

## Girdling Affects Carbohydrate-related Gene Expression in Leaves, Bark and Roots of Alternate-bearing Citrus Trees

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Effects of girdling on carbohydrate status and carbohydrate-related gene expression in citrus trees were investigated. Alternate-bearing 'Murcott' (a *Citrus reticulata* hybrid of unknown origin) trees were girdled during autumn (25 Sep. 2001) and examined 10 weeks later. Girdling brought about carbohydrate (soluble sugar and starch) accumulation in leaves and shoot bark above the girdle, in trees during their fruitless, 'off' year. Trees during their heavy fruit load, 'on' year did not accumulate carbohydrates above the girdle due to the high demand for carbohydrates by the developing fruit. Girdling caused a strong decline in soluble sugar and starch concentrations in organs below the girdle (roots), in both 'on' and 'off' trees. Expression of *STPH-L* and *STPH-H* (two isoforms of starch phosphorylase), *Agps* (ADP-glucose pyrophosphorylase, small subunit), *AATP* (plastidic ADP/ATP transporter), *PGM-C* (phosphoglucomutase) and *CitSuSI* (sucrose synthase), all of which are associated with starch accumulation, was studied. It was found that gene expression is related to starch accumulation in all 'off' tree organs. RNA levels of all the genes examined were high in leaves and bark that accumulated high concentrations of starch, and low in roots with declining starch concentrations. It may be hypothesized that changes in specific sugars signal the up- and down-regulation of genes involved in starch synthesis. © 2003 Annals of Botany Company

**Key words:** Alternate bearing, carbohydrates, *Citrus*, girdling, soluble sugar, starch.

### INTRODUCTION

Girdling has been, and is still, widely used in citrus, grape, peach and other fruit tree crops, mainly to increase flowering, fruit set and fruit size (Goren *et al.*, 2003). Girdling consists of removal of a strip of bark from the trunk or major limbs of a fruit tree, thereby blocking the downward translocation of photosynthates and metabolites through the phloem.

The best-known effects of girdling are presumably brought about by accumulation of assimilates above the girdle. Tree parts beneath the girdle, on the other hand, suffer from shortage of assimilates. Following trunk girdling, the roots are gradually depleted of their carbohydrate reserves, sometimes reaching serious root starvation (Weaver and McCune, 1959). Cytokinin and gibberellin content of shoots is also modified by girdling (Cutting and Lyne, 1993).

The common, general explanation for the effects of girdling is that the development of the reproductive organs benefits from the higher concentrations of assimilates available in the canopy following girdling. The precise biochemical changes resulting from girdling have been studied only in some particular cases (Beruter *et al.*, 1997), and a detailed interpretation of the physiological effects of girdling is still lacking.

Progress in plant research during the past decade has led to the understanding that, beyond their nutritional and

energetic contribution, sugars play regulatory roles, which involve up- and down-regulation of gene expression (Koch, 1996; Jang and Sheen, 1997; Smeekens, 2000; Koch and Zeng, 2002). The feast/famine hypothesis of Koch (1996), in particular, provides a model of whole plant carbohydrate-mediated gene regulation.

The main purpose of the present study was to check whether girdling, which modifies carbohydrate levels in canopy and roots of fruit trees, also involves changes in gene expression. Since starch is the main storage carbohydrate in citrus tissues, we focused on the expression of genes related to starch biosynthesis.

### MATERIALS AND METHODS

#### *Plant material and experimental design*

Field grown Murcott (a *Citrus reticulata* hybrid of unknown origin) trees grafted onto a sour orange (*Citrus aurantium*) rootstock, about 5 years old, were selected for these experiments. Trees of this cultivar reveal an extreme alternate-bearing habit, with 'off' (without fruit) and 'on' (with a heavy fruit load) trees present in the same orchard each year. Eight uniform 'off' and six uniform 'on' trees were used, and each group was divided into two sub-groups (four each for 'off' trees and three each for 'on' trees); one was girdled (girdling) and the other not girdled (control). Girdling was performed on 25 Sep. 2001 on the trunk 20 cm above the ground. A ring of bark (about 3 mm in width)

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around the trunk was removed using a sharp knife and the girdle was renewed every 3 weeks to prevent rapid closure of the girdle. Roots (0.5 cm in diameter), bark (peeled from 1-year-old shoots) and mature leaves were sampled on the day of girdling (zero time control) and then on 9 Dec. 2001 from girdled and control trees for evaluation of girdling effects.

