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A larva of the tobacco hornworm feeding on a tobacco leaf. But, as reported on p.33 of this issue, transgenic tobacco plants that are genetically engineered to produce a bacterial protein toxic to insects are protected from hornworm attack in greenhouse tests. See also News and Views.

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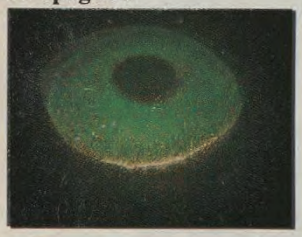
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■ Maternal mRNAs

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■ Short-term selection

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■ Skaergaard challenge

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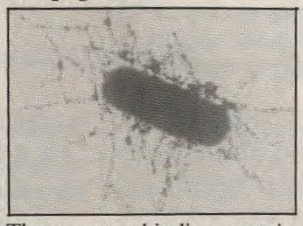
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■ Adhesin localization

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The receptor-binding protein adhesin is located at the tip of the filaments that a bacterium uses to stick to membranes of its host.

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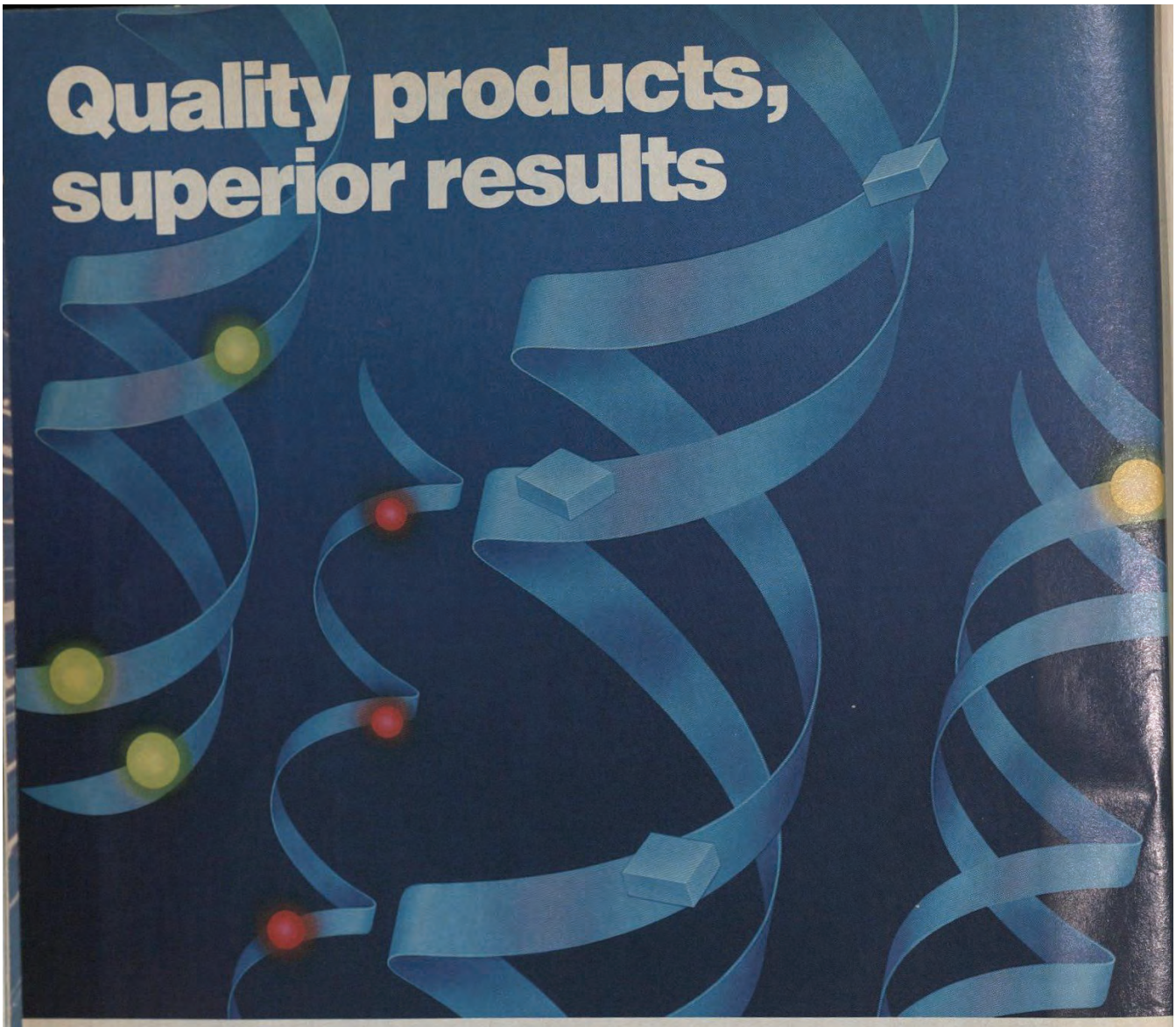
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with many other much studied problems, is centrally important to theoretical computer science as an NP-complete problem[4]. Here we consider the matter of intractable problems in information-based complexity.

