als laatste bedankt ik Janine, de belangrijkste warmbloedige factor van de afgelopen jaren.

Hans

LIST OF ABBREVIATIONS

ABA	abscisic acid
ADP, ATP	adenosine 5' di-, triphosphate
ADPGlc	ADP-glucose
BAP	benzylaminopurine
BSA	bovine serum albumine
BSTA	N,O-bis(trimethylsilyl)acetamide
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
BTP	bis-tris propane
cv.	cultivar
DEAE	diethylaminoethyl
DM	dry matter
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
e.g.	exempli gratia, for example
et al.	et alii
FPLC	fast protein liquid chromatography
Fru	fructose
Fru1,6P_2	fructose 1,6-bisphosphate
Fru2,6P_2	fructose 2,6-bisphosphate
Fru6P	fructose 6-phosphate
GC-MS	gas chromatography-mass spectrometry
Glc	glucose
Glc1P	glucose 1-phosphate
Glc6P	glucose 6-phosphate
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonoc acid
HK	hexose kinases
HPAEC-PED	high pH anion exchange chromatography-pulsed electrochemical detection
13(S)-HPOT	13(S)-hydroperoxy-9(Z),11(E),15(Z)-octadecatrienoic acid
i.e.	id est
JA	jasmonic acid
kD	kilodalton (named after J. Dalton (1766-1844))

L.	C. Linnaeus (1707-1778)
Lindl.	J. Lindley (1799-1865)
LD	long day
LOX	lipoxygenase
MeJA or JA-Me	methyl jasmonate
Mes	2-(N-morpholino)ethanesulfonic acid
NAD, NADH	nicotinamide adenine dinucleotide, oxidized and reduced
NADP, NADPH	nicotinamide adenine dinucleotide phosphate, oxidized and reduced
PAR	photosynthetically active radiation
PFK	phosphofructokinase
PFP	pyrophosphate:fructose 6-phosphate phosphotransferase
P_i	inorganic phosphate
PP_i	inorganic pyrophosphate
PVP	polyvinylpyrrolidone
RIA	radioimmunoassay
sd	standard deviation of series
SD	short day
SHAM	salicylhydroxamic acid
spp.	subspecies
TLC	thin-layer chromatography
TMCS	trimethylchlorosilane
TSIM	N-(trimethylsilyl)-imidazole
UDP, UTP	uridine 5' di-, triphosphate
UDPGlc	UDP-glucose
viz.	videlicet, namely

Chapter 1

GENERAL INTRODUCTION

The potato species *Solanum demissum* Lindl.

'... But I believe myself to be in a condition to show that it is a mistake to say that the cultivated potato, that is to say *Solanum tuberosum*, is unknown in a wild state in Mexico. To the kindness of Mr. C.A. Uhde, a German gentleman, who has resided for many years in the west of Mexico, the Society (The British Horticultural Society; JH) has been indebted for various samples of wild Mexican potatoes. ... The first of these was marked 'Native Mexican Potatoes, growing at 8000 to 9000 feet elevation'. This provided a particularly dwarf sort. It was planted May 2, and was in flower in the end of June. Its flowers are produced close to the ground, and fruit soon succeeds them; branches then push up, and blossom at the height of 12 to 15 inches. ... Very few tubers were formed. Many stems had none; and where they did occur they were small, flattened, somewhat kidney-shaped, and of a white colour, with white, crisp, semi-transparent flesh. This appears to be distinct from the potato, and may be called *Solanum demissum*, because of its dwarfness.'

This part of the first description of *Solanum demissum* Lindl. was written by Professor J. Lindley (1799-1865) in an article named 'Notes on the wild potato'. It was published in 1848 in the Journal of the Royal Horticultural Society (London), volume III (pp. 65-72). A French translation of this article was published in 1854 in the fourth volume of Professor C. Lemaire's 'Jardin Fleuriste, contenant l'histoire, la description, la figure et la culture des plantes les plus rares et plus méritants nouvellement introduites en Europe' (pp. 20-27). The drawing of *Solanum demissum* Lindl. on the cover of this thesis is adapted from Professor C. Lemaire's book, and identical to the first drawing published by Professor J. Lindley.

The flexible response of *Solanum tuberosum* on changes in duration of the night length

Night length and night temperature are two major factors that are involved in tuber initiation

in potato plants. A tuber-bearing *Solanum* species will start to form tubers after a number of cycles in which the duration of the dark period is longer than a species-dependent, minimal night length, provided that the night temperature is adequately low (5°C-20°C). The minimal night length is commonly referred to as the maximal or critical daylength. To obtain more insight in this species-dependent critical daylength it is useful to consider where and at what latitude tuber-bearing *Solanum* species are indigenous.

The genus *Solanum*, to which all the tuber-bearing potato species belong, contains about 1000 species. *Solanum* species are found all over the world, with strong concentrations of species diversity in Central and South America, and in Australia (Hawkes 1992). Tuber-bearing *Solanum* species all originate from Central and South America. Two separate centres of diversity can be distinguished: one in central Mexico (15°-22° north latitude (NL)) and the second in the high Andes from Colombia to the north-west of Argentina (5° NL -45° southern latitude (SL)) (Hawkes 1990).

Earlier this century, there was a considerable controversy about which subspecies (spp.) of *Solanum tuberosum* was brought first to Europe in about 1570: *S. tuberosum* spp. *tuberosum* or *S. tuberosum* spp. *andigena*. In the 19th century *S. tuberosum* plants grown in Europe (35°-60° NL) gave a good tuber production under relatively long-day (LD) conditions. In Chile and Argentina the daylength during the growth season is comparable with the daylength in Europe. *Solanum tuberosum* subspecies *tuberosum* originates from coastal Chiloé region in South Central Chile (42°-44° SL). Hence, the Russian botanists Juzepczuk and Bukasov (1929) concluded the potato plants brought to Europe necessarily belonged to the subspecies *tuberosum*. They considered critical daylength to be a fixed characteristic of every tuber-bearing *Solanum* species.

The first indications for human consumption of potatoes in Europe were found in the account books from 1576 of the Hospital de la Sangre at Seville (Spain). In 1650, the Italian Giulio Carpioni made an oil painting called 'Putto alato che fa le bolle di sapone' (winged Cupid blowing soap bubbles). On the foreground he painted vegetables, including potato tubers. Up to the present, this is the oldest European depiction of potato tubers (Zeven 1992). Presumably these tubers were introduced in Italy via Spain. In 1937 Salaman put forward historical arguments that made it unlikely that the first potato plants introduced in Europe belonged to the subspecies *tuberosum*, as claimed by Juzepczuk and Bukasov (1929). The Spaniards reached the Chilean region of Chiloé in 1559. The original inhabitants, the Araucanian, offered substantial resistance to the Spanish army till a truce was called by the

Spaniards in 1568. In 1576 the hostilities started again and lasted for about 200 years. Hence, the first argument of Salaman was that in those days the conditions for the collection of botanical material were suboptimal. But even if some tubers of the subspecies *tuberosum* were collected in March or April in the Chiloé region, it would have taken too long to transport them to Europe as living material. The first journey from Chile to Europe via the Magellan Straits was made in 1579. Hence, the tubers should have been shipped via Lima to Panama, carried through the jungle across the Isthmus to Nombre the Dios (the stretch of, nowadays, the Panama channel). It could, at first, arrive at the end of September in Seville, and be planted the next spring. According to Salaman (1937) it was unlikely that any tuber would have survived this journey. It is not known why Salamans bases his reasoning on the transport of tubers from Chile. After all he questioned in the same article whether true seeds or tubers were brought to Europe. True seeds may have a better chance to survive under adverse conditions than tubers. Salaman (1937) suggested that the *S. tuberosum* subspecies *andigena*, indigenous to Colombia, Ecuador and Peru (5° NL- 20° SL), was transported to Spain via the harbour of Cartagena in the north of Colombia.

Better arguments on the character of the early European potato are put forward by Salaman and Hawkes (1949). The authors considered early European descriptions and figures of potato plants from the 16th and 17th century. The various depictions of the early potato plants consistently had the morphological characteristics of the *andigena* subspecies. Salaman and Hawkes (1949) concluded that presumably potatoes of the subspecies *andigena* were the first *S. tuberosum* plants introduced in Europe. In those days potato plants, just introduced in Europe, only formed tubers in November and December and the potato plants, therefore, were grown only in the milder regions of Spain, Italy and France (Hawkes 1990). This short-day (SD) dependency forms an additional indication that the early *S. tuberosum* plants were of the subspecies *andigena*. Simmonds (1966) showed that selection for earliness in strongly SD dependent *S. tuberosum* spp. *andigena* plants resulted in very few generations in lines with a typical *tuberosum* spp. *tuberosum*-character. So, strong indications are available that SD dependency is not a fixed, unchangeable characteristic of an *andigena* plant, as presumed by Juzepczuk and Bukasov (1929).

One of the aims of this historical overview is to show that critical daylength is not a 'fixed' characteristic of *S. tuberosum*. This is also illustrated by one of the first experiments I did as part of the PhD-project entitled 'Hormonal regulation of tuber initiation in potato'. A prerequisite for the experiments to come was the availability of, towards tuber induction,

absolutely SD dependent potato plants. Plants of the *S. tuberosum* cultivars Désirée and Bintje were grown under 24 h daylength conditions. After about two months, tuber formation was at most reduced as compared to the tuber yield from plants grown under 12 h daylength conditions. Hence, it was decided not to use *S. tuberosum*. Absolutely SD dependent *Solanum* species were obtained from the Institut für Pflanzenbau und Pflanzenzüchtung, Braunschweig (Germany). Because of its morphological resemblance to *S. tuberosum*, *S. demissum* was selected amongst a number of other wild potato species. This resemblance is illustrated by the Index Kewensis (Hooker and Jackson, 1895), where *Solanum demissum* is indicated '*S. demissum* = tuberosum'. By doing so, the authors claimed that *S. demissum* was (a subspecies of) *S. tuberosum*. Nowadays, taxonomists consider both the hexaploid *S. demissum* and the tetraploid *S. tuberosum* to be member of the *Solanum* superseries *Rotata*. However, *S. demissum* belongs to the series *Demissa*, whereas *S. tuberosum* is a species belonging to the series *Tuberosa* (Hawkes 1990).

The translation of night length information into (a) transportable signalling compound(s)

In potato, information about the duration of the dark period is perceived in the leaves (Gregory 1956). A pigment in leaves that could be involved in the perception of this information is phytochrome. Phytochrome exists as two photoisomers, the red light absorbing form (P_r) and the far-red light absorbing form (P_{fr}). Red light can induce the conversion P_r to P_{fr}, this can be reversed by far-red light. P_{fr} is considered to be the biological active form of phytochrome. Batutis and Ewing (1982) obtained evidence for the involvement of phytochrome in the perception of night length information in the potato plant. Tuberization could be reduced by exposure of the plants to 5 min red light in the middle of the dark period, and this effect could be reversed significantly by 2 min subsequent far-red light. Hence, when phytochrome was converted partially into the biologically active form, tuber formation was reduced.

Gregory (1956) surmised that under SD conditions a tuber-inducing factor was synthesized or activated in the leaves and transported basipetally to the stolon tips. Since 1956 extensive research was done to identify the(se) tuber-inducing, transportable compound(s). It was investigated whether the tuber-inducing principle could be identified as (a combination of) 'classical' plant hormones. Two approaches should be distinguished: (1) studies on the tuber-inducing or inhibiting effect of exogenously applied phytohormones and

(2) investigations on changes in the endogenous plant hormone content during the development of a non-swollen stolon tip into a tuber.

(1) A combination of sucrose and a cytokinin clearly promoted *in vitro* tuber initiation in *S. tuberosum* explants (Palmer and Smith 1969). However, *in vivo* application of cytokinins to a diageotropically growing stolon tip failed to induce tuber formation, and converted the tip into a negatively geotropic shoot (Kumar and Wareing 1972). Both *in vitro* and *in vivo* application of gibberellins inhibited tuber initiation (Koda and Okazawa 1983a, and Okazawa 1960). Applications of other 'classical' phytohormones did not unequivocally affect tuber initiation.

(2) From studies on the endogenous phytohormone content of stolon tips, it is clear that a relatively low gibberellin activity in stolon tips is a prerequisite for tuber initiation in *S. tuberosum* (*e.g.* Okazawa 1960). Activities of other 'classical' phytohormones changed during tuber development, but these changes occurred only after the onset of subapical swelling (Koda and Okazawa 1983b). Hence, it is unlikely that these hormonal changes are involved in tuber induction.

The role of the 'classical' plant hormones in tuber induction is discussed *in extenso* in a number of recently published reviews (Ewing 1987, Ewing and Struik 1992, Vreugdenhil and Helder 1992).

The possible role of (glycosides of) hydroxylated jasmonic acids as transportable signalling compounds

In the fifties and the early sixties Tagawa, Okazawa and Nishiyama in Sapporo, Japan, studied changes in gibberellin activities in relation to tuber induction in potato (*e.g.* Okazawa 1959, Okazawa 1960). In 1983, Koda and Okazawa published data on activity changes in several endogenous plant hormones, and apparently the authors concluded that tuber induction could not be explained adequately by changes in activities of 'classical' plant hormones. In 1988, Koda *et al.* succeeded in the isolation of 2.7 mg of a specific tuber-inducing compound from 100 kg fresh leaves of *S. tuberosum*, cv. Irish Cobbler. The active principle was determined to be 3-oxo-2-(5'-ß-D-gluco-pyranosyloxy-2'-Z-pentenyl)-cyclopentane-1-acetic acid, *i.e.* a glucoside of 12-OH-jasmonic acid (12-OH-JA) (Yoshihara *et al.* 1989). Jasmonic acid itself was already described as a representative of a new group of endogenous plant growth regulators by the group of Professor Sembdner in Halle

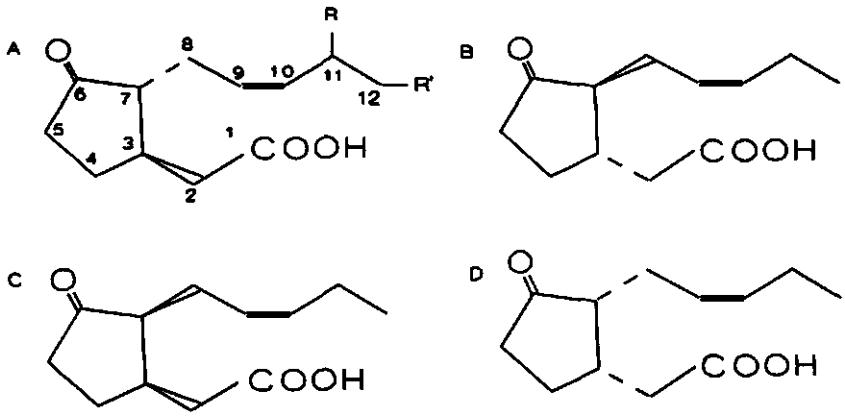
(Germany, *e.g.* Sembdner and Gross 1986). Remarkably, no 11-OH-JA was detected in leaves of the *S. tuberosum* cultivar Irish Cobbler, since metabolic studies of [2-^{14}C]-(±)-JA and [2-^{14}C]-9,10-dihydro-JA, fed to 6-day-old barley seedlings, revealed that in both cases hydroxylation preferentially took place at C-11 and to a lesser extent at C-12 (Sembdner *et al.* 1990).

Using cuttings of potato, Struik *et al.* (1987) showed differences in tuber-inducing activity in extracts from leaves of *S. tuberosum* plants grown under SD and LD conditions. Therefore, we decided to compare the nature and contents of hydroxylated JAs in leaflets of *S. demissum* leaflets collected from plants grown under SD and LD conditions.

The identification of the glucoside of 12-OH-JA as a tuber-inducing principle from potato leaves was complicated by the substantial losses in tuber-inducing activity during the isolation procedure (Yoshihara *et al.* 1989). This decrease was explained by a possible *cis/trans* isomerization at C-7, which is generally known for α-substituted cyclopentanones (Vick and Zimmermann 1979, Miersch *et al.* 1986). They considered the glucoside of 12-OH-(+)-7-*iso*-jasmonic acid (a *cis*-isomer) to be the biologically most active compound. Such an explanation presupposes a relatively high *cis/trans* ratio in the plant. Due to isomerization, occurring under weakly acidic or alkaline conditions, this ratio decreases (Miersch *et al.* 1987).

Considering two substituents on a cyclopentanone, four stereoisomers are conceivable. For example, commercially available JA, a racemic mixture, consist of two *trans* isomers, (-)-JA and (+)-JA, and two *cis* isomers, (+)-7-*iso*-JA and (-)-7-*iso*-JA (Fig. 1.1). In plants

Fig. 1.1. Stereoconfiguration of jasmonic acid (JA), 11-OH-JA (R=OH, R'=H), and 12-OH-JA (R=H, R'=OH). A; (-)-JA, B; (+)-JA, C; (+)-7-*iso*-JA and D; (-)-7-*iso*-JA.



and fungi only one *trans* and one *cis* isomer are detected (in case of, *e.g.*, JA: (-)-JA and (+)-7-*iso*-JA). The *trans* configuration is apparently more stable than the *cis* configuration since isomerization under normal conditions results in a *cis/trans* equilibrium of about 1:9. GC-MS analyses of 11-OH-JA and 12-OH-JA from the fungus *Botryodiplodia theobromae* revealed a similar equilibrium ratio between the two pairs of diastereoisomers (1:9) (Miersch *et al.* 1991). *In planta* the *cis/trans* ratio of (hydroxylated) JAs is probably higher than 1:9. After a very careful isolation procedure of JAs from *Vicia faba* fruits, a (+)-7-*iso*-JA : (-)-JA ratio was obtained of about 1:2 (Miersch *et al.* 1986).

Evaluation of the tuber-inducing activity of the two naturally occurring stereoisomers of methyl jasmonate (JA-Me) revealed considerable difference between (-)-JA-Me (the two substituents on the cyclopentanone are in the *trans* orientation) and (+)-7-*iso*-JA-Me (the two substituents on the cyclopentanone are in the *cis* orientation). Koda *et al.*, (1992) tested *in vitro* tuber-inducing activities of various isomers of JA-Me including (-)-JA-Me and (+)-7-*iso*-JA-Me on single-node segments of etiolated potato shoots. At concentrations of 10^{-7}, 10^{-6}, and 10^{-5} M in the culture medium the tuber induction rates were 0%, 13% and 47% for (-)-JA-Me, while the rates for (+)-7-*iso*-JA-Me, at the same concentrations, were 27%, 60% and 92%. It can be questioned to what degree (+)-7-*iso*-JA-Me is isomerized to the less active (-)-JA-Me during the three weeks that the testing took. Hence, the difference in the *in vitro* tuber-inducing activity between (-)-JA-Me and (+)-7-*iso*-JA-Me is at least as large as indicated by the data presented by the authors.

The *in vitro* potato tuber-inducing activities of JA, JA-Me, 12-OH-JA and the glucoside of 12-OH-JA are about equal (Koda *et al.* 1991). It was shown that (+)-7-*iso*-JA-Me had *in vitro* considerably more tuber-inducing activity than (-)-JA-Me. When the *cis/trans* ratio of the glucoside of 12-OH-JA from potato leaves *in planta* was higher than 1:9, like in, *e.g.*, *Vicia faba* fruits (Miersch *et al.* 1986), it is conceivable that the decrease in potato tuber-inducing activity observed by Yoshihara *et al.* (1989) was indeed due to *cis/trans* isomerization during the isolation procedure.

After extraction of (hydroxylated) JAs from leaflets of *S. demissum* a decrease in tuber-inducing activity was observed (Helder, unpublished results). Since it was for practical reasons impossible to overcome these problems by extraction of a huge quantity of leaves (*e.g.* 100 kg fresh weight) another approach was chosen. The (hydroxylated) JA content of leaflets of *S. demissum* grown under SD conditions was compared with the content of leaflets from plants grown under LD conditions. Chapter 2 of this thesis deals with this comparison.

A disadvantage inherent in this approach is the impossibility to prove that hydroxylated JAs are the only tuber-induction related compounds in leaflets of *S. demissum*.

The perception of a tuber-inducing signal in the stolon tip

Chapter 3 is about the perception of a tuber-inducing principle in the stolon tip and translation into actual tuber initiation. Since only indications were available about the nature of a tuber-inducing principle, its perception could not be studied on receptor level.

One of the intriguing characteristics of potato tuber induction is its irregularity: under tuber-inducing conditions only some stolon tips will start to swell subapically (Vreugdenhil and Struik 1989). Apparently, in some stolon tips the presence of a tuber-inducing signal is registered and triggers subapical swelling, whereas in other stolon tips it is not registered or does not trigger subapical swelling. Characteristics of stolons and stolon branches were monitored with minimal disturbance of normal growth. By this approach it was investigated whether the ability to translate the presence of a tuber-inducing signal in a stolon tip in actual tuber initiation could be correlated with external characteristics of stolons or stolon branches.

Changes in the mono- and disaccharide contents of stolon tips accompanying subapical swelling

In the course of this research project it became clear that the problem of the almost inevitable decrease of tuber-inducing activity in extracts from *S. demissum* leaflets, presumably due to isomerization, was hard to solve. Since there was not the slightest doubt about the limited duration of this research project, it seemed better not to put all my eggs in the hydroxylated-JA basket.

The exposure of *S. demissum* plants to SD conditions causes profound developmental changes in the plant. Before any subapical swelling of the stolon tips was visible, a reduction of shoot growth was observed as compared to shoot growth of LD plants. Assimilates are no longer directed to, *e.g.*, developing leaves, but mainly distributed to stolon tips, *viz.* the sites where the vegetative surviving structures of the potato plant, the tubers, will appear. The main form in which assimilates, carriers of energy and carbon, are (re)distributed in potato plants is sucrose. Concomitant with the onset of tuber initiation starch synthesis starts. Imported sucrose is osmotically inactivated by starch synthesis, and starch will accumulate

subapically. Subapically non-swollen stolon tips, that can be considered as moderate sucrose sinks, develop into strong sucrose sinks.

High sucrose concentrations in the medium can induce tuber formation *in vitro* in some tuber-bearing *Solanum* species. Ewing and Senesac (1981) tested *in vitro* the tuber-inducing activity of sucrose, glucose and fructose on nodal cuttings from pot-grown plants. Equimolar concentrations of either glucose or fructose were about half as active as sucrose. It is not known whether sucrose or related sugars play a role in tuber induction *in planta*. It would be interesting to know whether, before subapical thickening is visible, an (transient) increase of the concentration of one or more mono- and disaccharides can be observed in stolon tips.

Sugar concentrations in developing tubers from several *S. tuberosum* cultivars were determined by, *e.g.*, Davies (1984), and Morrell and ap Rees (1986). Davies (1984) observed a decrease of both the glucose and fructose levels, and an increase of the glucose to fructose ratio in developing stolon tips from the cultivar Maris Piper. The sucrose concentration was variable, but no consistent change was observed during tuber development. Morrell and ap Rees (1986) investigated the sucrose, glucose and fructose contents of developing tubers from three cultivars, *viz.* King Edward, Maris Bard and Pentland Javelin. In none of these cultivars similarly high ratios of glucose to fructose were found in developing tubers as in Maris Piper by Davies (1984). The sucrose concentrations were highly variable, and tended to decrease upon tuber development. An explanation for these apparently conflicting observations could be that Davies (1984) investigated developing stolon tips (up to 8 g fresh weight), whereas Morrell and ap Rees (1986) analyzed the sugar content of growing tubers (up to 30 g fresh weight).

To obtain more insight in the sucrose metabolism in stolon tips, we monitored the neutral, water-soluble sugar content of stolon tips from *S. demissum* during tuber development. Sensitivity of the sugar detection equipment (HPAEC-PED; high pH anion exchange chromatography combined with pulsed electrochemical detection) allowed us to detect minute sugar quantities in individual stolon tips. First, it was investigated which neutral, water-soluble sugars were detectable in subapically non-swollen and swollen stolon tips. Next, quantitative sugar determinations were done to see whether consistent changes in the concentrations of these sugars could be observed. An additional goal was to obtain insight in the apparently conflicting observations as discussed above. In the view of the observed changes in the glucose and fructose contents it was hypothesized what enzyme shift could underlie these changes (Chapter 4).

Qualitative changes in sucrose metabolism in stolon tips accompanying subapical swelling

On the basis of the observed changes in the glucose and fructose contents in developing stolon tips, changes in the way sucrose is metabolized were postulated in Chapter 4. Hypotheses concerning changes in the relative importance of invertases and sucrose synthase were tested in Chapter 5. Evidence was obtained for both an enzyme and a spatial shift in sucrose metabolism during the onset of tuber initiation. Two models on sucrose unloading in *S. demissum* stolon tips are presented.

Comparison of hexose monophosphate levels in LD and SD stolon tips, showing various degrees of subapical swelling

Starch in plant cells resides in intracellular membrane-bounded compartments called amyloplasts. Thus, starch synthesis takes place in an environment that is spatially separated from the cytosol. It is not known which building blocks are imported from the cytosol into the amyloplast. Carbon for starch could enter as triose phosphate (*e.g.* Mohabir and John 1988) or as hexose monophosphate (*e.g.* Keeling *et al.* 1988). A strong indication in favour of the latter possibility is the absence of plastid fructose 1,6-bisphosphatase in a range of higher-plant organs, including potato tubers (Entwistle and ap Rees 1990). Fructose 1,6-bisphosphatase activity in the amyloplast is essential for the conversion of triose phosphates to ADP-glucose. Another approach was used by Hatzfeld and Stitt (1990). Potato tubers were fed with [1-^{14}C]glucose or [6-^{14}C]glucose, and the degree of randomization in glucosyl residues from starch was determined. Only 16%-26% of the molecules were randomized and it was concluded that mainly hexose units are imported into the amyloplast. Hexose monophosphates such as glucose 1-phosphate, glucose 6-phosphate and fructose 6-phosphate are candidates for import into the amyloplast. Among these hexose monophosphates, glucose 1-phosphate was reported to be taken up most efficiently into isolated amyloplasts from wheat endosperm (Tyson and ap Rees 1988). On the other hand, glucose 6-phosphate was the only hexose monophosphate that was taken up at physiologically relevant rates into amyloplasts isolated from developing pea embryos (Hill and Smith 1991).

In Chapter 6 hexose monophosphates were separated and detected by HPAEC-PED (high pH anion exchange chromatography combined with pulsed electrochemical detection). A

comparison is made between levels of hexose monophosphates in individual LD and SD stolon tips, the latter showing various degrees of subapical swelling. The main goal was to see if we could relate any changes in hexose monophosphate level to the onset of starch synthesis.

Finally, in Chapter 7 hormonal and metabolic aspects of tuberization in *S. demissum* are discussed.

Chapter 2

OCCURRENCE OF HYDROXYLATED JASMONIC ACIDS IN LEAFLETS OF *SOLANUM DEMISSUM* PLANTS GROWN UNDER LONG- AND SHORT-DAY CONDITIONS

Summary

Under short-day (SD) conditions both 11-OH-jasmonic acid (11-OH-JA) and a smaller quantity of 12-OH-JA occurred in leaflets of *Solanum demissum* Lindl. plants which had formed tubers. This is the first time that 11-OH-JA has been detected as a native substance in higher plants. Under long-day (LD) conditions no tubers were formed and none of these compounds were detectable. A positive correlation was found between the occurrence of 11-OH-JA and 12-OH-JA in leaflets of *S. demissum* and tuber formation, but a causal relation has yet to be proved. The (-)-JA content in leaflets was not significantly different under short and long days. Mild stress applied to detached SD and LD leaflets caused a rapid accumulation of JA in these leaflets. Upon this treatment an increase in the levels of hydroxylated JAs was detected in SD leaflets only. JA was a potent promoter of tuber formation *in vitro* in *S. demissum* explants. Lipoxygenase (LOX; EC 1.13.11.12) is involved in the biosynthesis of JA. Under SD conditions, application of SHAM, an inhibitor of LOX activity, to the roots did not prevent tuber formation *in vivo*. It is suggested that daylength controls the hydroxylation of JA. The enzyme(s), responsible for the hydroxylation of JA, would only be effective under SD conditions.

Introduction

Tuber-bearing *Solanum* species will form tubers under tuber-inducing conditions. Daylength and night temperature are among the most important factors which affect tuber formation in the potato plant. Long nights, referred to as short days in the rest of this paper, and relatively low night temperature (5°C-20°C) will give rise to tuber formation (Ewing 1987).

Grafting experiments were done by Gregory (1956) to investigate whether daylength is perceived in the leaves of the potato plant. He surmised that under short-day conditions a

tuber-inducing factor was synthesized or activated in the leaves and transported basipetally. Whether the principle itself reaches the stolon tip and effectively induces tuber formation or whether a secondary messenger is involved, is not known. Recently a tuber-inducing compound was isolated from the leaves of *Solanum tuberosum* L. cv. Irish Cobbler (Koda *et al.* 1988) and characterized (Yoshihara *et al.* 1989). The structure of the active compound was determined to be 3-oxo-2-(5'-ß-D-glucopyranosyloxy-2'-Z-pentenyl)-cyclopentane-1-acetic acid. The aglycon of this glucoside is 12-OH-jasmonic acid (12-OH-JA), which the authors named tuberonic acid. The purification of this tuber-inducing compound was complicated by a rapid decrease in biological activity during the isolation procedure. Yoshihara *et al.* (1989) explained this decrease by a possible *cis/trans* epimerization at C-7, which is generally known for α-substituted cyclopentanones (Vick and Zimmermann 1979, Miersch *et al.* 1987). Miersch *et al.* (1986) found that (+)-7-*iso*-JA (*cis*-epimer) is more active than JA in different bioassays. Koda *et al.* (1992) confirmed this with regard to potato tuber induction and the senescence promotion of oat leaves using (+)-7-*iso*-JA-Me.