#### Carbohydrate determinations

Samples were dried in the oven (80 °C) for 48 h and then ground into fine powder. Dry tissue samples (100 mg each) were extracted four times with 80 % (v/v) ethanol at 55 °C. All the extract solutions were collected and brought to a final volume of 50 ml. Soluble sugar was determined using anthrone, according to the method of Yemm and Willis (1964). Starch was measured after glucoamylase hydrolysis of the insoluble fraction and the released glucose determined according to Hassid and Neufeld (1964). Three tissue samples were extracted for each treatment. The amount of total non-structural carbohydrates (TNC) was calculated by summing the amount of starch and soluble sugar. Where appropriate, Student's *t*-test was used to determine the statistical significance.

#### Detection of starch with iodine

Roots (0.5 cm in diameter) and 1-year-old shoots (0.5 cm in diameter) were collected from girdled and non-girdled 'on' and 'off' trees on 11 Dec. 2001. Samples were hand-sectioned into 4-mm-thick cylinders and immediately immersed in Lugol solution (Sigma, St Louis, MO, USA) containing iodine and potassium iodide. Optimal staining was obtained within 3 min, after which the cylinders were photographed under an Olympus binocular system (Olympus SZX12).

#### RNA isolation and Northern blot analysis

Total RNA was extracted using a standard SDS-phenol method described in Current Protocol Unit 4.3 (Ausubel *et al.*, 1989). For RNA blot analysis, total RNA samples (20 µg per lane) were separated by electrophoresis in 1 % agarose gels containing formaldehyde and blotted onto Hybond-NX nylon membrane (Amersham Pharmacia Biotech, Little Chalfont, UK) by capillary transfer. The RNA blots were hybridized with specific cDNA probes (*Agps*, AF184597; *CitSuS1*, AB022092; *STPH-L*, AY098892; *STPH-H*, AY098895; *AATP*, AY098893; and *PGM-C*, AY112996) labelled using a random priming method (Boehringer Mannheim, Germany). Hybridization was carried out at 42 °C in a solution containing 10 % dextran sulfate (Na salt, MW 500 000), 1 % SDS, 2× SSC, 50 % formamide and 5× Denhardt's solution for 16 h followed by high stringent wash. The hybridized membranes were exposed and analysed by Phosphorimager (FUJI BAS 1000). Each membrane was re-probed several times after stripping and finally re-hybridized with citrus 18S rRNA. All hybridization experiments have been carried out at least twice, with identical results.

