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CHLORAMPHENICOL ACETYL TRANFERASE ACTIVITY IN *BRASSICA* spp.

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Upon discovery that *Brassica campestris* leaf extracts harbour some chloramphenicol acetyl transferase (CAT) activity, a systematic screening of plant tissue for this activity, so far only reported for prokaryotic microorganisms, has been conducted. Results were negative for three solanaceous plants as well as for the Cruciferae *Arabidopsis thaliana* and *Orychophragmus violaceus*. By contrast, the three tested species of the Cruciferae genus *Brassica* exhibit significant CAT activity. The *Brassica* CAT activity is much more heat labile than the enzyme encoded by the bacterial transposon, Tn9, that is commonly used as a reporter in gene fusion experiments.

Key words: chloramphenicol acetyl transferase (CAT); *Brassica*; reporter gene

Introduction

During the past years several technologies have been developed to introduce foreign genes into higher plant cells. Nowadays, experiments are performed using either virus-mediated [1,2], Ti plasmid-mediated (e.g. Refs. 3—5), or direct gene transfer [6—10]. Several bacterial genes for antibiotic resistance have been adapted as selectable and screenable markers for animal and higher plant cells; typical examples are neomycine phosphotransferase (APH(3)II, Refs. 3, 6, 11) and chloramphenicol acetyl transferase (CAT, EC 2.3.1.28, Refs. 3, 8, 12). The latter activity can be easily assayed and quantified [13], and the CAT gene from bacterial transposon Tn9 has become a popular reporter for gene fusion studies. Typically, the reading frame of the gene of interest is substituted for that of the Tn9 CAT gene (which has been cloned in the multicopy plasmid pBR 325, Ref. 14). The resulting hybrid gene is then artificially introduced into cells, where the CAT activity will reflect the expression of the chimeric gene, thus providing a clue in gene regulation studies. Reporter gene activity can be followed either in stable transformed cell lines or in a population of cells shortly after DNA delivery ('transient expression' experiments, Ref. 8), and the two methods may give complementary results. In higher eukaryotes described so far, CAT activity can be assayed against a zero background. Here we report that CAT activity is not naturally restricted to prokaryotic organisms, but can also be found in the plant kingdom, namely in the *Brassica* genus.

Materials and methods

Plants were grown in normal greenhouse conditions. Fresh leaf tissue (100 mg) was frozen in Eppendorf tubes at −20°C. Frozen samples were added to 100 μl of 0.25 M Tris—HCl (pH 7.8), crushed in the presence of sterile seasand, and centrifuged for 5 min to remove the cell debris. Supernatants were transferred into new Eppendorf tubes, heated at 65°C for 10 min, and centrifuged another 10 min.

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Forty microliters of the resulting supernatants were used for assay of CAT activity according to Gorman et al. [13]: the 180-µl reaction mixture contained 1 µCi of D-threo-dichloroacetyl-1-[^{14}C]chloramphenicol (1.85 MBq/mmol, Amersham), 0.45 mM acetyl coenzyme A and 0.14 M Tris—HCl pH 7.5. The reaction (37°C, 1 h) was stopped by adding 1 ml of ice-cold ethylacetate. After shaking and centrifugation, the organic phase was transferred into a new Eppendorf tube and evaporated in a vacuum centrifuge. Extracted compounds were redissolved in 15 µl of ethylacetate, spotted onto 0.25 mm silica gel thin layer plates (Polygram R Sil G/UV 254) and submitted to ascending chromatography in chloroform-methanol (95:5, v/v). Plates were dried and the separated spots of chloramphenicol and its acetylated forms revealed by autoradiography.

Results and discussion

Results of our screening for CAT activity in dicot plants are shown in Fig. 1. Activity was detected neither in crude leaf extracts of plants from three different Solanaceae genera, nor in those from the Cruciferae *Arabidopsis thaliana* and *Orychophragmus violaceus*. By contrast, leaf extracts of *Brassica campestris*, *napus* and *oleracea* (turnip, rapeseed and cauliflower, respectively) all exhibit the spots characteristic of the mono-acetylated derivatives of chloramphenicol. These spots comigrated with those produced by the Tn9 encoded CAT activity and were not produced in a control reaction where acetyl coenzyme A was omitted (data not shown), thus identifying a CAT activity beyond any doubt. Acetylated forms were also found with extracts of sterile, in vitro grown *B. napus* plantlets (data not

