ACTA SOCIETATIS
BOTANICORUM POLONIAE
Vol. 53, nr 1: 59-65
1984

Phases of crown-gall transformation susceptible to hydroxyurea

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(Received: April 19, 1983. Accepted: June 30, 1983)

Abstract

With the use of bacterial strains, both sensitive and resistant to hydroxyurea the action of this inhibitor on tumour formation on the leaves of *Kalanchoe daigremontiana* infected with *Agrobacterium tumefaciens* was tested for five days after inoculation. The results are in agreement with the opinion that the anti-tumour effect of hydroxyurea applied in the induction phase (between 18 and 60 h after inoculation) is the result of its direct action on plant cells, whereas inhibition of tumour formation by the inhibitor in the inoculation period depends on its action on the pathogenic bacteria.

Key words: crown-gal, transformation, hhydroxyurea

INTRODUCTION

Crown-gall is a plant tumour caused by *Agrobacterium tumefaciens*. It is known at present, owing to the detection of Ti *Agrobacterium* plasmids and their role in tumour induction, that bacteria transmit to the host plant cells genetic information indispensable for tumour formation (Hooykaas et al. 1979, Gelvin et al. 1982). The course of neoplastic transformation lasting several days is, however, still but little known. The use of metabolic inhibitors makes possible inhibition of tumorigenesis in several of its stages (Bopp 1965, Beiderbeck 1970a, b, 1971, 1972, Gribnau and Veldstra 1969) and throws more light on the molecular foundations of this process. Most known inhibitors, however, act both on plant and bacterial cells, this making interpretation of the results difficult.

It has been recently demonstrated that hydroxurea (HU), an inhibitor of DNA synthesis, inhibits formation of bacterial tumours in several experimental systems (Rennert 1978, 1980, Kowalczyk 1983). According to the hypothesis advanced, HU blocks the crown-gall induction phase acting in this period exclusively on the host cells which synthesize DNA indispensable for tumour formation. To confirm this hypothesis experiments are necessary with the use bacterial mutants resistant to the above named inhibitor, and this was the aim in view in the present study.

METHODS

Bacteria. The same virulent *Agrobacterium tumefaciens* strain (CCM 1037) was used as in earlier experiments (Rennert 1978, 1980, Kowalczyk 1983). N-hydroxyurea inhibits *in vitro* the growth and viability of these bacteria. The range of concentrations of the inhibitor reducing by 50 per cent growth after 48 h of incubation of the bacteria in several different media is 1-2 $\times$ 10^{-2} M (0.75-1.5 mg·cm^{-3}) and is much lower than the range of concentrations causing a 50 per cent decrease of viability (1.5-3.5$\times 10^{-2}$ M; 1.14-2.66 mg·cm^{-3}). In order to obtain a strain resistant to HU, portions of 0.1 cm^3 of *A. tumefaciens* (CCM 1037) culture of density 2 $\times$ 10^9 cells per cm^3 in the logarithmic phase of growth were sown in several different dilutions onto agar plates with universal medium and illuminated for 10 sec with a quartz Type GMB H, Hanau lamp (distance 30 cm, 130 W, 1.2 A). After exposure to this light the plates were transferred to darkness in an incubator (27°C) for 48h. The cells which survived illumination and formed colonies (0.2%) were plated into liquid L medium (Lippincott and Heberlein 1965) for 36 h, then centrifuged and suspended in the same volume of L medium without sugar. Portions of 0.1 cm^3 of this suspension were transferred onto agar plates (L medium) containing HU in the amount of 4 mg·cm^{-3}. From several colonies which grew after 48 h on the medium with inhibitor a bacterial strain was isolated capable of growth on L medium in the presence of HU of 3.5 mg·cm^{-3} concentration used for inducing tumours on the leaves of *K. daigremontiana*. Bacteria of both strains — the wild one susceptible to HU and the resistant mutant — were kept and multiplied for inoculation of plants on L medium. Other details are given in an earlier paper (Kowalczyk 1983).

Plants. *Kalanchoe daigremontiana* Ham et Ferr. (Balley 1949) were vegetatively reproduced and cultivated to the stage of 5-7 pairs of leaves at constant temperature of 22° ± 1°C. Then one part of the plants for experiments were left at this temperature, while the others were transferred to 29° ± 1°C. The remaining details concerning the conditions of plant cultivation and the method of infection of leaves

within bacteria (inoculation) have been previously described (K o w a l c z y k 1983).

Treatment of leaves with inhibitor. N-hydroxyurea (Schuchardt, München) was applied as aqueous solution of 3.8 mg·cm^{-3} (5 × 10^{-2} M) concentration. HU dosage and determination of the number of tumours are described earlier (K o w a l c z y k 1983).

Each experimental variant comprised 10 leaves and the series of experiments were replicated two to three times. The vertical lines in the diagrams represent standard errors.