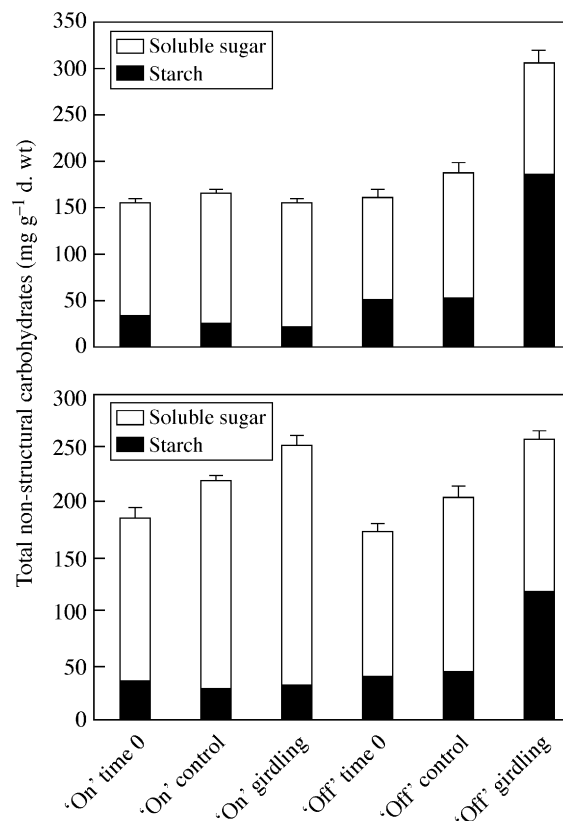


FIG. 1. Total non-structural carbohydrates (TNC) (soluble sugar and starch) in leaves (top) and bark (bottom) of girdled and non-girdled 'on' and 'off' citrus trees (means  $\pm$  s.d. of three independent samples,  $n = 3$ ).

## RESULTS

#### Carbohydrate concentrations in leaves and bark of girdled trees

Removal of a strip of phloem from the main trunk by girdling actually blocks the transport of sugars to the roots; large amounts of carbohydrates produced by photosynthesis will accumulate in vegetative organs above the girdle or be utilized for fruit development. In 'on' trees, no increase in TNC concentration was found in girdled tree leaves and only a small increase in TNC was found in girdled tree bark (Fig. 1), which was mainly the result of accumulation of soluble sugar (Fig. 1). In 'off' trees, TNC concentration increased in leaves and bark following girdling (Fig. 1), with leaves showing a remarkable increase ( $P < 0.01$ ) in comparison with non-girdled control trees. The increase in carbohydrates was mainly in the form of starch. The concentration of starch in girdled tree leaves was three times that of non-girdled control tree leaves, and in girdled tree bark twice that of control tree bark (Fig. 1). Soluble sugar concentrations in girdled tree leaves and bark did not change significantly ( $P > 0.05$ ). Iodine staining of shoot cross-sections showed that girdled 'off' trees accumulated a large amount of starch in the bark and in the pith (Fig. 2A and C). 'On' shoots, on the other hand (Fig. 2B and D), appeared to be devoid of starch, except for the outer bark in girdled trees as well as in controls.