Given an error tolerance $\varepsilon > 0$, it has been shown for many problems in mathematical analysis that the time complexity (number of operations) is proportional to $(1/\varepsilon)^{d/r}$ where d is the dimension of the problem and r measures the smoothness[2,46]. Note that, for fixed smoothness, the complexity is exponential in d. Among the problems with complexity proportional to $(1/\varepsilon)^{d/r}$ are multivariate integration[35–37], multivariate approximation[47], and systems of nonlinear equations[40].

A numerical example illustrates the implications of this complexity function. If one wants eight-place accuracy in computing triple integrals of functions that are once differentiable, then $\varepsilon = 10^{-8}$, $d = 3$, $r = 1$, and $(1/\varepsilon)^{d/r} = 10^{24}$. Even if we generously assume the existence of a sequential computer that performs 10^{10} operations per second, the computation will take of the order of 10^{14} seconds or some 100,000 years.

By comparing what we have said about TSP with multivariate problems of the type discussed here, we note two important general differences. Exponential complexity for TSP (and many other problems) is currently only conjectured, but exponential complexity has been established for the indicated problems in information-based complexity. Secondly, the role of the problem size n in combinatorial complexity is, in information-based complexity, often taken by the dimension d. We observe that problems of high dimensionality occur in such diverse disciplines as physics and nonlinear economic modelling.

These conclusions are based on complexity considerations, meaning that they are intrinsic to the problem we are trying to solve. No algorithm, no matter how clever, can sidestep these computational barriers.

Can intractable problems be solved?

In spite of the seemingly absolute nature of these conclusions on intractability, there are several ways in which their consequences might be mitigated.

Physicists have long known about the power of randomness. Classical Monte Carlo methods, for example, may be regarded as random processes for making the cost of certain computations independent of dimension. When randomness is used, one must be willing to settle for probabilistic rather than worst-case assurances about error. Researchers in information-based complexity are now asking basic questions about the power and limitations of randomness. What problems can be solved more easily by using randomness? How much can the complexity of such problems be lowered? How much can their uncertainty be reduced? Are there problems for which randomness can be shown to be of no help[54]?

The intractability results discussed in the last section are based upon a worst case setting for our problem formulation. If one is willing to live with the assurance that the uncertainty is no greater than ε on average, can that make tractable a problem that is intractable in the worst case? Average case analysis of problems and algorithms is an increasingly important area of study in theoretical computer science. Much current work in this area is also taking place in information-based complexity. The investigation of the extent to which complexity is lowered in the average case setting is an important part of this work[2,3,12,48,49].

Another approach to measuring uncertainty and complexity uses a probabilistic setting, in which one allows uncertainty to exceed ε, but only with a small predefined probability. Questions of error and intractability in this setting are also the object of current research[14,15,48].

Parallel computation

In the integration example discussed earlier and in many other important problems the information operators used have the form $N(f) = [L_1(f), L_2(f), \ldots, L_n(f)]$, for a fixed number n of linear functionals $L_i : \mathbf{F} \to \mathbb{R}$ (the problem domain $\mathbf{F}$ is assumed to be a linear space). Each component of such information can be computed independently so that $N(f)$ can be separated into n 'parallel' computations. Information with the above property is called nonadaptive information and is of considerable importance in facilitating parallel computation. Indeed, if n nonadaptive evaluations are required to achieve the desired accuracy we may use n processors to perform the desired evaluations simultaneously. This will essentially lower the information cost by a factor of n.

If, on the other hand, the choice of the L_i depends on the previously computed information or if the number of evaluations n varies with f, the information is called adaptive and will tend to be unsuitable for parallel computation. A question that naturally arises is the extent to which nonadaptive information can be computationally as powerful as adaptive information. This question can be carefully formulated in the context of information-based complexity. It has been shown[11,50,51] that for linear problems (S and N are linear operators), nonadaptive information is essentially as powerful as adaptive information. This result is surprisingly robust, holding in both worst-case and average-case settings[48,51].

Thermodynamics and information theory

We began this article by observing that computational complexity and thermodynamics share two characteristics. We conclude by discussing how information-based complexity relates to the notion of entropy, a term whose origins come from thermodynamics. Our interest here is in a more recent use of the term entropy and its by-product, mutual information, as defined in classical information theory.