If hydroxylated JAs are indeed responsible for, or at least involved in the process of tuber induction, it is to be expected that these compounds are absent or far less abundant in leaves from potato plants grown under long day than under short-day conditions, provided the experiment is done with an absolutely SD-dependent *Solanum* species. In this paper we present data on the JA and hydroxylated JA contents of leaflets of *Solanum demissum* plants grown under LD and SD conditions. *S. demissum* was preferred to *S. tuberosum* because the latter will form tubers even under 24 h daylength conditions, provided that the night temperature is adequately inductive. *S. demissum* is an absolutely SD-dependent *Solanum* species.

The isolation of a tuber-inducing compound from the leaflets of a potato plant grown under tuber-inducing conditions does not imply that such a compound is causally related to tuber induction. Two approaches were chosen to obtain more insight into the nature of this relationship.

(1) The ability of JA to induce tuber formation *in vitro* in *S. demissum* explants was tested in a bioassay. Only minute amounts of 11- and 12-OH-JA were available. Therefore, the tuber-inducing activity of these substances could not be tested.

(2) If tuber formation is prevented when enzymes involved in the biosynthesis of (hydroxylated) JAs are inhibited, then this would indicate that these compounds are (co-) responsible for tuber formation. Since inhibitors of enzymes catalyzing the hydroxylation of

JA are not known, we chose to inhibit enzymes of JA biosynthesis. Vick and Zimmerman (1983) suggested that lipoxygenase (LOX) initiates the reaction leading to JA. LOXs catalyze the conversion of *cis,cis*-1,4-pentadiene structures in polyunsaturated fatty acids, *e.g.* linolenic acid, into a conjugated hydroperoxide [*e.g.* 13(S)-HPOT]. LOXs are characterized by their insensitivity to cyanide and inhibition by SHAM and n-propyl gallate (Park and Polacco 1989). Vegetative storage protein mRNA in soybean leaf explants was increased by wounding, petiole girdling, and treatment with 10 μM MeJA (Staswick *et al.* 1991). When gene expression of vegetative storage protein was induced by wounding or heat girdling, this could be effectively inhibited by 50 μM SHAM, an inhibitor of LOX activity. Therefore, we chose SHAM to test whether tuber formation under SD conditions could be prevented.

JA and MeJA can act as intra- and interplant signalling-compounds (Farmer *et al.* 1992, Farmer and Ryan 1990, respectively). JA and MeJA can induce the expression of wound-inducible proteinase inhibitor genes in tomato and tobacco leaves (Farmer and Ryan 1990). If JA and MeJA are mediators in plants reaction on stress, it would be interesting to know whether mild stress can induce rapid formation of these compounds. Hence, we examined the effect of a non-destructive extraction treatment, the rinsing of the intercellular spaces of leaflets, on the endogenous concentration of (hydroxylated) JAs in the leaflets of *S. demissum*.

Materials and methods

Plant material. True seeds (BGRC 9981) of *S. demissum* were obtained from the Inst. für Pflanzenbau und Pflanzenzuchtung der FAL (Braunschweig, Germany). Seeds were germinated and seedlings grown in potting soil in a greenhouse. Each plant was considered as a separate clone. From several clones stem parts with an axillary bud were surface sterilized and grown *in vitro* according to Vreugdenhil and Van Dijk (1989). The clones were propagated every 4 weeks on MS-medium (Murashige and Skoog 1962) supplemented with 1.5 % sucrose and 0.8% agar. Plants were grown in culture jars (87 x 85 mm, Fa Meli, Brussels, Belgium) containing 70 ml medium, kept in a growth cabinet (Ecophytschrank VTPH 5/1350, Heraeus Vötsch, Germany) at 16 h daylength and 20°C.

Tuber induction *in vitro*. Single-node subapical cuttings were obtained from 4-week-old *S. demissum* plants grown *in vitro*. In each culture jar 10 cuttings were grown on MS-medium,

containing 6% sucrose and supplemented with 10 mM $CaCl_2$ (Koda and Okazawa 1983). BAP and (±)-JA were added at the concentrations indicated. A solution of (±)-JA (K-salt) was sterilized by filtration through a 0.22 μm filter (Millex-GV, Millipore, France), and added to the medium just before solidification. Explants were kept in the dark at 20°C. After 3 to 4 weeks the effects of the various treatments on tuberization were observed.

Tuber induction *in vivo*. For *in vivo* experiments 2-week-old *in vitro* plants were transferred to potting soil. Initially the plants were covered with wet cheesecloth to prevent wilting. After 4 days the cheesecloth was removed and 60 plants were transferred to a walk-in growth chamber at 20°C. Photosynthetically active radiation (PAR) was supplied by SON-T 400 W AGRO and HPI-T 400 W lamps (Philips, The Netherlands) in a ratio 1:1. Irradiance at plant level was 100 W m^{-2} (400-700 nm). The photoperiod was 16 h.

After 4 weeks *in vivo* growth, PAR period was reduced from 16 to 10 h. Half of the number of plants were grown further under these SD conditions. The day temperature was 20°C, the night temperature 15°C. The other half was exposed to LD conditions. The temperature in LD conditions was the same as that in SD conditions. Only the photoperiod was extended from 10 to 16 h using low-intensity (< 2 W m^{-2}) incandescent lamps. One week after onset of tuber formation in SD plants, leaflets from the compound leaves of SD and LD plants were collected. From each condition 1 kg leaflets was frozen in liquid nitrogen immediately after cutting.

Mild stress was applied to freshly harvested leaflets by the collection of extracellular extracts from these leaflets as described by Rathmell and Sequeira (1974) modified according to Mäder (1976). To facilitate handling, the leaflets were halved in the middle with a razor-blade perpendicular to the central vein. After vacuum infiltration with demineralized water, leaflets were centrifuged for 15 min at 740 g. The activity of malate dehydrogenase (a cytoplasmic marker) in the extracellular extract was lower than 0.15% of the total activity in the leaflets (Struik *et al.* 1987), indicating that the cells were not damaged. From both LD and SD plants 1 kg of mildly stressed leaflets was frozen in liquid nitrogen. Leaf material was freeze-dried and kept at -80°C until further analysis.

Detection and quantification of (-)-jasmonic acid. Extraction of the freeze-dried leaflets and quantification of (-)-jasmonic acid was done by a radioimmunoassay according to Knöfel *et al.* (1990).

Application of an inhibitor of the synthesis of (-)-JA. *S. demissum* plants were grown in pot assemblies which allowed non-destructive observation of roots, stolons and upper plant parts (Krauss and Marschner 1971), modified according to Struik and Van Voorst (1986)) under SD conditions as described above. In this system the stolons grow in a separate compartment filled with a sand/perlite mixture, while the roots hang in a continuously aerated nutrient solution, pH 6.0 (Struik *et al.* 1989).

From the onset of stolon initiation, SHAM was added to the nutrient solution in the concentrations indicated. The nutrient solution was similar to that during initial growth, except for the absence of Fe, which would be chelated by SHAM (Rich *et al.* 1978). To avoid Fe deficiency, Fe-containing nutrient solution (without inhibitor) was given to the plants for 2 days after every 5 days of SHAM application. The same was done to the control plants. After 4 weeks the plants were harvested.

Isolation and detection of (glycosides of) hydroxylated JAs

Extraction and solvent fractionation. Freeze-dried leaflets (100 g) were homogenized (3x) in 200 ml ethanol using an Ultra Turrax homogenizer (Janke & Kunkel KG, Germany) at pH 4.0 and filtered. The combined filtrates were vacuum-evaporated and 100 ml H_2O was added. The pH was adjusted to 8.0 with 1 M NH_4OH. The aqueous solution was extracted with n-hexane (3x 50 ml). The n-hexane layer was discarded, the aqueous layer adjusted to pH 3.0 with 4 N HCl, and extracted with ethyl acetate (3x 30 ml). The aqueous layer was discarded. Acidic compounds in the pooled ethyl acetate fractions were separated by extraction into a saturated $NaHCO_3$ solution (3x 20 ml). The extracted ethyl acetate fraction which contained neutral compounds was discarded. The pH of the saturated $NaHCO_3$ solution was readjusted to 3.0 with 4 N HCl. Thereafter, this aqueous phase was extracted with chloroform (3x 20 ml). The acidic chloroform fractions were dried (Na_2SO_4) and the chloroform was evaporated under vacuum.

Purification of (glycosides of) hydroxylated JAs. The redissolved residue was chromatographed on a 50 ml DEAE Sephadex A-25 (Pharmacia, Sweden) column, with acetate as counter ion, using a discontinuous gradient of acetic acid in 80% methanol (v/v) (Gräbner *et al.* 1976). Acids of the jasmonic acid type will elute from the column using 0.25 N acetic acid in 80% methanol (v/v) (fraction 3+4, Meyer *et al.* 1984). Fractions eluting

with 0.75 N and 1 N acetic acid, known to contain glycosylated jasmonates, were combined, evaporated and the residue hydrolyzed with cellulase (24 h, 37°C, McIlvain buffer, pH 3.0). The mixture was extracted with chloroform and the evaporated extract further separated on DEAE-Sephadex A-25 as described before. After evaporation of the combined fractions further purification was achieved by TLC on silica gel GF_{254} (Merck, Germany) with a solvent system chloroform-ethyl acetate-acetic acid (5:4:1).

Derivatization. The fractions corresponding to hydroxylated jasmonic acids (R_f-values: 0.20-0.25) were methylated with ethereal diazomethane. Trimethylsilylation was done as indicated using either Deriva-sil [BSTFA/TMCS/TSIM /pyridine 3:2:3:10 by volume (Regis, USA)] at 85°C for 15 min or a mixture BSTA/TMCS/pyridine (1:1:0.1 by volume) at room temperature (21°C) for 1-2 h.

Detection. Detection of hydroxylated jasmonic acids by GC-MS - An HP 5890 gas chromatograph equipped with a mass selective detector (Hewlett-Packard model 5970 B) was used. GC was performed on a 25 m x 0.2 mm i.d. cross-linked methylsilicone fused-silica column, film thickness 0.11 μm, phase ratio 450 (HP Ultra 1), with helium as carrier gas (2.5 ml min^{-1}) and splitless injection. The following temperature program was used; injection: 275°C, direct inlet interface: 230°C, ion source: 250°C. The oven temperature was kept at 60°C for 1 min, and increased at 25°C min^{-1} to 110°C (1 min), then at 10°C min^{-1} to 250°C.

Results

Morphological responses

Daylength had profound effects on the morphology of *S. demissum* plants. Plants grown under LD conditions did not form tubers, whereas those in SD did. Plants grown for 3 weeks under SD conditions were shorter than plants in LD (data not shown). In SD plants the angle which petioles make with the main stem was about 90°. In LD plants this angle was approximately 70° (data not shown).

(-)-Jasmonic acid

Both LD and SD leaflets contained relatively small amounts of endogenous (-)-jasmonic acid as compared to a number of other plant species (see Meyer *et al.* 1984). There was no significant difference between the (-)-JA content of LD and SD unstressed leaflets (Tab. 2.1).

Leaflets to which a mild stress, *viz.* the collection of extracellular fluid, had been applied (treated leaflets), showed a significantly higher (-)-JA concentration than control leaflets, both under SD and LD conditions. Apparently the collection of extracellular extracts, which took about 2 h, was enough to more than double the endogenous (-)-JA concentration.

Table 2.1. Concentrations of (-)-jasmonic acid (ng g FW^{-1}) in leaflets of *S. demissum* determined by radioimmunoassay. Treated leaflets are leaflets to which a mild stress, *viz.* the collection of extracellular fluid, had been applied. Data are mean ± standard deviation of 3 determinations.

	SD conditions	LD conditions
Untreated leaflets	10 ± 3	8 ± 3
Treated leaflets	28 ± 7	18 ± 5

Hydroxylated jasmonic acids

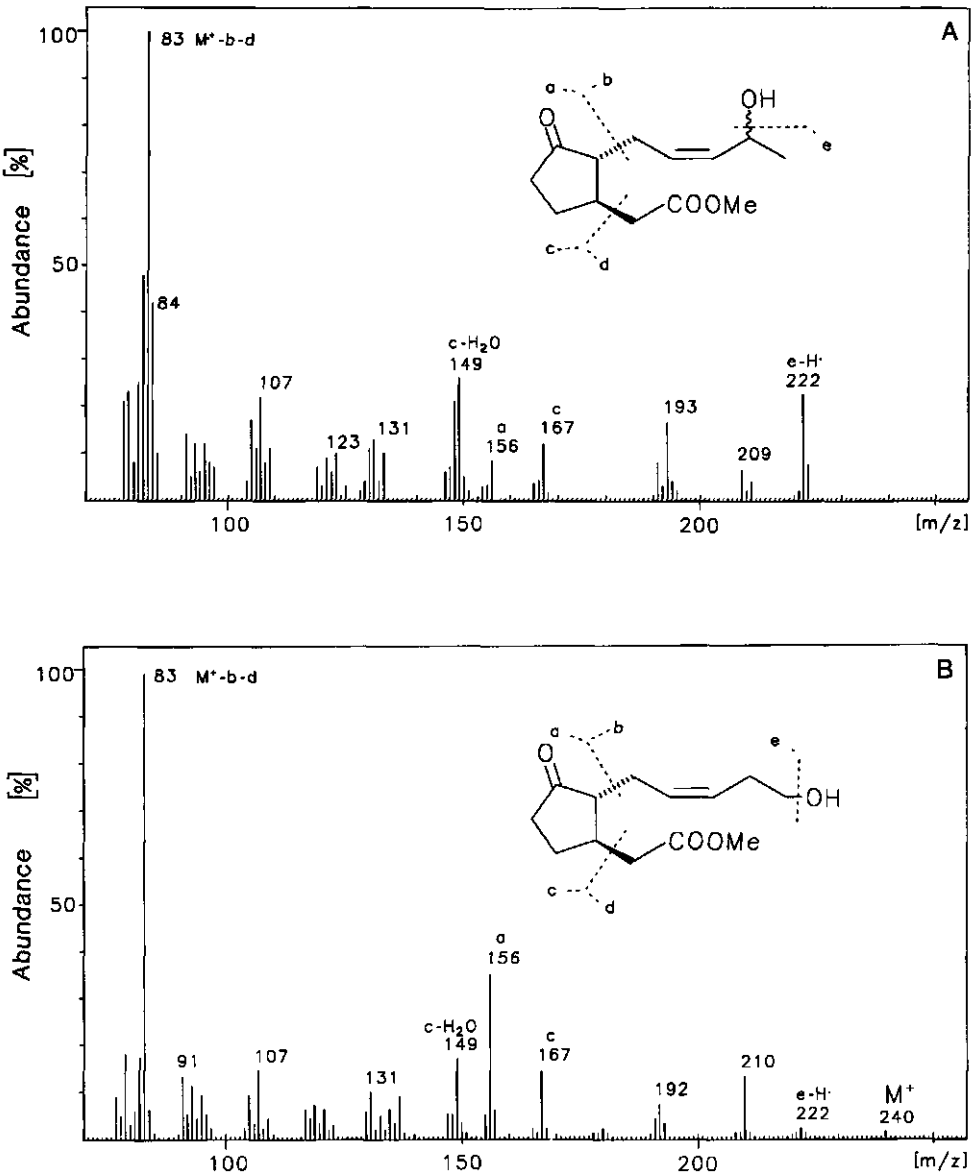
Both 11-OH-JA and 12-OH-JA were detected in SD leaflets. Identification was based on comparison with retention times and mass-spectra of 11-OH-JA and 12-OH-JA earlier isolated from *Botryodiplodia theobromae* (Miersch *et al.* 1991). The mass-spectra of 11-OH-JA-Me (R_t= 12.5 min) and 12-OH-JA-Me (R_t= 14.5) are given in Fig. 2.1.

Contrary to hydroxylated JAs, glucosides of these substances (as found by Yoshihara *et al.* 1989) need to be trimethylsylilated before GC-MS analysis. When 11-OH-JA and 12-OH-JA from *B. theobromae* were trimethylsilylated by Deriva-sil, only 12-OH-JA was detectable by GC-MS. However, both compounds were successfully derivatized using a mild mixture (BSTA/TMCS/pyridine; data not shown).

The 11-OH-JA concentration was higher than the 12-OH-JA concentration in SD leaflets. None of these compounds could be detected in LD leaflets. Similar to what was found for (-)-JA, mild stress induced an increase in the 11- and 12-OH-JA concentration in SD leaflets. In mildly stressed LD leaflets none of these compounds could be detected (Tab. 2.2). No

deuterated hydroxylated JAs were available so we could not quantify the concentrations

Fig. 2.1. Mass-spectra of two hydroxylated JAs isolated from leaflets of S. demissum plants grown under SD conditions. A, 11-OH-JA-Me; B, 12-OH-JA-Me.



exactly. Glycosides of 11- and 12-OH-JA were not detected in either SD or in LD leaflets, as the hydrolysis of the corresponding fraction (see Materials and methods) yielded no free acids.

Table 2.2. Detection of hydroxylated jasmonic acids in leaflets of *S. demissum* by GC-MS. Treated leaflets are leaflets to which a mild stress, *viz.* the collection of extracellular fluid, had been applied.
++ > + > ± > --; -- = not detectable by GC-MS

	SD conditions	LD conditions
Untreated leaflets		
11-OH-JA (1)	+	--
12-OH-JA (2)	±	--
Glycosides of (1) or (2)	--	--
Treated leaflets		
11-OH-JA (1)	++	--
12-OH-JA (2)	+	--
Glycosides of (1) or (2)	--	--

Effect of JA on *S. demissum* explants *in vitro*

No tuber formation was observed when *S. demissum* explants were exposed to a high sucrose concentration. Additions of (±)-JA or BAP to the medium gave rise to some tuber formation. A number of tubers were formed when a combination of (±)-JA and BAP was applied (Tab. 2.3).

Effect of a LOX inhibitor

The tested concentrations of SHAM did not effectively inhibit or reduce tuber formation in

S. demissum under SD conditions (Tab. 2.4). Application of 1 mM SHAM caused severe browning of the roots, and normal growth was impeded.

Table 2.3. Effect of (±)-JA on tuber formation *in vitro* in *S. demissum*. Apart from JA and BAP, the composition of the medium is as described in Materials and Methods. Data are the number of tubers per 10 single-node explants ± standard deviation, n=4.

	(±)-JA concentration, mg l^{-1}	
BAP concentration (mg l^{-1})	0	2
0	0 ± 0	1.8 ± 1.0
2	0.5 ± 0.6	5.3 ± 1.3

Table 2.4. Effect of SHAM on growth and tuber formation in *S. demissum* plants *in vivo*. Data are mean ± standard deviation (n=6 for each treatment).

	Dry weight roots (g)	Dry weight shoot (g)	Number of tubers per plant	Average tuber diameter (mm)
Control	7.7 ± 2.6	25.4 ± 6.3	28 ± 17	6.2
0.01 mM SHAM	7.3 ± 1.2	22.0 ± 1.8	30 ± 9	6.5
0.1 mM SHAM	6.5 ± 0.8	22.0 ± 2.5	32 ± 23	7.2
1.0 mM SHAM	3.5 ± 1.3	7.4 ± 1.8	31 ± 9	6.6

Discussion

This is the first time that 11-hydroxy-jasmonic acid has been detected as a native substance in higher plants. Earlier, it was found to occur in a fungus, *Botryodiplodia theobromae* Pat. (Miersch *et al.* 1991), and to be formed metabolically after application of JA to higher-plant cell cultures (A. Kehlen 1991. Thesis, Martin Luther Univ., Halle-Wittenberg, Germany).

We found a clear positive correlation between tuber formation and the occurrence of 11- and 12-OH-JA in leaflets of a *Solanum demissum* clone. *S. demissum* is, with respect to tuber formation, an absolutely SD-dependent *Solanum* species, never forming any tubers under LD conditions. Hydroxylated JAs could only be isolated from SD leaflets (both control and stressed), whereas JA occurred both in LD and in SD leaflets. This indicates that hydroxylated JAs, rather than JA itself, might play a role in tuber formation.

Two distinct differences could be seen between the data from *S. tuberosum* (Yoshihara *et al.* 1989) and our results on *S. demissum*:

(1) 11-OH-JA was more abundant than 12-OH-JA in SD leaflets of *S. demissum*. Yoshihara *et al.* (1989) did not detect (glycosides of) 11-OH-JA in leaves of *S. tuberosum*; and

(2) glucosides were not detected in SD leaflets of *S. demissum*, whereas a glucoside of 12-OH-JA was the active compound isolated from *S. tuberosum* leaves by Yoshihara *et al.* (1989).

A question arises concerning the reason for these two discrepancies. First, it cannot be excluded that the occurrence of 11-OH-JA is species-dependent, although this seems unlikely. Metabolic studies of [2-^{14}C]-(±)-JA and [2-^{14}C]-9,10-dihydro-JA, fed to 6-day-old barley seedlings, revealed that in both cases hydroxylation preferentially took place at C-11 and to a lesser extent at C-12 (Sembdner *et al.* 1990). The discrepancy might also be caused by technical problems. Allylic groups, as in 11-OH-JA, are known to be very reactive. Rearrangements and different chemical modifications are to be expected (Miersch *et al.* 1991), which result in notable losses during isolation or derivatization procedures. Hence, (glycosides of) 11-OH-JA should be isolated using an internal standard and have to be trimethylsylilated under mild conditions. We did not have deuterated hydroxylated JAs to quantify the losses, but when 11-OH-JA was trimethylsylilated under mild derivatization conditions, the losses were clearly reduced. Considering these facts we assume that 11-OH-JA may play a more important role than 12-OH-JA in tuberization.

Secondly, glycosylation is generally known in plants as a means of making nonpolar substances more polar in order to facilitate transport. The reason why no glycosides were detected in SD leaflets of *S. demissum* is not known. An explanation could be that in this case hydroxylated JAs are only glycosylated just before phloem loading and export out of the leaves.

It is not known whether there is a causal relation between the occurrence of hydroxylated JAs and tuber formation. Hydroxylated JAs are metabolites of JA. No inhibitors of JA

hydroxylating enzyme(s) are available, therefore we have tried to prevent tuber formation under tuber-inducing conditions indirectly, using an inhibitor of LOX activity. The application of SHAM had no apparent effect on tuber formation. SHAM (1 mM) was not compatible with normal plant growth in *S. demissum*. Parrish and Leopold (1978) found that 5 mM SHAM inhibited LOX activity from soybean by 90%. On the other hand Staswick *et al.* (1991) fed 50 μM SHAM to petioles of soybean leaves for 24 h, and thus prevented the synthesis of vegetative storage protein mRNA. The lack of observable inhibition of tuber formation by SHAM does not necessarily rule out a role for LOX in the synthesis of an inducing compound. A low diffusion rate of SHAM to the intracellular site of JA biosynthesis could account for the lack of inhibition. Definitive inhibitor studies will have to await the identification of highly specific, translocatable inhibitors of enzymes of the lipoxygenase pathway.

Endogenous (-)-JA and (+)-7-*iso*-JA are found in many plant species. The physiological function of these and related compounds is not yet established. In many aspects the role of these compounds is apparently similar to that of ABA (Parthier *et al.* 1992, and literature cited therein). ABA is known as a stress-related, probably stress-mediating phytohormone (Skriver and Mundy 1990). JA apparently has similar characteristics. The rinsing of the intercellular spaces with water, which can be considered as a mild stress, induced a rapid increase in the (-)-JA concentration in both SD and LD leaflets of *S. demissum*. A function for this rapid synthesis could be the JA-induced expression of proteinase inhibitor encoding genes. Proteinase inhibitors can be considered as part of the defense system of plants. Farmer *et al.* (1992) showed that application of JA to leaves of young tomato plants led to a systemic induction of proteinase inhibitor I. Ewing (1987) stated that there is no clear relation between endogenous ABA concentrations and tuber induction. Droopy, an abscisic acid deficient mutant of *S. tuberosum* L., group *Phureja*, does form tubers (Quarrie 1981). If indeed there is a causal relation between tuber formation and the occurrence of 11-OH-JA and 12-OH-JA, this would be an example of a function for JA metabolites, but not JA, that in plants is unrelated to the physiological role of ABA.

On the basis of the observation that mild stress caused a rapid accumulation of JA both in SD and in LD leaflets, and that hydroxylated JAs accumulate in stressed SD leaflets, one might predict that the levels of hydroxylated JAs would increase in stressed LD leaflets. However, in stressed LD leaflets no hydroxylated JAs were detectable. Therefore, LD leaflets may at most contain very low concentrations of 11- and 12-OH-JA. If these

compounds are involved in tuber formation, the JA-hydroxylating enzyme(s) likely play a key role in this process.

Acknowledgements. I am indebted to Mrs. C. Gebhardt, Mrs. E.-M. Schneider and Dr. C. Brückner for the radioimmunological determinations of JA.

Chapter 3

STOLON CHARACTERISTICS AND TUBER INITIATION IN A WILD POTATO SPECIES (*SOLANUM DEMISSUM* LINDL.)

Summary

Solanum demissum Lindl. is a short-day dependent, tuber-bearing species. *In vitro* propagated genetically identical plants were grown under long-day (16 h) conditions at 20°C day and 15°C night temperatures. Subsequently, from day 0 onwards, plants were exposed to short-day (10 h) conditions and grown in a multi-compartment system that allowed stolon initiation, stolon branching, stolon growth and tuber initiation to be monitored twice weekly. These frequent measurements resulted in detailed life-histories of all 841 stolon tips formed until day 46. Tuber initiation was irregular under these well-defined conditions; at day 46 only 106 stolon tips showed subapical swelling. This irregularity of tuber initiation was also observed within stolons. It is hard to conceive how a tuber-inducing factor produced in the leaves could only reach a selected number of tips within a stolon, therefore it is suggested that actual tuber initiation depends on characteristics of the stolon or stolon branch itself. Several such characteristics were analyzed to obtain information on their potentially determining role in tuber initiation. No positive correlations were found between subapical swelling and branching order, stolon and stolon-branch age, longitudinal growth rates of stolons and stolon branches, and attachment of the stolon to the main stem.

Introduction

Tuberization in potato plants is a continuous process. Developmental stages preceding tuber initiation, *viz.*, stolon induction, stolon growth, cessation of the longitudinal growth and tuber induction, and tuber initiation itself, may occur simultaneously within an individual plant (for definitions of the developmental stages, see Vreugdenhil and Struik 1989). Hence, when a potato plant without tubers is exposed to conditions which favour tuber initiation, not all the available stolon tips will start to tuberize at the same time. To obtain insight into this

intriguing irregularity, it is useful firstly to consider tuber induction.

Short days (SD; in fact long nights) are tuber-inducing in tuber-bearing *Solanum* species, provided that the night temperature is adequately low. Daylength is detected in the leaves of the potato plant. Under SD conditions a tuber-inducing factor is probably synthesized or activated in the leaves and transported basipetally via the phloem (Gregory 1956). Substances that might be involved in tuber induction *in planta* have been isolated from the leaves of tuberizing potato plants and characterized as (glucosides of) hydroxylated jasmonic acids (Yoshihara *et al.* 1989, Chapter 2). However, it was not shown that these compounds are responsible for tuber induction.

A tuber-inducing compound from the leaves will be transported basipetally but only some of the stolon tips will start to swell subapically. There are two ways to explain the irregularity of tuber initiation. Firstly, a tuber-inducing factor reaches a restricted number of stolon tips and tubers are formed at these tips only. Chapman (1958) suggested that in *Solanum tuberosum* L., cv. Triumph, a tuber-inducing factor is produced only in certain leaves which correspond with certain stolons. This implies that the movement of a stimulus via the phloem is predominantly basipetal with little or no lateral movement. Studies on the vasculature of the stem of two *S. tuberosum* cultivars, Irish Cobbler and Russet Burbank, do not support a very restricted lateral movement of some compound(s) in the phloem. In both cultivars the stem contains only three large and three small vascular bundles. Moreover, the vascular system in the potato stem is highly interconnected (Artschwager 1918, McCauley and Evert 1988). On the other hand, assimilates exported from one source leaf of a single-stem potato plant entered two of the three main vascular bundles in the stem, and remained confined to the same side of the stem (Oparka and Davies 1985). The possibility of lateral movement within a stem does not necessarily implicate a completely even distribution of assimilates over the vascular bundles. Nevertheless, it is hard to conceive how a single vascular bundle of an induced sprout could be free of a tuber-inducing signal, when a compound exported from one leaf will enter two of the three available main vascular bundles.