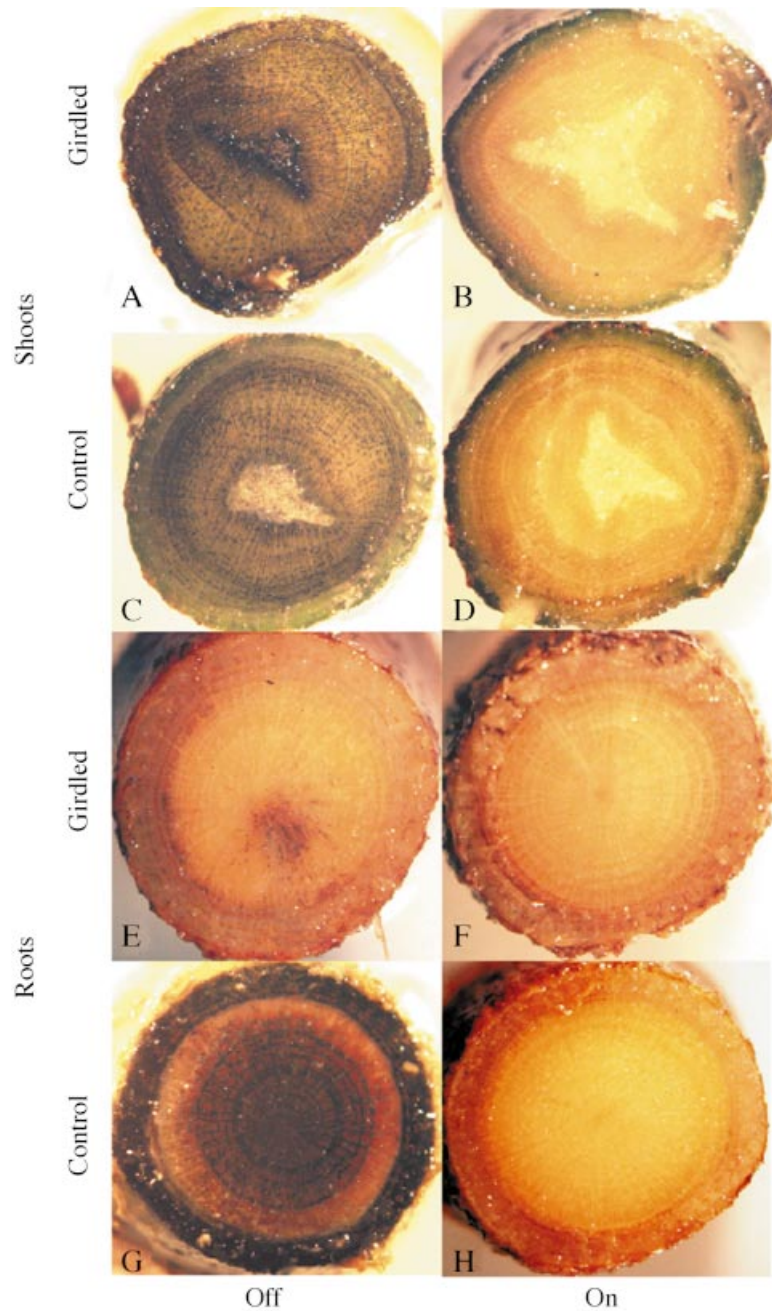


FIG. 2. Iodine staining of shoot (A–D) and root (E–H) cross-sections. A and E, Girdled ‘off’ trees; B and F, girdled ‘on’ trees; C and G, non-girdled ‘off’ trees; D and H, non-girdled ‘on’ trees. The cross-sections of shoot and roots are 0.5 cm in diameter.

#### Relative mRNA levels in leaves and bark of girdled trees

To investigate whether girdling effects involve regulation at the RNA level, expression of several genes related to carbohydrate metabolism was analysed: *STPH-H*, *STPH-L*, *Agps*, *PGM-C*, *AATP* and *CitSuS1*. *STPH-H* and *STPH-L* code for two isoforms of starch phosphorylase, type-H and type-L, respectively, *Agps* codes for a small subunit of ADP glucose pyrophosphorylase, *PGM-C* encodes a cytosolic phosphoglucomutase, *AATP* is a plastidic ATP/ADP transporter gene and *CitSuS1* encodes a sucrose synthase.

Girdling induced the expression of *STPH-H*, *STPH-L*, *Agps*, *PGM-C* and *CitSuS1* in both leaves and bark of ‘off’ trees (Fig. 3), corresponding to starch accumulation in these organs (Fig. 1). In ‘on’ trees, however, the changes in mRNA transcription levels did not correspond to the changes in starch concentration.

#### Carbohydrate concentrations in roots of girdled trees

Opposite seasonal trends of root carbohydrate concentrations were found in ‘on’ and ‘off’ trees (Fig. 4). In ‘on’ trees