Concepts to measure uncertainty, disorder and information content have arisen in a variety of scientific disciplines. Entropy, introduced by Clausius in thermodynamics, was first used in a probabilistic setting by Boltzmann in his treatment of statistical mechanics. In statistics, the variance and R. A. Fisher's technical definition of information are noteworthy. In mathematics, Kolmogorov used the term entropy to define a measure of 'capacity' in abstract metric spaces. Finally, Shannon introduced the logarithmic definitions of information and its negative, entropy, as the basis for information theory.

Information-based complexity has connections with several of the concepts discussed above. Thus, in a worst-case setting, there are connections between ε-complexity and Kolmogorov entropy[11]. Working in an average case setting, there is a class of problems for which the radius information $r^{\mathrm{ave}}(N_n)$ can be expressed as the standard deviation of a related distribution[48].

Finally, one can define a value of information $V(N) = \log_2(r^{\mathrm{ave}}(\mathbf{0})/r^{\mathrm{ave}}(N))$, where the notation indicates an average-case setting and $r^{\mathrm{ave}}(\mathbf{0})$ denotes the radius computed with no information available. It can be shown[52] that $V(N)$ provides interesting connections and contrasts with the corresponding average mutual information, an entropy-based concept from information theory. The two values agree for an important class of problems. In a variety of other situations, however, the average mutual information is not well-defined or the two values disagree. The value of information $V(N)$ is virtually always defined and has a natural interpretation in terms of 'bits of accuracy gained'. Thus for problems with incomplete information, where information provides a mechanism for refining what we know about the space of problem elements, information-based complexity provides a new approach to measuring the value of information.

We thank the National Science Foundation for partial support.

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ARTICLES

Transgenic plants protected from insect attack

Mark Vaeck, Arlette Reynaerts, Herman Höfte, Stefan Jansens, Marc De Beuckeleer, Caroline Dean*, Marc Zabeau, Marc Van Montagu & Jan Leemans

Plant Genetic Systems NV, Jozef Plateaustraat 22, B-9000 Gent, Belgium

The Gram-positive bacterium Bacillus thuringiensis *produces proteins which are specifically toxic to a variety of insect species. Modified genes have been derived from* bt2, *a toxin gene cloned from one* Bacillus *strain. Transgenic tobacco plants expressing these genes synthesize insecticidal proteins which protect them from feeding damage by larvae of the tobacco hornworm.*

MODERN agriculture uses a wide variety of insecticides to control insect damage. Most of them are chemically synthesized. Notable exceptions are the insect toxins produced by *Bacillus thuringiensis*: spore preparations of this Gram-positive bacterium have been used for more than 20 years as a biological insecticide[1]. The insecticidal activity resides in crystalline inclusion bodies produced during sporulation of the bacteria, which are composed of proteins (termed delta endotoxins) specifically toxic against a variety of insects. Different strains of *B. thuringiensis* differ in their spectra of insecticidal activity. Most are active against Lepidoptera, but some strains specific to Diptera[2,3] and Coleoptera[4,5] have been identified. The crystals dissolve in the alkaline conditions of the insect midgut and release proteins of relative molecular mass 65,000–160,000 (M_r 65K–160K)[2,5,6] which are proteolytically processed by midgut proteases to yield smaller toxic fragments[7]. *B. thuringiensis* insect toxins are highly specific, in that they are not toxic to other organisms. Hence, they are safe insecticides and present an interesting alternative to chemical control agents. Their commercial use however is limited by high production costs and the instability of the crystal proteins when exposed in the field.

We have used *Agrobacterium*-mediated T-DNA transfer[8] to express chimaeric *B. thuringiensis* toxin genes in tobacco plants with the objective of protecting the plants from insect attack.

* Permanent address: Advanced Genetic Sciences Inc., San Pablo Avenue, 6701, Oakland, California 94608, USA.

We show here that a defence mechanism against phytophagous insects can be devised by genetic engineering.

Modified *Bacillus* toxins

We have reported[9] the cloning of the *bt2* gene from *B. thuringiensis* strain berliner 1715 and the characterization of the recombinant polypeptide expressed in *Escherichia coli*. This protein, termed Bt2, is 1,155 amino acids long and is a potent toxin to several lepidopteran larvae, such as those of *Manducta sexta*, a pest of tobacco. Bt2 is a protoxin and generates a smaller polypeptide of M_r 60K which retains full toxic activity[9]. The smallest fragment of Bt2 that is still fully toxic has been mapped in the NH_2-terminal half of the protein, between amino-acid positions 29 and 607 (ref. 9) (and see Fig. 1*a*).

