Feasibility of landmine detection using transgenic plants

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ABSTRACT

Genetically modified plants that detect TNT and its degradation products are potentially powerful aids in humanitarian demining and detection of unexploded ordnance. Although the feasibility of TNT detection by plants and microorganisms has been demonstrated by several research teams world wide, thus far, none of these previously demonstrated systems has the sensitivity and specificity to be effective under field conditions. We are using two approaches to increase the potential effectiveness of these and related biological detection systems. First, we are expanding the repertoire of explosive-responsive promoters by conducting DNA microarray experiments with plants treated with TNT-degradation products, and characterizing the inducibility of reporter gene expression by these promoters. Second, we are evaluating the dynamics and limiting factors in the transmission of artificial signals from roots to shoots. This will increase the ability of soil-based TNT perception strategies to effect human-readable changes in shoot morphology as part of a practical plant-based explosives detection system.

Keywords: Biosensors, Transgenic plants, Signal transduction, Inducible expression, Landmine, Explosive, Trace, Detection

1. INTRODUCTION

Landmine clearance is a complex, slow and expensive process, which is also extremely dangerous. While improved clearance techniques and increased funding and support would help countries afflicted by mines to return more land to safe civilian use, the sheer size of the problem diminishes the effect of any incremental improvements. What is needed is a method to minimize the total area to be cleared. With accurate surveys of suspected areas, truly contaminated areas can be identified for clearing, while all other areas can be returned to productive use. For example, through such surveying techniques, the Croatia Mine Action Centre reported[1] that it was able to reduce the total area suspected of being mined from 13,000 km^2 to 4,500 km^2, saving many decades of clearing effort and billions of dollars, based on current clearance rates and costs. Funding and efforts can then be focused on safely clearing known contaminated sites.

Currently available techniques to aid in the survey process are quite limited. While many improvements can be envisioned, one must bear in mind that in order for a proposed technique to achieve any realizable benefit, the operating costs and infrastructure required to field it must be affordable enough that many units could be employed throughout impoverished and isolated areas. It is clear that minefield surveys could benefit from more innovative approaches focusing on low-tech techniques.

In this paper, we will present a short description of the humanitarian demining problem and identify how a genetically modified plant system could be used to identify landmine-affected areas. We will then discuss our intitial experimental efforts in the development of a proof-of-concept system, and conclude by identifying characteristics of a practical plant-based TNT sensor.

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1.1. Minefield Detection

Were minefields laid in prescribed patterns, with meticulous recording and maintenance, the humanitarian landmine problem would largely be one of simple removal. Unfortunately, the current crisis situation arose from the proliferation of unmarked minefields. Hasty defences, poorly trained soldiers and terrorism all contribute to the problem of unrecorded mines and mine affected areas. A humanitarian landmine problem can arise even when mines were employed in an established defensive position. This results when details about their locations are lost due to natural conditions such as erosion and flooding, as well as more common causes in war zones; maps are lost or destroyed, and the soldiers who planted the mines are either no longer available or simply have no interest in helping.

While many techniques have been developed for military use, the military problem is quite distinct from that of humanitarian demining. The military is concerned mainly with minefield breaching - rapidly clearing a route through a minefield, leaving most of the mines in place. To achieve these ends, military systems tend to rely on high-technology and highly trained operators. This is clearly insufficient for humanitarian use, as even the fear of the presence of landmines will result in land remaining uninhabited. To return mine affected land to productive use, humanitarian efforts must systematically find and remove every mine. Technology for use in developing countries must be affordable and simple enough to be maintained and operated by local people who are trained and supervised by demining experts from non-government organizations (NGOs).

Minefield surveys are typically classified into three levels.[2] Level I surveys are designed to identify the general location of mined or suspected mined areas. Information is collected about the areas affected or not affected by mines. These areas must then be categorized, and the reliability and credibility of data recorded. Level I surveys may also measure the humanitarian and socio-economic impact of landmine contamination. This allows activities and resources to be concentrated on the areas of greatest need.