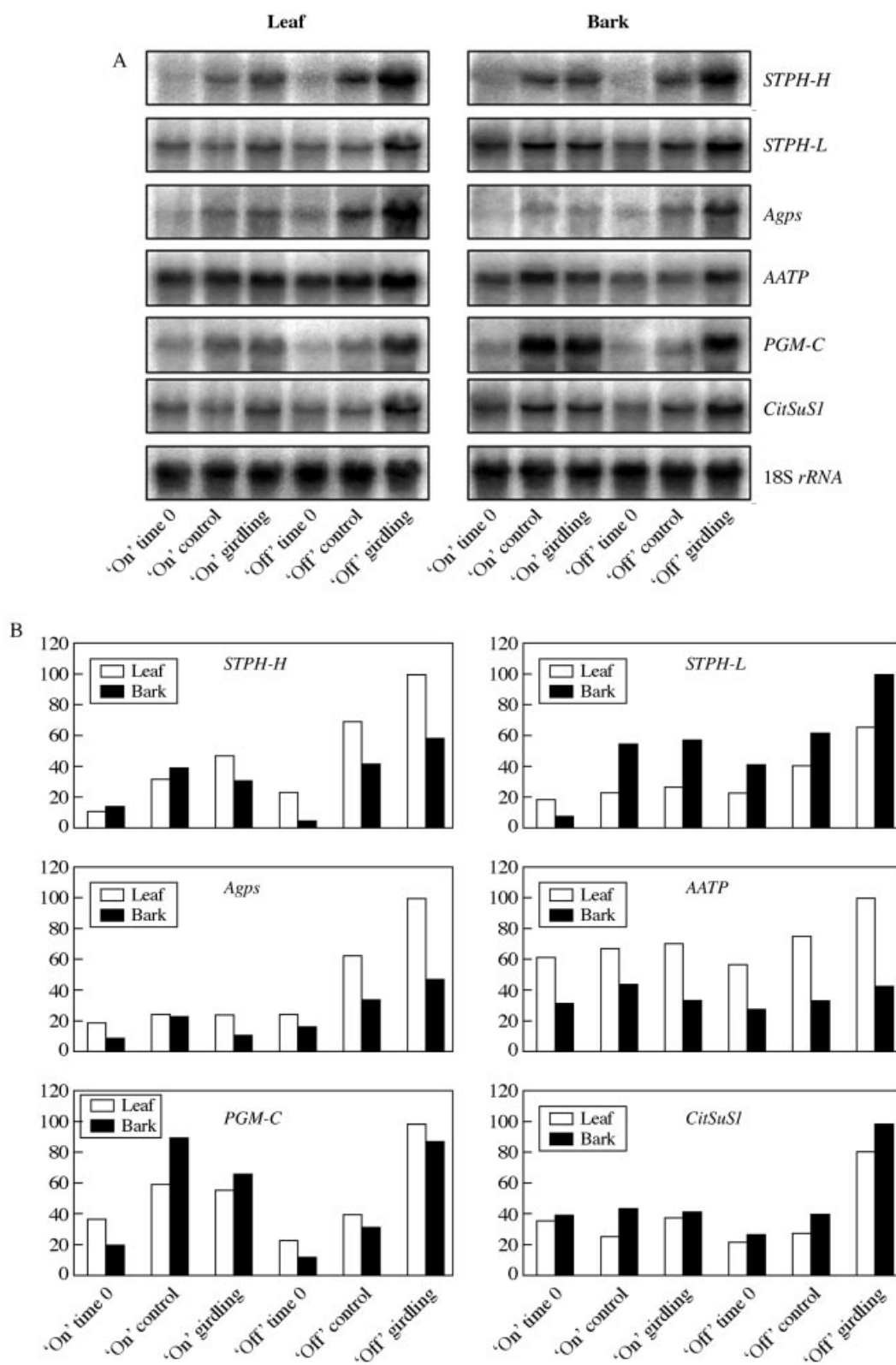


FIG. 3. Relative transcript levels of *STPH-H*, *STPH-L*, *Agps*, *AATP*, *PGM-C* and *CitSuS1* in leaves and bark of girdled and non-girdled 'on' and 'off' citrus trees (A). Total RNAs (20 µg) isolated from leaves and bark were separated and analysed by RNA blot. 18S *rRNA* from citrus was used as an internal control for loading differences. Relative levels of transcripts (B) were estimated by quantitation of signals on the blotted membranes with a Phosphorimager BAS1000 (Fuji Photo Film, Tokyo, Japan). The results are expressed as a percentage of the respective maximum level for each gene. Results are representative data of two independent experiments, the same as for Fig. 5.

