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ent-Kaurene and squalene synthesis in *Fusarium fujikuroi* cell-free extracts

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Abstract

Sterols and gibberellins are the main terpenoids in the Ascomycete *Fusarium fujikuroi*. Their respective precursors squalene and *ent*-kaur-16-ene (henceforth called kaurene) were the main terpenoids synthesised from radioactive mevalonate by extracts of *F. fujikuroi* in vitro. Kaurene predominated when the extracts were obtained from mycelia engaged in gibberellin production. Squalene predominated in all other cases, and particularly when the extracts were obtained from mutants with various defects in gibberellin synthesis or nitrogen-fed wild-type cultures. New protein synthesis was required to maintain the production of gibberellins in vivo and of kaurene in vitro, but not to maintain the capacity to produce squalene in vitro. Addition of a nitrogen source to cultures engaged in gibberellin production caused a large, transient increase in the mycelial concentration of L-glutamine and abolished the accumulation of gibberellins immediately and the capacity to produce kaurene later.

Keywords: *Fusarium fujikuroi*; *Gibberella*; Terpenoids; Gibberellins; Sterols; Kaurene; Squalene

1. Introduction

The gibberellins (Crozier, 1983; MacMillan, 1997) are a group of tetracyclic terpenoids present in low concentrations in plants, where they regulate different steps of growth and development. The Ascomycete *Fusarium fujikuroi* (O'Donnell et al., 1998), previously called *Fusarium moniliforme* and *Gibberella fujikuroi*, is the industrial source for the various applications of gibberellins in agriculture and brewing.

Both *F. fujikuroi* and plants make not only gibberellins, but also other terpenoids, such as sterols and carotenoids. In *F. fujikuroi* these terpenoids derive from hydroxymethylglutaryl CoA (Fig. 1); although gibberellins and sterols share all the early intermediates, up to farnesyl pyrophosphate, the two pathways are physically separated from the beginning in different subcellular compartments (Domenech et al., 1996). The various terpenoids differ in function and timing of synthesis. While the sterols are essential components of cell membranes, the gibberellins are dispensable secondary metabolites secreted to the culture medium of the fungus only after depletion of the nitrogen source; addition of fresh nitrogen source to the cultures stops gibberellin biosynthesis (Candau et al., 1992). The intracellular signal for nitrogen inhibition is glutamine (Muñoz and Agosín, 1993) or a metabolite thereof.

Kaurene is the first tetracyclic diterpene of the gibberellin pathway and squalene is the first triterpene precursor of sterols. Both are produced by cell-free extracts of *F. fujikuroi* incubated with R-[^{14}C] mevalonate (Avalos et al., 1988; Fernández-Martín et al., 1995). To investigate the regulation of the biosyntheses of gibberellins and sterols in *F. fujikuroi* we have examined the production of kaurene and squalene in cell-free extracts from mycelia from wild-type and mutant strains grown under different conditions.

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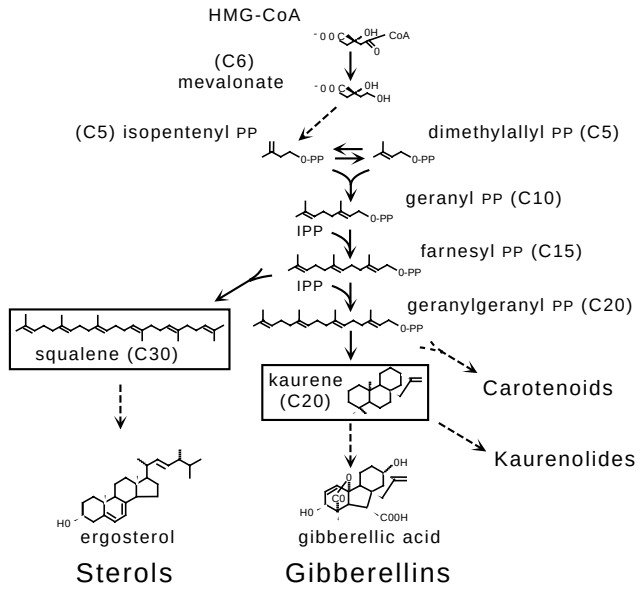

Fig. 1. Main steps in the biosyntheses of gibberellins and sterols in *F. fujikuroi*. Dotted arrows represent several reactions.