A second possibility is that a tuber-inducing factor reaches every stolon tip, but tubers are only formed at tips where the signal-transduction chain is able to register the signal and to trigger tuber initiation. This explanation is more likely; it presumes that characteristics of the stolon or stolon branch itself determine whether a tuber is initiated or not under tuber-inducing conditions. The ability to register a signal presumes an adequate number of sensitive, accessible receptors. Since the tuber-inducing factor still has to be identified,

nothing is known about a signal-transduction chain. The ability to trigger tuber initiation might depend on certain preconditions. One of these could be a relatively low longitudinal growth rate of the stolon or stolon branch. Factors favouring longitudinal growth of stolon and stolon branches, *viz.*, a continuous supply of nitrogen or a high gibberellin activity, inhibit or delay tuber formation in plants growing under tuber-inducing conditions (Krauss and Marschner 1982).

Explanations for the irregularity of tuber initiation focus mainly on differences in longitudinal growth rates in stolon and stolon branches. Two potential mechanisms were presented by Vreugdenhil and Struik (1989):

1) Different levels of gibberellin activity, caused by, *e.g.*, unequal distribution of gibberellins from the mother tuber, might lead to variation in the rate of longitudinal growth of individual stolon branches. Tuber initiation will only occur in stolon tips with a sufficiently low gibberellin activity.

2) Different quantities of ethylene might be formed in elongating stolons due to the variation in the mechanical resistance of the soil. Mediated by ethylene, a relatively high mechanical resistance in the soil would slow down the longitudinal growth of individual stolon branches, and thereby meet a precondition for tuber initiation.

In this report we present data on the history of all individual stolons and stolon branches from plants of the wild potato species *Solanum demissum* grown under SD conditions. *S. demissum* is absolutely SD-dependent for tuber induction. Under long-day (LD) conditions (16 h daylength) no tuber formation was observed (Helder, unpublished results). Since the plants were pre-grown under LD conditions, the experiment could be started with completely uninduced plants. This would have been difficult with *S. tuberosum* cultivars, since, *e.g.*, Bintje and Désirée form tubers even under 24 h daylength, provided that the night temperature is sufficiently low.

Stolon characteristics were monitored to see whether there was any correlation between these characteristics and actual swelling of potential tuber sites. Stolon initiation, stolon branching, stolon growth and tuber initiation were observed under tuber-inducing conditions to test whether subapical swelling can occur in stolon tips of a certain branching order, or of a certain age, or in stolon tips with a reduced longitudinal growth rate, or in a certain position in relation to the stem.

Materials and Methods

Plant material. True seeds (BGRC 9981) of *Solanum demissum* were obtained from the Institut für Pflanzenbau und Pflanzenzüchtung der FAL (Braunschweig, Germany). One single clone was used in all experiments. This clone was propagated *in vitro* as described in Chapter 2 (p. 15).

***In vivo* growth and tuber induction.** Three-week-old *in vitro* plants were transferred to potting soil. The plants were initially covered with wet cheesecloth to prevent wilting. After 4 days the cheesecloth was removed and the plants were grown in a glasshouse for 2 weeks. Thereafter, six plants were transferred to a walk-in growth chamber. Photosynthetically active radiation (PAR) was supplied by SON-T 400 W AGRO and HPI-T 400 W lamps (Philips, The Netherlands) in a ratio of 1:1. The light intensity at plant level was 100 $W.m^{-2}$ (400-700 nm). The photoperiod was 16 h. The day temperature was 20°C and the night temperature 15°C.

After 4 weeks *in vivo* growth the PAR-period was reduced from 16 to 10 h. The plants were first exposed to SD conditions on day 0. The day and night temperatures were kept at 20°C and 15°C, respectively. This implies that the duration of the 20°C-period was shortened under SD conditions.

On day 0, before stolon formation had started, *S. demissum* plants were removed from the potting soil and further grown hydroponically in pot assemblies, allowing non-destructive observation of roots, stolons and upper plant parts (Krauss and Marschner (1971), modified according to Struik and Van Voorst (1986)). In this system the stolons grow in a separate compartment filled with a mixture of sand and perlite (ratio 1:1, v/v). Variation in the resistance of the soil encountered by individual stolons was reduced by the use of this homogeneous, low-resistance stolon medium. The roots hang in a continuously aerated nutrient solution (pH 6.0; Struik *et al.* 1989).

The stolon medium could easily be removed by a vacuum cleaner to allow observation of stolon initiation, branching, growth, and subapical swelling. The exposure of stolon and tubers to the light and open-air conditions was kept as short as possible and the sand/perlite mixture was renewed after each observation. Until day 46 the length and the thickness of each stolon branch, 3 cm behind the apex, were measured twice a week (except for the last week, day 39-46, in which one measurement was done) using a ruler and a pair of calipers.

When a stolon branch was observed for the first time, it was considered to be initiated in the middle of the preceding inter-observation period.

During the experiment the plants had a single stem, for other stems as well as sprouting axillary buds were removed. At day 46 the shoots of the plants were removed and the fresh and dry weights of the individual shoots were determined.

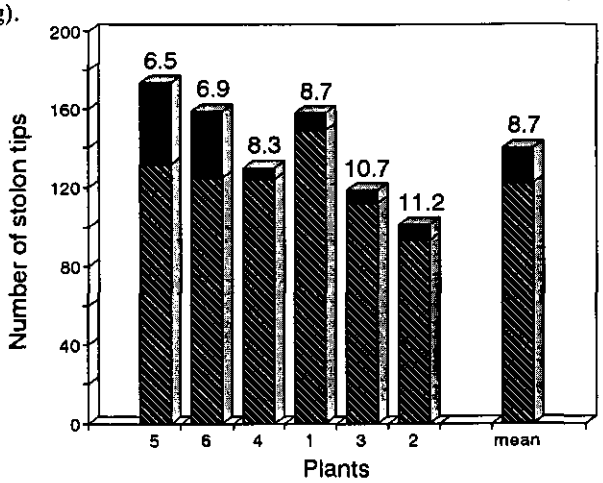
Results

General observations

When plants were transferred to tuber-inducing conditions at day 0, a few, heavily branched stolons were formed on each plant (average ($\bar{x}$) $\pm$ standard deviation (s.d.); 4 $\pm$ 1), which resulted in a large number of potential tuber sites (*i.e.* stolon tips). At day 46 the life histories of 841 individual stolon tips from six plants was known. The average number of potential tuber sites per plant was 140 $\pm$ 28. The negative correlation between the number of stolon tips and the dry weight of the shoot (Fig. 3.1) is noteworthy.

From the large population of stolon tips in *S. demissum*, 106 tips were swollen subapically at day 46. There was a considerable variation in the number of tubers formed per

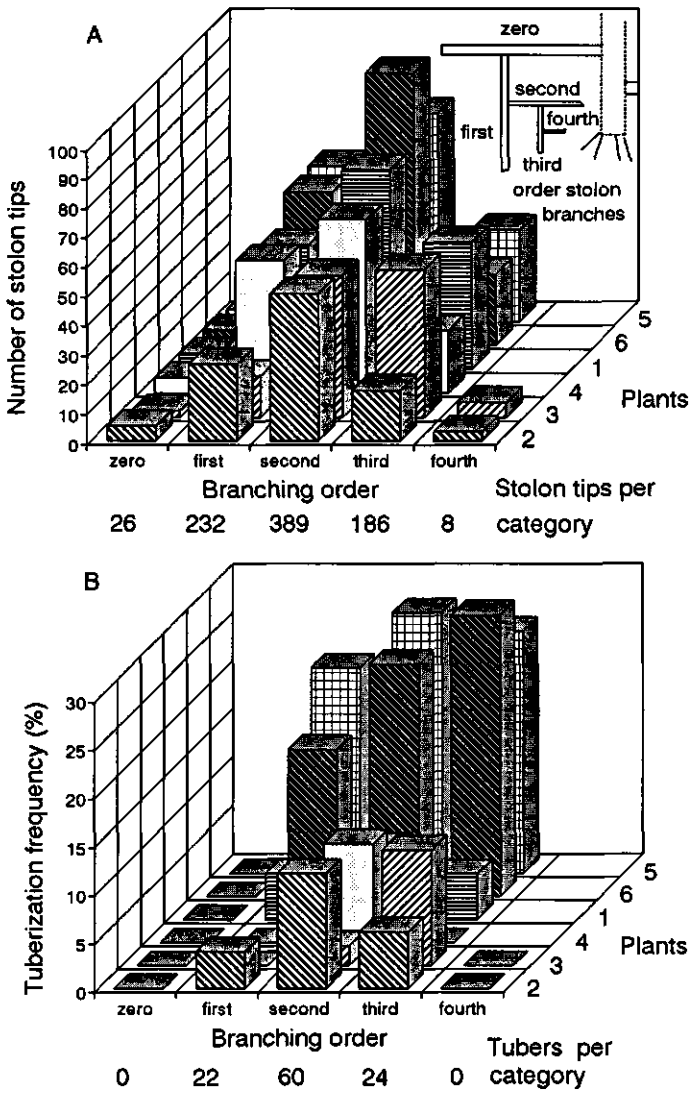
Figure 3.1. The number of stolon tips formed per plant at day 46. The length of the whole bar represents the total number of stolon tips. The fractions of stolon tips which were subapically swollen are indicated by black shadings, the non-swollen fractions by hatched shadings. The numbers above each bar indicate the shoot dry weight (g).



plant ($\bar{x} \pm$ s.d.; 18 $\pm$ 16). With one exception, all the tubers were initiated between days 39 and 46.

Tuber initiation was irregular; even within one stolon some stolon tips started to swell subapically whereas others, which were apparently identical, did not. We investigated

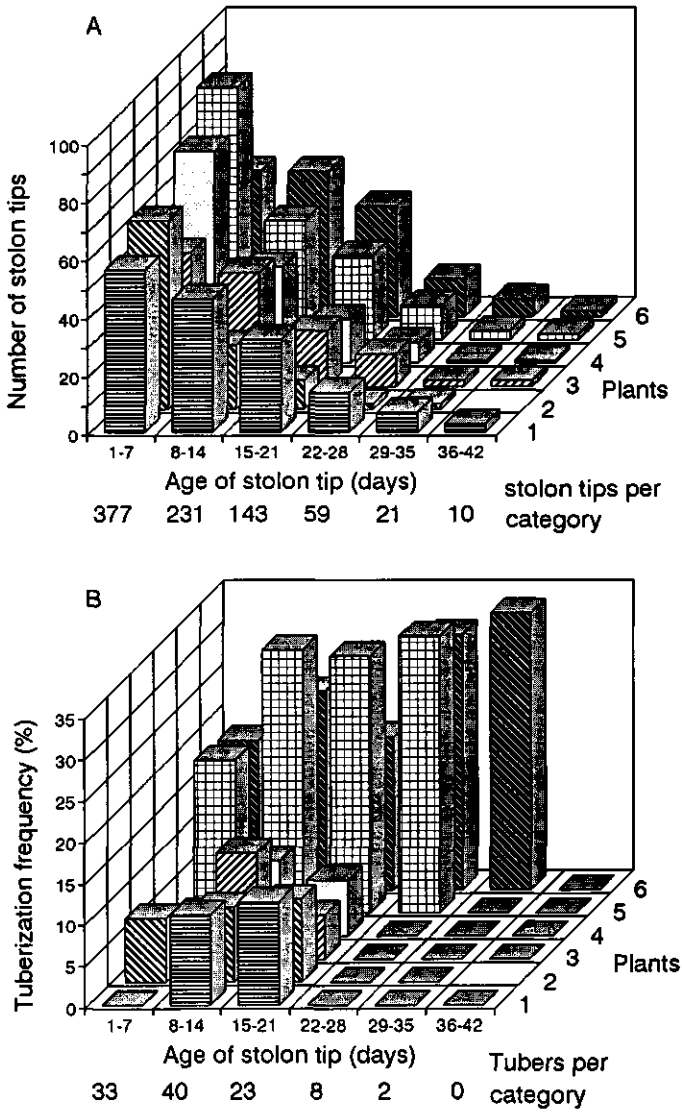
Figure 3.2. A. Number of stolon tips formed per branching order for each *S. demissum* plant at day 46. The different branching orders are defined in the upper right corner. B. Tuberization frequencies per branching order for individual plants.



whether some stolon characteristics could be correlated with this phenomenon.

Twice a week stolons were exposed to dim light and air for a short time. Changes in the pale colour of stolons and stolon branches can be expected when they are exposed too long to light, or when they are damaged. However, no changes were observed in the colour

Figure 3.3. A. Number of stolon tips formed per age category for individual plants at day 46. B. Tuberization frequencies per age category for individual plants.



of the stolons and stolon branches during the experiment.

Effect of branching order on tuber initiation

The potential correlation between branching order and the proportion of stolon tips producing a tuber (named 'tuberization frequency' after Svensson 1962) was investigated. Definition of the branching orders is given in Fig. 3.2A. The stolon tips were far from evenly distributed over the different branching orders. In these *S. demissum* plants, the relatively modest apical dominance resulted in extensive branching of the stolons. The apical dominance was also modest in the first-order branches and a steep increase of the number of first- and second-order stolon tips was observed (Fig. 3.2A).

Tuberization frequencies at day 46 are shown in Fig. 3.2B. The lack of subapical swelling in zero-order branches is noticeable. No clear differences in tuberization frequencies were observed between branching orders 1, 2 and 3.

Effect of age of a stolon tip on tuber initiation

The age distribution of the stolon tips per plant at day 46 is shown (Fig. 3.3A). The uneven distribution of the stolon tips over the age categories is an indirect result of the modest apical dominance. There is no prove that stolon tips of *S. demissum* have to reach a certain minimum age before tuberization can take place. On the contrary, stolon tips belonging to the oldest category did not form any tuber (Fig. 3.3B). The variation in tuberization frequency within the age categories was considerable. Tuberization frequencies in stolon tips of the youngest age category were remarkable; also very young stolon tips can start to swell subapically (Fig. 3.3B). The idea that tuber initiation occurs preferentially in older, mature stolon tips should be rejected.

Effect of longitudinal growth rate on tuber initiation

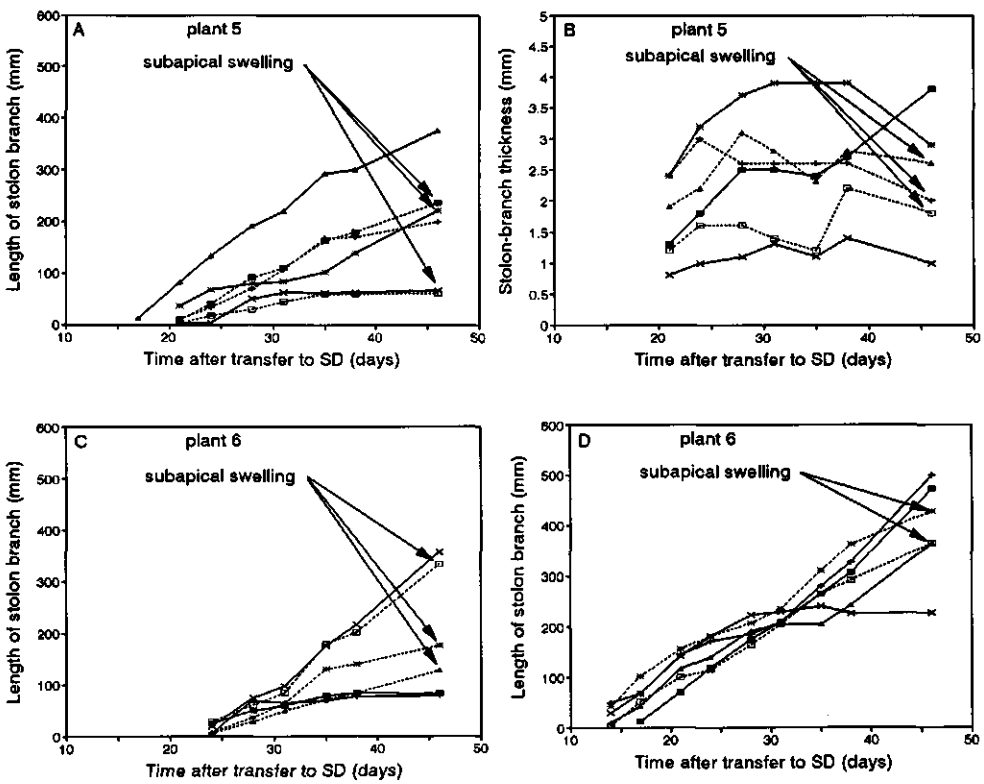
The length of the stolon branches was monitored twice weekly and this resulted in growth curves for individual stolon branches. After 46 days exposure to SD conditions, curves were obtained for subapically swollen and non-swollen stolon branches. This allowed an intra-plant comparison between growth characteristics of these two types of stolon branches.
Figure 3.4 shows growth curves of stolon branches from two plants. No clear correlation was

found between longitudinal growth and subapical swelling. A stolon branch with a strongly reduced longitudinal growth may form a tuber, as well as a stolon branch with a non-reduced growth (Fig. 3.4A). No correlation was found between thickness of the stolon branch (or changes therein) and subapical swelling (Fig. 3.4B). Growth curves of stolon branches of two age categories, 22 and 32 days old, from plant 6 are shown in Figs. 3.4C and 3.4D. Again no correlation was found between growth rates and tuberization.

Effect of stolon position on tuber initiation

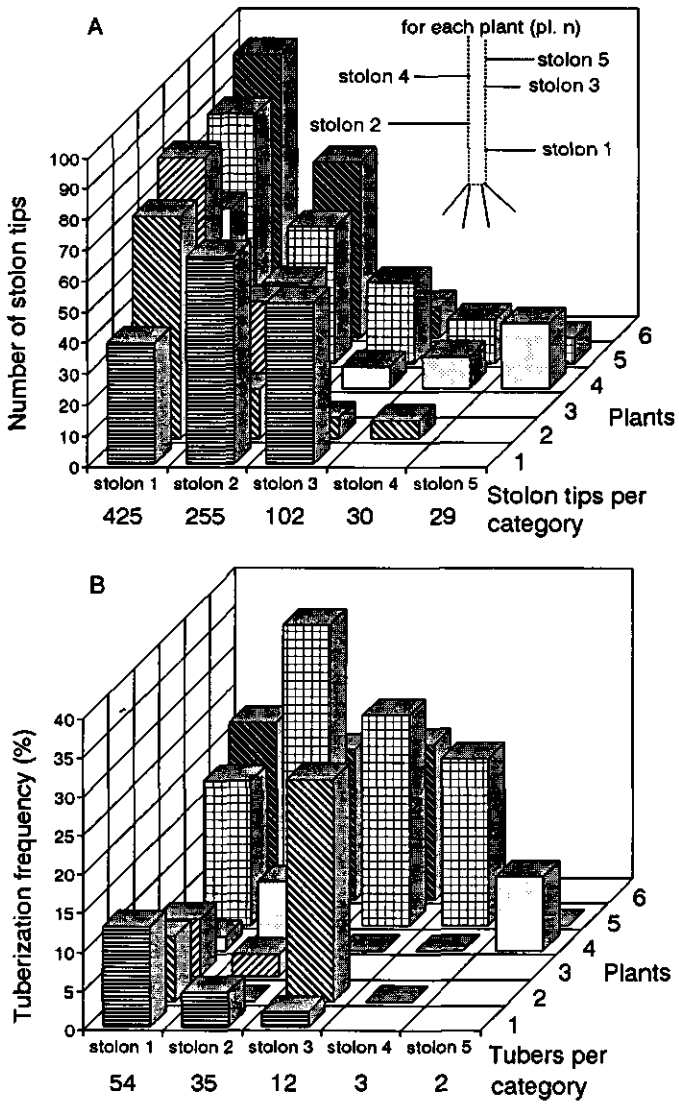
Stolon initiation started at the basal end of the stem and progressed acropetally. As a

Figure 3.4. A, C and D. Growth curves of three times 6 randomly chosen stolon branches of plants 5 and 6. B. Thickness of 6 randomly chosen stolon branches from plant 5. Stolon branches depicted in C developed later than those in D. Arrows indicate which of the stolon branches were subapically swollen at day 46.



consequence these older basal stolons had, on average, a larger number of stolon tips. Except for plant 1, a decreasing number of stolon tips was observed in stolons attached at a higher position on the stem (Fig. 3.5A). No clear correlation was observed between the relative position on the stem and the tuberization frequency (Fig. 3.5B).

Figure 3.5 A. Number of stolon tips in relation to the position of the stolon on the stem for individual *S. demissum* plants at day 46. B. The tuberization frequencies in relation to the position of the stolon on the stem for individual plants.



Discussion

The stolonization patterns described differ from the patterns found by Lovell and Booth (1969) for *S. tuberosum* cv. Majestic. The latter formed more stolons and did not show the extensive branching observed in *S. demissum*. Under identical SD conditions, *S. tuberosum*, cv. Désirée, showed less branching than *S. demissum* (data not shown). This could be a result of a stronger apical dominance in *S. tuberosum* plants, or of the difference in starting material. *S. demissum* was grown from *in vitro* explants, whereas seed-tubers were the starting material for cv. Désirée.

Both the irregular mechanical resistance of the soil and the uneven distribution of plant hormones from the mother tuber are thought to be involved in the irregularity of tuber initiation. In this experiment the first factor was strongly reduced by the use of a homogeneous medium with a low mechanical resistance, and the second factor was circumvented by the use of *in vitro* multiplicated plantlets as starting material. Nevertheless, tuber initiation in *S. demissum* was irregular under tuber-inducing conditions.

If the stem vasculature of *S. demissum* is similar to that of the *S. tuberosum* stem, which is very likely, a tuber-inducing factor should be present in all stolon tips. Hence, the irregularity must be explained in terms of differences in the ability of the signal-transduction chain to perceive and translate the presence of this compound into actual tuber initiation. Apart from the ability to monitor the presence of a tuber-inducing substance, about which virtually nothing is known (see Introduction), competition for assimilates might co-determine whether or not a certain stolon tip develops into a tuber. When starch synthesis was almost completely inhibited in tubers and partially in the leaves by the anti-sense inhibition of ADP-glucose pyrophosphorylase in transgenic *S. tuberosum*, cv. Désirée, the transgenic plants formed 41-91 tubers per plant, whereas only 8-16 tubers were formed in control plants (Müller-Röber *et al.* 1992). The authors suggested that starchless, sucrose-containing tubers represented weaker sinks than starch-containing tubers, and were therefore not able to prevent the formation of other tubers on the same stem.

Irrespective of whether or not competition for sucrose is decisive for the site and the number of tubers initiated, we wished to study tuber initiation in a mildly competitive environment. After 6 weeks exposure to SD conditions the average fresh weight of the tubers from *S. demissum* was 150 mg. The average number of tubers formed per plant was 18, and the tubers were, with one exception, less than one week old. The total sink size was small

during the onset of tuber initiation in *S. demissum*, the phenomenon we focused on in this experiment, and hence the competition for assimilates was relatively mild.

No clear positive correlation was found between subapical swelling in stolon tips from *S. demissum* and branching order, stolon and stolon-branch age, longitudinal growth rates of stolons and stolon branches, and attachment of the stolon to the main stem. The absence of a correlation between longitudinal growth and tuber initiation in *S. demissum* is remarkable, since differences in longitudinal growth rates in stolon branches, mediated by GA and ethylene activities, are used to explain the irregularity of tuberization (Vreugdenhil and Struik 1989). Considering the more or less oval shape of a 'normal' potato tuber, the longitudinal growth of a stolon branch should be reduced during tuber initiation and growth. However, the high longitudinal growth rates observed in stolon branches of *S. demissum* did not interfere with tuber initiation. Presumably the reduction in longitudinal growth occurs just before the start of subapical swelling. If so, the inter-observation period was apparently too long to register this final growth rate reduction.

None of the simple, non-destructive measures that were used to characterize stolons and stolon branches from *S. demissum* could be positively correlated with tuber initiation within a population of potential tuber sites. Some hypotheses concerning why some stolon tips start to swell subapically can clearly be rejected. Tuber initiation in *S. demissum* depends on metabolic and hormonal conditions in stolons or stolon branches, which are insufficiently reflected in external characteristics of these stolons or stolon branches to indicate the change of longitudinal growth into radial growth.

Chapter 4

MONO- AND DISACCHARIDES IN STOLON TIPS OF THE WILD POTATO SPECIES *SOLANUM DEMISSUM* GROWN UNDER LONG- AND SHORT-DAY CONDITIONS

Summary

An absolutely short-day dependent tuber-bearing *Solanum* species, *S. demissum* Lindl., was exposed to short-day (SD) or long-day (LD) conditions. Under these conditions, the mono- and disaccharide contents of stolon tips were monitored by high pH anion exchange chromatography with pulsed electrochemical detection and by enzymatic sugar analyses. The dry-matter (DM) percentage was presented as a novel measure to indicate early stages of development of stolon tips. Glucose and fructose were the main monosaccharides detected in non-tuberizing and tuberizing stolon tips. Subapically non-swollen LD and SD stolon tips both had glucose concentrations of about 170 mg g^{-1} dry matter. The fructose levels of these stolon tips were 90 and 54 mg g^{-1} dry matter, respectively. With an increasing DM percentage, SD stolon tips showed a gradually decreasing glucose concentration. In SD stolon tips with a DM percentage between 9 and 12, the fructose level dropped rapidly and remained low during further tuber development. The only detectable disaccharide in the stolon tips was sucrose, at a concentration of about 8 mg g^{-1}. In early developmental stages, no changes in the sucrose level were observed in stolon tips.

The Michaelis constant of hexose kinases from stolons at pH 8.0 for glucose was 44 μM, the apparent Michaelis constant for fructose 127 μM. The V_{max} for glucose phosphorylation is about one-tenth of the apparent V_{max} for the substrate fructose. In subapically non-swollen stolon tips, both the relatively high fructose concentration and the glucose to fructose ratio of 1.9-3.1 indicated that sucrose is probably not hydrolysed in the cytosol, but in a compartment where fructose (and glucose) is spatially separated from hexose kinase activity.

Introduction

In potato, the developmental change from a longitudinal growing stolon tip into a tuber is

mainly regulated by daylength and night temperature. Long nights and a relatively low night temperature favour tuber formation (for recent review see Ewing and Struik 1992). A mere extension of the dark period can cause a dramatic change in the partitioning of assimilates. Consequently, shoot growth slows down, and a major part of the photosynthate is directed towards the developing tubers.

Under tuber-inducing conditions, major changes are to be expected in the turnover of soluble, neutral sugars in stolon tips developing into a tuber. Tuberization coincides with the accumulation of assimilates. Sucrose is the form in which the photosynthates are transported to the developing tuber (Oparka 1986, and literature cited therein). The imported photosynthates are osmotically inactivated by starch formation. This guarantees the feasibility of a prolonged photosynthate import. Tuber-inducing conditions cause an increased sucrose import into stolon tips that are developing into a tuber.

Only two types of enzyme can metabolize sucrose, invertase (EC 3.2.1.26) and sucrose synthase (EC 2.4.1.13). In non-tuberizing stolon tips of *Solanum tuberosum* L. sucrose synthase activity is not detectable (Sung *et al.* 1989). Hence, sucrose is presumably split by an invertase. Young developing tubers of the *S. tuberosum* cultivars Kennebec and Norchip have a high level of sucrose synthase activity and low invertase activity (Pressey 1969). Therefore, it is likely that tuber initiation coincides with an enzyme shift. Such a shift should be reflected in the glucose content of developing tubers. The glucose concentration should decrease in developing tubers since the glucose moiety of sucrose is, in case of invertase, released as glucose, whereas, in case of sucrose synthase, UDP-glucose is released.

As far as is known, no data have been published on the comparison of sugar levels in stolon tips between induced and non-induced potato plants of the same age. Some data on sugar concentrations in developing tubers of induced plants are available.

A) The sucrose level was relatively low in developing tubers of *S. tuberosum*, cv Maris Piper, and did not significantly differ from sucrose concentrations in stolons (Davies 1984).

B) A decrease in glucose concentration was reported in developing tubers of *S. tuberosum* cv Maris Piper (Davies 1984). No changes in the glucose level have been observed by Morrell and ap Rees (1986) in developing tubers of the *S. tuberosum* cultivars King Edward, Maris Bard and Pentland Javelin.

C) Davies (1984) found that tuber initiation was accompanied by a drastic decrease in the fructose concentration. The possibility that fructose was preferentially used in the synthesis of fructans was excluded. Davies and Oparka (1985) analyzed the hexokinase (EC

2.7.1.1) activity in tuber extracts of the *S. tuberosum* cultivar Maris Piper. The high glucose to fructose ratio in young tubers was explained by the preference of a raw tuber extract containing hexose kinases to phosphorylate fructose rather than glucose. No rationale was given for the change of this ratio upon the onset of tuberization.

Our interest is the effect of daylength on carbohydrate metabolism in stolon tips and developing tubers. Cultivars of *S. tuberosum* adapted to the temperate zone are not appropriate for this purpose since, *e.g.*, the cultivars Bintje and Désirée form tubers even under 24 h daylength conditions (Helder, unpublished data). *S. demissum* is an absolutely SD-dependent species. This allows an adequate control in which tubers are absent under LD conditions. In this report we compare, both qualitatively and quantitatively, the mono- and disaccharide contents of stolon tips and developing tubers from induced and non-induced *S. demissum* plants. High pH anion exchange chromatography with pulsed electrochemical detection (HPAEC-PED) was used mostly for the qualitative analysis of neutral sugars in individual stolons and small tubers. Enzymatic sugar analyses of individual stolon tips were performed to confirm the HPAEC-PED data, and to further quantify the changes observed.