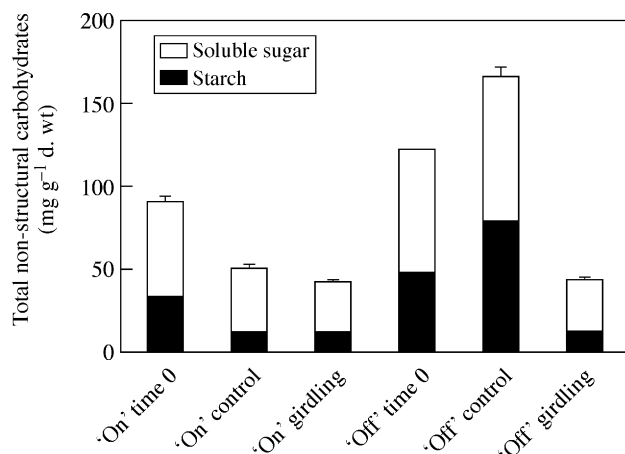


FIG. 4. Total non-structural carbohydrates (TNC) (soluble sugar and starch) in roots of girdled and non-girdled 'on' and 'off' citrus trees (means  $\pm$  s.d. of three independent samples,  $n = 3$ ).

the concentration of TNC and each of its components, starch and soluble sugar, declined between 'time 0' (25 Sep. 2001) and the end of the experiment (9 Dec. 2001). Girdling caused a further, slight decline. In 'off' tree roots, TNC concentrations, and those of starch in particular, were already high at 'time 0' and continued to rise in non-girdled trees during the experiment. However, girdling markedly reduced both starch ( $P < 0.01$ ) and soluble sugar ( $P < 0.01$ ) concentrations, down to levels comparable with those of 'on' roots. These findings were also confirmed by the iodine staining of root cylinders (Fig. 2E–H). Non-girdled 'off' tree roots (Fig. 2G) were loaded with starch in the bark as well as in the vascular cylinder of root cross-sections. Girdled 'off' tree roots (Fig. 2E), and girdled as well as non-girdled 'on' tree roots (Fig. 2F and H) stained poorly, indicating a low concentration of starch.

#### Relative mRNA levels in roots of girdled citrus roots

Expression of *STPH-H*, *STPH-L*, *Agps*, *AATP*, *PGM-C* and *CitSuS1* in girdled tree roots was analysed by Northern blot analysis. Results showed that all the genes examined were down-regulated in girdled 'off' tree roots (Fig. 5). Among them, *STPH-H*, *STPH-L* and *Agps* showed an 80–95 % reduction, whereas *AATP*, *PGM-C* and *CitSuS1* had only a <50 % reduction in transcript levels compared with non-girdled control roots. The transcript levels of all the genes examined increased in girdled 'on' tree roots in comparison with non-girdled control roots (Fig. 5), with *Agps* and *STPH-L* showing a marked increase.

## DISCUSSION

The results of the present investigation confirm earlier observations but, at the same time, provide some new insight into the study of girdling. Girdling results in accumulation of carbohydrates above the girdle, but such an accumulation takes place only when there is an excess of

carbohydrates, as occurs during 'off' years. As shown by Garcia-Luis *et al.* (1995), the presence of fruit prevents the girdling-induced accumulation of carbohydrates. During 'on' years the high, barely saturable demand by the developing fruit (Fishler *et al.*, 1983; Goldschmidt, 1999) consumes all the available carbohydrates and prevents any accumulation of carbohydrates in leaves and bark (Fig. 1).

Starch has been shown to be the major storage carbohydrate in citrus (Goldschmidt and Golomb, 1982). Assuming that starch accumulation in shoots is confined to the bark, bark samples were used for shoot carbohydrate determinations. However, the iodine staining of shoot sections revealed that starch accumulation in the shoot is not confined to the bark; considerable starch accumulation takes place in the xylem rays (Fig. 2C). Indeed, carbohydrates can be stored in ray parenchyma and other parenchymatous cells associated with the xylem. When there is a large excess of carbohydrates, as in 'off' trees, starch accumulates even in the innermost part of the shoot, the pith (Fig. 2A).

The effects of girdling on root metabolism have only rarely been studied in detail (Wallerstein *et al.*, 1978). During 'on' years root carbohydrate concentrations are relatively low and the effects of girdling are minimal (Fig. 4). During 'off' years, there is a dramatic increase in root starch and soluble sugar concentrations in non-girdled control trees (Fig. 4). Girdling curtails this accumulation, and carbohydrates drop to very low levels, presumably due to the respiratory and metabolic needs of the root system. Starch quantitative determinations are corroborated by iodine staining: in non-girdled control roots large amounts of starch are deposited in the bark and in the vascular cylinder of the root (Fig. 2G).