2. Results and discussion

Cell-free extracts of wild-type mycelia grown for 6 days in low-nitrogen medium converted mevalonate to kaurene; squalene was produced when the cultures had been grown in high-nitrogen medium (Fig. 2). Squalene and kaurene were the final in vitro products because their conversion to later metabolites requires NADPH and other cofactors (Ono and Imai, 1985; Ashman et al., 1990) that were absent in our in vitro tests. Low-nitrogen cultures were producing gibberellins at the time of extraction, while high-nitrogen cultures were not, as expected from the regulation of gibberellin production by nitrogen (Candau et al., 1992; Avalos et al., 1999). The nature of the nitrogen source was not critical; it was NH_4NO_3 in the experiments in Fig. 2, but similar results were obtained when various amino acids (alanine, glycine, leucine, glutamine, or glutamate) were used instead.

The *gib* mutants were isolated because they produce no gibberellins, or smaller amounts than the wild type (Candau et al., 1991). Extracts from low-nitrogen cultures of the *gib* mutants produced squalene, while

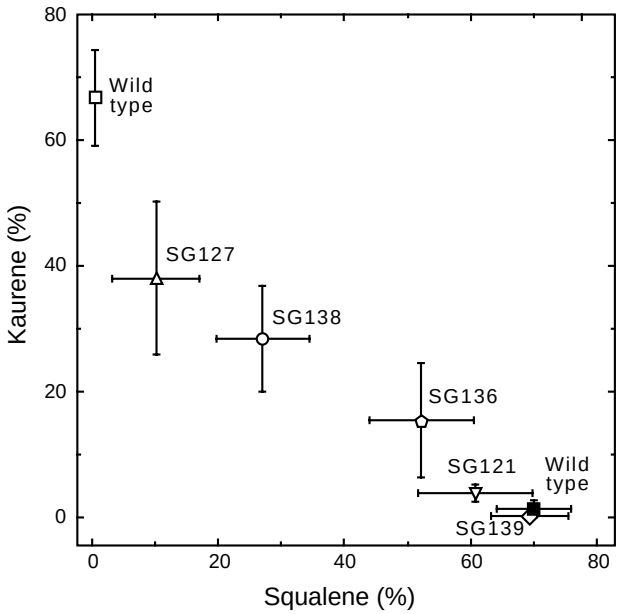

Fig. 2. Biosynthesis of terpenoids in cell-free extracts. Radioactivity in kaurene or squalene as a fraction of total radioactivity in hydrophobic terpenoids (2.1 kBq per experiment in the average) after the incubation of cell-free extracts from wild type and *gib* mutant mycelia with [2-^{14}C]mevalonate. Cultures were grown for 6 days in low-nitrogen medium (open symbols) or high-nitrogen medium (closed symbols).