In plant transformation experiments, we used chimaeric genes containing the entire coding sequence of *bt2* as well as truncated genes. A diagram of the chimaeric genes is shown in Fig. 1*b*. Some of our T-DNA constructs include a chimaeric neomycin phosphotransferase gene (*neo*) as a marker selectable in plants[10]. Others carry translational fusions between fragments of *bt2* and the *neo* gene. Fusions to the 5′ end of the *neo* gene still confer kanamycin resistance in bacteria[11] and in plants[12]. Plasmid pLB884 (ref. 9) contains the truncated gene *bt884* and encodes an NH_2-terminal fragment of Bt2 up to amino-acid position 610. In *E. coli* it produces a polypeptide of the expected size which is fully toxic towards insect larvae[9].

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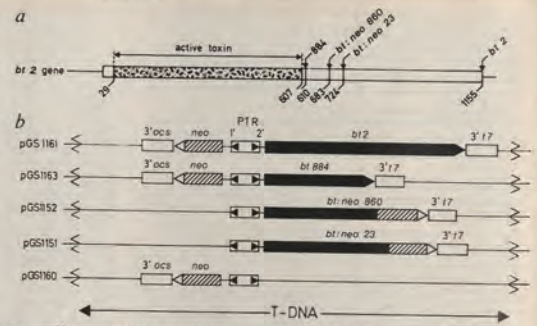


Fig. 1 *a*, Structure of the *bt2* gene. The smallest gene fragment encoding an active insect toxin is indicated, and the 3′ end positions (codon numbers) of the different truncated genes. *b*, Chimaeric genes derived from *bt2* present in plant expression vectors. The 5′ end of the *bt2* coding sequence is fused to the 2′ promoter fragment of the TR DNA. pGS 1161 contains the intact *bt2* gene. The *bt2* segment in pGS1163 ends at nucleotide position 1,830 of the *bt2* coding sequence. In pGS1151 and pGS1152, the *neo* gene has been fused to 5′ fragments of the *bt2* gene at positions 2,173 and 2,050 of the *bt2* coding sequence, respectively. PTR, a 482-base pair (bp) fragment containing the TR DNA 1′ and 2′ promoters, isolated from pOP443 (ref. 15); 3′t7, a 211-bp fragment containing the polyadenylation site of the TL DNA gene 7 (ref. 23); 3′ocs, a 706-bp *Pvu*II fragment containing the polyadenylation site of the octopine synthase gene[24]. Plasmids pGS1160, pGS1161 and pGS1163 were made using the intermediate plasmid pGSH160 which contains a chimaeric *neo* gene[14]. For pGS1151 and pGS1152, containing the *bt2*:*neo* fusion genes pGSH150, a derivative of pGSH160, lacking the *neo* gene and 3′ ocs, was used.

Plasmids pLBKm860 and pLBKm23 (H.H. *et al.*, in preparation) contain fusion genes *bt*:*neo860* and *bt*:*neo23* encoding NH_2-terminal fragments of Bt2 up to amino-acid position 683 and 724, respectively. *E. coli* cells harbouring these plasmids are resistant to kanamycin and produce fusion proteins Bt:NPT860 and Bt:NPT23 which have the expected M_r of 106K and 110K respectively and which react with anti-Bt2 (Fig. 2A) and anti-NPTII antibodies. At least 50% of the fusion proteins is present in a soluble form in the bacterial cells. The neomycin phosphotransferase activity of the fusion proteins, as determined in an *in situ* assay[13], is comparable to wild-type NPTII activity. Little or no enzymatic activity was exhibited by polypeptides of lower M_r (Fig. 2*b*). We conclude that the fusion proteins are relatively stable and responsible for the kanamycin-resistant phenotype. Insect assays revealed that, on a molar basis, the fusion proteins exhibit the same toxicity towards *M. sexta* larvae as intact Bt2 protein which has an LD_{50} value on first instar larvae of 4 ± 2 ng per larva.

Plant expression vectors

The intact and modified *B. thuringiensis* toxin genes were inserted between the T-DNA borders of plant expression vector pGSH160 (for *bt2* and *bt884*) or pGSH150 (ref. 14) (for *bt*:*neo860* and *bt*:*neo23*). These plasmids contain the promoter of the 2′ gene, a constitutive promoter which directs expression of mannopine synthase in the TR DNA of plasmid pTiA6 (ref. 15). The *Bacillus* genes are followed by a termination signal provided by the 3′ end of gene 7 of pTiA6. The resulting plasmids were mobilized into the *Agrobacterium* recipient C58C1 Rif^R pGV2260. The latter contains an octopine Ti plasmid from which the whole T-DNA region has been deleted and replaced by pBR322 (ref. 14). Recombination between pGV2260 and the expression vector through the homologous pBR322 sequences produced Ti plasmids pGS1161, pGS1163, pGS1151 and pGS1152 containing *bt2*, *bt884*, *bt*:*neo23* and *bt*:*neo860*, respectively (Fig. 1).