Materials and Methods

Plant material. True seeds (BGRC 9981) of *S. demissum* were obtained from the Institut für Pflanzenbau und Pflanzenzüchtung der FAL (Braunschweig, Germany). One single clone was used in all experiments. This clone was propagated *in vitro* as described in Chapter 2 (p. 15).

Tuber induction and sampling. After 4 weeks *in vivo* growth in a walk-in growth chamber, the PAR period was reduced from 16 h to 10 h. Half of the plants were further grown under LD conditions. The other plants were exposed to SD conditions. Climatal conditions were as described in Chapter 2, p. 16. From the day the PAR period was reduced onwards, a limited number of LD and SD stolon tips (length $\approx$ 2 cm) were collected every other day. Furthermore, stolon tips of two sets of six plants each were harvested after three and six weeks of exposure to SD conditions. All harvests were done 6-7 h after the start of the PAR period. Immediately after collection, the stolon tips were frozen in N_2 (l), and freeze-dried. After freeze-drying the samples were stored at -80°C until further analysis.

Extraction of the stolon tips. Individual, lyophilized stolon tips were again frozen in liquid

N_2, and ground to powder. Homogenized stolon tips were essentially extracted as described by Foley *et al.* (1992). After addition of 1 ml methanol:water (4:1, v/v), the samples were heated for 10 min at 75°C. Subsequently, the solvent was removed by vacuum centrifugation, followed by over-night freeze-drying. Sample extraction was done in H_2O, adding 50 μl H_2O per mg DM. Undissolved residue was removed by centrifugation for 20 min in an Eppendorf table centrifuge at maximal speed. For neutral sugar analyses by HPAEC-PED the supernatant was diluted 256 times in water.

Qualitative sugar determinations by HPAEC-PED

Chromatography. The system used for high pH anion exchange chromatography with pulsed electrochemical detection (HPAEC-PED) consisted of a Dionex (Sunnyvale, USA) BioLC gradient pump module and a pulsed electrochemical detector (PED). Sample injection was via a Spectra Physics (San Jose, USA) autosampler (SP 8880) equipped with a 20 μl sample loop. The Rheodyne injection valve on the autosampler was equipped with a Tefzel rotor seal to withstand the high pH of the eluents. Inserts (0.1 ml) were used in the autosampler vials. Neutral sugars were chromatographed on a CarboPac PA1 (4 x 250 mm) column and a companion guard column (CarboPac PA1, 4 x 50 mm). The flow rate used was 1 ml/min at ambient temperature.

Elution. The eluents were prepared by suitable dilution of 50% NaOH solution with high purity water (specific resistance > 10 MΩ.cm). A Dionex eluent degas module (EDM-2) was employed to sparge and pressurize the eluents with helium. Mono- and disaccharides were separated by isocratic elution in 100 mM NaOH for 35 min. Thereafter, the column was washed for 5 min in 100 mM NaOH containing 1100 mM sodium acetate, followed by re-equilibration for 15 min with 100 mM NaOH.

Chemicals. NaOH solution (50% w/w) was purchased from J.T. Baker B.V. (The Netherlands). Sodium acetate (anhydrous) was from Merck (Germany).

Detection. Integrated amperometry was used to detect the carbohydrates using a gold working-electrode and a Ag/AgCl reference electrode. The following pulse potentials and durations were used for the detection of neutral sugars: t= 0-0.5 s, E= 0.10 V; t= 0.51-0.59 s, E= 0.60 V; t= 0.60-0.65 s, E= -0.60 V. The integration period was t= 0.30-0.50 s. Sugars were measured at an analog range of 3 μC.

Data processing. Chromatographic data were collected and integrated on a Spectra Physics

integrator (SP4400) connected to a PC. WINner/386 software (Spectra Physics, San Jose, USA) was used for further data processing.

Enzymatic sugar and starch determination. Sucrose, glucose and fructose were determined by a test kit purchased from Boehringer Mannheim GmbH (Germany), No.716260. Starch was solubilized and quantified according to the description delivered with testkit No. 207748 from Boehringer Mannheim GmbH (Germany).

Hexose kinase assay. Lyophilized, subapically non-swollen stolons were frozen in liquid N_2, and ground to powder. Stolon powder (80 mg) was extracted in 1 ml 50 mM BTP (pH 8.0), 5 mM $MgCl_2$ and 1 mM DTT. Insoluble PVP (10 mg per ml extraction buffer) was added. A 40%-80% $(NH_4)_2SO_4$ pellet was used as described by Doehlert (1989). The pellet was re-suspended in extraction buffer. Extraction and fractionation were carried out at 4°C. The capacity of a raw extract to phosphorylate various concentrations of glucose or fructose using ATP as a phosphoryl donor, was monitored according to Doehlert (1989). A spectrophotometric assay coupled hexose phosphate production with glucose-6-P dehydrogenase (EC 1.1.1.49, from *Leuconostoc mesenteroides*, Boehringer Mannheim GmbH (Germany), and NAD reduction. In case fructose was used as substrate, phosphoglucose isomerase (EC 5.3.1.9, Boehringer Mannheim GmbH, Germany) was added. The enzyme activity was monitored every 2 min during 20 min by measuring the absorbance at 340 nm after addition of glucose or fructose. The linear part of the curve was used for the quantification of the activity. The assays were done at 30°C.

Results

Determination of the developmental stage of stolon tips

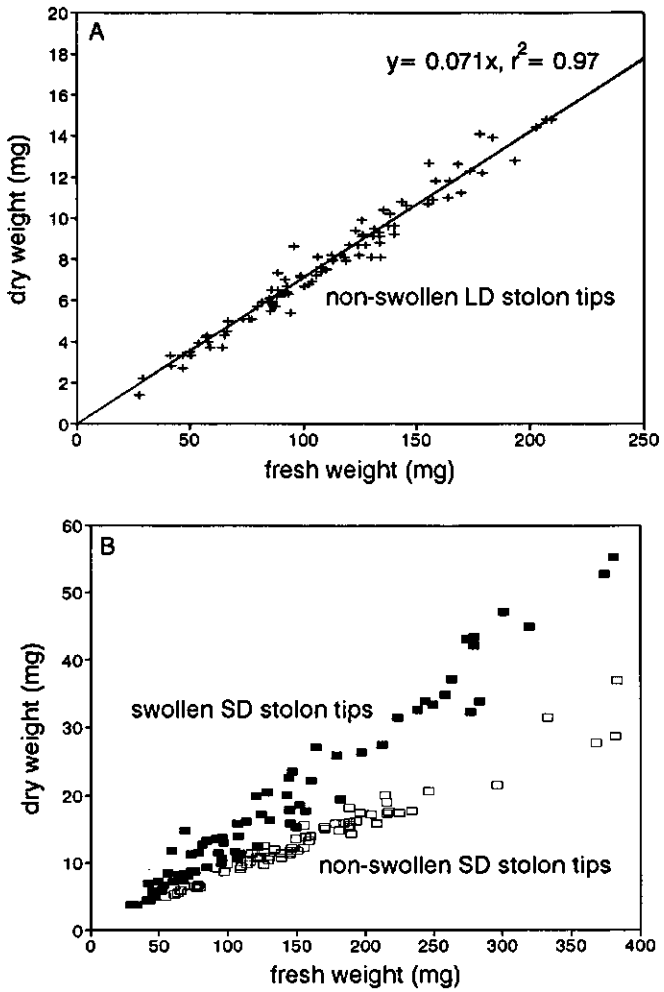
When investigating the correlation between mono- and disaccharide contents of stolon tips and their stage of development, the question raises how to quantify stage of development. Davies (1984) and Morrell and ap Rees (1986) correlated sugar content of stolon tips with fresh weight.

After six weeks exposure to SD conditions, only a part of the stolon tips of *S. demissum* plants was subapically swollen. SD stolon tips showing visible subapical thickening (n= 78)

had fresh and dry weights of 147 ± 107 (x ± s.d.) and 20 ± 15 mg, respectively. The fresh and dry weight of subapically non-swollen SD stolon tips (n= 69) were 160 ± 72 mg and 12 ± 6 mg. It was concluded that fresh weight nor dry weight were suitable measures for the quantification of early tuber development in *S. demissum*.

In early developmental stages, a rapid increase in the starch content of stolon tips from

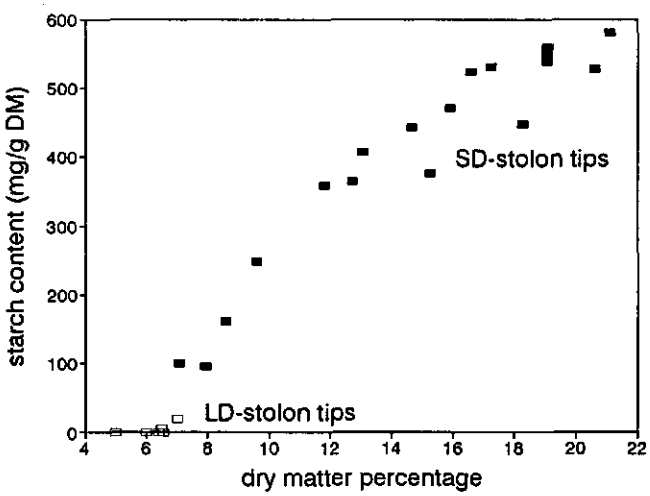
Figure 4.1. The relationship between fresh weight and dry weight of stolon tips (length ≈ 2 cm) from *S. demissum* grown under LD conditions (A), or under SD conditions (B, non-swollen tips; □, swollen tips; ■). The average DM percentage of subapically non-swollen (+) LD stolon tips was 7.1.



S. tuberosum was observed (Obata-Sasamoto and Suzuki 1979, Hawker *et al.* 1979). Starch determinations in individual stolon tips are laborious and time-consuming. Hence, it was tested whether DM percentage was a reliable indicator for the starch content. If changes in the DM percentage of a stolon tip are mainly the result of starch accumulation, this parameter should be constant in LD stolon tips, that never formed tubers, and whose starch content was below the detection limit (detection limit ≈ 5 mg g^{-1} DM). Figure 4.1A shows that the DM percentage of individual LD stolon tips was remarkably constant at about 7. The relation between fresh weight and dry weight was linear (r^2 = 0.97) (Fig. 4.1A). The stolon tips were all cut at 2 cm from the tip, so stolon tips with a higher fresh weight had a larger diameter. Hence, Fig. 4.1A shows indirectly that the DM percentage of the stolon tips was independent of stolon thickness. Neither the age of the plants, nor the number of LDs to which the plants were exposed affected the DM percentage of LD stolon tips (data not shown).

If changes in the DM percentage of a stolon tip are mainly the result of starch accumulation, subapically swollen SD stolon tips should have higher DM percentage than non-swollen SD stolon tips. Figure 4.1B clearly shows that such is the case. Under SD conditions visible subapical swelling coincided with a DM percentage higher than 10 (Fig. 4.1B). Incidentally, subapical thickening was also observed in stolon tips with a DM percentage between 8 and 10.

Fig. 4.2. The relationship between DM percentage and starch content in stolon tips from *S. demissum*. Tips were collected from plants that had been exposed to LD (□) or SD (■) conditions for six weeks.



To investigate whether the dry-matter percentage of a stolon tip is a reliable indicator for the starch content in early stages of tuber development, the starch content of SD stolon tips with various DM percentages was determined. Figure 4.2 shows that the relation between starch content and DM percentage is unequivocal in small tubers. In relatively large tubers (starch content > 450 mg g^{-1} DM), the starch content is apparently no longer the dominant factor that causes the increase of the DM percentage (Fig. 4.2).

It was concluded that DM percentage adequately reflected starch content in early developmental stage of stolon tips, and thus could be used as a measure to indicate their stage of development.

Neutral sugar content of stolons and developing tubers, qualitatively

A standard mixture of mono- and disaccharides was analyzed on a CarboPac PA1 column using 100 mM (Fig. 4.3) or 20 mM NaOH (data not shown) as eluent. Identification of sugars were made in both solvent systems. Figure 4.3 shows that apparently the most abundant neutral sugars in LD stolon tips were glucose and fructose. A smaller amount of sucrose was also detected. Two minor peaks in the HPAEC-PED chromatogram, with retention times of about 3.4 and 3.8 min, were not identified. In developing tubers the neutral, soluble mono- and disaccharides did not qualitatively differ from those in stolons (Fig. 4.4). Enzymatic analyses confirmed the identification of the glucose, fructose and sucrose peaks from the HPAEC-PED chromatogram (data not shown).

Neutral sugar content of stolons and developing tubers, quantitatively

LD stolon tips. The glucose concentration in LD stolon tips is high; 170 ± 24 mg g^{-1} DM (x ± s.d.). The fructose and sucrose concentrations were approximately 90 ± 18 and 8 ± 7 mg g^{-1} DM, respectively. The age of the plants hardly affected the glucose, fructose and sucrose concentration in individual stolon tips (data not shown).

SD stolon tips. Three types of SD stolon tips can be distinguished: type 1; tips collected from plants not bearing tubers, type 2; tips from tuber-bearing plants, but not swollen themselves, and type 3; swollen tips or small tubers. After 3 weeks exposure to SD conditions, tuberization was not yet observed. In Fig. 4.5A glucose and fructose data are given of these type 1 SD stolon tips. The average glucose and fructose levels in the tips were 165 ± 27 mg g^{-1} DM (x ± s.d.) and 54 ± 18 mg g^{-1} DM, respectively. After 6 weeks

Fig. 4.3. Analyses of a mixture of standard sugars, and three water-extracts from different *S. demissum* LD stolon tips using HPAEC-PED. Each chromatogram represents the neutral sugars extracted from 1.56 μg dry matter. Composition of standard sugar mixture; 1, inositol (100 ng); 2, fucose (100 ng); 3, glucose (100 ng); 4, fructose (100 ng); 5, sucrose (200 ng); 6, melezitose (200 ng); 7, raffinose (200 ng); 8, stachyose (200 ng).

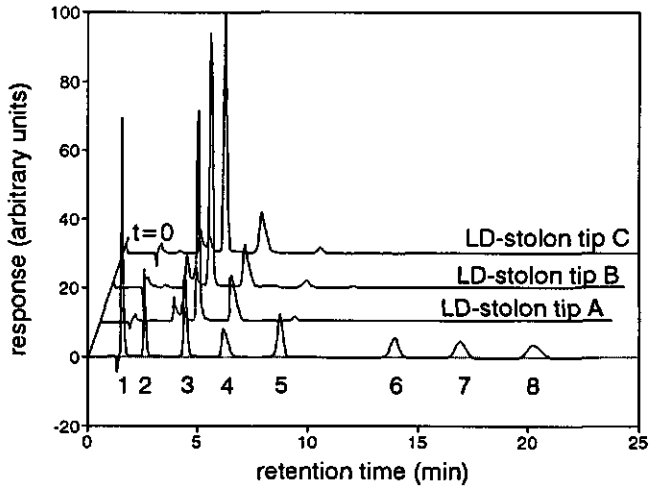


Fig. 4.4. Sugar analyses on four SD stolon tips with an increasing DM percentage from a single plant by HPAEC-PED. Each chromatogram represents the water-soluble, neutral sugars extracted from 1.56 μg dry matter.

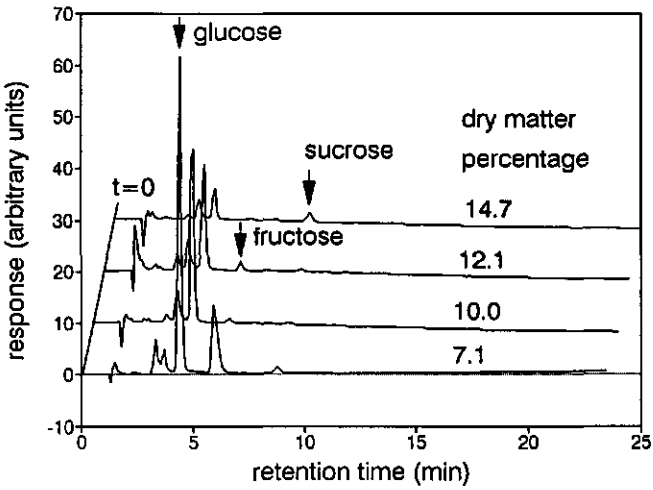
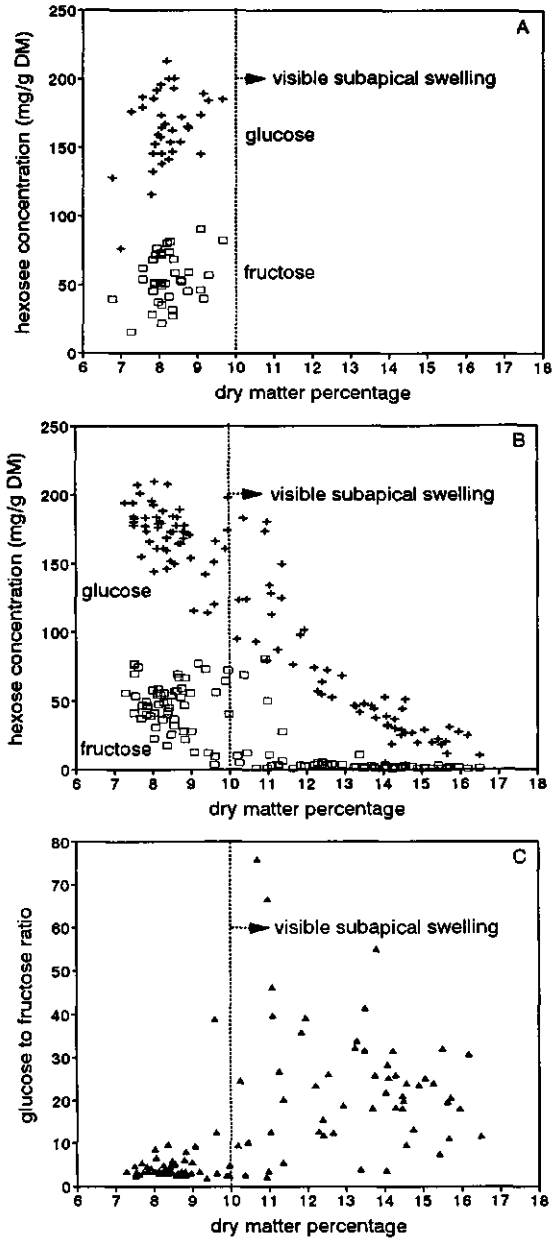


Fig. 4.5. Enzymatic glucose (+) and fructose (□) determinations on SD stolon tips from *S. demissum*. A. After 3 weeks exposure to SD conditions before tuberization occurred. B. After 6 weeks exposure to SD conditions. C. The glucose to fructose ratio after 6 weeks exposure to SD conditions.



exposure to SD conditions all plants were bearing a large number of small tubers. In Fig. 4.5B type 2 SD stolon tips are represented by data points with an accompanying DM percentage ranging between 7 and 10. The glucose and fructose concentrations, 173 ± 24 and 47 ± 18 mg g^{-1} DM, found in type 2 SD stolon tips do not significantly differ from type 1 tips.

Development of an SD stolon tip into a tuber. The glucose concentration started to change at a DM percentage of about 9, and it gradually decreased till a minimum was reached at about 15. The fructose concentration followed a different pattern; above a DM percentage of 9, it dropped rapidly and from a DM percentage of 12 onwards it was constantly very low (Fig. 4.5B).

The data presented in Figs. 4.5A and 4.5B were determined enzymatically. The changes in the glucose and fructose levels in stolon tips with an increasing DM percentage were consistent with the observations by HPAEC-PED (Fig. 4.4). As shown in Fig. 4.4, indications were obtained for an increase of the sucrose concentration (Fig. 4.4). The sucrose concentration further increased in stolon tips with a dry matter percentage above 15 (data not shown). This phenomenon was not further investigated since it only occurred after the onset of visible subapical swelling.

Hexose kinase (HK) activity in stolons

The rapid decrease of the fructose concentration (Fig. 4.5B) and the increasing glucose to fructose ratio in developing tubers (Fig. 4.5C) might be explained by changes in the phosphorylation rates of fructose and glucose. The phosphorylation of glucose and fructose catalyzed by hexokinases, glucokinases (EC 2.7.1.2), or fructokinases (EC 2.7.1.4) can be referred to collectively as hexose kinases (HK) (Doehlert 1989).

HK exhibited a broad pH optimum around 8.0 both for glucose and fructose (data not shown). At similar hexose concentrations, the rate at which fructose was phosphorylated by hexose kinases from stolons of *S. demissum* was about tenfold higher than the phosphorylation rate of glucose. Substrate inhibition by fructose occurred at concentrations above 0.25 mM (Fig. 4.6). Eadie plots show that the phosphorylation of fructose by HK did not obey Michaelis Menten kinetics (Fig. 4.7A). The phosphorylation of glucose did (Fig. 4.7B). The K_m and the apparent K_m for glucose and fructose were about 44 μM and 127 μM, respectively (Table I). The apparent K_m of HK for fructose is comparable with the K_m values

Fig. 4.6. Hexose monophosphate production from glucose and fructose catalyzed by hexose kinases from *S. demissum* stolons using ATP as a phosphoryl donor.

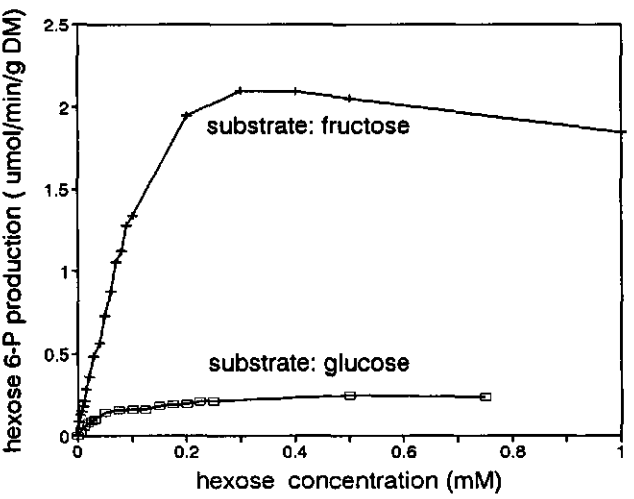
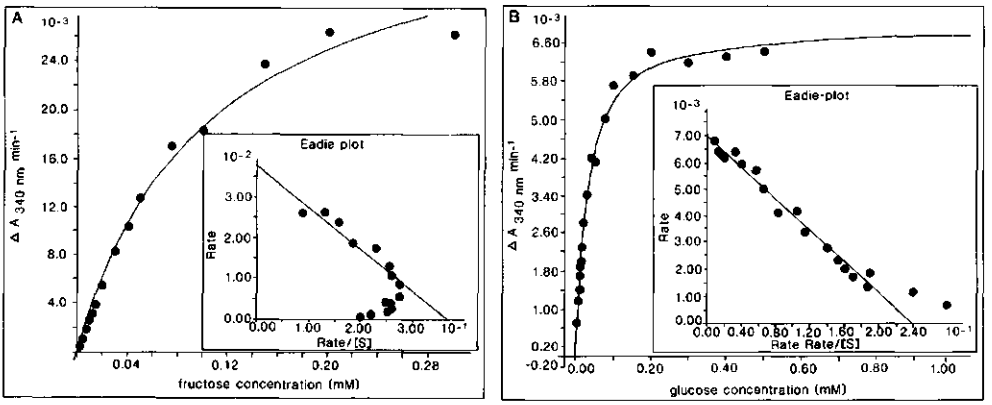


Fig. 4.7. The response of hexose kinases activity on increasing fructose (4.7A) and glucose (4.7B) concentrations and Eadie transformations of the data.



found for three fructokinases from developing tubers of *S. tuberosum* (ranging from 0.041 to 0.128 mM) (Gardner *et al.* 1992).

Discussion

The use of the absolutely SD-requiring potato species *S. demissum* allowed the comparison

of tuberizing and non-tuberizing plants of the same age which had received the same quantity of PAR. As expected, the difference in daylength had a profound effect on the development of the stolon tips. Tubers were only formed under SD conditions.

Quantification of the developmental stage of a stolon tip

It was shown that fresh weight or dry weight do not adequately reflect early developmental stages of stolon tips from *S. demissum*, and therefore we chose starch content as a measure to quantify the stage of development of a stolon tip. The DM percentage was shown to reflect adequately the starch content of stolon tips in early developmental stages. Hence, DM percentage was a good and reliable indicator for the stage of development of stolon tips. As far as known this is the first time that DM percentage is used as a measure to quantify the stage of development of potato stolon tips.

Effects of tuber initiation on the sugar levels

HPAEC-PED revealed that glucose and fructose were the most abundant neutral sugars in stolons of *S. demissum*. This was confirmed by enzymatic sugar analyses.

A) The sucrose level in non-tuberizing stolon tips of *S. demissum* was low. Indications were obtained for an increase in later developmental stages (DM% > 14). In developing tubers of *S. tuberosum*, the variation in the sucrose levels was considerable. No correlation was found between fresh weight of the stolon tip and sucrose level (Davies 1984).

B) The glucose concentration was remarkably high. The glucose concentration in non-tuberizing stolon tips was constant at about 170 mg g^{-1} DM, independent of whether LD, SD type 1 or SD type 2 stolons were analyzed. In SD stolon tips from *S. tuberosum*, cv Maris Piper, similar glucose levels were reported by Davies (1984); based on his data, we calculated that the glucose level was 143 mg g^{-1} DM, assuming a DM percentage of 7.

After 6 weeks exposure to SD conditions each plant was bearing tubers. The DM percentages of subapically swollen stolon tips varied between 10 and 16. As stolon tips developed the glucose level gradually decreased from 170 mg g^{-1} DM to about 25 mg g^{-1} DM. Such a gradually changing glucose level was not observed by Morrell and ap Rees (1986) in developing tubers of the *S. tuberosum* cultivars King Edward, Maris Bard and Pentland Javelin, with a fresh weight between 1 and 30 g. An explanation could be that these

authors did sugar analyses on tubers in which the glucose concentration had already dropped. In *S. demissum* the glucose concentration in developing tubers had already reached a minimum value at a fresh weight of about 300 mg.

In non-swollen stolon tips sucrose is probably hydrolysed by an invertase, whereas presumably the relatively importance of sucrose synthase will increase in developing tubers (see introduction). Such a switch would imply that a large part of the glucose moiety of sucrose is released as UDP-glucose instead of glucose. The observed steady decrease of the glucose concentration during tuber development could be interpreted as a result of an enzyme switch, that takes place gradually during the development of stolon tips.

C) In subapically non-swollen LD stolon tips, the fructose levels were high, about 90 mg g^{-1} DM. In type 1 and 2 SD stolon tips the fructose concentration was significantly lower. We do not have an explanation for this observed difference.

In subapically non-swollen LD and SD stolon tips, the glucose to fructose ratios were about 2 and 3, respectively. Two questions raise: 1) Since phosphorylation of fructose is much more rapidly than of glucose, why isn't this ratio substantially higher? 2) Since hydrolysis of sucrose by a soluble or insoluble acid invertase releases equimolar quantities of glucose and fructose, why isn't this ratio about one?

1) Assuming sucrose to be unloaded symplastically in non-swollen stolon tips, like in potato tubers (Oparka 1986), and split by a neutral invertase, equimolar quantities of glucose and fructose would be released in the cytosol. The phosphorylation rate of fructose by HK from stolons of *S. demissum* is about tenfold higher than the phosphorylation rate of glucose (Fig. 4.6). The K_m and apparent K_m for glucose and fructose, respectively, were both relatively low. If the hydrolysis products glucose and fructose were exposed to HK activity, the glucose to fructose ratio certainly would have been higher than 2 or 3. Therefore, we assume that fructose and glucose, and HK activity were spatially separated. Sucrose hydrolysis in the vacuole by a soluble acid invertase, or in the apoplast by a cell wall-bound acid invertase, would be compatible with a relatively low glucose to fructose ratio in non-swollen stolon tips. Circumstantial support for this view comes from the observation that no specific neutral invertase was detected in subapically swollen (Richardson *et al.* 1990) or in non-swollen stolon tips (Dr. H.V. Davies, pers. commun.) from *S. tuberosum*.

2) Hydrolysis of sucrose by a soluble or cell wall-bound acid invertase releases equimolar quantities of glucose and fructose. Nevertheless, the glucose to fructose ratio in non-tuberizing stolon tips is without exception higher than one (*e.g.* Fig. 4.5A). The

hydrolysis products glucose and fructose will have to cross the tonoplast or the cell-membrane to reach the cytosol. An explanation for the relatively high glucose to fructose ratio could be that fructose is transported more efficiently over one of these membranes than glucose.