A Level II survey is designed to reduce the large areas identified in a Level I survey. This is achieved by accurately defining the outer perimeters of the landmine contaminated areas. The marked perimeter forms the area for future mine clearance operations. This level of survey requires trained and properly equipped mine clearance personnel with the necessary skills to undertake and accurately record the survey work.

Level III surveys are conducted during the actual clearance of the areas identified in the Level II survey, and involves the accurate recording of the areas cleared. This level of survey is done in conjunction with clearance activities and is not considered suitable for the technology to be discussed.

Current Level I and II survey techniques are quite limited. Information in a Level I survey is typically gathered through a country-wide, door-to-door survey and is based on local knowledge. As one might imagine, this procedure is slow, very costly, and can provide little or no information on the extent of the contaminated areas, thus causing very large areas to be classified as contaminated based on limited anecdotal evidence. In the unfortunate cases where the local inhabitants have abandoned the area, such limited local knowledge may not even be available. Level II surveys, which define the perimeter of the minefields, are currently done by methodical visual inspections, detailed local information, when available, and on occasion with the use of dogs.

Advances in the ability to directly detect landmines will help localize areas of high landmine density, providing valuable information to a Level II survey. Advanced electro-optical airborne techniques, while promising and potentially very useful for aiding specific scenarios, are currently too expensive for wide spread use throughout the world.

1.2. Detection Methods

Landmine detection methods can typically be classified as stand-off or close-in. Close-in detection is often preferred for mine detection as the effect of the landmine on its surroundings is often minimal. If it were otherwise, the mine detection problem would not be such a concern. However, remote detection capability is required for any Level I and II survey technique, as the time and cost involved with a close-in inspection of vast areas is too high.

Of the close-in detection methods, we can further classify techniques based on the properties used to locate the landmine in the soil matrix and distinguish it from other objects. One can positively detect the bulk explosive

in the landmine, or depend on secondary properties such as electromagnetic signature or thermal contrast. As one can imagine, the removal of a stone or metal shard is as mentally taxing and emotionally draining for the deminer as is the removal of a more deadly mine. Therefore, minimizing the number of objects to be removed is paramount in maintaining the deminer's focus and safety. The most promising way of doing this is to positively identify the explosive content of the mine.

Many developing techniques are making an attempt to sense the explosive compounds and vapours which are leached from the mine and are present in the surrounding soil. Some novel methods attempt to analyse the vapours samples for the chemical compounds associated with buried explosives. These electronic 'Dog's Nose' devices, as they are known, have had limited success to date detecting the explosive vapour in air. Indeed, based on work done by Defence R&D Canada – Valcartier and US Cold Region Research and Engineering Laboratory (CRREL), it is likely that techniques based on this approach will suffer a limited probability of detection due to the variability of the already low vapour concentrations.[3,4]

1.3. Bacterial Sensing of Soil

The concentrations of explosive-derived compounds locked in the soil itself, however, are much higher. Short of assaying soil samples, one direct method of sensing the soil is through the use of explosive-sensitive bacteria.[5,6] This technique is promising in that it can be employed as a large area, remote sensing method, while also providing for the direct detection of explosive compounds. The bacteria would be spread over an area from an airborne platform and would subsequently become luminescent in the presence of explosive compounds in the surface soil. The area could then be passively, or actively, observed with an airborne hyperspectral camera.

The problems identified with this system have limited its development: The bacteria need to be activated on-site in large quantities, and sophisticated large-area dissemination equipment needs to be available. More importantly, once spread over an area of interest the bacteria sense only a thin layer of soil near the surface, where explosive concentrations may be quite low. Bacteria are also subject to environmental conditions which may hinder their growth, such as undesirable temperatures and UV light. Without an ability to gauge the survival probability of the deployed bacteria it is difficult to assess their effectiveness, leading to a greater risk of false negative measurements. Finally, sensing the bacterial response has required additional access to airborne platforms and special camera systems. While the latter point is not necessarily a hindrance, it may be beneficial to have alternative sensing options available.