Two approaches were used to increase the probability of obtaining high levels of toxin expression in plants. First, the expression levels directed from the 1′ and 2′ promoters from the TR DNA were found to be coordinated[15]. Consequently we expected that expression of the *neo* gene controlled by the 1′ promoter in plants transformed with pGS1161 and pGS1163, would be correlated with transcription of the toxin gene. Second, we anticipated that plant cells transformed with pGS1151 or pGS1152, containing *bt*:*neo* fusions, would produce fusion proteins expressing NPTII activity. Selection for high levels of kanamycin resistance would allow us to select directly for transformed clones producing substantial amounts of *B. thuringiensis* protein.

Transformation of tobacco plants

Transgenic tobacco plants were obtained by leaf disk infection[14,16] of *Nicotiana tabacum* var. Petit Havana SR1 (ref. 17). Shoots resistant to 50, 100 or 200 μg ml^{-1} kanamycin were selected in all transformation experiments, indicating that the fusion genes indeed confer NPTII activity on transformed plant cells. Individual transformed plants were grown up and subsequently assayed for kanamycin resistance by testing their ability to produce callus from leaf disks on increasing concentrations of kanamycin and the more toxic aminoglycoside antibiotic G418. Most of the transgenic plants expressing an intact NPTII protein produced highly resistant calli, growing on 1,000 μg ml^{-1} kanamycin and on 100 μg ml^{-1} G418. In contrast, plants that expressed a Bt:NPTII fusion protein readily fell into different

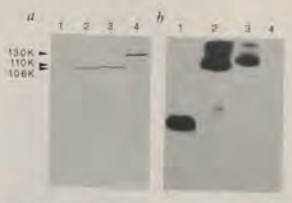


Fig. 2 Antigenic properties and enzymatic activity of Bt:NPTII fusion proteins. *a*, Western blot analysis[25] of crude extracts of *E. coli* clones producing intact NPTII enzyme (lane 1), Bt:NPT860 (lane 2) or Bt:NPT23 fusion protein (lane 3). Lane 4, purified Bt2. Blots were incubated with a diluted anti-Bt2 serum and subsequently with alkaline phosphatase-labelled anti-rabbit immunoglobulin. Substrates were 5-bromo-4-chloro-3-indolyl phosphate-toluidine salt and *p*-nitro-blue tetrazolium chloride (Sigma). *b*, Detection of NPTII activity in samples identical to those in *a*, by *in situ* phosphorylation of kanamycin with ^{32}P-ATP (ref. 13). All samples in *a* and *b* contain 0.25 μg protein per lane.

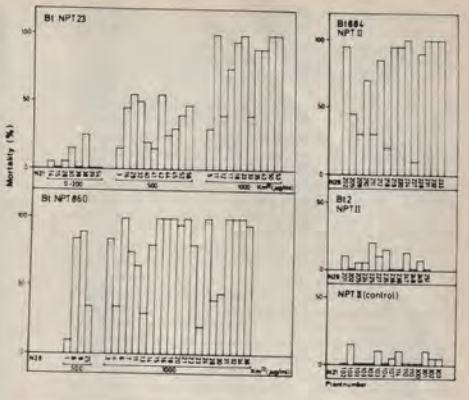


Fig. 3 Insect toxicity of transgenic tobacco plants expressing *B. thuringiensis* protein. Mortality of *Manduca sexta* larvae after 6 days of feeding on tobacco leaves is shown. The serial number of each plant is given below each column, and in the left-hand panels plants are grouped according to the concentration (in μg ml^{-1}) of kanamycin to which they are resistant. Each panel is labelled with the protein expressed (see Fig. 1). Plants were tested 4–6 weeks after transfer to the greenhouse, when they were about 40-cm high and had between six and eight full grown leaves. Two leaf disks of 4 cm in diameter were placed on wet filter paper in Petri dishes and infested with two batches of 10 first instar larvae of *M. sexta*. Leaf disks were replaced daily. Tests were conducted in a growth chamber at 25 °C, 75% relative humidity and under a 16 h light/8 h dark cycle.