The onset of tuber formation is accompanied by a dramatic decrease of the fructose level, and a rapidly increasing glucose to fructose ratio. Unlike the observed changes in the glucose level, the decrease of the fructose concentration can not be explained by an enzyme shift. After all, fructose is released irrespectively whether sucrose is hydrolysed by an invertase or sucrose synthase. The fructose concentration would decrease rapidly when this hexose was no longer spatially separated from hexose kinases. If sucrose is indeed hydrolysed by a soluble acid invertase or a cell wall-bound acid invertase in non-swollen tips, and by cytosolic sucrose synthase in developing tubers, tuber initiation would imply a rapid spatial shift. Such a shift would be compatible with the observed drop of the fructose level during tuber development for fructose would be rapidly metabolized to fructose 6-P in developing tubers.

The changes in the glucose level suggest a gradual enzyme shift, whereas the changes in the fructose level suggest a rapid spatial shift. These two interpretations are conflicting unless we assume that the glucose in developing tubers with a DM percentage between 11 and 15 belongs to a decreasing pool of residual glucose which is gradually taken up into the cytosol by a moderately efficient transfer mechanism. This would be a second indication for such a difference in efficiency of fructose and glucose uptake in the cytosol.

Our observations concerning changes in glucose and fructose levels during tuber development in *S. demissum* are compatible with a radical change in the way and the site sucrose is hydrolysed.

Chapter 5

INVERTASE AND SUCROSE SYNTHASE ACTIVITIES IN STOLON TIPS OF THE WILD POTATO SPECIES *SOLANUM DEMISSUM* GROWN UNDER LONG- AND SHORT-DAY CONDITIONS

Summary

Tuber formation in the wild potato species *Solanum demissum* Lindl. is absolutely short-day dependent. Activities of invertases and sucrose synthase were determined in non-tuberizing long- (LD) and short-day (SD) stolon tips, and in SD tips showing various stages of subapical swelling. No evidence could be found for a specific neutral invertase in stolon tips. The soluble acid invertase activity in stolon tips collected from plants exposed to LD conditions was 4-5 fold higher than the activity in non-swollen SD stolon tips. Tuber initiation in SD stolon tips, characterized by an increase of the dry matter (DM) percentage, coincided with a decrease of the activities of soluble acid invertase and of the 1 N NaCl solubilizable part of cell wall-bound acid invertase. The Michaelis constants of soluble and cell wall-bound acid invertases from stolon tips for sucrose at pH 5.2 were 2.6 $\pm$ 0.6 mM ($x \pm$ s.d.) and 1.8 $\pm$ 0.8 mM, respectively. In LD stolon tips, sucrose synthase activity was barely detectable. In SD stolon tips, the sucrose synthase activity increased concomitant with the DM percentage and the maximal activity was observed in small tubers with a DM percentage of 15. In tubers with a DM percentage above 15 no clear correlation was observed between DM percentage and sucrose synthase activity. The Michaelis constant of sucrose synthase from stolon tips for sucrose at pH 7.5 was 60 $\pm$ 18 mM. Two models are presented on sucrose metabolism in stolon tips of *S. demissum* before and during tuber development.

Introduction

Sucrose, the main transport sugar in plants, will be either stored or metabolized in a sucrose sink. Metabolization of sucrose starts with its splitting. Sucrose can be hydrolysed either by an invertase (EC 3.2.1.26) or by sucrose synthase (EC 2.4.1.13). The irreversible splitting of sucrose by an invertase releases equimolar quantities of glucose and fructose. The

reversible hydrolysis of sucrose by sucrose synthase releases equimolar quantities of UDP-glucose and fructose, at the expense of UDP. Sucrose synthase activity resides in the cytosol, whereas acid invertase activity is restricted to an environment with a low pH, such as the vacuole or the cell wall. The glucose and fructose released can be phosphorylated into glucose 6-P and fructose 6-P, respectively, by hexo- (EC 2.7.1.1), gluco- (EC 2.7.1.2), or fructokinases (EC 2.7.1.4).

In Chapter 4, changes were reported in the mono- and disaccharide contents of stolon tips from the wild, tuber-bearing potato species *Solanum demissum* Lindl. grown under SD and LD conditions. It was shown in Chapter 4 that the DM percentage of a stolon tip can be a simple and reliable measure to indicate starch accumulation and, thereby, early stages of tuber development in stolon tips of *S. demissum*. An increase of the DM percentage in SD stolon tips was accompanied by a decrease of the glucose and fructose concentrations in these tips. The gradual decrease of the glucose concentration was interpreted as a result of an increase in the relative importance of sucrose synthase activity as compared to invertase activity during the onset of tuber formation. The dramatic decrease of the fructose concentration upon tuber initiation was explained as a result of a spatial shift, from the apoplast or vacuole to the cytosol, that accompanied the above-mentioned enzyme shift (Chapter 4).

No literature is available about the sucrose metabolism in stolon tips from *S. demissum*. From *S. tuberosum* tubers, cv. Russet Burbank, a soluble acid invertase was purified and partially characterized (Bracho and Whitaker 1990b). The apparent molecular mass of the soluble acid invertase from potato, a glycoprotein, was 60 kD. In potato tubers, the *in planta* activity of soluble acid invertase might be regulated by a non-dialysable inhibitor (Schwimmer *et al.* 1961). Both Pressey (1967) and Bracho and Whitaker (1990b) reported the molecular mass of the proteinaceous inhibitor to be 17 kD. Binding of invertase inhibitor to invertase was reported to be slow (Bracho and Whitaker 1990a). By intensive mechanical agitation, named 'foaming', this inhibitor of soluble acid invertase was inactivated, whereas invertase itself was not (Pressey 1966). The invertase activity measured before foaming is called basal activity, that after foaming total activity (Richardson *et al.* 1990). Because of their presumed spatial separation, the proteinaceous inhibitor would reside in the cell wall whereas the soluble acid invertase would be located in the vacuole, the *in vivo* regulation of soluble acid invertase activity in *S. tuberosum* tubers by this inhibitor was questioned by Isla *et al.* (1992).

Sucrose synthase was partially purified from immature *S. tuberosum* tubers, cvs. Kennebec and Norchip. At pH 7.0 the K_m of sucrose synthase was 130 mM (Pressey 1969). The molecular cloning of sucrose synthase cDNA was done by Salanoubat and Belliard (1987).

In the present report, we verified whether the interpretation of the observed changes in glucose and fructose levels in stolon tips from *S. demissum* as a results of a change in the way sucrose is hydrolysed (Chapter 4), was supported by data on the enzymes involved in sucrose hydrolysis.

Materials and methods

Plant material. True seeds (BGRC 9981) of *Solanum demissum* were obtained from the Institut für Pflanzenbau und Pflanzenzuchtung der FAL (Braunschweig, Germany). One single clone was used in all experiments. This clone was propagated *in vitro* as described in Chapter 2 (p. 15).

Tuber induction and sampling. After 4 weeks *in vivo* growth in a walk-in growth chamber, the PAR-period was reduced from 16 h to 10 h. Half of the plants were further grown under LD conditions. The other plants were exposed to SD conditions. Climatal conditions were as described in Chapter 2. Subapically swollen and non-swollen SD stolon tips were collected one week after the onset of tuber initiation. LD stolon tips were harvested simultaneously with the SD stolon tips. SD stolon tips with a DM percentage above 15 were harvested two weeks later. All harvests were done 6-7 h after the start of the PAR period. The stolon tips (length $\approx$ 2 cm) were immediately frozen in N_2 (l), and freeze-dried. Special care was taken to avoid intermittent thawing. After freeze-drying the samples were stored at -80°C until further analysis.

Measurement of basal and total soluble acid invertase activity. Freeze-dried *S. demissum* material (300 mg) was extracted in 15 ml extraction buffer. Just before use, insoluble PVP (10 mg/ml) and DTT (final concentration: 2.5 mM) were added to the extraction buffer, consisting of 20 mM BTP (pH 6.5) and BSA (1 mg/ml). From *S. tuberosum* cv. Bintje tubers fresh material (6 g) was extracted in 15 ml extraction buffer. Extracts were centrifuged at 10,000 g for 20 min. Essentially according to Richardson *et al.* (1990), the supernatant was

dialysed over-night against 10 mM NaAc (pH 5.0), and subsequently agitated in a Sorvall Omni-mixer (model 17220, Sorvall Inc., Newtown, Conn., USA) at room temperature. The intensive mixing was interrupted several times to cool the extract. Within 1 h after foaming the invertase activity was measured. All sample handling was done at 4°C.

Invertase activity was essentially determined according to Giaquinta *et al.* (1983). Aliquots (50 μl) were added to 200 μl of a 25 mM citrate-phosphate buffer that contained 25 mM sucrose. Acid and neutral invertase activities were assayed at pH 5.2 and 7.5, respectively. After 45 min incubation at 37°C the reaction was stopped by immersion in a boiling water bath for 4 min. The assays were corrected with heat-denatured enzyme controls, which always represented less than 5% of the experimental values. In a sample from the reaction mixture glucose was determinated enzymatically using a glucose test kit (No. 716251, Boehringer Mannheim GmbH, Germany).

Extraction and preparation of invertases from stolon tips. Freeze-dried stolon tips were homogenized in N_2(l). Dry stolon-tip powder (80 mg, representing 8 to 10 stolon tips) was incubated in 1 ml low-salt buffer (20 mM BTP (pH 6.5), DTT (final concentration 2.5 mM), BSA (0.1 mg/ml) and insoluble PVP (10 mg/ml)) to extract soluble acid and neutral invertases. After 30 min centrifugation in an Eppendorf table-centrifuge at maximal speed, the supernatant was collected. The pellet was washed three times with low-salt extraction buffer without DTT and PVP. The effectiveness of these washing steps was checked by protein determinations according to Bradford (1976) in the supernatants. Subsequently, the pellet was incubated over-night with 1 ml high-salt buffer (20 mM Mes (pH 6.0) containing 1 N NaCl) for the extraction of cell wall-bound acid invertase.

From both the low-salt and the high-salt extracts 500 μl fractions were applied to a Superose-12 column (HR 10/30; Pharmacia, Sweden), which had been equilibrated with high-salt buffer. The column was eluted with the same buffer by fast protein liquid chromatography (FPLC) at a flow rate of 0.5 ml/min. As indicated, fractions of 0.4 or 1 ml were collected in test-tubes that contained 0.1 or 0.25 ml of a BSA solution (0.4 mg/ml). Within 6 h the invertase activities in the fractions were determined. The recovery of invertase activity was always close to 100%. The Superose-12 (HR 10/30) column was calibrated with ribonuclease A (13.7 kD), chymotrypsinogen A (25.0 kD), ovalbumin (43 kD), bovine serum albumin (67 kD) and aldolase (158 kD) from a gel-filtration calibration kit (Pharmacia, Sweden).

Measurement of sucrose synthase activity. Freeze-dried individual stolon tips (4-12 mg dry weight) were extracted in 1 ml 100 mM Hepes (pH 7.5), 10 mM MgAc, 1 mM EDTA and 1.0 mg/ml BSA. Just before use, the extraction buffer was supplemented with DTT to a final concentration of 2 mM. The extracts were centrifuged for 15 min in an Eppendorf table-centrifuge at maximal speed. A Sephadex G-50 fine column (Pharmacia, Sweden), with a bed volume of 2 ml, was used to desalt (or better: desugar) 0.9 ml of the supernatant. The column was eluted with 0.9 ml modified extraction buffer; 10 mM Hepes (pH 7.5) was used instead of 100 mM.

The determination of sucrose synthase activity was essentially performed as described by Xu *et al.* (1986). Eluent (50 μl) was added to 650 μl assay buffer. After the addition of 50 μl concentrated substrate solution, the sucrose, UDP and PP_i concentrations were 50, 1 and 1 mM, respectively. The release of UDP-glucose (UDPGlc) was indirectly monitored in this assay. UDPGlc-pyrophosphorylase (EC 2.7.7.9) catalyzed the conversion of UDP-glucose to glucose 1-P and UTP at the expense of PP_i. Subsequently, glucose 1-P was converted into glucose 6-P by phosphoglucomutase (EC 5.4.2.2). Glucose 6-P dehydrogenase (EC 1.1.1.49, from *Leuconostoc mesenteroides*) catalyzed a reaction which released gluconate 6-P and NADH at the expense of glucose 6-P and NAD. NADH-formation was monitored every 2 min during 20 min by measuring the absorbance at 340 nm after addition of the substrates. The linear part of the curve was used for the quantification of the activity. The assays were done at 30°C. In stolon-tip extracts UDPGlc-phyrophosphorylase activity was so high that the enzyme could be omitted without a measurable effect (see also Xu *et al.* 1989).

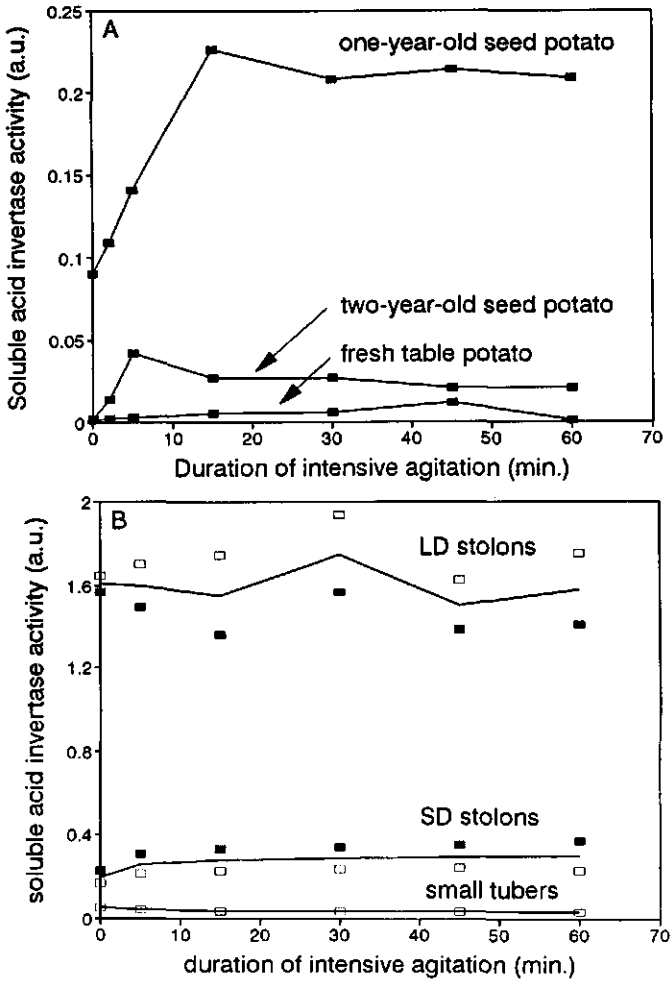
Enzymes. All enzymes used were purchased from Boehringer Mannheim GmbH, Germany.

Northern analysis. Two weeks after the onset of tuber initiation 12 stolons tips (length ≈ 2 cm) were collected from two, randomly chosen *S. demissum* plants. Six stolon tips from each plant were harvested at the same day, and frozen in liquid nitrogen. Within each group of stolon tips, 3-4 stolon tips showed subapical swelling. Total RNA was isolated from individual stolon tips (50-150 mg fresh weight). Equal amounts of stolon tip RNA were applied per lane (5 μg). RNA was blotted onto Hybond N (Amersham, UK) and hybridized with a ^{32}P-labelled 1.3 kb EcoRI fragment of clone λ10a, corresponding to the C-terminus of the potato sucrose synthase (Salanoubat and Belliard 1987). The isolation and analysis of RNA was performed as described by Salehuzzaman *et al.* (1992).

Results

Activities of invertases and sucrose synthase were measured in LD and SD stolon tips from *S. demissum*. A complicating factor for the measurement of soluble acid invertase activity could be the partial inhibition of the activity by a proteinaceous inhibitor, analogous to tubers

Figure 5.1. A. Effect of foaming on the soluble acid invertase activity (in arbitrary units) in seed and table potatoes from the *S. tuberosum* cultivar Bintje. B. The effect of foaming on the soluble acid invertase activity (in arbitrary units) in subapically non-swollen LD and SD stolons, and small tubers from *S. demissum*. The lines interconnect averages of two independent measurements.



of *S. tuberosum*.

Basal and total soluble acid invertase activities in stolons and developing tubers from *S. demissum*

By intensive mechanical agitation, so called 'foaming', the proteinaceous inhibitor of potato acid invertase can be inactivated (Pressey 1966). It was investigated whether foaming could substantially increase the soluble acid invertase activity, leaving aside whether enzyme-inhibitor complexes were formed before or after homogenization of the plant tissue.

The effectiveness of foaming was illustrated by extracts from three kinds of *S. tuberosum* tubers. In Fig. 5.1A it is shown that the soluble acid invertase activity in one-year-old seed potatoes from *S. tuberosum*, cv. Bintje, reached its maximum after about 15 min foaming. The total invertase activity in these seed potatoes was more than two times the basal activity (for definitions, see Introduction). Basically, the same pattern was observed in two-years-old seed potatoes. The basal and total soluble acid invertase activities were very low in fresh table potatoes (Fig. 5.1A).

In Fig. 5.1B the effect of foaming on soluble acid invertase activity in stolons and developing tubers from *S. demissum* is shown. Remarkable is the relatively high soluble acid invertase activity in LD stolons as compared to the activity in SD stolons. Except for SD stolons where a slight increase was observed, foaming had hardly a measurable effect on the invertase activity. LD-stolon-tip extracts were used for further characterization of *S. demissum* invertases because extracts from these tips showed a relatively high enzyme activity, and no measurable difference was found between basal and total invertase activity. This implicates the absence of a measurable inhibition of soluble acid invertase activity.

Separation of invertase and its proteinaceous inhibitor

Inactivation of the proteinaceous inhibitor in small samples is technically difficult and laborious. As argued above no evidence was found for a substantial *in planta* inactivation of soluble acid invertase in LD stolon tips. To prevent inhibition of the enzyme after decompartimentalization, LD-stolon-tip extracts were applied to a Superose-12 (HR 10/30) column. Assuming that the molecular mass of the inhibitor from *S. demissum* is comparable to the mass of the invertase inhibitor from tubers of *S. tuberosum* (17 kD), this gel-filtration

Fig. 5.2. Two elution patterns of soluble acid invertase activity after application of low-salt extracts from LD stolon tips of S. demissum to a Superose-12 gel-filtration column. Enzyme determinations were done to the 0.4 ml fractions collected. The column was calibrated with 1; aldolase (158 kD), 2; bovine serum albumin (67 kD), 3; ovalbumin (43 kD), 4; chymotrypsinogen A (25.0 kD) and 5; ribonuclease A (13.7 kD).

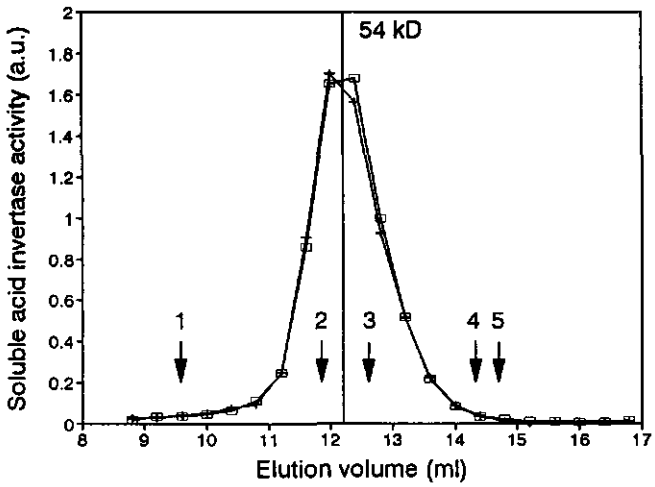
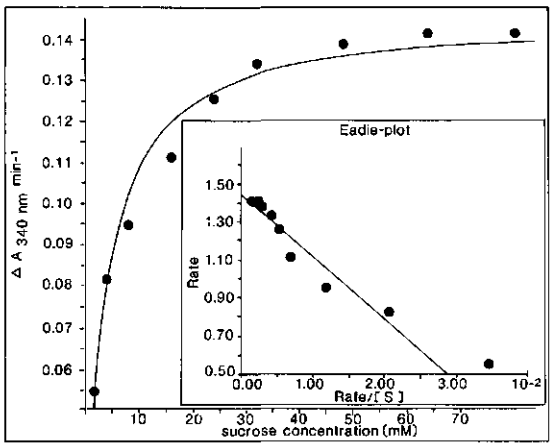


Fig. 5.3. Relation between soluble acid invertase activity extracted from *S. demissum* LD stolon tips and the sucrose concentration. In the insert, Eadie transformations of the data are given.



step should be efficient in separating the invertase from its proteinaceous inhibitor. The molecular mass of soluble acid invertase from stolon tips of *S. demissum* was apparently about 54 kD (Fig. 5.2). All invertase activity in LD-stolon-tip extracts was bound to a Con A-Sepharose column (Pharmacia, Sweden) and released by elution with 250 mM methylmannoside. Similar to invertase activity from leaves of *Urtica dioica* L. (Fahrendorf and Beck 1990), the invertase activity appeared as a broad peak from this column (data not shown). Hence, the soluble acid invertase from *S. demissum* stolons presumably is a glycoprotein. Because of its nature, the afore-mentioned estimation of the molecular mass refers to the apparent molecular mass.

Active, inhibitor-free LD-stolon-tip fractions were used to determine the affinities of soluble and cell wall-bound acid invertase for sucrose. Both acid invertases from stolon tips from *S. demissum* did obey Michaelis-Menten kinetics (soluble acid invertase: Fig. 5.3, cell wall-bound acid invertase: data not shown). The K_m of soluble acid invertase for sucrose was calculated to be 2.6 $\pm$ 0.6 mM (n=3). This K_m was comparable with K_m's found for soluble acid invertases from tubers of several *S. tuberosum* cultivars, that were varying between 3 and 16 mM (Bracho and Whitaker 1990b, and literature cited therein). The K_m of cell wall-bound acid invertase for sucrose was 1.8 $\pm$ 0.8 mM (n=3).

Neutral invertase activity in LD stolon tips

Active, inhibitor-free LD-stolon-tip fractions were tested for soluble and cell wall-bound acid and neutral invertase activities. Figure 5.4A shows the soluble acid invertase activity per fraction.

The pH dependence of soluble acid invertase from *S. demissum* stolon tips after purification as described above is given in Fig. 5.5. Maximal activity was observed at about pH 5. The shape of the curve does not indicate the presence of invertases with a different optimal pH. However, at pH 7.5 the invertase activity was still 5-10% of the maximal activity. Apparently, a soluble neutral invertase activity peak was indicated in Fig. 5.4A. Because of the broad pH optimum of this soluble acid invertase, and the exact co-elution of soluble acid and neutral invertase activities, it was concluded that there is no evidence for a specific neutral invertase in LD stolon tips from *S. demissum*. The finding of an insoluble neutral invertase activity peak (Fig. 5.4B) should be considered also as rest-activity, a consequence of the presumably broad pH optimum of cell wall-bound acid invertase of LD

stolon tips from *S. demissum*. An insoluble neutral invertase has not been described in literature.

Figure 5.4. A. Soluble acid and neutral invertase activities determinated in 1.0 ml fractions collected after application of a low-salt extract from LD stolon tips of *S. demissum* to a Superose-12 gel filtration column. Both activities were measured in each fraction at the pH 5.2 and 7.5, respectively. B. Idem for fractions of high-salt solubilizable invertase activities.

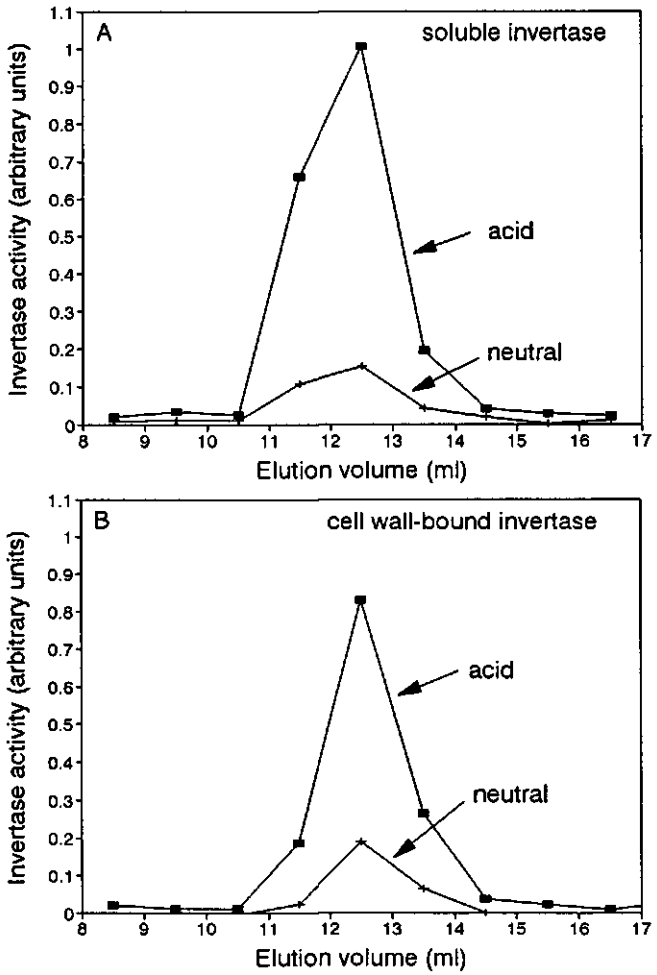


Fig. 5.5. Determination of the invertase activity at different pH values between 4 and 8. Measurements were performed using pooled, active fractions that were collected after gel filtration of a low-salt extract of LD stolon tips from *S. demissum*.

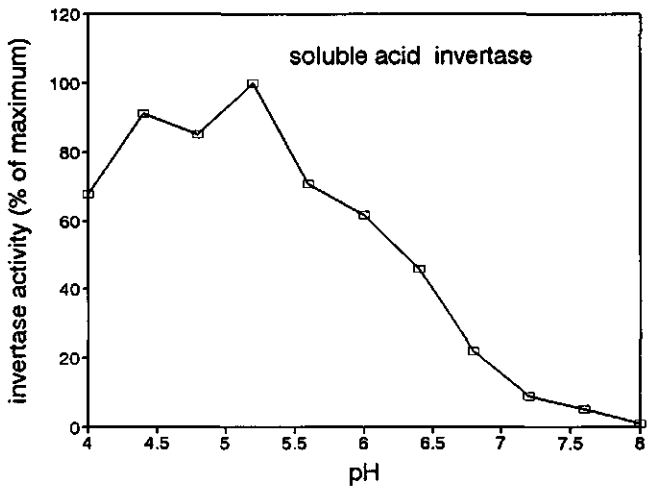
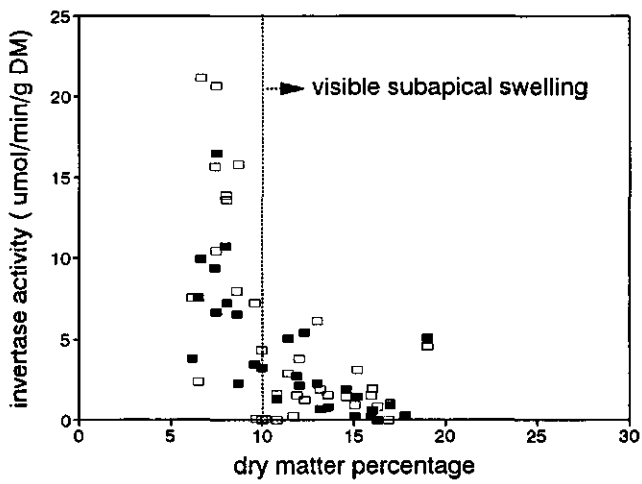


Fig. 5.6. Basal activities of soluble acid invertase (□) and activities of high-salt solubilizable, cell wall-bound acid invertase (■) extracted from individual stolon tips that were simultaneously collected from *S. demissum*. Invertase activities were measured as the release of glucose from added sucrose.



Soluble and cell wall-bound acid invertase activities in stolon tips and developing tubers

Figure 5.6 shows invertase activities in individual stolon tips in various developmental stages, harvested at the same day from the same plant. The activities of soluble acid invertase decreased concomitant with an increasing DM percentage of the stolon tips. After over-night high-salt incubation, cell wall-bound acid invertase activity was measured in the incubation buffer (Fig. 5.6). Subapical swelling of the stolon tip could also be correlated with a decrease of activity of high-salt solubilizable enzyme. After several extraction cycles with a high-ionic-strength solution acid invertase activity was still present in the pellet (data not shown). Only a part of the cell wall-bound invertase could be solubilized. Hence, the cell wall-bound acid invertase activity, and its decrease upon tuber initiation, could not be quantified accurately.

Sucrose synthase activity in individual stolon tips and developing tubers

Sucrose synthase from developing tubers of *S. demissum* did obey Michaelis-Menten kinetics (Fig. 5.7). The K_m of sucrose synthase for sucrose was 60 ± 18 mM (n=3). Sucrose synthase activities were determined in individual stolon tips and developing tubers from *S. demissum* grown under SD and LD conditions. Sucrose synthase activity was quantified by the rate of UDP-glucose release in the presence of sucrose. In LD stolon tips, that never

Fig. 5.7. Relationship between sucrose synthase activity extracted from subapically swollen stolon tips from *S. demissum* and the sucrose concentration. In the insert, Eadie transformation of the data is given.