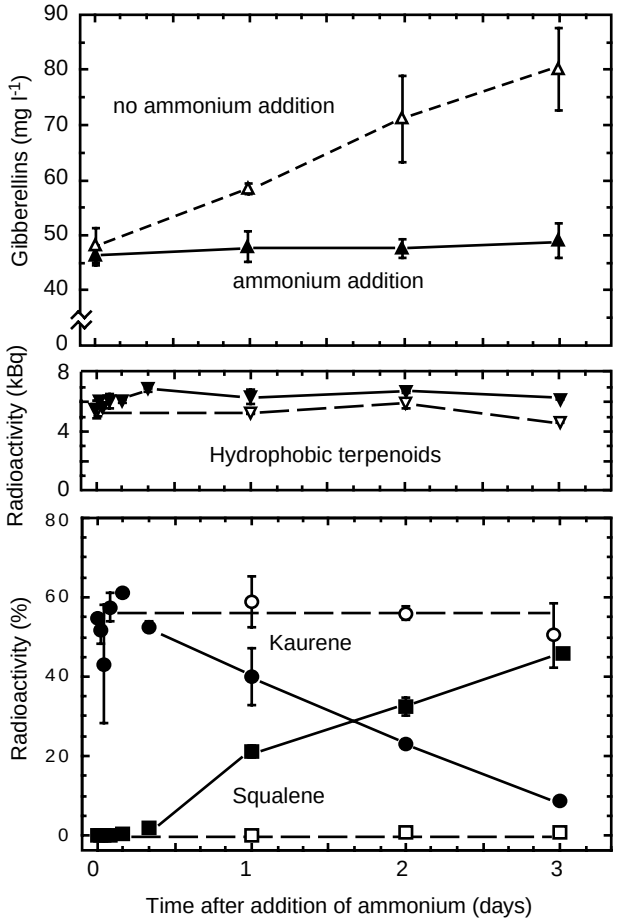

Fig. 3. Gibberellin production and in vitro terpenoid biosynthesis after addition of nitrogen source NH_4Cl (final concentration 100 mM) was added (closed symbols) or not (open symbols) to wild-type cultures grown for 8 days in low-nitrogen medium. Upper panel, concentration of gibberellins in the medium. Central panel, total incorporation of radioactivity from mevalonate into hydrophobic terpenoids. Lower panel, incorporation into kaurene (circles) and squalene (squares), as a fraction of total radioactivity in hydrophobic terpenoids.

those of the wild type did not (Fig. 2). The in vitro squalene and kaurene productions correlated negatively with each other. The more extreme phenotype was that of strain SG139, a mutant that does not accumulate gibberellins or kaurenolides in vivo. Low-nitrogen-culture extracts of this mutant behaved exactly as high-nitrogen-culture extracts of the wild type. Very similar results were obtained with strains SG121 and SG136. Less extreme was the in vitro phenotype of strain SG138, practically devoid of gibberellins because of its inability to carry out various oxidative reactions in the pathway (Barrero et al., 1999). The mutant that was closest to the wild type was strain SG127, which has a partial block of the gibberellin pathway.

Squalene appears to be synthesised 'by default' in various mycelial extracts, both wild type and mutant, unless the original mycelia were engaged in gibberellin production; in this case squalene was displaced by kaurene as the main in vitro product of mevalonate.

The capacity to synthesize kaurene in vitro was lost gradually after addition of fresh nitrogen source to the cultures (Fig. 3). Squalene synthesis in vitro increased steadily from initially undetectable levels and mirrored the decrease in kaurene synthesis. These changes started a few hours after the addition of the nitrogen source and continued for several days, until squalene became the predominant in vitro terpenoid product. The accumulation of gibberellins ceased immediately after the addition of fresh nitrogen source to cultures that were synthesising them actively (Fig. 3), as expected (Candau et al., 1992). The nitrogen source had both immediate and delayed inhibitory effects.

The addition of ammonium provoked a transient increase in the mycelial concentration of L-glutamine (Fig. 4), which, at its peak, constituted about one tenth of the mycelial dry mass. Lesser variations were found in the concentrations of L-alanine and L-glutamic acid. The concentrations of other amino acids remained approximately constant.

Our results support the belief that the intracellular nitrogen signal in the regulation of gibberellin biosynthesis is glutamine (Muñoz et al., 1993) or one of its metabolites. An additional effect through the depletion of 2-oxoglutarate is not excluded.

Mycelia grown in high-nitrogen medium started gibberellin production a few hours after they were washed and transferred to a glucose solution (Fig. 5). Gibberellins accumulated at an approximately constant rate for several days. At the same time the mycelia took a reddish tint owing to the accumulation of bikaverin, a polyketide compound (Balan et al., 1970). The in vitro production of squalene, found in extracts of mycelia

