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The *Agrobacterium tumefaciens* T-DNA gene 6^b is an *onc* gene

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Abstract

In this article it is shown that the T-DNA of *Agrobacterium tumefaciens* contains besides the well-known *cyt* and *aux* genes another gene with an oncogenic effect in plants. The gene in question is called 6^b and causes the formation of small tumors in plant species such as *Nicotiana glauca* and *Kalanchoe tubiflora*.

Introduction

The soil bacterium *Agrobacterium tumefaciens* is a phytopathogen by virtue of its ability to transform normal plant cells into tumor cells. This eventually results in tumor formation in dicotyledonous plants. The molecular mechanism underlying this bacterium-plant interaction has been described in some detail (for a recent review see [11]). Transfer of a piece of oncogenic DNA, the T-DNA, originating from the large, bacterial Ti plasmid, to plant cells at the infection sites is the key event of the process. The T-DNA contains *onc* genes, the expression of which is responsible for the tumorous character of the transformed plant cells. Mutational analysis has shown that three *onc* genes are of prime importance, viz. the genes *cyt* (*ipt*), *aux1* (*iaa*M) and *aux2* (*iaa*H). These genes code for enzymes involved in the biosynthesis of the cytokinin isopentenyl-AMP (*cyt*) and the auxin indole acetic acid (*aux1, aux2*). Mutations in *Agrobacterium* T-DNA genes other than *cyt, aux1* and *aux2* did not lead to avirulence [4]. Therefore, it is questionable whether any of the other genes that naturally are present in the T-DNA, are involved in tumorigenesis. Only some indirect data point to a possible accessory or regulatory role of some of these genes (e.g. T-DNA genes 5 and 6^a, 6^b) in tumorigenicity. Mutations in the area covering genes 6^a and 6^b of the wild-type octopine Ti plasmid were found to lead to an increased size of tumors induced on kalanchoe [4] but similar mutations in the nopaline Ti plasmid had no such effect [9]. Recent data showed that gene 6^a determines a permease system for the excretion of octopine and related opines from transformed plant cells [12], which makes a role of this gene in determining tumor size difficult to understand. In this paper we show that T-DNA gene 6^b is an *onc* gene by itself, capable of inducing tumor formation in a limited set of plant species.

Materials and methods

Bacterial strains and plasmids

Escherichia coli strain KMBL1164 *thi pro lac* (P. van de Putte) was used as a recipient for plasmid constructs in transformation experiments. Transformed plasmids were subsequently transferred to *Agrobacterium* via conjugation in triparental matings with HB101 (pRK2013). The binary vector pBin19, which contains a Km^r marker, was described by Bevan [2].

Agrobacterium strain LBA4404 [6] was used as a recipient for the binary constructs. Strain LBA1834 is a *cyt aux* double mutant [5], while LBA4443 is a derivative of LBA4404 containing a binary vector, which still contains octopine Ti T-DNA genes 5, 6^b and 3 [7]. Strain LBA1010 contains the wild-type Ti plasmid and is used as a control in virulence assays.

Conjugation, cloning, DNA isolation

Cloning experiments, DNA isolations etc. were done according to the protocols in the laboratory manual of Maniatis *et al.* [10]. Conjugations were performed on Sartorius filters as described previously [8]. Transconjugants were selected by the rifampicin resistance (20 μg/ml) of the *Agrobacterium* recipient LBA4404 and the kanamycin resistance (50 μg/ml) of the pBin19 vector on SM minimal medium plates [8].

Virulence assays

These were done using *Kalanchoe tubiflora* and *Nicotiana glauca* plants as described previously [8].