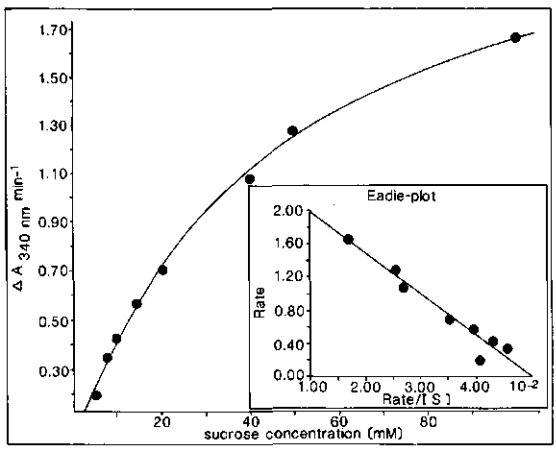


Fig. 5.8. Relationship between DM percentage and sucrose synthase activities in LD (□) and SD stolon tips (+) from *S. demissum*. Stolon tips with a DM percentage higher than 10 showed visible subapical swelling.

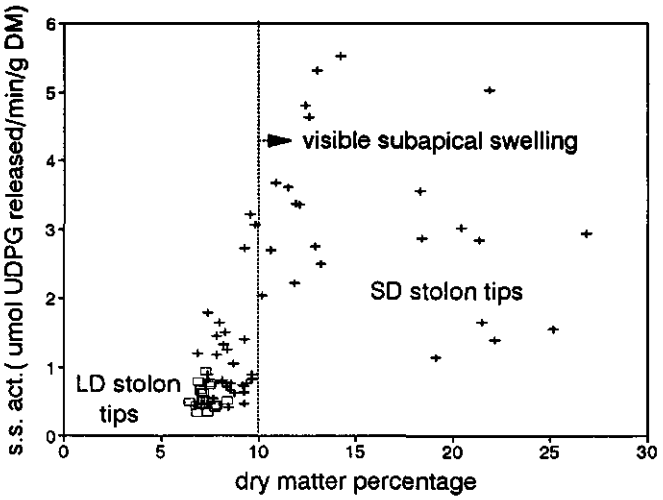
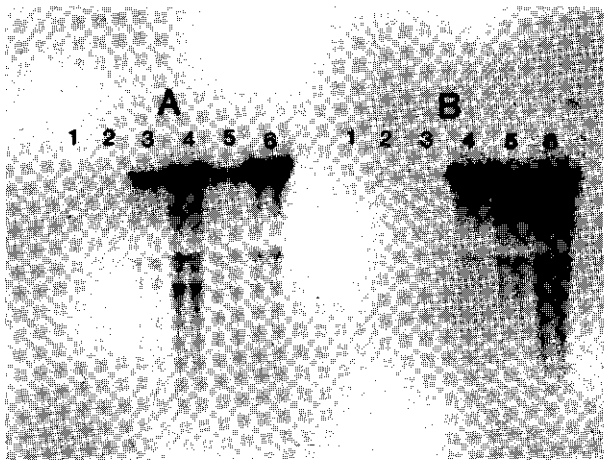


Fig. 5.9. Northern analysis of RNA preparations from two times 6 stolon tips at the same day collected from two SD plants. Stolon tips 1 and 2 from plant A and 1, 2 and 3 from plant B were non-swollen. Per lane approximately 5 μg RNA was loaded onto the gel. RNA was blotted onto Hybond N (Amersham, UK) and hybridized with a sucrose synthase cDNA fragment (SuSy) from *S. tuberosum* (Salanoubat and Belliard, 1987).



showed any subapical swelling, sucrose synthase activity was too low to be quantified accurately (Fig. 5.8). In SD stolon tips with a DM percentage below 10, a substantial increase in the sucrose synthase activity was observed in a part of the population. A sharp increase in the sucrose synthase activity was observed in stolon tips with a DM percentage between 9 and 15. In tubers with a DM percentage above 15 no clear correlation was observed between enzyme activity and DM percentage.

To see whether sucrose synthase activity in non-swollen SD stolon tips was regulated on transcription or translation level, total RNA was extracted from two times 6 individual subapically swollen and non-swollen stolon tips harvested at the same day from two plants. Nothern blotting with a ^{32}P-labelled sucrose synthase cDNA fragment (SuSy) from *S. tuberosum* showed hardly any signal with RNA from non-swollen SD stolon tips from *S. demissum*, whereas the signal with RNA from swollen tips was relatively high (Fig. 5.9).

Discussion

Under 16 h-daylength (LD) conditions *Solanum demissum* did not form tubers. Extension of the dark period from 8 h to 14 h induced subapical swelling and an increase of the DM percentage in some stolon tips. Activities of invertases and sucrose synthase were determined in non-swollen LD stolon tips and in SD stolon tips at different stages of tuberization.

As in *S. tuberosum* tubers (Richardson *et al.* 1990) no evidence was found for a specific neutral invertase activity in stolon tips from *S. demissum*. LD stolons showed a remarkably high soluble acid invertase activity as compared to subapically non-swollen SD stolon (Fig. 5.1B). In *S. tuberosum* cv. Bintje, both stolon elongation and stolon branching are stimulated by LD conditions (Struik *et al.* 1988). LD conditions had similar effects on stolon growth in *S. demissum* (data not shown). Hence, the high activity of soluble acid invertase in stolons is associated with strong longitudinal stolon growth in *S. demissum*.

Early phases of tuber development in *S. demissum*, as determined by an increasing DM percentage of the stolon tip, were accompanied by a decrease of the (basal) activities of soluble and cell wall-bound acid invertase (Fig. 5.6). Presumably, this decrease was not the result of inactivation of the enzyme by a proteinaceous inhibitor, since inactivation of the inhibitor in, *e.g.*, small tubers, could not recover the invertase activity (Fig. 1B). Essentially the same low basal and total invertase activities were found by Richardson *et al.* (1990) for relatively large, freshly harvested tubers from *S. tuberosum*.

Initially, the subapical swelling of stolon tips from *S. demissum* was accompanied by a sharp increase of the sucrose synthase activity. The sucrose synthase activity reached its maximum at a DM percentage around 15. In later developmental stages, characterized by a DM percentage above 15, no clear correlation between DM percentage and sucrose synthase activity was observed (Fig. 5.8). Tuber growth is accompanied by a constant increase of the amount of relatively inert storage tissue. Sucrose synthase is presumably not distributed evenly in developing tubers, but concentrated around the sites of phloem unloading. For sucrose synthase measurements entire tubers were homogenized. We suggest that in tubers with a DM percentage above 15, the decrease of the sucrose synthase activity is caused by a dilution of the enzyme-containing tissue by relatively inert storage tissue. When tubers with a DM percentage above 15 are considered, the data on sucrose synthase activities reported here are comparable with enzyme activities found in developing *S. tuberosum* tubers (Sung *et al.* 1989). The authors found no clear correlation between sucrose synthase activity and fresh weight in *S. tuberosum* tubers. The difference between our results on small tubers with a DM percentage below 15 and the data obtained for *S. tuberosum* tubers presumably results from the tuber sizes. Sung *et al.* (1989) used tubers with a fresh weight up to 210 g, whereas we focused on tuber initials, *viz.*, tubers with a fresh weight between 50 and 350 mg.

Two different models for the unloading of sucrose in stolon tips of *S. demissum* might explain both the changes in mono- and disaccharide contents (Chapter 4) and the observed changes in invertase and sucrose synthase activities. Ultrastructural (Oparka 1986) and physiological (Oparka and Prior 1988) evidence was presented for passive and symplastic unloading of sucrose in tubers of *S. tuberosum*. In these models, sucrose unloading in *S. demissum* tubers is assumed to be similar to sucrose unloading in *S. tuberosum* tubers.

Model 1) In subapically non-swollen stolon tips, sucrose unloading from the phloem occurs symplastically. In the cytosol no specific neutral invertase or sucrose synthase is available to catalyze the hydrolysis of sucrose. For metabolization sucrose has to cross either the tonoplast or the plasmalemma. In the vacuole sucrose is exposed to soluble acid invertase activity. In the apoplast a cell wall-bound acid invertase catalyzes the hydrolysis of sucrose. The products glucose and fructose will be imported into the cytosol where further metabolization takes place. In developing tubers sucrose unloading also will occur symplastically, and cytosolic sucrose synthase will catalyze the hydrolysis of sucrose.

Model 2) In subapically non-swollen stolon tips, sucrose unloading from the phloem occurs apoplastically. In the apoplast, sucrose is exposed to cell wall-bound acid invertase

activity. Sucrose is split into glucose and fructose, and both reaction products are imported into the cytosol. Tuber initiation would coincide with a shift from apoplastic to symplastic unloading. In a review on sieve element unloading, Patrick (1990) described the feasibility of such a switch. Sucrose unloaded in the cytosol will be split by sucrose synthase.

Apoplastic sucrose unloading in non-swollen stolon tips, as proposed in the second model, presupposes cell wall-bound acid invertase activity in the apoplast. The activity of this invertase is considered to be regulated by proton secretion to the apoplast by ATPases located on the plasma membranes of the sink cells (Eschrich 1980). Invertase activity in the apoplast in developing and mature leaves of *Urtica dioica* was questioned by Möller and Beck (1992). The apoplastic pH was determined to be 7.0-7.2. This pH range was not compatible with activity of cell wall-bound acid invertase. In our eyes, the second model is the more attractive one. However, the *in planta* activity of cell wall-bound acid invertase in the stolon tips of *S. demissum* remains to be proven.

Acknowledgement. Northern analyses were performed at the Deparment of Plant Breeding, Wageningen, The Netherlands, with help of Dr. Richard Visser and Irma Straatman-Engelen.

Chapter 6

COMPARISON OF PHOSPHORYLATED HEXOSE CONTENTS OF INDIVIDUAL STOLON TIPS FROM *SOLANUM DEMISSUM* USING HPAEC-PED

Summary

Small quantities of phosphorylated intermediates can be effectively separated and detected by high pH anion exchange chromatography using pulsed electrochemical detection (HPAEC-PED). HPAEC-PED allows the comparison of phosphorylated intermediate contents of small plant samples (1-2 mg DM). Retention times of phosphorylated intermediates in both standard solutions and in crude plant extracts were highly reproducible. Identification of peaks was based on repeated co-migration using various elution conditions, and on the evaluation of peak-size and shape in chromatograms from stolon tip extracts with and without the addition of a standard solution of phosphorylated intermediates.

Levels of phosphorylated intermediates were determined in individual stolon tips (dry weight: 2-50 mg) of *Solanum demissum*, a wild potato species which only forms tubers when it is exposed to short-day (SD) conditions. Comparisons were made between the phosphorylated intermediate contents of subapically non-swollen stolon tips collected from plants exposed to long-day (LD) and SD conditions, and between SD stolon tips showing various stages of subapical swelling. The Fru1,$6P_2$ concentration dropped dramatically in subapically swollen SD stolon tips as compared to LD tips. The physiological consequences of this and other observed changes are discussed.

Introduction

As in many other plants, sucrose is the main transport sugar in potato. In photosynthetically inactive sink tissue, like in a stolon tip, the first step in sucrose metabolisation is its splitting. Sucrose is metabolised either irreversibly by an invertase (EC 3.2.1.26), or reversibly by sucrose synthase (EC 2.4.1.13).

In case of sucrose hydrolysis by an invertase, the reaction products are glucose (Glc) and

fructose (Fru). Hexokinases (EC 2.7.1.1), glucokinases (EC 2.7.1.2), or fructokinases (EC 2.7.1.4) are often referred to collectively as hexose kinases (Doehlert 1989). At the expense of one NTP per hexose unit, cytosolic hexose kinases will irreversibly phosphorylate Glc and Fru into Glc6P and Fru6P, respectively. One unit of NDP will be released for every phosphorylated hexose.

Depending on the concentrations of UDP, sucrose, UDPGlc and Fru, cytosolic sucrose synthase will catalyse the conversion of sucrose and UDP into UDPGlc and fructose, or the reverse reaction. The fructose moiety of sucrose will enter glycolysis as Fru6P.

For tuberization *Solanum demissum* Lindl. is an absolutely short-day (SD) dependent potato species; tuber formation does not occur under LD. Tuber initiation in *S. demissum* coincides with a shift in the way sucrose is split. In non-swollen stolon tips acid invertase is the predominant sucrose-metabolising enzyme, whereas sucrose synthase is the main enzyme catalysing the splitting of sucrose in radially growing stolon tips (Chapter 5). One purpose of the present experiments is to investigate whether this change in enzyme activity is reflected in changes in pool size of phosphorylated intermediates.

In contrast with starch synthesis in leaves, starch accumulation in photosynthetically non-active tissue is accompanied by the import into the amyloplast of phosphorylated hexoses, such as Glc1P, Glc6P or Fru6P (Keeling *et al.* 1988, Hatzfeld and Stitt 1990). In isolated amyloplasts from pea, Glc6P is the most efficiently incorporated substrate (Hill and Smith 1991), whereas Glc1P is imported efficiently in amyloplasts from wheat endosperm (Tyson and ap Rees 1988). We assume that in developing tubers of *S. demissum*, too, phosphorylated hexoses are imported into the amyloplast. In the amyloplast, the readily reversible conversion of Glc1P and ATP into ADPGlc and PP_i (inorganic pyrophosphate) by ADPGlc pyrophosphorylase (EC 2.7.7.27) is effectively irreversible due to the high pyrophosphatase (EC 3.6.1.1.) activity. By this mechanism, accumulation of Glc1P in the amyloplast is prevented. If the glycolytic enzymes, required for the conversion of Glc6P and Fru6P into Glc1P, are present in the amyloplast, then these phosphorylated hexoses can be a substrate for starch synthesis, and also their levels will presumably be low. Therefore, overall measurements of levels of phosphorylated intermediates could provide information about the nature of the imported compound(s).

In case sucrose is split by sucrose synthase, the glucose moiety of sucrose is released as UDPGlc. UDPGlc is converted by UDPGlc-pyrophosphorylase (EC 2.7.7.9) into Glc1P and UTP at the expense of one unit of PP_i. The earlier-mentioned shift from invertase to

sucrose synthase activity implies that one unit of each UDP and PP_i are needed for the metabolisation of the glucose moiety of sucrose into Glc1P. How does a plant meet this high demand for UDP and PP_i? UDP is produced in the conversion of Fru into Fru6P, if UTP is used as a phosphoryl donor. Fructokinase activity in developing potato tubers is not selective with regard to NTPs (Xu *et al.* 1989). Alternatively, UDP and PP_i could be provided by a futile cycle in which Fru6P is converted into Fru1,$6P_2$ by NTP-phosphofructokinase (NTP-PFK, EC 2.7.1.11), and Fru1,$6P_2$ is converted back into Fru6P by PFP. PFP can operate both in the glycolytic and gluconeogenic directions (Xu *et al.* 1989). In this cycle UTP and 2 P_i (inorganic phosphate) are converted into UDP and PP_i. PFP activity is regulated by, *e.g.*, Fru2,$6P_2$ and PP_i (Stitt 1987, Stitt 1989, respectively). Therefore, it is interesting to see whether levels of Fru6P and Fru1,$6P_2$ change together with the shift from invertase into sucrose synthase activity.

In this report two comparisons are made. Firstly, levels of phosphorylated intermediates are compared between non-swollen stolon tips from LD and SD plants. Sucrose metabolism in LD and SD stolon tips differs quantitatively; acid invertase activity is significantly higher in LD stolon tips as compared to the activity in non-swollen SD stolon tips. In both types of stolon tips sucrose synthase activity is below detection level (Chapter 5). Secondly, concentrations of phosphorylated intermediates are compared between SD stolon tips showing different stages of subapical swelling. The consequences of the increasing activity of sucrose synthase during subapical swelling are considered.

When potato plants are exposed to tuber-inducing conditions not all stolon tips will start to swell simultaneously. Potato tuber initiation is irregular; only a subset of the SD stolon tips starts to swell subapically (Vreugdenhil and Struik 1989, Chapter 3). This irregularity is a serious drawback because stolon tips, harvested at the same time from a single plant, will be in different stages of development, and thus cannot be pooled for analyses. However, this irregularity can also be interpreted as an advantage, because stolon tips in different stages of development can be collected from a single plant on the same day.

Since the dry weight of these tips is low, ranging from 2 to 50 mg, and the level of the phosphorylated intermediates is also low, a very sensitive technique was needed. In this report we show that high pH anion exchange chromatography combined with pulsed electrochemical detection (HPAEC-PED, for basic principles see Lee (1990)) allows the levels of several phosphorylated intermediates to be compared between individual stolon tips and small tubers.

Materials and methods

Plant material. True seeds (BGRC 9981) of *Solanum demissum* Lindl. were obtained from the Institut für Pflanzenbau und Pflanzenzuchtung der FAL (Braunschweig, Germany). One single clone was used in all experiments. This clone was propagated *in vitro* as described in Chapter 2 (p. 15).

***In vivo* tuber induction.** For *in vivo* experiments about 70 two-week-old *in vitro* plants were transferred to potting soil. After 4 weeks *in vivo* growth in a walk-in growth chamber, the PAR period was reduced from 16 h to 10 h. Half of the plants were further grown under LD conditions by using low-intensity (< 2 W m^{-2}) incandescent lamps. The other plants were exposed to SD conditions. Climatal conditions were as described in Chapter 2. After six weeks of exposure to LD or SD conditions, non-swollen LD and subapically non-swollen and swollen SD stolon tips were harvested. All harvests were done 6-7 h after the start of the PAR period. Immediately after collection, the stolon tips were frozen in N_2 (l), and freeze-dried. After freeze-drying the samples were stored at -80°C until further analysis.

Sample preparation. To compare two sample preparation methods, single, relatively large, freeze-dried tubers (n=3, dry weight: 100-200 mg) were homogenized and the homogenate was divided.

One half of each sample was extracted essentially as described by Foley *et al.* (1992). After addition of 1 ml methanol:water (4:1, v/v), the samples were heated for 10 min. at 75°C. Subsequently, the solvent was removed by vacuum centrifugation, followed by overnight freeze-drying. Sample extraction was done in H_2O, adding 50 μl H_2O per mg DM. Undissolved residue was removed by centrifugation for 20 min. in an Eppendorf table centrifuge at maximal speed. The supernatant was analyzed without further dilution.

The other half of each sample was extracted as described by Viola and Davies (1991). In this procedure, homogenized plant tissue is extracted two times with a 1.41 M perchloric acid solution. No sample heating is needed.

Qualitative sugar determinations by HPAEC-PED

Chromatography. The system used for high pH anion exchange chromatography with pulsed

electrochemical detection (HPAEC-PED) consisted of a Dionex (Sunnyvale, USA) BioLC gradient pump module and a pulsed electrochemical detector (PED). Sample injection was via a Spectra Physics (San Jose, USA) autosampler (SP 8880) equipped with a 20 μl sample loop. The Rheodyne injection valve on the autosampler was equipped with a Tefzel rotor seal to withstand the high pH of the eluants. Inserts (0.1 ml) were used in the autosampler vials. Sugars were chromatographed on a CarboPac PA100 (4 x 250 mm) column and preceded by a guard column (CarboPac PA100, 4 x 50 mm). The flow rate used was 1 ml/min. at ambient temperature.

Elution. The eluants were prepared by suitable dilution of 50% NaOH solution with high purity water (specific resistance > 10 MΩ.cm). A Dionex eluent degas module (EDM-2) was employed to sparge and pressurize the eluants with helium.
Two eluants were used: eluant A was a 100 mM NaOH solution containing 50 mM NaAc, eluant B contained 1100 mM NaAc in 100 mM NaOH. The following elution program was applied: t= 0-10 min., 0% B; t= 10-30 min., linear gradient from 0% to 16% B; t= 30-50 min., linear gradient from 16% to 48% B; t= 50-65 min., 48% B. Thereafter, the column was washed for 5 min. with 79% B, followed by 20 min. re-equilibration with 0% B.

Chemicals. NaOH solution (50% w/w) was purchased from J.T. Baker B.V. (The Netherlands). Sodium acetate (anhydrous) was from Merck (Germany). Glc1P, Glc6P, Fru6P and Fru1,6P_2 were purchased from Boehringer Mannheim GmbH (Germany). Fru2,6P_2, ADPGlc and UDPGlc were from Sigma (USA).

Detection. Integrated amperometry was used to detect the carbohydrates using a gold working-electrode and a Ag/AgCl reference electrode. The following pulse potentials and durations were used for the detection of neutral sugars: t= 0-0.5 s, E= 0.10 V; t= 0.51-0.59 s, E= 0.60 V; t= 0.60-0.65 s, E= -0.60 V. The integration period was t= 0.30-0.50 s. Phosphorylated hexoses were detected at an analog range of 1 μC.

Results

Separation and detection of a standard mixture of phosphorylated sugars

Figure 6.1 shows that several phosphorylated hexoses were separated adequately by HPAEC.

Quantities in the 10 pmol range can be detected for a number of phosphorylated hexoses. Contrary to peaks representing the other phosphorylated hexoses, the ADPGlc peak shows tailing.

In Table 6.1, the relative PED response per nmol is given for several phosphorylated hexoses. The response is expressed in relative terms because the absolute response, *viz.* peak area, is variable and depends on the condition of the gold-electrode. This does not imply that only relative data on sugars level can be obtained by HPAEC-PED. Every third or fourth sample run was alternated by the analysis of a standard mixture of P-sugars, and quantification of a phosphorylated hexose was done by comparison of the peak area with the analogous peak area in the nearest standard chromatogram.

Each of the separated substances showed a specific relative PED response per nmol. It is noted that even phosphorylated hexoses with the same functional groups, such as Glc1P and Glc6P, give different relative responses. The standard deviation of the PED response of ADPGlc is relatively high. Presumably ADPGlc does not stand well the exposure to strongly alkaline elution conditions. Hence, no ADPGlc determinations were done in plant extracts. The retention times for a given compound were remarkably constant (Tab. 6.1).

Fig. 6.1. A mixture of phosphorylated hexoses was chromatographed on a Dionex CarboPac PA-100 column (4.6 x 250 mm) and a companion guard column (CarboPac PA-100, 4 x 50 mm), and detected by PED. The mixture contained: A, Glc1P (0.77 nmol); B; Glc6P (0.96 nmol); C, Fru6P (0.88 nmol); D, ADPGlc (0.37 nmol); E, Fru2,6P_2 (0.20 nmol); F, Fru1,6P_2 (0.33 nmol), G, UDPGlc (0.43 nmol).

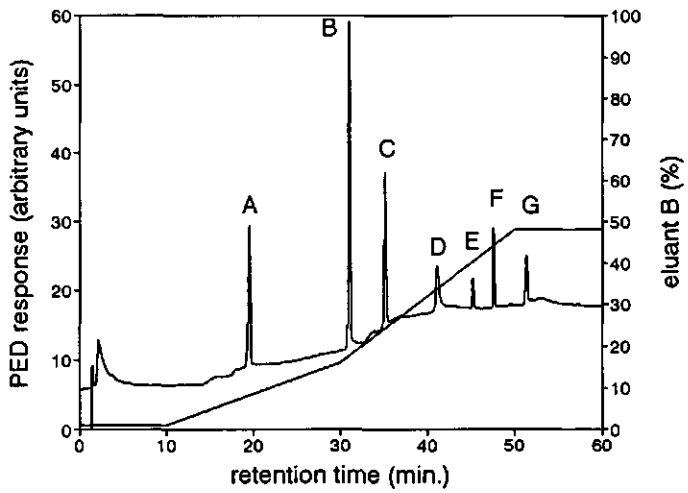


Table 6.1. Average retention times and relative PED responses of phosphorylated hexose in a standard mixture separated by HPAEC-PED. Data are obtained from six representative chromatograms.

	average retention time (min.) ± sd	PED response per nmol relative to the PED response of Glc6P
Glucose 1-P (Glc1P)	19.55 ± 0.13	52 ± 6 %
Glucose 6-P (Glc6P)	31.01 ± 0.02	(100%)
Fructose 6-P (Fru6P)	35.29 ± 0.15	57 ± 5 %
ADP-glucose (ADPGlc)	41.57 ± 0.70	53 ± 28 %
Fructose 1,6 P_2 (Fru1,6P_2)	47.57 ± 0.03	65 ± 10 %
UDP-glucose (UDPGlc)	51.75 ± 0.39	34 ± 3 %

Comparison of methods to extract phosphorylated hexoses

Two extraction methods were compared: extraction in 80% methanol, followed by heating at 75°C (80% meth.), and extraction with perchloric acid at 4°C (PCA). A small tuber sample was split and extracted using both methods and analyzed by HPAEC-PED. Fig. 6.2 shows a 5 min. part of four chromatograms with a peak that co-migrates with Glc1P. No significant differences in peak area can be observed. Also in the other parts of the chromatograms no differences between the phosphorylated intermediate contents occurred (data not shown). The first method (80% meth.) was used in further experiments, since in this procedure no extra salt was added to the samples.

Separation and detection of phosphorylated sugars in stolon tips

The water-soluble fraction of a stolon tip was chromatographed without any precolumn cleanup. The absence of cleanup steps excluded the possibility of precolumn losses. Therefore, internal standards were not routinely added to the water-soluble fraction of the stolon homogenate. The water-soluble fraction of stolon tips from *S. demissum* contains high concentrations of glucose and fructose (Chapter 4). The presence of these neutral sugars

could interfere with the detection of low levels of phosphorylated hexoses in stolon tips from *S. demissum*. Therefore, the first 10 min. of the elution program were used to elute neutral compounds. The high resolution of HPAE chromatography is illustrated in Fig. 6.3. Per sample, the number of peaks that could be detected between t= 10 min. and t= 65 min. in the water-soluble fraction of stolon tips varied between 46 and 62. Identification *sensu stricto* of substances separated by HPAEC is possible by on-line mass spectrometry. However, at present only molecular mass information is obtained by HPAEC-MS (Van der Hoeven *et al.* 1992), which obviously is not enough for a proper identification of phosphorylated hexoses.

Co-elution with a standard can be used as a relatively strong indicator for the identity of a certain peak, because the variation in the retention times between different chromatograms is minute in this system (Tab. 6.1). The identity of the peaks was confirmed using a modified elution program (data not shown). In an alternative approach, small quantities of several phosphorylated hexoses were added to the sample. The identity of a certain peak was reconfirmed by evaluation of the peak area and shape with and without the addition of the internal standards. In Fig. 6.4, parts of chromatograms of LD stolon tip extracts are shown with (+) and without (-) the addition of Glc1P. The shape and the

Fig. 6.2. Homogenates of two freeze-dried tubers (indicated '1' and '2') from *S. demissum* were split. One half of each sample was extracted with perchloric acid (PCA), whereas enzyme activity in the other half was eliminated by heating the sample for 10 min. in 80% MeOH (80% meth.). A part of the chromatograms is shown including a peak that co-migrates with Glc1P (arrow).