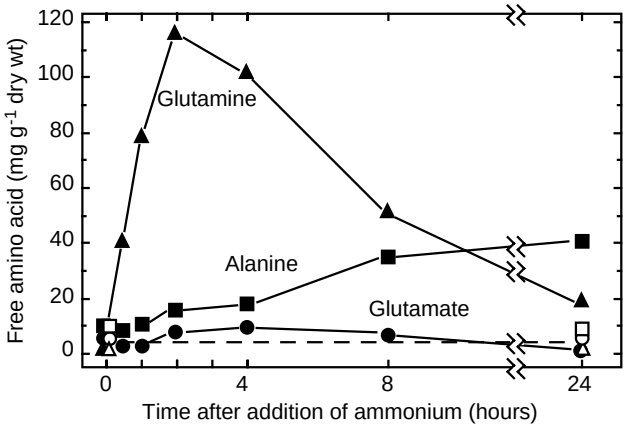

Fig. 4. Intracellular concentrations of free amino acids after addition of nitrogen source. NH_4Cl (final concentration 100 mM) was added (closed symbols) or not (open symbols and dashed line) to wild-type cultures grown for 8 days in low-nitrogen medium. Triangles, L-glutamine; squares, L-alanine; circles, L-glutamic acid.

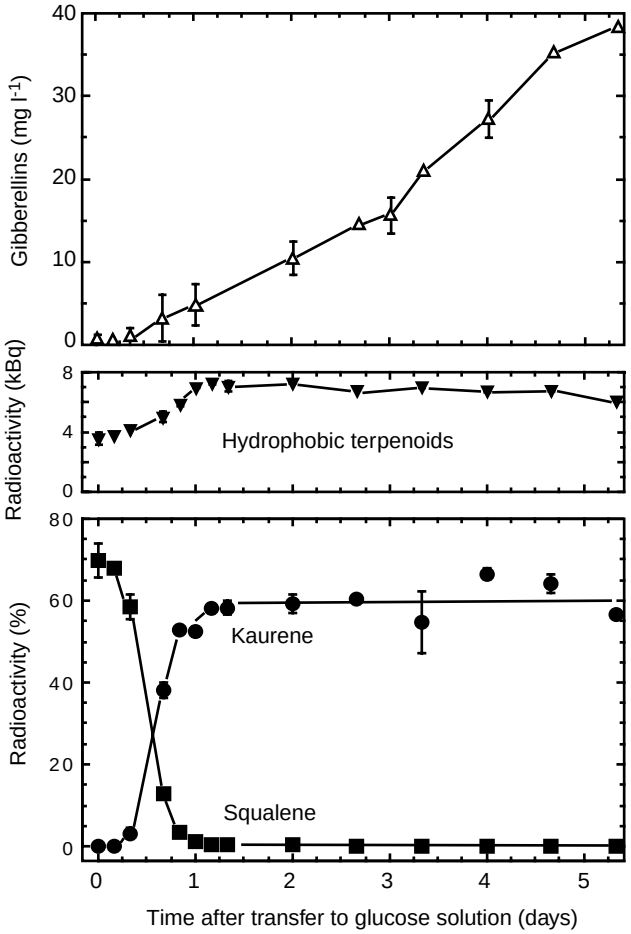

Fig. 5. Gibberellin production and in-vitro terpenoid biosynthesis after nitrogen deprivation. Mycelia grown for 6 days in high-nitrogen medium were transferred to a glucose solution. Upper panel, concentration of gibberellins in the solution. Central panel, total incorporation of radioactivity from [2-^{14}C]mevalonate into hydrophobic terpenoids. Lower panel, incorporation into kaurene (circles) and squalene (squares), as a fraction of total radioactivity into hydrophobic terpenoids.

that had not yet started to produce gibberellins, was replaced by that of kaurene following nitrogen removal (Fig. 5).

Addition of cycloheximide blocked the production of gibberellins in mycelia that had been transferred to a glucose solution (Fig. 6). No gibberellins were produced when cycloheximide was added in the first hours after transfer, before the onset of gibberellin production in the mycelia and of kaurene production in their extracts. Accumulation of gibberellins stopped a few hours after addition of cycloheximide to mycelia actively engaged in gibberellin synthesis. This suggests that new protein synthesis was required for the onset and for the maintenance of gibberellin biosynthesis.