The expression of several genes (*STPH-H*, *STPH-L*, *Agps*, *AATP*, *PGM-C* and *CitSuS1*) was investigated in the present study; the proteins encoded by all these genes seem to play a role in starch synthesis. Both *STPH-L* and *STPH-H* encode for starch phosphorylase, an enzyme that catalyses the reversible reaction for degrading or synthesizing starch polymers (Stitt and Steup, 1985). Starch phosphorylase activity has been shown to correlate with rapid starch synthesis in buttercup squash (Irving *et al.*, 1999). High expression of plastidic starch phosphorylase genes (*STPH-L*) was found to correlate with starch biosynthesis in potato (Duwenig *et al.*, 1997a), spinach (Duwenig *et al.*, 1997b), pea (van Berkel *et al.*, 1991) and maize (Yu *et al.*, 2001). ADP-glucose pyrophosphorylases (AGPs), encoded by *Agps*, catalyse the rate-limiting step for starch biosynthesis in both photosynthetic and non-photosynthetic tissues (Weber *et al.*, 2000). The plastidic ATP/ADP translocator, encoded by *AATP*, is involved in starch synthesis by providing ATP, which is required for AGPs activity, to the plastid (Neuhaus *et al.*, 1997). The cytosolic phosphoglucomutase, encoded by *PGM-C*, was found to play a critical role in starch accumulation in the potato tuber (Ferne *et al.*, 2002). Sucrose synthase, encoded by *SuSy*, is also associated with starch synthesis (Dejardin *et al.*, 1997).

All six genes were highly expressed in leaves and bark of girdled 'off' trees (Fig. 3), as well as in non-girdled 'off' roots (Fig. 5), all of which accumulated high concentrations