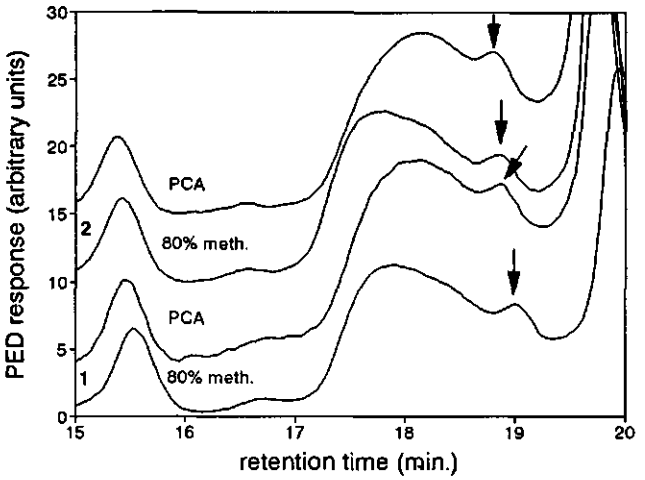
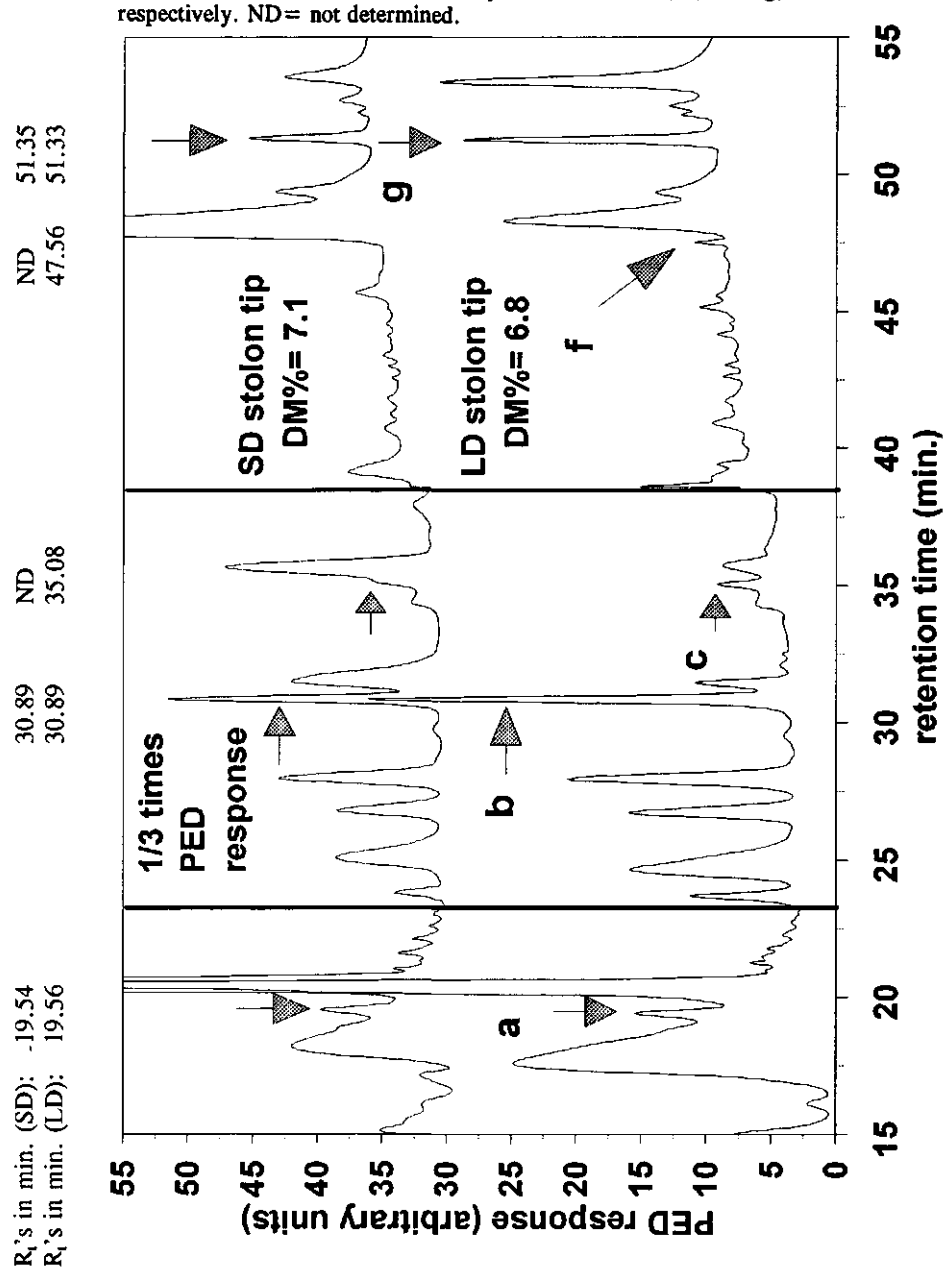


Fig. 6.3. Comparison of chromatographs of water-soluble fractions from individual, representative subapically non-swollen LD and SD stolon tips from *S. demissum*. The positions of Glc1P, Glc6P, Fru1,6P_2, and UDPGlc are indicated by the lower cases a, b, f and g, respectively. ND= not determined.



retention time are unchanged. For other phosphorylated intermediates similar results were obtained.

Phosphorylated hexoses in subapically non-swollen SD and LD stolon tips

Chromatograms were compared of water-soluble fractions of non-swollen stolon tips that were collected from plants grown under LD and SD conditions. Non-swollen SD stolon tips were collected from tuber-bearing plants. In Fig. 6.3 a part is shown of two representative chromatograms of the water-soluble fractions of LD and SD stolon tips.

Peak 'a' in Fig. 6.3 shows that Glc1P was not properly separated from an other unknown compound. Therefore, no quantification was possible. After comparison of 5 chromatograms of each treatment, it is concluded that the Glc1P levels of non-swollen SD and LD stolon tips did not differ.

The concentration of Glc6P (peak 'b') in SD stolon tips was significantly lower than in LD stolon tips (6.7 $\pm$ 1.1 nmol mg^{-1} DM and 4.2 $\pm$ 0.8 nmol mg^{-1} DM, respectively).

Peak 'c' in Fig. 6.3 shows that Fru6P was not properly separated from an other unknown compound. Variation of the elution conditions did not improve the separation to an extent that (relative) quantification was possible.

The concentration of an unknown (group of) compound(s), that eluted shortly after

Fig. 6.4. The effect of the addition of 0.1 nmol Glc1P to two extracts of LD stolon tips (indicated 'A' and 'B') on peak surface and peak shape of the presumed endogenous Glc1P.

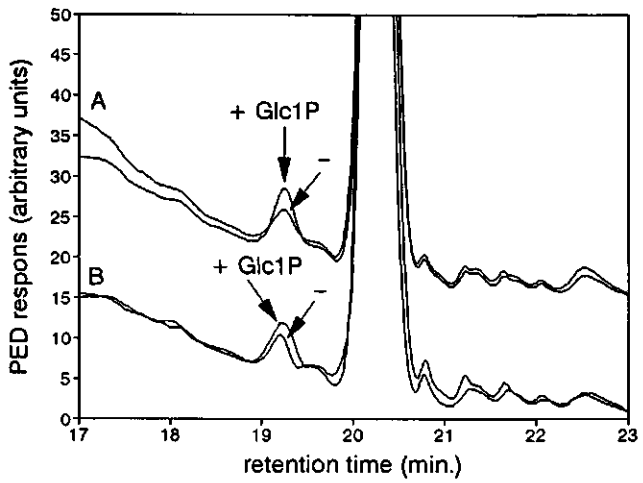
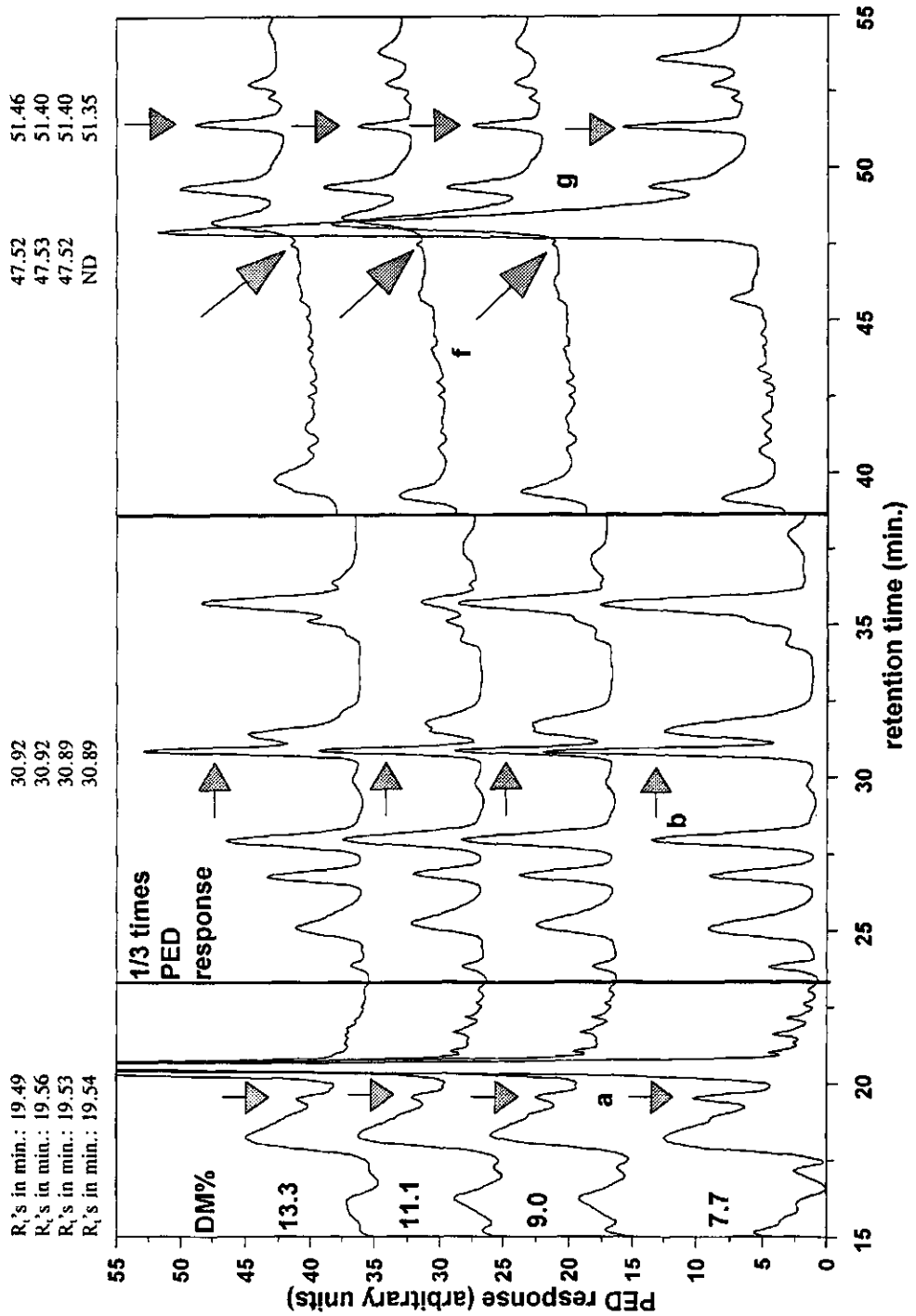


Fig. 6.5. Comparison of chromatographs of water-soluble fractions of SD stolon tips from *S. demissum* with various dry matter percentages, representing different developmental stages. The positions of Glc1P, Glc6P, Fru1,6P_2, and UDPGlc are indicated by the lower cases a, b, f and g, respectively.



Fru1,6P_2, was drastically increased in SD stolon tips. Contrary to LD stolon tips, Fru1,6P_2 (peak 'f') was not separated from this group in SD stolon tips (Fig. 6.3). In LD stolon tips, the concentration Fru1,6P_2 was 214 $\pm$ 85 pmol g^{-1} DM .

The concentration of UDPGlc (peak 'g') in SD stolon tips was about 4 times lower than in LD stolon tips (0.6 $\pm$ 0.2 g^{-1} DM and 2.8 $\pm$ 0.5 nmol respectively).
All concentrations given are the average of at least 5 replicates ($\pm$ sd).

Phosphorylated hexoses in tuberizing stolon tips

Because tuber initiation is irregular in *S. demissum*, both subapically non-swollen and swollen stolon tips can be collected from a single SD plant at the same moment. The dry-matter content was used to indicate the developmental stage of a stolon tip. Stolon tips with a DM content higher than 10% are visibly swollen (Chapter 4). Four representative chromatograms of water-soluble fractions of stolon tips of different developmental stages were compared (Fig. 6.5).

The Glc1P level tended to decrease during the process of tuber initiation. As can be seen in Fig. 6.5, the Glc1P peak is superimposed on a peak of an unknown compound. Therefore, no proper quantification was possible. From the peak areas, it is estimated that the Glc1P level was about halved during the process of tuber initiation.

The Glc6P level shows considerable variation during tuber development. The transient decrease observed at intermediate DM%, was not consistent. Glc6P determinations in other samples revealed other, although still relatively variable, patterns. The average Glc6P concentration in the stolon tips was 3.7 $\pm$ 0.9 nmol mg^{-1} DM.

In SD stolon tips with a low DM%, Fru1,6P_2 could not be separated from an unknown substance(s) which level decreased strongly during tuber initiation. After the onset of tuber initiation the concentration of this unknown substance decreased sufficiently to make Fru1,6P_2 detectable. The average Fru1,6P_2 concentration in subapically swollen stolon tips was 25 $\pm$ 4 pmol mg^{-1} DM. The Fru1,6P_2 concentration was strongly reduced as compared to the concentration in LD stolon tips (Figs. 6.3 and 6.5).

From the different experiments, no indications were found for a drastic change in the UDPGlc concentrations in stolon tips during the process of tuber initiation. The average UDPGlc concentration was 0.57 $\pm$ 0.21 nmol mg^{-1} DM.
All concentrations given are the average of at least 5 replicates ($\pm$ sd).

Discussion

Closely related phosphorylated hexoses such as Glc1P and Glc6P can be separated underivatized by high pH anion exchange chromatography (HPAEC) on the basis of small differences in acidity. Pulsed electrochemical detection (PED) is sufficiently sensitive to determine glycolysis intermediates in relatively small quantities of plant material (1-2 mg DM). For example, in a crude extract of a developing tuber, 5-10 pmol Fru1,6P_2 was detectable. The molar response of PED is compound-dependent. Identification *sensu stricto* of phosphorylated hexoses is not possible by HPAEC-PED. However, strong indications about the identity of several peaks were obtained on the basis of repeated, very strict co-elution of these peaks with phosphorylated hexoses from standard solutions under various elution conditions (Fig. 6.4). HPAEC-PED is a relatively fast technique to detect phosphorylated intermediates in a complex solution such as a crude plant extract, but it cannot replace enzymatic metabolite measurements as described by, *e.g.*, Stitt *et al.* (1989). Using several elution programs it was unsuccessfully tried to separate Glc1P and Fru6P from other, unknown compounds. Therefore, a comparison of levels of these compounds could be made, but proper quantification was not possible by HPAEC-PED.

When subapically non-swollen SD stolon tips were compared to LD stolon tips, the Glc6P concentration was significantly lower in SD tips (Fig. 6.3). This change can be interpreted as a result of the earlier observed decrease of the acid invertase activity under SD conditions (Chapter 5). As a consequence less glucose is released, and less Glc6P is available for dissimilatory energy production. Accordingly, the decrease of the acid invertase activity coincides with a strong decrease in longitudinal growth of the stolons (Helder and Van der Maarl, unpublished results). A decrease in stolon growth under SD conditions is also found for *S. tuberosum* (Struik *et al.* 1988).

Although tuber initiation in *S. demissum* is accompanied by a shift from acid invertase activity into sucrose synthase activity (Chapter 5), no increase of the UDPGlc concentration was observed during the process of subapical swelling (Fig. 6.5). Geigenberger and Stitt (1993) showed that sucrose synthase catalyses a readily reversible reaction *in vivo* in developing tubers from *Solanum tuberosum*. Assuming the levels of the reactants to be comparable to those in developing *S. demissum* tubers, it is possible that also sucrose synthase activity was involved in the observed buffering of the UDPGlc concentration in stolon tips of *S. demissum*. However, the UDPGlc was probably metabolised immediately

by UDPGlc-pyrophosphorylase. The activities of this enzyme were found to be higher than 1 unit/mg protein in several actively filling sucrose sinks (Sung *et al.* 1989). Similar high activities were found in LD and SD stolon tips from *S. demissum* (Chapter 5).

As argued in the Introduction, massive import of (a) phosphorylated intermediate(s) into amyloplasts and subsequent utilization for starch synthesis, might be reflected in a decrease of the overall concentration of that intermediate in stolon tips. In SD stolon tips the increase of the DM%, indicative for tuberization, was accompanied by a distinct decrease of the Glc1P concentration (Fig. 6.5). No correlation was found between the fluctuating Glc6P levels and the DM% of the tips. As stated before, Fru6P could not be quantified in some developmental stages. These results might be interpreted as an indication that Glc1P is (one of) the phosphorylated hexose(s) imported into *S. demissum* amyloplasts.

Although no data are available for Fru1,$6P_2$ levels in non-swollen SD tips, a drastic drop of the Fru1,$6P_2$ concentration was observed when subapically swollen SD stolon tips were compared with LD tips. Unlike in LD stolon tips, relatively large quantities of UDP and PP_i are needed for starch synthesis in swollen SD tips (see Introduction). A way to produce these quantities could be the stimulation of PFP activity in the gluconeogenic direction. A moderate increase of NTP-PFK activity might be expected, too, but this increase need not to keep step with the increase of the PFP activity because UDP is also released in the irreversible phosphorylation of fructose. Contrary to both PFKs, aldolase activity is not known to be strictly controlled. Therefore, the observed drop of the Fru1,$6P_2$ concentration can be explained by an increased activity of PFP in the reverse direction to yield PP_i.

The results indicate that Glc1P is taken up by amyloplasts as a substrate for starch synthesis, and point to the existence of a futile phosphorylated-fructose cycle for the generation of co-enzymes for the conversion of the glucose moiety of sucrose into Glc1P, and at least partly agree with the earlier established change in sucrose-metabolising enzymes at the onset of tuberization.

Chapter 7

GENERAL DISCUSSION

Introduction

In this chapter the results presented in the preceding chapters will be discussed in a broader context in which findings will be linked to knowledge about other fields in plant physiology. Some essential results that were presented in preceding chapters will be repeated briefly, to make the content of this chapter accessible (understandable) with minimal knowledge about the rest of this thesis.

Environmental conditions and tuber induction in potato plants

The SD dependency of potato plants originally means that tubers will be formed after a number of diurnal cycles in which the duration of the dark period has been longer than a species-dependent, minimal night-length, provided that the night-temperature is adequately low (5°C-20°C). The duration of the dark period is monitored in the leaves. By an unknown mechanism, in which phytochrome is involved, the plant is able to 'remember' the number of cycles it has been exposed to. It is conceivable that a sufficiently long night is translated into a certain amount of an accumulatable quantity. Actual tuber initiation would start if a certain threshold value is surpassed.

The minimal night-length for such domesticated *Solanum tuberosum* L. cultivars as Bintje and Désirée, has been reduced to 0 h. Therefore, for commercial potato cultivars, it is hard to create non-inducing conditions. In order to have a non-tuber-bearing control object, another *Solanum* species was selected. Tuber induction in the Mexican, wild potato species *Solanum demissum* Lindl. is short-day (SD) dependent. *S. demissum* plants reached the point at which tuber initiation is triggered after at least three weeks exposure to a night-length of 14 h. Although it is hard to understand how plants were able to sense seasons while they were continuously grown and multiplied in growth chambers for more than two years, more

short-day cycles (four to five weeks) were needed when experiments were done in winter.

Even after two months exposure to 8 h night-length (= 16 h day-length) conditions, *S. demissum* plants did not form tubers. From this datum, it cannot be excluded that 16 h day-length is still tuber inducing. A small amount of the quantity might accumulate over this period without reaching the threshold value needed for actual tuber initiation. Nevertheless, plants exposed to 16 h day-length conditions were considered as practically non-induced.

As already indicated by its name, *S. demissum* is small as compared to, *e.g.*, *Solanum tuberosum*. Except for this characteristic, the habits of *S. demissum* resemble those of *S. tuberosum*.

The aspecificity of a tuber-inducing principle

Nitsch (1965) did grafting experiments using a non-tuber-forming and a tuber-forming *Helianthus* species. Sunflower (*Helianthus annuus* L., variety Mammoth Russian) was grafted onto Jerusalem artichoke (*H. tuberosus* L., variety Vilmorin D-19), and this combination was exposed to long or short days. Flowering in *H. annuus* is day-length independent. The stem part of Jerusalem artichoke was covered with black polyethylene, so the rootstock could not monitor day-length. Tuber formation in the *H. tuberosus* variety is absolutely short-day dependent. In this experiment, the rootstock only formed tubers when the sprout was exposed to SD conditions. Nitsch (1965) concluded that tuber induction in Jerusalem artichoke is triggered by a relatively unspecific (group of) compound(s), *viz.* (a group of) compound(s) that can also be synthesized and transported basipetally by sunflower, a plant that never forms tubers.

In a number of plant species, tuber and flower induction are triggered by the duration of the dark period. The relation between flowering and tuber induction was studied by Chailakhyan *et al.* (1981) and Martin *et al.* (1982). The towards flowering absolutely long-day dependent tobacco species *Nicotiana sylvestris* L. was grafted onto the towards tuber-induction absolutely short-day dependent *Solanum* species *S. andigena*. Most remarkable was the following observation. When *N. sylvestris* was grafted onto *S. andigena* L. and exposed to long-day conditions (16 h), flowers were formed in the upper-plant parts, whereas the *S. andigena* rootstock formed tubers (Chailakhyan *et al.* 1981). This result not only confirms that a tuber-inducing principle is synthesized or activated in the leaves (Gregory 1956), but it shows that the presumably non-specific tuber-inducing principle can also be synthesized

and transported under LD conditions.

The identity of (a) non-specific tuber-inducing compound(s)

In Chapter 2 it is shown that the occurrence of 11-hydroxy-jasmonic acid (11-OH-JA) and, to a lesser extent, 12-OH-JA is closely correlated with tuber induction in *S. demissum*. In leaflets from LD plants, no hydroxylated JAs were detectable, even not after application of mild stress. Mild stress was shown to induce rapid (hydroxylated) JA synthesis (Chapter 2).

While a tuber-inducing principle should be non-specific, it was impossible to verify this for 11-OH-JA because it was the first time that this hydoxylated JA was detected as an endogenous compound in higher plants.

Earlier, a glucoside of 12-OH-JA was detected in leaves from *S. tuberosum* cv Irish Cobbler plants that were exposed to tuber-inducing conditions (Yoshihara *et al.* 1989). The aglycone of this substance was called 'tuberonic acid' by the authors. The same glucoside was detected in leaves from tuber-forming Jerusalem artichoke (*H. tuberosus*) plants (Yoshihara *et al.* 1992).

In leaves of the tuber-forming monocotyledon yam (*Dioscorea batatas* Decne.), Koda and Kikuta (1991) found no hydroxylated jasmonic acids, whereas the JA concentration increased with the growth of the plants. When JA was applied to yam plants *in vitro*, it induced tuber formation in concentrations higher than 10^{-7} M.

From our results it should be concluded that if one wishes to name 12-OH-JA 'tuberonic acid', it would be more appropriate to call this substance 'a tuberonic acid'. In that case, the 'tuberonic acids' would at least comprise 11-OH-JA and 12-OH-JA.

The relationship between (hydroxylated) JAs and tuber induction in *S. demissum*

Whether the relationship between the occurrence of hydroxylated JAs and tuber induction in *S. demissum* is causal, remains to be proven. JA was shown to induce tuber formation *in vitro* in *S. demissum* explants where no translocation is required (Chapter 2). Since only minute amounts of 11- and 12-hydroxy-JA were available for calibration of the GC-MS, it was not possible to test these compounds *in vitro*, too.

Assuming JA to be tuber-inducing *in planta*, it is hard to explain why similar concentrations JA were found in leaflets from non-tuber-bearing and tuber-bearing potato plants. A clue could be that JA itself is too apolar to be exported from the leaflets. JA would

be hydroxylated only to facilitate glycosylation. The resulting, relatively polar product will be transported by the phloem without any problem. Hydroxylated JAs are metabolization products of jasmonic acid (*e.g.* Parthier *et al.* 1992). The conversion of JA into 11- and 12-OH-JA is likely to be catalyzed enzymatically. If hydroxylated JAs are responsible for tuber induction, SD conditions would results in:

- the formation or activation of JA hydroxylating enzymes or,
- the neutralization of the spatial separation between enzyme and substrate.

This role of JA-hydroxylating enzymes would comprise the facilitation of the transport of the tuber-inducing principle, and not its synthesis.

An indication for a causal relationship between the occurrence of hydroxylated JAs and tuber induction could be obtained if tuber formation is suppressed when (one of) the enzyme(s) involved in the hydroxylation of JA is inhibited. However, the mere existence of JA-hydroxylating enzymes still has to be proven and, consequently, no specific inhibitors are available yet.

A powerful approach to unravel the consequences of exposure to SD conditions could be differential screening of the polyA-mRNA contents of LD and SD leaflets from an absolutely SD-dependent *Solanum* species. Although this approach is laborious, it is quite feasible since genetically identical plants can be easily obtained and grown under well-defined conditions.

The irregularity of tuber initiation

When potato plants are exposed to tuber-inducing conditions, only a subset of the stolon tips will start to swell subapically; tuber initiation in *S. tuberosum* is irregular (Vreugdenhil and Struik 1989). The SD-dependent *Solanum* species *S. demissum* is suitable to investigate this phenomenon (Chapter 1). Since plants were pre-grown under 16 h day-length conditions, the experiments could be started with non-induced plants. From the same day onwards, well-grown, 'virgin' potato plants were exposed to tuber-inducing conditions. Despite of the rigorous measures taken to minimize interplant variation, the variation in the number of tubers formed per plant was considerable. All *S. demissum* plants formed heavily branched stolons, and no parameter was found that correlated with tuber initiation. It is concluded that it is still premature to design a more fundamental approach to unravel the reception of the tuber-inducing principle in the stolon tip.

Presumably hydroxylated jasmonic acids are involved in tuber induction. It remains to be demonstrated that this compound(s) is transported basipetally. If so, one could think about the characterization of (a) receptor(s). From our data it can be deduced that this will be a hard job. Receptor molecules may be only functional or accessible in a subset of the available stolon tips. Intensive monitoring of a large set of stolon tips did not reveal any indicator for the availability of a functional receptor in a stolon tip (Chapter 3).

Sucrose metabolism in stolon tips during tuber initiation

It is unclear how the registration of a tuber-inducing principle in a stolon tip leads to the expression of genes encoding for (semi-)tuber-specific proteins. It was decided to investigate sucrose metabolism in developing stolon tips for two reasons: sucrose can induce tuber formation *in vitro* in *S. tuberosum* explants (*e.g.* Hussey and Stacey 1984), and sucrose can induce the expression of genes coding for patatin and proteinase inhibitor II *in vitro* (Wenzler *et al.* 1989, Johnson and Ryan 1990). At the present day we know that *in planta* this (semi-) tuber-specific gene expression is not triggered by sucrose (Müller-Röber *et al.* 1992) and probably the same applies for tuber induction by sucrose.

We started to measure pool-sizes of neutral sugars in developing stolon tips. It was found that the (freeze-)dry-matter content of a stolon tip is a good measure for the quantification of early developmental stages of stolon tips. In these stages, the dry matter content reflected unequivocally the starch content of a stolon tip. Evidently, starch synthesis is not the first event in tuber initiation. Several developmental changes, such as an increase of the concentrations of mRNAs encoding for sucrose synthase and ADPG-pyrophosphorylase (Dr. D. Vreugdenhil, pers. commun.) will occur in the stolon tip before starch accumulates. However, in this study on sugar metabolism in stolon tips, which is closely related to starch biosynthesis, dry matter content was a useful measure.

A possible function for an enzyme shift during tuber development

Tuber induction changes a photosynthetically non-active stolon tip from a moderate sucrose sink into a strong sucrose sink. In the Chapters 4, 5 and 6, it is clearly shown that tuber induction implicates more than a quantitative change in sucrose metabolism. Whereas sucrose was split by an acid invertase in the apoplast or in the vacuole, this function is taken over by cytosolic sucrose synthase after the onset of tuber initiation.

Is this enzyme shift functional? It can be argued that sucrose synthase splits sucrose energetically more efficiently than an invertase. However, if one considers that one sucrose molecule converted to CO_2 and H_2O by respiration produces 76 molecules of ATP, it is hard to conceive how an $\approx 1\%$ efficiency increase (one ATP molecule) could be relevant as an explanation of the above-mentioned enzyme shift.

Sucrose splitting by invertase is highly exothermic and irreversible. *In vitro* the reaction catalyzed by sucrose synthase is reversible. If one of the reaction products is removed immediately, such a reaction could still *de facto* be irreversible. Geigenberger and Stitt (1993) showed for developing potato tubers from *Solanum tuberosum* that sucrose synthase catalyzes a readily reversible reaction *in vivo*. Assuming sucrose splitting in developing tubers from *S. demissum* to be reversible, too, the question raises whether a shift from irreversible to reversible sucrose splitting could be functional. Geigenberger and Stitt (1993) argued that if sucrose in the cytosol is split by a neutral invertase into fructose and glucose, this would have a drastic osmotic effect. Only by controlling the rate of sucrose unloading from the phloem, the plant would be able to regulate the invertase activity in the stolon tip.

Sucrose splitting by sucrose synthase could prevent the accumulation of UDPGlc and fructose, since the direction of sucrose synthase activity is regulated by the levels of its reaction products. This self-regulation is presumably functional in developing tubers where large quantities of sucrose are transported to the stolon tips, and the danger of 'osmotic accidents' is realistic.

A possible function for a spatial shift during tuber development

Acid invertases and sucrose synthase are active in different compartments. If it is assumed that sucrose splitting is catalyzed by cell wall-bound acid invertase, then tuber initiation would implicate a change from apoplastic sucrose unloading to symplastic sucrose unloading.

Does the change in the site where sucrose is split, offers advantages to the plant? In subapically non-swollen stolon tips that show fast, longitudinal growth, the site of phloem unloading will shift. If phloem unloading would be symplastic, then plasmodesmata connecting sieve elements with surrounding parenchyma cells would function only for a short period. Hence, the cost for apoplastic unloading apparently would be lower than the cost for symplastic unloading in fast, longitudinally growing stolons. On the contrary, in radially growing organs, such as a developing tuber, the sites of phloem unloading will be less

subject to spatial shift. Hence, the plasmodesmatal connections could function for a relatively longer period. Although tuber initiation implicates a change from apoplastic to symplastic phloem unloading, this might explain why a high, cell wall-bound acid invertase activity is still found in potato tubers (Dr. K.J. Oparka, pers. commun.). Assuming cell wall-bound acid invertase to have a relatively slow turnover, this apparently non-functional enzyme could be the remainder from the time that phloem unloading was apoplastic, in the non-swollen stolon tip. It was beyond the scope of this research program to show properly the absence of plasmodesmatal connections between the distal end of the phloem and the surrounding tissue in non-swollen stolon tips. In this thesis, it is shown that glucose and fructose measurements are relatively simple and time-effective additional tools, that could be used side by side with more conventional techniques, such as microinjection of Lucifer Yellow CH in the phloem.