1.4. Transgenic Plants for Sensing of Explosive Compounds

Following the demonstration of the bacteria-based approach, the authors began a research programme in the fall of 2001 to investigate the general concept of the use of biological systems as chemical sensors. The Canadian Centre for Mine Action Technologies[7] (CCMAT) began funding the project in the spring of 2003.

In the proposed system, a suitable benign plant or bacteria species local to an area of interest would quickly be identified through purposefully designed genetic tests. The species would then be genetically modified (GM) to include an expressed response in the presence of specified chemical compounds. Observed changes in the structure, appearance, or other physical characteristic, would indicate the presence of hazardous materials. Sterility could be added to mitigate concerns associated with GM organisms. It is difficult to foresee the limitations of this new type of sensor, but biological systems are known to be exquisitely sensitive and it is anticipated that this approach will be broadly applied in the detection of numerous hazardous compounds, from traditional chemical agents and explosives, to novel and emerging threats such as toxic industrial chemicals.

As an illustration of the more general concept of GM chemical biosensors, we chose to address the humanitarian demining problem. We proposed to design a proof-of-concept process by which plants could be genetically modified to be sensitive to the chemical compounds known to permeate the soil around emplaced landmines. The plant's genes would also be designed to include a reporting mechanism, signalling the presence of these compounds through a change in the plant's structure, appearance (such as flower colour) or some other physical characteristic. The project also involves investigating the potential to develop genetic tests which could be employed in the field to efficiently identify candidate plants local to an area of interest, suitable for rapid modification to sense any number of desired compounds. Such a system would provide a mass-deployable, wide area, explosive sensing capability that would find applications in a humanitarian and military scenarios.

This project builds on the results of the previous bacteriological approach to landmine detection, while addressing some of the limiting problems of that system. The robustness of the plants would guarantee that they would survive the transportation and dissemination process in large numbers. By selecting local plant species, one would have detailed knowledge of their growth properties and could assess the quality of the survey data. Further, plant roots permeate the soil structure and therefore can sample concentrations of target compounds directly around the mine. This would result in an environmental amplification of the active compounds, providing a detection method where low concentrations of target chemicals could cause a large change in plant structure, function, or appearance.

After development costs, this low technology system would be inexpensive and very scalable, even in countries with minimal infrastructure. The specific plant selection and development could be sponsored by an international demining agency, and the starter seed delivered to the afflicted area for cultivation. Low cost production facilities could then be set-up and the required seed quantities produced by local concerns for delivery throughout the area.

1.5. Systems approach for plant-based TNT detection

TNT and its closely-related derivatives are xenobiotics that are not naturally found in the environment. Therefore, there is no reason to expect that plants might have evolved mechanisms to sense the presence of TNT or its derivatives. Even if natural plants were able to sense TNT in the soil, there is no reason to expect that they would respond to that stimulus by altering the appearance of their above-ground structures in a way that might be obvious to a human observer. If we are to use plants as biosensors, it is therefore necessary to introduce into the plants, mechanisms for both sensing and reporting TNT. Moreover, because sensing and reporting occur in different organs of the plant, it is also necessary to introduce a system for transmitting specific signals between these organs. We will explain the existing and proposed strategies for plant-based TNT detection in the context of these three modules: a root-based TNT sensor, a shoot-based reporter, and a root-to-shoot signal transmission system