RESULTS AND DISCUSSION

To estimate the effect of HU on the formation of tumours induced by bacteria resistant to this inhibitor, a second series of experiments was performed simultaneously with HU-susceptible bacteria under identical conditions as in the earlier paper (K o w a l c z y k 1983). The results are shown in Fig. 1. In the system with susceptible bacteria (curve A) there are two periods during the five days after infection of the leaves in which the system is highly sensitive to the inhibitory influence of HU. The first period lasts only several hours, while the second one comprises the time between 18 and 60 h after inoculation. Application of HU in each of these periods leads to a considerable reduction of the number of tumours. These results agree with earlier ones (K o w a l c z y k 1983). In the system with resistant bacteria (curve B) inhibitor application before inoculation and several hours after it did not affect the number of forming tumours. The susceptibility of the host-pathogen system to the inhibitory action of HU appears exclusively in the second period, and the course of tumour inhibition is very similar to that with susceptible bacteria.

It may, therefore, be concluded that in the first period after inoculation, the active participation of bacteria in the process of tumour formation is decisive. HU applied in this period affects directly the pathogen cells making their interaction with the host cells impossible. Therefore, the use of HU-resistant bacteria abolishes the anti-tumour effect of the inhibitor. This period of crown-gall transformation corresponds to the period of metabolic activity of the bacteria in the wounds as earlier described (K o w a l c z y k 1983).

In the second period (between 18 and 60 h after inoculation) the bacterial activity in neoplastic transformation seems of little consequence: decisive here is the activity of the plant cells. HU used in this period acts, mainly on the plant cells and the process(es) inhibited is the factor restricting tumour formation. Therefore, the use of HU-resistant bacteria does not prevent the anti-tumour action of the inhibitor.

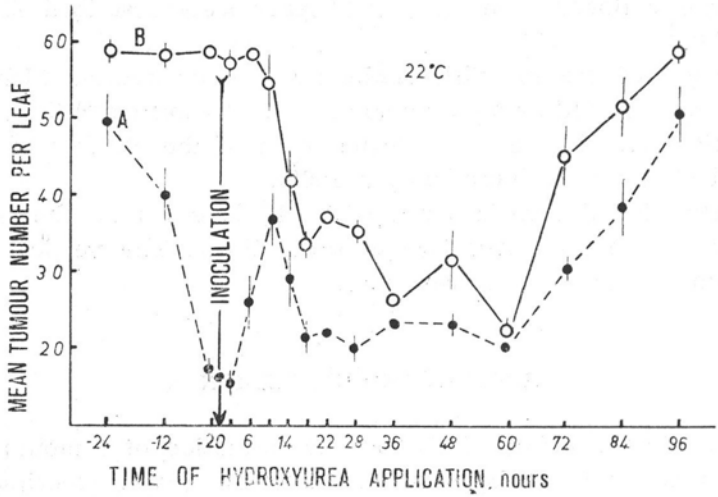

Fig. 1. Influence of the time of hydroxyurea application on initiation of tumours evoked by bacteria sensitive (A) and resistant (B) to this inhibitor. The *K. daigremontiana* leaves were moistened with a HU aqueous solution (5×10^{-2} M) in the amount of 0.05 cm^3 (190 µg) per leaf. The treatment was performed once, but at various times before or after inoculation of the leaves with a virulent *A. tumefaciens* strain susceptible (A) or resistant (B) to HU. In the control series, instead of the inhibitor solution water was used. Preparation of the plants for the biotest and the inoculation procedure was done according to Beiderbeck (1970c). The experiment was run at 22° ± 1°C. The number of tumours was estimated 14 days after inoculation. Each curve in the diagram represents results from two series. One experimental variant of a single series comprised 10 replications (10 leaves). Vertical lines denote standard errors. The mean number of tumours in the control experiment was for A — 54.9 ± 2.7 and for B — 59.6 ± 0.4

It has been earlier demonstrated that the second period of anti-tumour activity of HU in the process of crown-gall transformation includes the phase of induction (Rennert 1978, Kowalczyk 1983). In this phase DNA synthesis occurs in the plant cells, induced by the wounding (Kupila and Stern 1961, Kupila-Ahvenniemi and Therman 1971, Broekaert and Van Parijs 1973), and the preceding wound divisions (Lipetz 1967). One may, therefore, assume that the process inhibited by HU in the induction phase is plant DNA synthesis, as also sugested by the results of investigations with the use of other metabolic inhibitors (Kowalczyk 1983, Bopp 1965).

The course of wound healing processes connected with crown-gall induction in *Kalanchoe daigremontiana* was studied by Lipetz (1965, 1966, 1967). By determining the number of nuclei on the wound surface undergoing DNA synthesis after injection of tritiated thymidine, he demonstrated that a temperature raised in the range 21°-32°C accelerates the appearance of the first wound divisions and shortens the course of

the DNA synthesis preceding them (phase S) (Lipetz 1967). Raising of the temperature also shortens the time during which the plant cells in the wound region remain susceptible to tumorigenic influences. Maximum susceptibility precedes the appearance of the first observable oriented cell divisions (Lipetz 1965, 1966).