In the last chapter (Chapter 6), comparisons are made of the phosphorylated-hexose contents of individual stolon tips from *S. demissum* plants that were exposed to SD or LD conditions. As a consequence of the enzyme shift from acid invertase to sucrose synthase, one could expect an increase of the UDP-glucose level during tuber development. Furthermore, the conversion of the glucose-moiety of each sucrose molecule needs equimolar amounts of UDP and PP_i in case of sucrose splitting by sucrose synthase. A futile cycle in the glycolysis serves in *S. tuberosum* tubers for the conversion of UTP and $2P_i$ into UDP and PP_i (for details see Chapter 6). We were wondering whether these changes were reflected in the levels of phosphorylated sugars in individual stolon tips. The UDP-glucose level was well-buffered during tuber development in *S. demissum*. This is logical if one considers the apparently constitutively high UDPGlc-pyrophosphorylase activity found in stolon tips (Chapter 5). It is also functional because substantial accumulation of UDP-glucose in the cytosol would result in sucrose synthesis. Presumably as a consequence of the activity of the futile cycle, a sharp decrease was observed in the fructose 1,6-bisphosphate concentration. The data presented in Chapter 6 not only show the feasibility of phosphorylated-sugar determinations in minute amounts of plants tissue, but also the usefulness of pool-size determinations for monitoring a dynamic process, like tuber induction in potato.

A short retrospective

This research project on the hormonal regulation of tuber induction in potato started in 1988. Tuber-inducing activity had been detected in extracellular extracts from leaflets of *S.*

tuberosum. It was my task to characterize and possibly identify tuber-inducing compounds from these extracts. In retrospect, I think we were lucky that the group of Dr. Yoshihara (Sapporo, Japan) identified a tuber-inducing compound from potato-leaves in 1989. Using 100 kg fresh potato leaves, they were able to overcome the problem of the dramatic loss of biological activity in extracts. We encountered similar problems and it would have been impossible for us to solve the problem in that way. Being aware that the activation could be caused by *cis/trans* epimerization of jasmonic acid-like substances, we could think about a strategy to circumvent this problem. In cooperation with the group of Prof. Sembdner (Halle, Germany) we succeeded to identify 11-OH-JA, a substance that could be (co-)responsible for tuber induction. In 1989, however, it was still not sure if we would be able to tackle the inactivation problem and it seemed better not to put all my eggs in the tuber-induction basket. Since it was found that sucrose can trigger the expression of genes encoding for tuber-specific proteins *in vitro*, it was decided to investigate whether an (transient) increase of the sucrose concentration could be found in stolon tips just before swelling. No such an increase could be detected, but interesting changes in other sugars were found. This finally resulted in the Chapters 4, 5 and 6 of this thesis.

Conclusions

In *S. demissum*, tuber formation is positively correlated with the occurrence of hydroxylated jasmonic acids in the leaflets. The relationship between tuber induction and jasmonates is not unique for *S. demissum*. Jasmonates were shown to be involved in tuber induction in potato (*S. tuberosum*, Yoshihara *et al.* 1989), Jerusalem artichoke (*H. tuberosus*, Yoshihara *et al.* 1992) and yam (*Dioscorea batatas*, Koda and Kikuta 1991). Many other physiological processes in plants are influenced by jasmonates in a low dose - response relationship (Parthier *et al.* 1992). Therefore, there is good reason to consider jasmonates as a sixth group of phytohormones.

Formation of hydroxylated jasmonic acids in the leaflets is not only correlated with a change in the way sucrose is split but also with a spatial shift in enzyme activity. In non-swollen stolon tips sucrose is hydrolysed in the cell wall or in the vacuole, whereas in developing tubers it is hydrolysed in the cytosol. However, it remains an enigma why sucrose synthase activity increases in one stolon tip, while it remains low in a 'neighbour' tip, while both are part of the same stolon.

SUMMARY

1. How does a potato plant form tubers?

Potato plants produce sexual multiplication and survival structures, true seeds, and asexual multiplication and survival bodies, tubers. Berries of the potato plant contain a large number of minute seeds. Relatively large tubers are formed in the soil in the subapical part of the stolons. The genetically heterogeneous seeds of a potato plant will spread in a larger area than its tubers, whose radius of spread is restricted by the length of the stolons. Potato tubers from one plant are genetically identical (to each other and to the mother plant) and reside in the soil, a relatively sheltered environment. Contrary to germinating seeds, the sprouts on a potato tuber have access to a relatively large quantity of storage food. A potato plant invests a lot of dry matter into the tubers. Apparently, tubers are, apart from being a valuable agricultural product, important structures for the potato plant itself. In this thesis I focused on tuber induction (Chapters 2 and 3), and changes in sucrose metabolism in stolon tips as a result of tuber induction (Chapters 4, 5 and 6).

A series of long nights and a relatively low night temperature favour tuber induction in tuber-forming *Solanum* species. As a result of exposure to these conditions a potato plant synthesizes a (set of) compound(s) in the leaves, which is (are) transported basipetally to the stolon tips. During the last thirty years, it was surmised that this (set of) compound(s) consisted of a (mix of) classical plant hormones. However, no (mix of) plant hormone(s) could be identified as the tuber inducing principle yet.

The starting point for this research project was the finding of Struik *et al.* (1987) that tuber inducing activity is present in extracellular extracts from leaflets of tuber-bearing *Solanum tuberosum* plants. An important indication that this activity is related to tuber induction would be its absence in non-tuber-bearing plants from the same age. However, tuber formation in commercial West-European *S. tuberosum* cultivars cannot be prevented by a simple shortening of the nightlength. Therefore, it was decided to switch to a wild

Solanum species that only forms tubers under short-day conditions. *S. demissum*, a Mexican *Solanum* species which habits resemble *S. tuberosum*, meets this prerequisite. Tubers are formed when plants are exposed to 10 h daylength, whereas no tuber formation is observed when the daylength is extended to 16 h.

2. The role of (hydroxylated) jasmonic acids in tuber induction

Yoshihara *et al.* (1989) isolated and characterized a substance from leaves of tuber-bearing potato plants (*Solanum tuberosum*, cv. Irish Cobbler) which they called 'tuberonic acid'. 'Tuberonic acid' is a glucoside of 12-hydroxy-jasmonic acid (12-OH-JA). This finding prompted us to investigate whether the tuber-inducing activity found in extracts from potato leaflets could be attributed to the presence of 'tuberonic acid'. Assuming 'tuberonic acid' to be involved in tuber induction, it is to be expected that this substance is absent in leaflets from non- tuber-bearing *S. demissum* plants.

Extracts of leaflets from *S. demissum* plants grown under long- (LD) and short-day (SD) conditions were analyzed. The aglycon of 'tuberonic acid', not its glucoside, was detected in leaflets from SD plants. Moreover, a second hydroxylated jasmonic acid was detected: 11-OH-JA. It was the first time that this compound was detected in higher plants (Chapter 2). As a native substance it was detected before in a fungus, *Botryodiplodia theobromae*, by Miersch *et al.* (1991). Because no deuterated hydroxylated JAs were available, we could not determine the absolute concentrations of 11- and 12-OH-JA in SD leaflets. However, the level of 11-OH-JA of SD leaflets was higher than the 12-OH-JA level. No hydroxylated JAs were detected in LD leaflets.

Hydroxylated JAs are metabolization products of JA. The JA concentrations in LD and SD leaflets did not differ significantly. Hence, tuber induction in *S. demissum* is correlated with the hydroxylation of JA. A number of diurnal cycles of long nights and relatively cool temperatures will finally result in either the formation or activation of JA hydroxylating enzymes or the neutralization of the spatial separation between enzyme and substrate.

Whether hydroxylated JAs are causally related to tuber induction remains to be proven. JA was found to induce the formation of tubers on *S. demissum* explants *in vitro*. Hydroxylated JAs could not be tested because only minute amounts of these substances were available. The reason why JA itself is apparently not involved in tuber induction *in planta* could be its apolarity. Apolarity prevents basipetal transport of a compound via the phloem.

Hydroxylated JAs can be easily glycosylated, and become transportable. This hypothesis is only apparently conflicting with the observation that JA can induce tuber formation *in vitro*. In the bioassay, stem pieces with axillary buds were placed in the JA-containing, solidified nutrient medium, so no transport of JA via the phloem was needed.

3. Reception of a tuber-inducing substance in a stolon tip

Tuber induction in potato plants is irregular: only a subset of the stolon tips available will start to swell subapically after exposure to tuber-inducing conditions. The vasculature of potato plants excludes the possibility that a subset of stolon tips would be solely connected with a certain subset of leaves that does or does not synthesize a tuber-inducing signal. Nevertheless, this misconception persists in the literature.

We monitored external characteristics of 841 stolon tips from 6 plants exposed to SD conditions. It was investigated whether a correlation could be found between subapical swelling and branching order, stolon and stolon-branch age, longitudinal growth rates of stolons and stolon branches, and attachment of the stolon to the main stem. No correlation was found between tuber formation and one of these external characteristics (Chapter 3). It is concluded that tuber initiation in *S. demissum* depends on metabolic or hormonal conditions in stolons or stolon branches, which are insufficiently reflected in external characteristics of these stolons or stolon branches to indicate the change of longitudinal growth into radial growth.

4. Tuber induction and concomitant changes in sucrose metabolism in stolon tips

High concentrations of sucrose induces tuber formation in *S. tuberosum* explants *in vitro* (*e.g.* Hussey and Stacey 1984) as well as the expression of genes coding two semi-tuberspecific proteins, patatin (Wenzler *et al.* 1989) and proteinase inhibitor II (Johnson and Ryan 1984). We decided to investigate whether changes in the sucrose metabolism could be observed in the stolon tip before or during tuber initiation.

The mono- and disaccharide contents of individual stolon tips of different developmental stages were determined qualitatively and quantitatively. No (transient) increase of the sucrose concentration could be detected concomitant with tuber initiation. The sucrose concentration was constantly low in developing stolon tips, and only tended to increase in relatively large

tubers. However, we could not be conclusive about this since sucrose measurements were done at stolon tips as a whole. Very local (transient) sucrose accumulations could not be excluded. Recently, Müller-Röber *et al.* (1992) showed that a constitutively high sucrose concentration in potato tuber does not lead to an enhanced expression of patatin or proteinase inhibitor II. It illustrates that phenomena observed in *in vitro* systems do not necessarily occur *in planta*. Apart from sucrose, glucose and fructose were the main sugars in stolon tips. The glucose concentration decreased gradually during the process of subapical swelling whereas the fructose concentration dropped (Chapter 4).

Invertases catalyze the irreversible hydrolysis of sucrose into glucose and fructose. Acid invertase activity is high in non-swollen stolon tips and decreases during subapical swelling. A specific neutral invertase could not be detected in stolon tips (Chapter 5).

Sucrose synthase catalyzes a reversible reaction in which UDP and sucrose are converted into UDP-glucose and fructose. Sucrose synthase activity is barely detectable in non-swollen stolon tips. From the onset of subapical swelling onwards sucrose synthase activity increases rapidly (Chapter 5).

The decreasing glucose concentration could be attributed to an increase of the sucrose synthase activity relative to the acid invertase activity in the stolon tip. On the other hand, sucrose splitting results in the formation of fructose irrespective of the nature of the enzyme involved. The steep decrease of the fructose concentration could be explained by a spatial shift: acid invertase activity is restricted to the cell wall or the vacuole, an environment where hexose kinases are relatively inactive, whereas sucrose synthase activity occurs in the cytosol. The fructose level in stolon tips drops in developing tubers, because sucrose splitting takes place in the cytosol. Here, fructose is exposed to a high activity of hexose kinases. The rate of fructose phosphorylation in extracts from stolon tips of *S. demissum* is manifold higher than the rate of glucose phosphorylation (Chapter 4). In Chapter 6, the consequences of the above mentioned enzyme and spatial shift on levels of several phosphorylated sugars were determined.

Potato tubers; everybody can tell you what they look like and how they taste. Most people will recognize a potato crop in the field. At the same time, nobody can tell you how a potato plant starts to form tubers. It is a fascinating subject to do research on, and maybe this thesis comprises a few little steps towards a better understanding of this phenomenon.

SAMENVATTING

1. Waardoor vormt een aardappelplant knollen?

Aardappelknollen zijn overlevingsstructuren die onafhankelijk van de moederplant een ongunstige periode kunnen overleven. Na een zekere rustperiode zullen de ogen van de aardappelknol uitlopen op een moment dat de condities voor groei weer gunstig geworden zijn. Eén van de vragen die centraal stonden in dit onderzoek luidt: wat maakt dat een aardappelplant op een gegeven moment knollen vormt?

Ondanks het feit dat er gedurende tientallen jaren veel onderzoek is gedaan naar dit fenomeen, is het niet bekend welke signaalstoffen hierbij betrokken zijn. Wel is bekend welke condities maken dat een aardappelplant knollen gaat vormen. Knolvorming wordt geïnduceerd als de nachten gedurende enige tijd lang genoeg geweest zijn. Bovendien moeten deze nachten tamelijk koel zijn (5-20°C). De meeste knolvormende *Solanum*-soorten komen van oorsprong voor in het Andesgebergte in Zuid-Amerika en op de hoogvlakten van Mexico en Guatemala. Gedurende het groeiseizoen zijn de nachten in deze streken lang en koel. Toen de eerste aardappelplanten naar Europa gebracht werden, vormden deze nauwelijks knollen; de nachten waren hier te kort. De aardappelplant heeft echter een grote genetische variatie voor wat betreft de lange-nachtbehoefte. Selectie heeft geresulteerd in aardappelrassen die schijnbaar helemaal geen behoefte meer hebben aan lange nachten. Het bekende aardappelras Bintje maakt nog knollen bij een nachtlengte van 0 uur. Als men het mechanisme van knolinductie wil bestuderen dan kan dat het beste gebeuren met een aardappelsoort die alleen na blootstelling aan een bepaald aantal lange nachten knollen vormt. Knolvorming dient achterwege te blijven na blootstelling aan een identiek aantal korte nachten. 'Gewone' aardappelplanten die in onze streken verbouwd worden staan schijnbaar permanent aan. *Solanum demissum* is een wilde, uit Mexico afkomstige aardappelsoort die wel aan en uit te schakelen is door middel van een verlenging of een verkorting van de nacht.

2. Welke stoffen induceren knolaanleg?

Eén van de hoofddoelen van dit onderzoek was uit te zoeken welke hormoonachtige stoffen in de aardappelplant verantwoordelijk zijn voor de knolaanleg. Toen ik net goed en wel gestart was met dit onderzoek verscheen er een artikel van een groep Japanners die claimden in de bladeren van knoldragende aardappelplanten een plantehormoon gevonden te hebben die bij de aardappel verantwoordelijk was voor de inductie van knolaanleg. Zij noemden deze substantie (12-hydroxy-jasmijnzuur) 'knolzuur'. Dit 'knolzuur' is een omzettingsprodukt van jasmijnzuur, een hormoonachtige stof die in zeer veel plantesoorten voorkomt. Een vluchtige vorm van jasmijnzuur, methyljasmijnzuur, ruikt zeer aangenaam en is in belangrijke mate verantwoordelijk voor de prettige geur van jasmijnbloemen. De publikatie van deze groep uit Sapporo was enigszins verontrustend omdat het niet uit te sluiten viel dat zij datgene gevonden hadden waarnaar ik op zoek was. De Japanse onderzoekers hadden gewerkt met een commercieel aardappelras, dat wil zeggen een ras dat niet of nauwelijks nachtlengte afhankelijk is. Als 'knolzuur' inderdaad verantwoordelijk is voor knolaanleg, dan zou verwacht mogen worden dat deze stof afwezig is in bladeren van planten die voor wat betreft knolaanleg niet geïnduceerd zijn. Bij *S. demissum* is verkorting van de nachtlengte tot 8 uur voldoende om knolaanleg te voorkomen. Daarom zijn planten opgekweekt onder korte- en lange-nachtomstandigheden, en bladeren geanalyseerd op de aanwezigheid van jasmijnzuur-achtige verbindingen. Aangezien ik geen ervaring had met dit type verbindingen hebben we voor de analyses contact aanzocht met een groep organisch-chemici uit Halle (Duitsland), die veel ervaring met jasmijnzuur en verwante verbindingen hebben. In samenwerking met hen vonden we dat 'knolzuur' ook in bladeren van *S. demissum* voorkomt. Opvallend was dat de stof alleen voorkwam in knoldragende planten die blootgesteld waren aan lange nachten. In bladeren van planten die blootgesteld waren aan korte nachten vonden we deze stof niet. Het meest spectaculaire echter was dat nog een andere stof gevonden werd, namelijk 11-hydroxy-jasmijnzuur. Deze stof is nauw verwant aan 'knolzuur' en komt in grotere hoeveelheden in bladeren van knoldragende planten voor dan 'knolzuur' zelf. Ook deze stof was afwezig in planten die blootgesteld waren aan korte nachten. Jasmijnzuur zelf, een precursor van gehydroxyleerde jasmijnzuren, kwam in gelijke hoeveelheden in beide soorten bladeren voor. Het is daarom aannemelijk dat deze stof niet verantwoordelijk is voor knolaanleg.

11-hydroxy-jasmijnzuur was al eerder gevonden door Dr. Otto Miersch uit Halle in een schimmel die groeit op cacao. Het was echter de eerste keer dat deze stof in hogere planten

werd aangetroffen. Het lijkt logisch om de term 'knolzuur' te vervangen door 'de knolzuren'; een groepje stoffen waartoe 11- en 12-hydroxy-jasmijnzuur horen. In mijn onderzoek heb ik niet kunnen bewijzen dat deze knolzuren ook echt verantwoordelijk zijn voor knolaanleg, maar het lijkt zeer aannemelijk dat ze, op z'n minst, betrokken zijn bij dit fenomeen. Die betrokkenheid heb ik aannemelijk kunnen maken door jasmijnzuur te geven aan *in vitro* aardappelplantjes. Dit leidde na een paar weken tot de vorming van knollen. Dit is opvallend als men bedenkt dat de jasmijnzuurconcentratie in bladeren van knoldragende en knolloze aardappelplanten gelijk was. Waarom is de jasmijnzuurconcentratie niet heel laag in bladeren van knolloze planten? Jasmijnzuur is relatief apolair, en daarmee moeilijk transporteerbaar naar de ondergrondse delen. In gehydroxyleerde jasmijnzuren kan de OH-groep gezien worden als een haakje, waaraan gemakkelijk een (polair) suikermolekuul gekoppeld kan worden. De suiker-jasmijnzuur combinatie is wel goed transporteerbaar. Naar alle waarschijnlijkheid leidt blootstelling van de bladeren aan een bepaald aantal lange nachten indirect tot het transport van (een) knolinducerende substantie(s) naar de stoloonuiteinden.

3. De onregelmatigheid van knolaanleg

Als een aardappelplant geïnduceerd is wil dat nog niet zeggen dat alle stoloonuiteinden (een stoloon is een ondergronds, horizontaal groeiende stengel) gaan verdikken en uitgroeien tot een knol. Slechts bij een beperkt aantal uiteinden gebeurt dit. Wat men zich moet realiseren is dat er wel een prikkel tot knolaanleg gezonden kan worden naar een stoloonuiteinde, maar dat dit uiteinde de aanwezigheid van deze prikkel ook moet kunnen waarnemen en vertalen in een actie. Kennelijk is slechts een beperkt aantal stoloonuiteinden hiertoe in staat. Men zou zich bijvoorbeeld voor kunnen stellen dat een stoloonuiteinde een minimale leeftijd, een zekere rijpheid, moet hebben alvorens het in staat is de prikkel waar te nemen en te vertalen. We hebben een groot aantal stolonen gevolgd in de tijd om na te gaan of de groeikarakteristieken van een stoloon iets zeggen over de bereidheid om knollen te vormen. Geen enkele karakteristiek is gevonden die een correlatie vertoonde met de eerste knolaanleg. Het lijkt erop dat de eerste knollen aan een aardappelplant 'zomaar ergens' gevormd worden. De knollen die na deze eerste serie gevormd worden, worden waarschijnlijk wel systematisch gevormd. Men zou zich voor kunnen stellen dat concurrentie om suikers hierbij een rol speelt.

4. Veranderingen in het suikermetabolisme tijdens de knolontwikkeling

Een aardappelknol bevat veel zetmeel. Dit zetmeel dient onder andere als voedsel voor de kiemen vanaf het moment dat de moederplant geen suikers meer levert. Voordat een stoloonuiteinde gaat verdikken bevat het vrijwel geen zetmeel. Knolaanleg gaat gepaard met zetmeelsynthese. Zetmeel kan beschouwd worden als een kluwen van lange suikerketens. Omdat de knol zelf geen fotosynthese-activiteit bezit moeten deze suikers aangevoerd worden. Bij de aardappel gebeurt dat in de vorm van saccharose, riet- of bietsuiker. Dit betekent dat knolaanleg samengaat met een zeer forse toename van de saccharose-export naar de zwellende stoloonuiteinden. In het onderzoek is naar voren gekomen dat knolaanleg gepaard gaat met grote veranderingen in de saccharose stofwisseling. En dit zijn niet alleen kwantitatieve veranderingen maar ook kwalitatieve veranderingen.

Saccharose is als zodanig niet bruikbaar voor zetmeelsynthese. Saccharose is een disaccharide opgebouwd uit één glucose- en één fructose-eenheid. Saccharose moet gesplitst worden alvorens het kan worden gebruikt. Er zijn twee soorten enzymen die dit kunnen: invertasen en saccharosesynthetase. Invertasen hydrolyseren saccharose, de reactieprodukten zijn glucose en fructose. Saccharosesynthetase is een reversibel enzym dat afhankelijk van de omstandigheden saccharose kan afbreken of synthetiseren (saccharose + UDP $\rightleftarrows$ UDP-glucose + fructose). In dit onderzoek is naar voren gekomen dat knolaanleg samenvalt met een verschuiving van invertase-activiteit naar saccharosesynthetase-activiteit. Dit uitte zich onder andere in een sterke afname van de glucose-concentratie in zwellende stoloonuiteinden, een logisch gevolg van de toegenomen activiteit van saccharosesynthetase: er wordt nu UDP-glucose gevormd en geen glucose. Raadselachtiger was dat de fructoseconcentratie nog veel sterker afnam. Dit is vreemd als men bedenkt dat fructose altijd gevormd wordt, of saccharose nu gesplitst wordt door een invertase of door saccharosesynthetase. Ook fructose is als zodanig onbruikbaar in een plant. Fructose wordt enzymatisch omgezet in het bruikbare fructose-6-fosfaat. Tijdens het onderzoek kwam naar voren dat deze omzetting alleen in het cytoplasma plaatsvindt. De waargenomen drastische daling van het fructosegehalte kan dus het gevolg zijn van een verandering van de plaats waar saccharose gesplitst wordt. Deze gedachte werd bevestigd toen we de invertase-activiteit in stoloonuiteinden nader onder de loep namen. De gevonden invertasen functioneren namelijk alleen in een vrij zure omgeving, in zo'n zure omgeving is saccharosesynthetase juist niet actief. In ongezwollen stoloonuiteinden wordt saccharose gesplitst in een zure omgeving (celwand of vacuole), terwijl in zich ontwikkelende knollen saccharose gesplitst wordt in een wat zuurgraad betreft

neutrale omgeving (cytoplasma).

Als de knollen snel beginnen te groeien wordt de bovengrondse groei geremd. Een groot deel van de beschikbare suikers wordt naar de knollen getransporteerd. Door mijn onderzoek is duidelijk geworden dat knolaanleg meer is dan het omgooien van de saccharosewissel. In de zich ontwikkelende knollen wordt saccharose op een ander station uitgeladen, en op een andere manier verwerkt.

Samenvattend kunnen de volgende conclusies getrokken worden:

1) Gehydroxyleerde jasmijnzuren, in het bijzonder 11-hydroxy-jasmijnzuur, spelen hoogstwaarschijnlijk een belangrijke rol bij de inductie van knolaanleg in *Solanum demissum* Lindl. Lange-nachtcondities leiden niet zozeer tot de synthese van een knolinducerend principe in het blad alswel tot het transport ervan naar de stoloonuiteinden.

2) Aan de hand van uiterlijke karakteristieken van stolonen van een geïnduceerde aardappelplant kan niet worden afgeleid waar de eerste knolaanleg zal gaan plaatsvinden.

3) De daling van het glucose- en fructosegehalte in zich ontwikkelende stoloonuiteinden is gecorreleerd aan een afname van de zure invertase-activiteit en een toename van de saccharosesynthetase-activiteit.

4) Behalve een kwalitatieve verschuiving in de manier waarop saccharose gesplitst wordt, gaat knolinductie ook met een ruimtelijke verschuiving gepaard: saccharose wordt in ongezwollen stoloonuiteinden gesplitst in de celwand of in de vacuole, terwijl dit in zich ontwikkelende knollen plaatsvindt in het cytoplasma.

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PUBLICATIONS

Parts of this thesis have been published

A substantial part of Chapter 1:
Helder, H. and Vreugdenhil, D. (1994) Tuber induction in potato: the possible role of hydroxylated jasmonic acids. In: *Plant production on the threshold of a new century*. Edited by Struik, P.C., Vredenberg, W.J., Renkema, J.A., and Parlevliet, J.E., Kluwer Academic Publishers, Dordrecht (in press).

Chapter 2:
Helder, H., Miersch, O., Vreugdenhil, D., and Sembdner, G. (1993) Occurrence of hydroxylated jasmonic acids in leaflets of *Solanum demissum* plants grown under long- and short-day conditions. *Physiol. Plant.* **88**, 647-653.

Chapter 3:
Helder, H., Van der Maarl, A., Vreugdenhil, D., and Struik, P.C. (1993) Stolon characteristics and tuber initiation in a wild potato species (*Solanum demissum* Lindl.). *Potato Res.* **36** (in press)

The data presented in Chapters 4, 5 and 6 are to be published

Chapters 4 and 5 (combined):
Helder, H., Koot, E., Van der Maarl, A., and Vreugdenhil, D. (1994). Sucrose metabolism in stolon tips of the wild potato species *Solanum demissum* grown under long- and short-day conditions. In preparation

Chapter 6:
Helder, H., Tetteroo, F.A.A., Vreugdenhil, D. (1994) Comparison of phosphorylated hexose contents of individual stolon tips from *Solanum demissum* using HPAEC-PED. In preparation

Other publications:

Helder, H., D. Vreugdenhil, and Struik, P.C. (1989) Preliminary characterization of tuber-inducing acivity in extracellular extracts from *Solanum demissum*. In: *First international symposium on the molecular biology of the potato, Bar Harbor, Maine, USA*. p. 4 (abstract)

Helder, H., O. Miersch, D. Vreugdenhil, and Sembdner, G. (1991) The effect of day-length on the occurrence of hydroxylated jasmonic acids in the leaves of *Solanum demissum*. In: *Abstracts 14th International Conference on Plant Growth Substances, Amsterdam. The Netherlands*. p. 82 (abstract)

Van de Geijn, S.C., J. Helder, H.G. Van Hooren, and Hänisch ten Cate, Ch.H. (1989) Growth, root respiration and phosphorus utilization of normal and *Agrobacterium rhizogenes* transformed potato plants. In: *Structural and Functional Aspects of Transport in Roots*, p. 269-274. Edited by Loughman, B.C., Kluwer Academic Publishers, Dordrecht.

Vreugdenhil, D. and Helder, J. (1992) Hormonal and metabolic control of tuber formation. In: *Progress in Plant Growth Regulation*, 393-400. Edited by Karssen, C.M., Van Loon, L.C., and Vreugdenhil, D., Kluwer Academic Publishers, Dordrecht.

CURRICULUM VITAE

Mijn naam is Johannes Helder en ik werd op 25 november 1962 geboren in Hamilton (Great Britain). Na de lagere school heb ik 6 jaar op het Eindhovens Protestants Lyceum doorgebracht, een periode die afgerond werd met het behalen van het VWO-B diploma in 1981. In augustus van dat jaar ben ik in Wageningen planteziektenkunde (N-14) gaan studeren. Het kandidaatsexamen werd in 1985 behaald. Na een stage van 6 mnd. in Pasto en Bogotá (Colombia) voor de vakgroep Nematologie, ben ik begonnen aan een doctoraal programma bestaande uit twee hoofdvakken (6 mnd.) en één bijvak (3 mnd.). De hoofdvakken waren Nematologie (bij dr. ir. F.J. Gommers) en Bodemvruchtbaarheid en Plantenvoeding (bij prof. dr. G.R. Findenegg), het bijvak was Erfelijkheidsleer (bij prof. dr. ir. M. Koornneef). Het doctoraalexamen werd in januari 1988 met lof behaald. In maart van dat jaar ben ik als assistent in opleiding (AIO) begonnen bij de vakgroep Plantenfysiologie. Sinds 1 februari 1993 ben ik als toegevoegd onderzoeker werkzaam bij de vakgroep Nematologie in Wageningen.