Table 1 Kanamycin and G418 resistance in transgenic tobacco plants

Agrobacterium strain	Expression products of chimaeric genes	No. of plants resistant: Kanamycin (μg ml^{-1}) <50	50	100	200	500	1000	G418 (μg ml^{-1}) <10	10	100	Total
pGS1151	Bt:NPT23	3	1	7	17	14	10	51	1	0	52
pGS1152	Bt:NPT860	0	0	0	0	5	31	35	1	0	36
pGS1161	Bt2, NPTII	0	0	0	0	0	14	0	0	14	14
pGS1163	Bt884, NPTII	0	0	0	0	1	14	1	0	14	15
pGS1160	NPTII	0	0	0	0	2	12	2	0	12	14

Plants were scored according to the highest concentration of antibiotic on which callus could be induced from leaf disks from *in vitro* grown plants[21].

classes of kanamycin resistance (Table 1). Because the specific enzymatic activity of the fusion proteins is comparable to that of intact NPTII, we presume that these fusion proteins are present in lower amounts in the plant cells than intact NPTII protein.

Insecticidal activity in transgenic plants

Leaves of transgenic plants containing the four types of *B. thuringiensis* gene constructs were fed to *M. sexta* larvae in order to evaluate whether the levels of toxin in the plants would be insecticidal. Mortality rates of *M. sexta* larvae were monitored after 6 days of feeding on leaves of transformed plants (Fig. 3). We found that high toxicity to insects, resulting in 75–100% mortality of the larvae, was observed in about one quarter of the plants that expressed the longer fusion protein Bt:NPT23 and in about two-thirds of those with the shorter Bt:NPT860. Thus, the Bt:NPTII fusions allowed us to select transformants that express levels of the toxin sufficiently high to be insecticidal. Second, insect toxicity caused by the fusion proteins is directly correlated with the level of kanamycin resistance of the transformed plant. In addition, the short fusion generates a larger fraction of transformants expressing high kanamycin resistance and insect toxicity than the longer fusion, suggesting that the shorter *bt*:*neo860* gene provides higher levels of biologically active protein than the longer *bt*:*neo23*. Clear insecticidal activity was also detected in most of the 15 plants expressing the truncated *bt884* gene, of which two-thirds induced more than 75% larval death. None of the plants transformed with the full length *bt2* gene produced insect killing activity above levels obtained in NPTII-expressing control plants. These experiments indicate that for the promoter gene constructs we used, only truncated *bt2* genes give rise to expression levels that are strongly insecticidal in transgenic tobacco.

Protection from insect damage

To test whether expression of modified *bt2* genes in plants results in effective protection against insect damage, selected transgenic plants were grown in the greenhouse and were infested with freshly hatched larvae of *M. sexta*. Plants were kept under conditions that were optimal for survival and growth of the insects. Plants N21-11 and N28-16 were highly protected, because the larvae stopped feeding within 18 hours and all were killed within three days. The damage caused by single larvae was limited to areas of only a few square millimetres (Fig. 4). Other plants, such as N21–53 or N28-6, suffered slightly more damage. On these plants however all the larvae were killed after six days. Control plants such as N21–110, transformed with pGS1160, or untransformed SR1, were severely damaged within 4–6 days and were entirely consumed after 12 days.

Expression of chimaeric genes in plants

Quantitative detection of *B. thuringiensis* toxin in the leaves of transformed plants was performed using a sensitive ELISA, with a mixture of monoclonal antibodies specific for the NH_2-terminal region of the protein. A correlation was found between the quantity of *Bacillus* protein and insecticidal activity in the transgenic plants. Plants transformed with the truncated *bt2* gene or the fusion constructs contain approximately ten times

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more *Bacillus* protein than those transformed with the complete *bt2* sequence (Table 2). Thus, the failure to obtain insect-resistant plants using the intact *bt2* gene is most probably due to inefficient protein synthesis in these transformed plant cells.

Transgenic plants that express the shorter Bt : NPT860 protein are on average more effective in killing insects and express higher levels of toxin than those expressing the longer Bt : NPT23 protein (Table 2). The Bt : NPTII fusion proteins detected in leaf extracts of the transformed plants had the expected size, as determined in Western blots. NPTII enzyme activity was exclusively associated with these fusion proteins as determined by *in situ* NPTII assays. Production of incomplete proteins or degradation in the plant cells was not observed. Thus, transformed tobacco plants produced fully functional fusion proteins of the size of the intact gene product.