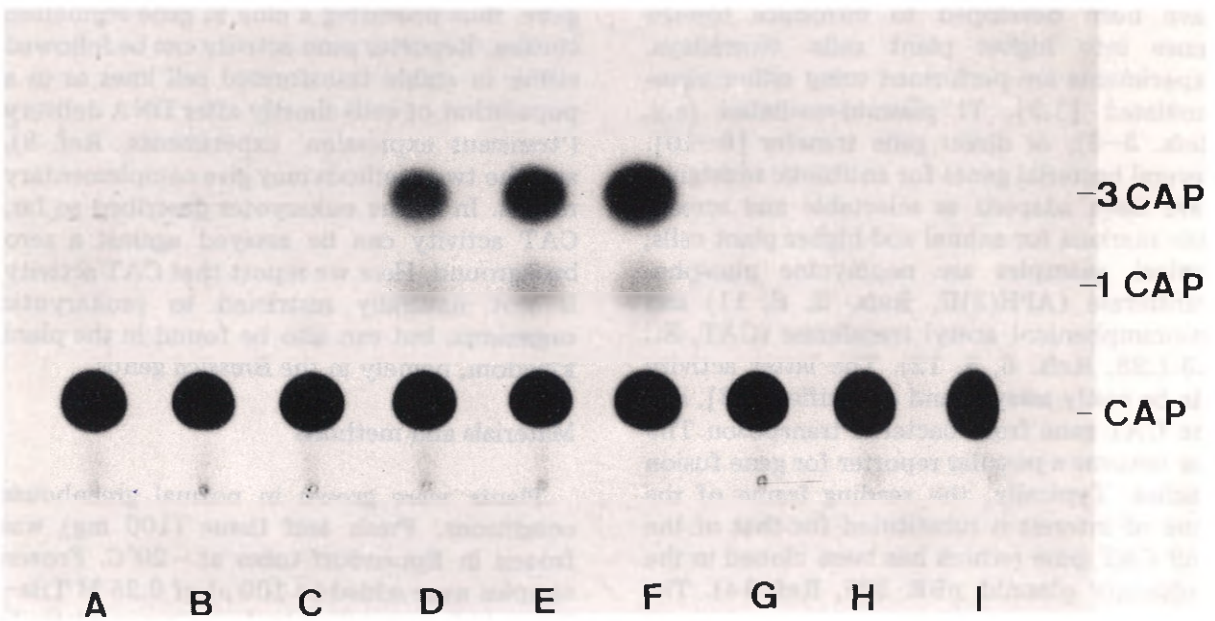

Fig. 1. Plant screening for CAT activity. Reaction cocktail (see Materials and methods) was incubated without plant extract (A), with extracts of *O. violaceus* (B), *A. thaliana* cv. Virginia (C), *B. oleracea* (D), *B. napus* cv. Brutor (E), *B. campestris* var. rapa cv. Just Right (F), *Nicotiana clevelandii* (G), *Petunia hybrida* (H), and *Datura stramonium* (I). Overnight exposure of an autoradiogram of the chromatographically separated substrate and products is shown. CAP: chloramphenicol, 1 CAP: 1-acetyl-chloramphenicol, 3 CAP: 3-acetyl-chloramphenicol.

shown). This rules out the possibility that the CAT activity is due to a microorganism contaminating specifically the *Brassica* genus. Thus, a CAT activity is truly coded for by the genome of at least some *Brassica* spp.

Reported observations, as well in our experiments (Fig. 1) show that CAT activity in plants is rather an exception than a rule, with the range of plants devoid of activity including monocotyledonous plants like *Zea mays* [8] and *Hordeum vulgare* [2], and dicotyledonous plants like *Lotus corniculatus* (Leguminosaeae, Ref. 15), *Daucus carota* (Umbelliferae, Ref. 8), and two solanaceous species, *Nicotiana tabacum* (Refs. 3, 8) and *Hyosciamus muticus* (our unpublished observations). Our data indicate that the CAT trait is not present in all members of the Cruciferae family, but is apparently restricted to the *Brassica* genus.