Results

Several years ago the Ti plasmid was disarmed in several laboratories including our own via the inactivation of the T-*cyt* gene as well as one or both of the T-*aux* genes. *Agrobacterium* strains carrying such plasmids were said to be disarmed, because they were no longer capable of inducing tumors on plant species such as tobacco and tomato. However, when analyzing such strains on a wider range of plant species we and others [14] found unexpectedly that some were able to form small tumors on *Kalanchoe tubiflora* and *Nicotiana glauca*. As an example strain LBA1834 can be mentioned. This strain contains a derivative of the octopine Ti plasmid with inactivated genes *aux2*, *cyt* and 6^a [5]. Therefore, in this case any of the T-DNA genes *aux1*, 5, 7, 6^b or 3 might be responsible for oncogenicity on the two plant species mentioned. Another example concerns strain LBA4443, which is a derivative of the helper virulence strain LBA4404 with a binary vector embracing the T-DNA genes 5, 6^b and 3 [7]. In order to find out directly whether gene 6^b had anything to do with this phenomenon, we cloned the *Bam* HI fragment 17^a, which contains genes 6^a and 6^b (Fig. 1) from the wild-type octopine Ti plasmid in both orientations into the binary vector pBin19. After transfer to the helper virulence strain LBA4404 we assayed the resulting strains LBA4463 and LBA4464 for virulence and found that both were able to induce small tumors on *K. tubiflora* and *N. glauca* indeed (Fig. 2). This shows that either gene 6^a or 6^b or both are *onc* genes by themselves. In order to determine whether gene 6^b was solely responsible for tumorigenicity, we deleted a *Pst* I segment – coordinates 9211–10069 [1] from fragment *Bam* HI 17^a (Fig. 1) in vector pACYC184, recloned the deleted *Bam* HI 17^a fragment, which carries gene 6^b but not 6^a, into pBin19 in both orientations and transferred the resulting plasmids to LBA4404. The resulting strains called LBA4465 and LBA4466 were indeed able to induce small tumors on *N. glauca* and *K. tubiflora* whereas the virulence helper strain (with or without the pBin19 vector) did not induce any proliferation at all. This latter result proves that in fact gene 6^b is an *onc* gene with a tumorigenic effect only on particular plant species such as *N. glauca* and *K. tubiflora*.

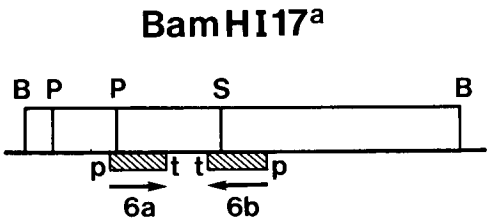

Fig. 1. On the map of the *Bam* HI fragment 17^a (coordinates 9062–13774) of the octopine Ti plasmid [2, 3], the cleavage sites for the restriction enzymes *Bam* HI (B), *Pst* I (P) – coordinates 9211 and 10069 – and *Sma* I (S) – coordinate 11207 – are indicated according to Barker *et al.* [1]. The symbols p and t denote promoter and terminator, respectively.

Discussion

In this paper we have shown that the T-DNA of the Ti plasmid contains, besides the three well-