The steady-state *Bacillus* messenger RNA levels in the transgenic plants were low and could not be reliably detected in Northern blot analysis. Therefore, they were quantified using ribonuclease protection experiments[16]. A probe containing the 5′ fragment of the *bt2* coding sequence up to nucleotide position 186 was synthesized using the SP6 transcription system and annealed to total RNA from the leaves of transgenic plants. After ribonuclease digestion, the protected fragments were run on a denaturing polyacrylamide gel. The results showed that RNA levels in the leaves of the transformed plants were correlated with *B. thuringiensis* protein levels (data not shown). The *B. thuringiensis* mRNA in N28-16, the plant that produces the highest level of protein, corresponds to ~0.0001% of the poly(A)$^+$ mRNA. The *Bacillus* protein detected in this plant represents 0.02% of the total soluble protein, or 3 μg of this protein per gram fresh leaf tissue. A fivefold lower level, as present in plant N28-34, was sufficient to induce 100% killing in a 6-day insect assay (Table 2). For comparison, the expression levels of the intact *neo* gene also driven by the TR 2′ promoter, varied between 10 and 40 μg of NPTII per gram leaf tissue (0.07–0.27% of total protein). This difference in amount of protein is consistent with the lower kanamycin resistance levels of plants transformed with the *bt* : *neo* fusions compared to those transformed with the intact *neo* gene (Table 1).

Inheritance of the protection

Eighty-five transgenic tobacco plants, transformed with the four types of chimaeric *B. thuringiensis* genes and expressing various levels of active insect toxin, were grown in the greenhouse. All grew normally and were indistinguishable from controls in morphology and vigour of growth. We analysed the inheritance of the kanamycin resistance in eleven plants expressing *bt* : *neo* genes. Most of these plants contained one (N21-23, N21-50, N28-31, N28-34, N28-21) or two (N21-35, N28-19, N28-24, N28-32) kanamycin resistance loci, as confirmed by Southern blot analysis. Interestingly, some plants that were recognized as producing a large amount of *Bacillus* protein, such as N21-11 and N28-16, generated exclusively kanamycin-resistant F_1 seedlings. DNA analysis showed the presence of at least five copies of the T-DNA in these plants.

F_1 progeny from some transgenic plants were assayed for the expression of insecticidal activity. Insect toxicity was correlated with the kanamycin resistance marker in the F_1 progeny of plants N21-23, N21-50 and N28-34. Insecticidal activity was similar to that observed in the parental plants. Approximately 15 F_1 progeny of N21-11 and N28-16 were analysed. They all induced 100% killing of *M. sexta* larvae in the standard 6-day assay. Toxin levels in the F_1 progeny of N21-11 varied between 20 and 50 ng per mg of total protein, comparable to the 30 ng per mg in the parental plant.

Discussion

Four chimaeric genes containing modified *Bacillus* toxin genes under the control of the 2′ promoter of the *Agrobacterium* TR DNA, have been transferred into tobacco plants. All contain the toxic core of the Bt2 protein; *bt2* encodes the complete M_r 130K protoxin, *bt884* is a 5′ fragment of *bt2* up to codon 610. *Bt* : *neo23* and *bt* : *neo860* encode fusion proteins which are relatively stable, both in bacteria and plants, and which retain full insect toxicity and NPTII enzyme activity.



Fig. 4 Protection from insect feeding damage in transgenic plants expressing *B. thuringiensis* protein. Plants 40–50-cm high were infested in the greenhouse with fifteen *M. sexta* larvae per plant. *a*, On N21-11, expressing Bt : NPT23, all larvae died within three days. Leaf damage is very limited. *b*, Damage on a control plant expressing NPTII. Pictures were taken after 11 days.

Insecticidal levels of toxin were produced when truncated *Bacillus* genes or fusion constructs were expressed in transgenic plants. Mortality rates among *M. sexta* larvae feeding on transgenic leaf material depended on the amount of toxic polypeptide produced. Typically, greenhouse grown plants producing more than 0.004% of their protein as the toxin produced 100% mortality in 6-day feeding assays. Some of the plants we have selected, contain toxin at three to five times this level (N21-11 and N28-16). In greenhouse tests, these plants were well protected from leaf damage caused by insects. Quantification also showed that the toxin expressed in plants has the same specific activity as in a bacterial host.

No significant insecticidal activity could be obtained using the intact *bt2* coding sequence, despite the fact that the same promoter was used to direct its expression. Intact Bt2 protein and RNA amounts in the transgenic plant leaves were 10–50 times lower than those for the truncated *B. thuringiensis* polypeptide or the fusion proteins. Expression levels were not significantly influenced by fusing the *neo* gene to the *bt2* sequence, but rather by the length of the *bt2* fragment. Why the complete *bt2* gene is not expressed at an equally high level in plant cells, is not known. Several parameters, such as differential RNA stability and translation efficiency might be important.

We observed in transgenic plants containing the *bt* : *neo* fusion constructs a correlation between insecticidal activity and resistance to kanamycin. Three-quarters of all plants resistant to 1,000 μg ml^{-1} kanamycin induced 75–100% insect mortality. Such fusion genes can be used to select efficiently for transformed plants expressing strong insecticidal activity through direct selection for high kanamycin resistance.