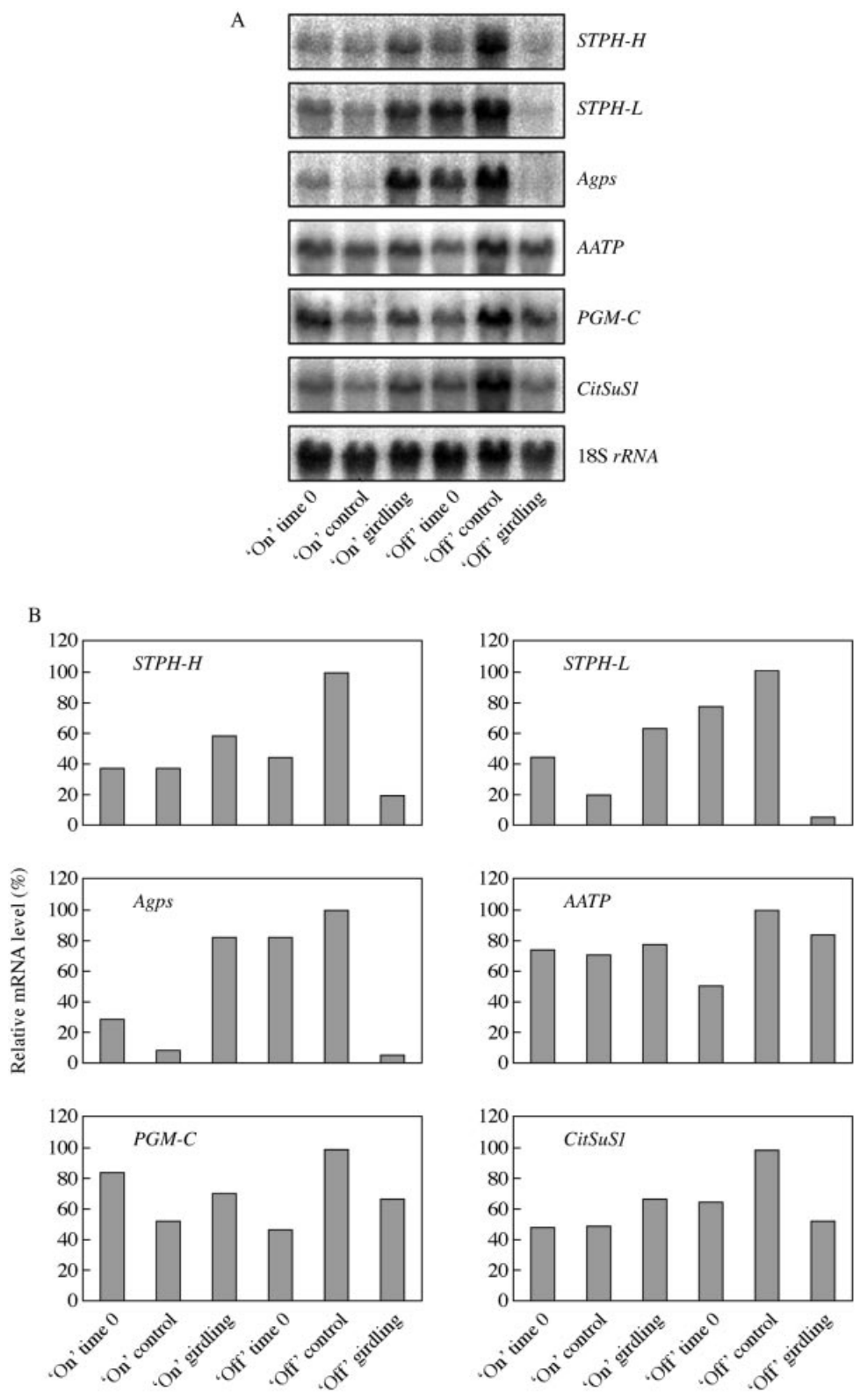


FIG. 5. Relative transcript levels of *STPH-H*, *STPH-L*, *Agps*, *AATP*, *PGM-C* and *CitSuSI* in roots of girdled and non-girdled 'on' and 'off' citrus trees (A). Total RNAs (20  $\mu$ g) isolated from roots were separated and analysed by RNA blot. 18S *rRNA* from citrus was used as an internal control for loading differences. Relative levels of transcript (B) were estimated as described in Fig. 3.

of starch. The up-regulation of these genes in starch-accumulating organs is supported by the scheme of Kossmann and Lloyd (2000) that illustrates the pathway for starch accumulation. When there is an excess supply of carbohydrates, as in the case of 'off' trees, sucrose can be converted into starch via a series of reactions involving sucrose synthase, cytoplasmic phosphoglucomutase and AGPs. Recent evidence indicates that the *CitSuS1* gene is up-regulated by exogenous sucrose and glucose in citrus leaf discs (C.-Y. Li, D. Weiss and E. E. Goldschmidt, unpubl. res.). *Agps* expression is also increased by sugars (Sokolov *et al.*, 1998). In girdled 'off' tree roots, the low concentrations of starch and soluble sugar (Fig. 4) are accompanied by down-regulation of *STPH-H*, *STPH-L* and *Agps*, which are apparently very responsive to sugar concentrations. *AATP*, on the other hand, is only slightly affected by the carbohydrate status in leaves, bark (Fig. 3) and root (Fig. 5) and seems to be constitutively expressed. *PGM-C* and, to a lesser extent, both forms of *STPH*, are strongly expressed in 'on' tree leaves and bark in spite of their low starch content, corresponding perhaps to the high concentration of soluble sugar in these tissues (Fig. 3).

In conclusion, it has been demonstrated that even seemingly simple agrotechnical manipulations like girdling involve extensive changes in gene expression. Following recent literature (Smeekens, 2000; Koch and Zeng, 2002; Rolland, 2002) it might be hypothesized that changes in specific sugar components, which have yet to be identified in citrus, signal the regulation of genes involved in starch biosynthesis (and other carbohydrate-related genes) in citrus tree organs.

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