New protein synthesis was apparently not needed to maintain the squalene-synthesising ability. Extracts were prepared from the mycelia that had been incubated in a glucose solution for 3 days (Fig. 6). When cycloheximide had been present in the glucose solution all the time, the extracts synthesised squalene, but not kaurene. The later cycloheximide was added, more kaurene and less squalene was synthesised in vitro. The break-even point was reached when cycloheximide was added 1.4 days after the transfer to the glucose solution.

Enzyme stability should be advantageous in a process that is maintained for many days in the absence of a nitrogen source. We found, however, that mycelia ceased to produce gibberellins a few hours after addition of cycloheximide and their extracts lost their ability to synthesise kaurene about 2 days later.

The results, particularly those with the mutants and with cycloheximide, indicate that squalene synthesising enzymes were always present in the cells, while kaurene synthesising enzymes were available under some circumstances only and had to be continuously renewed. Squalene and kaurene are produced in separate subcellular compartments (Domenech et al., 1996), but disruption of these compartments made the enzymes compete in vitro for the last common substrate, farnesyl diphosphate. Under these conditions, conversion to geranyl–geranyl diphosphate and kaurene predominates over conversion to squalene.

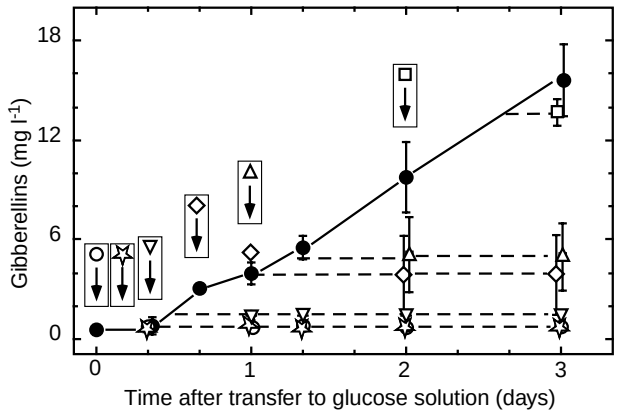

Fig. 6. Inhibition of gibberellin production by cycloheximide. Mycelia grown for 6 days in high-nitrogen medium were transferred to a glucose solution and cycloheximide was added at different times. Open symbols and dashed lines, concentration of gibberellins in the solution after cycloheximide addition at the time indicated by the arrows. Closed symbols and continuous lines, controls without cycloheximide.

The isolation of the genes for gibberellin biosynthesis in *F. fujikuroi* (Tudzynski et al., 1998; Tudzynski and Hölter, 1998) opens the way for a correlation between the biosynthesis in vivo and in vitro with gene expression. The gene cluster for gibberellin biosynthesis includes two contiguous genes, one for a specific geranyl–geranyl diphosphate synthase and the other for a bifunctional copalyl diphosphate and kaurene synthase whose transcription is enhanced by nitrogen starvation.

3. Experimental

F. fujikuroi IMI58289, a gibberellin producing wild type, was obtained from the Commonwealth Mycological Institute, Kew, England. The *gib* mutants defective in gibberellin biosynthesis were obtained after exposure of wild-type spores to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Candau et al., 1991).

The fungi were grown in 500-ml Erlenmayer flasks with 250 ml of minimal medium that contains D(+)-glucose (80 gl^{-1}), NH_4NO_3 (high-nitrogen, 4.8 g l^{-1}; low-nitrogen, 0.48 g l^{-1}), KH_2PO_4 (5 g l^{-1}), $MgSO_4{\cdot}7H_2O$ (1 g l^{-1}), and trace elements (Geissman et al., 1966). The flasks were inoculated with about 10^6 spores and incubated in the dark at 30°C in an orbital shaker (150 rpm). Spores were collected from cultures grown on sporulation agar (1 g l^{-1} yeast extract, 1 g l^{-1} D-glucose, 1 g l^{-1} NH_4NO_3, 1 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4{\cdot}$ $7H_2O$, 16 g l^{-1} agar) under white light (about 4 W m^{-2}). When required, NH_4NO_3 was replaced by amino acids, maintaining the total nitrogen concentration. When indicated, cultures grown in high-nitrogen minimal medium were washed thoroughly with water and transferred to a glucose solution that contained D(+)-glucose (20 g l^{-1}) and the trace elements of the minimal medium.