The presence of this endogenous activity limits the use of the CAT gene as a reporter for gene fusion experiments in *Brassica* spp. The most straightforward way of circumventing this problem is to use another marker for gene expression studies in this genus; challenging reporters include luciferase [16], neomycine phosphotransferase and dihydrofolate reductase [1]. Alternatively, the bacterial CAT gene can still be used by taking profit of the great thermoresistance of its product. Figure 2 illustrates the behaviour upon 68°C incubation of the CAT activity in a *Brassica* crude extract, either alone or mixed with a dilution of a bacterial extract containing an approximately equivalent amount of activity. As noted earlier [8], the heat inactivation is initially beneficial to the reading of the activity; a possible explanation for this phenomenon is that heating quickly denatures ribosomes that otherwise trap the radioactive substrate. Upon longer incubation however, the plant actively clearly drops, and is barely detectable after 150 min at 68°C. From 30 min on, the activity of the mixed extracts undergoes a

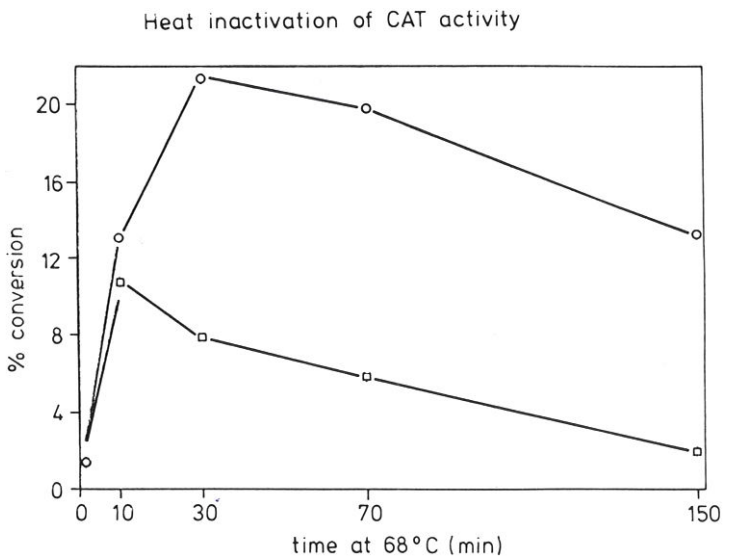

Fig. 2. Time course of heat inactivation of CAT activity in a crude extract of *B. campestris*, either alone (□—□—□) or mixed with a clear lysate supernatant of *Escherichia coli* harbouring pBR 325 (○—○—○). Active extracts were incubated at 68°C for increasing times, centrifuged 10 min at 0°C in Eppendorf tubes, and their supernatants used for CAT assay (37°C, 1 h). Radioactive spots were cut out and counted in a scintillation counter. CAT activity is expressed as the percentage of conversion to acetylated forms, calculated as $cpm_{(1\text{-CAP} + 3\text{-CAP})}/cpm_{(1\text{-CAP} + 3\text{-CAP} + \text{CAP})}$. Shown data are the average of the values obtained in two independent experiments; individual values never differ by more than 10%.

parallel decrease, that can then be attributed to the denaturation of the plant enzyme, with the bacterial one remaining unaffected. After 150 min at 68°C, the contribution of the *Brassica* activity to the total activity can be neglected. From this reconstruction experiment, we conclude that heat treatment allows the selective reading of the prokaryotic CAT activity. Therefore, the bacterial CAT gene can still be used as a faithfull reporter in *Brassica* spp. The choice of CAT as a reporter may nevertheless lead to ambiguous results if the gene fusion produces clearly less CAT activity than the endogenous gene (as detected after a 10-min heating).

The existence of a CAT activity in *Brassica* spp. makes us face a surprising biological situation where an enzyme is present and its key substrate most likely absent. We can advance two different explanations to this apparent futility. On one hand, chloramphenicol detoxification may be a fortuitous property of a plant transacetylase having a related but different normal substrate. On the other hand, the *Brassica* CAT enzyme may not play any functional role. If this were the case, the reason for its presence may be found in the genetic history of the concerned plants. This activity seems to be restricted to a single genus, which, in evolutionary terms, would correspond to a recent trait. One might then speculate that the responsible gene was exported from the bacterial kingdom to an ancestral *Brassica* through horizontal gene transfer. The existence of at least one example of such horizontal DNA transfer from an *Agrobacterium* Ti-plasmid to a plant species [17], together with the presence of CAT activity in some *Agrobacterium* strains [18], lends support to this hypothesis.

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