Table 2 Insecticidal activity and *B. thuringiensis* protein content of transformed plants

Toxic protein	Plant	*Bacillus* protein detected (ng per mg total protein)	Insecticidal effect (% mortality) Day 3	Day 4	Day 6	Weight reduction in surviving larvae (%)
Bt : NPT23	N21-11	33 (132)	90	100	100	—
	N21-35	6.9 (71)	0	40	90	57
	N21-17	2.6	0	15	75	71
	N21-18	5.7	25	50	90	80
	N21-32	2.5	5	40	50	39
	N21-41	4.3	0	0	15	34
	N21-43	4.7	10	40	80	66
Bt : NPT860	N28-16	42 (190)	100	100	100	—
	N28-34	6.9 (42)	70	85	100	—
	N28-6	13	80	90	100	—
	N28-15	10	75	95	100	—
	N28-19	6.2	35	65	100	—
	N28-21	7.0	45	75	100	—
	N28-24	12	60	90	100	—
	N28-31	6.3	55	80	100	—
	N28-32	14	85	90	100	—
Bt2	N21-105	1.3 (5.5)	0	5	15	7
	N21-225	1.2 (2.1)	5	10	20	17
	N21-201	<1	0	5	10	23
	N21-236	1.3	0	0	0	0
	N21-238	<1	0	0	0	0
	N21-249	1.8	0	10	10	10
Bt884	N28-212	30 (125)	100	100	100	—
	N28-220	11 (40)	60	95	100	—
	N28-219	11	55	65	95	85

Values in parentheses refer to amounts detected in greenhouse grown plants. Extracts were prepared from leaves of propagated *in vitro* plants or from leaves of plants grown in a greenhouse that had between six and eight fully expanded leaves. Leaf tissue was ground up and subsequently sonicated (10 s at 50 W) in extraction buffer (Na_2CO_3, 50 mM at *p*H10; dithiothreitol (DTT), 5 mM; leupeptin, 1 mg ml^{-1}; Triton X-100, 0.05%; EDTA 50 mM; phenylmethylsulphonyl fluoride (PMSF), 0.19 mg ml^{-1}). The extract was cleared by centrifugation and *B. thuringiensis* polypeptides in the supernatant were quantified using an indirect enzyme-linked immunosorbent assay[22] (ELISA). Polyvinyl microtitre plates were coated with a goat antibody against *B. thuringiensis* crystal protein. Plant extract dilutions were incubated at 4 °C for 2 h in the coated wells. After rinsing, bound antigen was reacted with a mixture of four distinct monoclonal antibodies against Bt2 and subsequently with an alkaline phosphatase conjugated goat anti-mouse immunoglobulin antibody. The bound enzyme conjugate was detected by adding *p*-nitrophenyl phosphate as a substrate, and relative quantities were determined by measuring absorbance values at 405 nm. The monoclonal antibodies used specify antigenic epitopes located between amino-acid positions 29 and 222 in the NH_2-terminal region of the Bt2 protein (H. Vanderbruggen *et al.*, in preparation). To quantify the *Bacillus* protein levels in transgenic plants, ELISA binding curves of leaf extracts were compared to standard binding curves, obtained by diluting known quantities of the purified homologous protein in control extracts from non-transformed SR1 tobacco plants. The detection limit of the test for purified, solubilized Bt2, was 0.1–1.0 ng ml^{-1}. Toxin levels in plant leaves are expressed as ng toxin per mg of total soluble protein. Insecticidal activity was determined using *Manduca sexta* larvae feeding on leaf material. Mortality after 3, 4, 6 days was determined and weight reduction in the surviving larvae measured after 6 days.

Our experiments illustrate the feasibility of engineering plants that defend themselves against lepidopteran insects which are sensitive to the *B. thuringiensis berliner* insect toxin. However some species, such as *Heliothis* and *Spodoptera* which belong to the *Noctuidae*, an important group of pest insects, are less sensitive to common strains of *B. thuringiensis*, including berliner 1715 (ref. 19). To protect plants fully against these insects, higher levels of expression will be required. This might be achieved using chimaeric *Bacillus* genes containing stronger plant-specific promoters. The 35S promoter of cauliflower mosaic virus[20], for example directs a 10–50-fold higher expression than the regular T-DNA promoters in plants. Alternatively, it may be possible to construct chimaeric toxin genes with higher specific activity against important target insects. Transfer of different chimaeric genes into a variety of crops may provide a new and environmentally safer method of controlling destructive insect pests.

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