Thus if the action of HU in the period between 18 and 60 h after inoculation with bacteria is limited to inhibition of the DNA synthesis stimulated by wounding, this period under elevated temperature is shortened. The results of the experiment with raised temperature (29°C) are shown in Fig. 2. In the system with bacteria susceptible to HU (curve A) also two periods of high sensitivity of the system to the inhibitor are noted. The first period as in the preceding experiment (Fig. 1) is limited to several hours after inoculation, whereas the second period starts several hours earlier and lasts a dozen or so hours less than was the case at lower temperature (22°C). The use of resistant bacteria abolishes the sensitivity of the system to the action of the inhibitor in the first period (curve B), and inhibition of tumours by HU applied in the second period occurs during a time identically shorter as in the system with susceptible bacteria. The shortening of the period of crown-gall transformation by a dozen or so hours when the temperature is raised by 7°C agrees more or less with the observations of Lipetz (1966, 1967). This author ascertained that in *K. daigremontiana* kept at 32°C peak of the first DNA synthesis wave after wounding appears 3 h earlier, and the first divisions occur 12h earlier than in plants kept at 25°C. The time of suscepti-

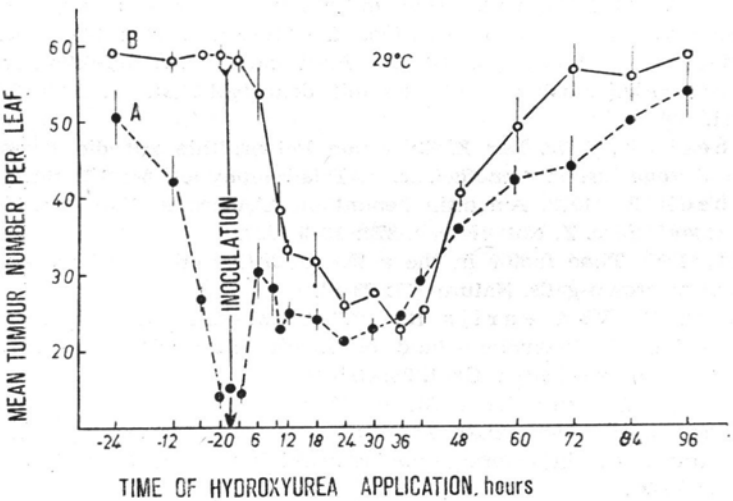

Fig. 2. Shortened course of crown-gall induction at raised temperature. The investigations were performed with plants kept at 29°C. The remaining details of the experiment were identical as those described in Fig. 1. The mean number of tumours in the control was for A — 55.6 ± 2.86 and for B — 59.3 ± 0.7

bility of the cells to the oncogenic factor (Lipetz 1965) was also correspondingly shorter.

Thus, during oncogenesis three events occurring in plant cells indicate sensitivity to temperature:

1) the process of DNA synthesis preceding wound divisions;
2) the process owing to which plant cells in the wound region become sensitive to the oncogenic stimulus. This process also precedes wound divisions;
3) the process inhibited by hydroxyurea after application of the latter in the induction phase.

All these phenomena occur in the middle period of the crown-gall transformation, corresponding to the phase of induction. It would seem that it is one and the same nontypical process of DNA synthesis which runs in two consecutive waves (Kupila-Ahvenniemi and Therman 1971, Broekaert and Van Parijs 1973, Lipetz 1967) and owing to which recombination of the plasmid DNA fragment of the bacteria with the plant cell genome becomes possible (Thomashow et al. 1980).

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Fazy transformacji crown-gall wrażliwe na hydroksymocznik

Streszczenie

Stosując system transformacji crown-gall z liśćmi *Kalanchoe daigremontiana*, badano przeciwtumorową aktywność hydroksymocznika (HU) w czasie kilku dni po zakażeniu zranień bakteriami wrażliwymi lub opornymi na ten inhibitor. W systemie z bakteriami wrażliwymi przeciwtumorowa aktywność HU ma charakter dwufazowy. Faza pierwsza ogranicza się do kilku godzin po inokulacji, druga obejmuje czas między 18 a 60 godz. po niej (faza indukcji). Stosowanie HU w każdej z tych faz silnie hamuje tworzenie tumorów. W systemie z bakteriami opornymi, hamowanie tumorów przez HU występuje tylko w fazie drugiej. W podwyższonej temperaturze, czas wrażliwości systemu na hamujące działanie HU w fazie drugiej ulega znacznemu skróceniu, niezależnie od szczepu bakterii użytego do inokulacji.

Wyniki te w pełni potwierdzają pogląd, że przeciwtumorowy efekt HU stosowanego w fazie pierwszej zależy od jego działania na komórki patogena, natomiast w fazie indukcji zjawiskiem krytycznym dla powstania tumora jest proces przebiegający w komórkach roślinnych i niezależny od patogena; charakteryzuje się on wrażliwością na HU a także na temperaturę. Procesem tym jest prawdopodobnie biosynteza roślinnego DNA.