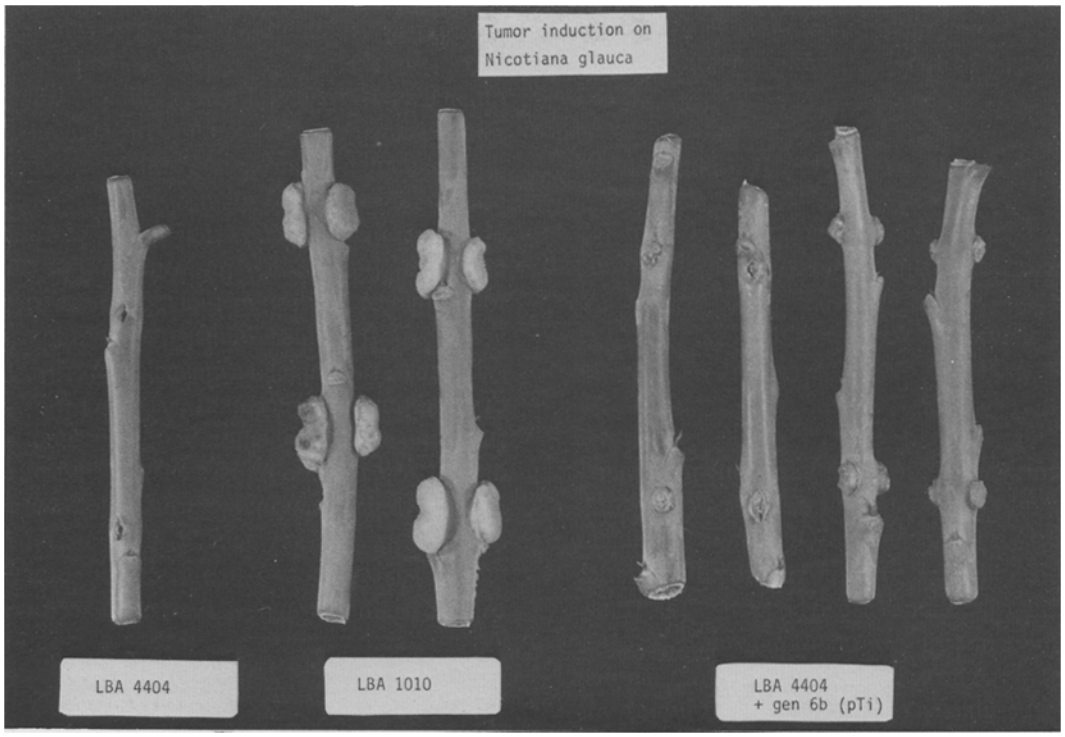

Fig. 2. Tumor formation on *Nicotiana glauca* by LBA4404 (left: no tumors formed), LBA1010 (middle: large tumors formed) and LBA4463, LBA4464 (right: small tumors induced). LBA4404 only contains the virulence helper plasmid; LBA1010 contains the wild-type Ti plasmid; LBA4463, LBA4464 are derived from LBA4404 and have the pBin19 vector with the *Bam* HI 17^a fragment in two orientations.

characterized *onc* genes *cyt, aux1* and *aux2*, at least one more gene called 6^b with an oncogenic effect on certain plant species. In the literature certain morphogenic effects have been ascribed to gene 6^b. Mutations in the area covering genes 6^a and 6^b of the octopine Ti plasmid were reported to result in the formation of larger tumors on kalanchoe [4], although a similar effect was not seen in the nopaline Ti system [9]. Any inhibitory role of gene 6^b on tumor formation on kalanchoe is difficult to reconcile with its oncogenic effect *per se* on the same plant species as shown in this article. Double mutants with a mutation in gene *cyt* as well as in 6^b induce tumors on *K. tubiflora* from which more roots proliferate than from tumors formed via *cyt* mutants [14]. Ream *et al.* [14] have shown also that double mutants with mutations in *aux* and the 6^b gene cause tumors that are less prone to shoots formation as compared to *aux* mutants. These latter data reveal that gene 6^b has an inhibitory effect on root formation and stimulates shooting. Such effects could be explained if the products determined by gene 6^b led to breakdown or inactivation of auxin or modify a cytokinin into a more potent form. Alternatively, the gene might make transformed plant cells more sensitive to cytokinin or less sensitive to auxin. The fact that gene 6^b acts as an *onc* gene by itself shows that if the products of gene 6^b are involved in the modification of a class of phytohormones, the target molecules are not (only) the plant growth regulators formed via the T-DNA genes, but rather comprise those formed by the plant cells naturally.

Gene 6^b is not only present in the octopine Ti plasmid T-DNA, but also in that of the nopaline Ti plasmid [9, 15]. Moreover, homologous sequences have also been detected in the T-DNAs of the limited host range type Ti plasmid pTiAG162 [16]. The widespread occurrence of these 6^b-like sequences suggests that the gene plays a role in the interaction of many different agrobacteria and plant cells. Although not essential for tumor formation on the plant species studied thus far, it might be that the

gene has a much more prominent role in tumorigenicity on some plant species.

Even on plant species where no direct oncogenic effect of 6^b is seen such as tobacco, its presence leads probably to growth aberrations. Tobacco plants containing a truncated T-DNA comprising only genes 6^b and 3 (octopine synthase), form a poor root system and after flowering show premature leaf abscission besides other abnormalities [13]. From all these results it is clear, therefore, that the presence of gene 6^b must be avoided in plant vectors which are to be applied for the improvement of crops. Further studies will hopefully reveal the precise function determined by gene 6^b.

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