Gibberellin concentrations in the media were estimated fluorometrically (Candau et al., 1991) using gibberellic acid (Sigma, St. Louis, MO, USA) as a reference and were expressed as the mean and its standard error in two to six independent experiments, with two or more separate determinations in each. Amino acid concentrations were determined by reverse-phase HPLC (Farfán et al., 1996). Proline cysteine, and tryptophan could not be measured by this method.

Cell-free extracts were obtained by lyophilising

mycelial samples and passing them immediately through a sieve (pores 0.5 mm in diameter). The powder was mixed with 8 vol extraction buffer (0.4 M Tris–HCl pH 8, 5 mM dithiothreitol, 200 ml l^{-1} glycerol) and centrifuged at $10^4 \times g$; 0.2 ml supernatant was mixed with 0.3 ml extraction buffer without glycerol containing (final concentrations) 18 μM (18.5 kBq) RS-[2–^{14}C]mevalonate, 10 mM ATP, 2 mM NAD, 2 mM NADP, 6 mM $MnCl_2$, 4 mM $MgCl_2$ and 10 mM KF and incubated at 35°C for 2 h. The required sodium mevalonate was obtained by drying the lactone (Amersham International, Amersham, UK) and dissolving it in 10 μl 10 mM NaOH. After stopping the reaction with 1 ml of MeOH, the mixture was extracted three times with petrol (bp 40–60°C). An aliquot was radioassayed to determine labelled hydrophobic terpenoids (4.3 ± 2.4 kBq, mean and s.e. in 136 tests.) The rest of the extract was dried with N_2 and developed with petrol on Silica Gel G TLC. Kaurene and squalene, the main products (Fig. 7), were scraped off and radioassayed. Radioactivity was determined with a liquid scintillation counter (Wallac 1409, Turku, Finland) with 4 ml Biogreen 1 (Sharlau SA, Barcelona, Spain). The results are the mean and s.e. of the ratio of radioactivity in kaurene and squalene to that in hydrophobic terpenoids in at least two independent experiments, with two separate determinations in each.

Cycloheximide (actidione, Serva Feinbiochemica, Heidelberg) was used at a final concentration of 50 mg l^{-1} which totally inhibits the growth of *F. fujikuroi* (Candau, 1991).

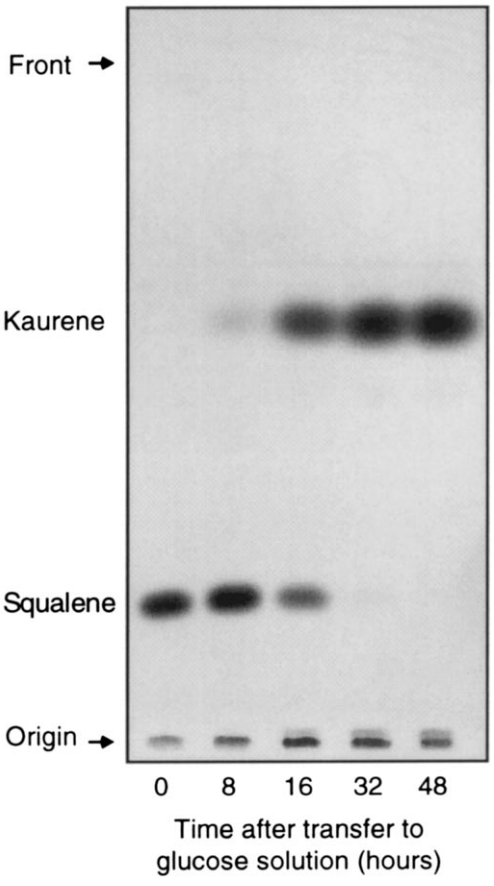

Fig. 7. Terpenoids synthesised from [2-^{14}C]mevalonate by cell-free extracts from mycelia incubated in high-nitrogen medium for 6 days and in a glucose solution for the time indicated. Autoradiography of a TLC plate exposed on X OMAT S film (kodak) for 14 days.

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