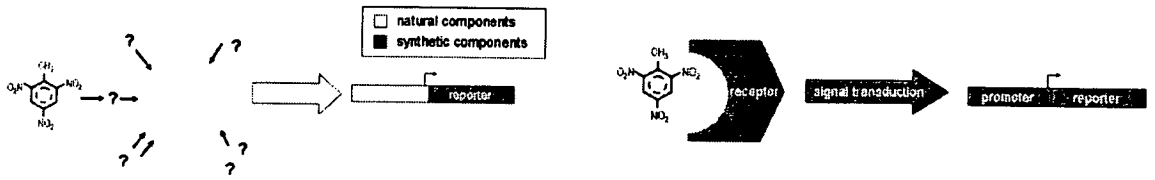
2. EVALUATION OF EXISTING TECHNOLOGIES

2.1. Sensor systems

To create TNT-sensing plants, we can exploit elements of their natural patterns of perception of specific molecules. In one strategy, a researcher can engineer a completely synthetic receptor that binds to TNT specifically, then initiates a signal transduction cascade and ultimately activates a gene of our choosing. This "synthetic receptor" is the strategy used by Defence Advanced Research Agency (DARPA) investigators including Dr. Homme Hellinga (Duke University) as part of the Biological Input-Output Systems (BIOS) project, and is expected to produce the most specific responses to TNT, but is also expected to present the greatest number of technical challenges.[8] The second strategy, used by Aresa (Copenhagen, Denmark), is to simply substitute a gene of their choosing for the genes that are normally activated by a plant in the presence of TNT. We refer to this as the "promoter fusion" strategy, since the switches that activate or deactivate genes are called promoters. Since plants do not seem to have specific receptors for TNT, the response of a normal, unmodified plant to TNT probably overlaps with the plant response to natural compounds with similar properties including nitrogenous compounds and neutral aromatic compounds. Thus, the promoter fusion strategy is expected to be inherently less specific than the synthetic receptor strategy. Each of these strategies, and their specific implementations and limitations, is outlined below.

2.2. Promoter Fusion

A given gene can generally be divided into two segments. One segment of the DNA is the "coding-region" which ultimately encodes the protein sequence. The other segment of a gene's DNA is called the promoter. This promoter acts something like a switch that determines when and where the coding region will be transcribed into RNA. Promoters and coding-regions are modular, meaning that the promoters and coding-regions of different genes can be recombined, and the expression pattern of the promoter will be conserved. For example, if gene A is normally transcribed in flowers, and gene B is normally transcribed in roots, the promoter of gene A can be attached to the coding region of gene B, resulting in ectopic transcription of gene B in flowers. In the context of



(a) Natural promotor fusion

(b) Synthetic receptor

Figure 1. Although the natural promoter strategy (a) is likely to be limited in specificity, it is conceptually simpler than any other strategy. Its specificity may be increased by the use of multi-partite reporter systems each controlled by a different inducible promoter, which is one application of the promoters we are characterizing through this project. We are also collaborating with US-DARPA funded researchers to implement a practical synthetic receptor strategy (b) with a variety of reporters. It is hoped that these strategies, alone or in combination, will contribute to practical TNT biosensors.

this paper, if Y is a gene whose transcription is activated in the presence of TNT, and Z is a gene that produces a red pigment, then replacing the native promoter of gene Z with the promoter from gene Y should produce a novel gene that causes red pigment to accumulate when stimulated by TNT. Such combinations are the basis of the Aresa strategy for TNT detection.

According to Aresa's patent,[9] the company tested the promoters of at least four different *Arabidopsis thaliana* genes as the basis of potential TNT-sensing systems. The promoters were all derived from genes involved in basic nitrogen metabolism, which are expected to be induced in the presence of their substrates (i.e. nitrate, or nitrite): Nitrate Reductase 1 (Nr-1), Nitrate Reductase 2 (Nr-2), Nitrite Reductase (Nii), and High-affinity nitrate transporter ACH2 (Ntr-2). The rationale for the selection of these promoters is that nitrate and nitrite would be expected to be present in the vicinity of a landmine, since they are among the breakdown products of TNT. However nitrate and, to a lesser extent, nitrites are also naturally abundant in soil. For example, most of the protein in a plant such as *Arabidopsis* is derived from nitrogen in the soil (mostly nitrate). This is a potentially critical limitation to the Aresa promoter fusion sensor system; as presently described, it cannot be expected to distinguish signal (i.e. TNT derived nitrate or nitrite) from background (i.e. naturally occurrence of these compounds). Final assessment of this specificity issue awaits publication of quantitative data by Aresa.

Although the general biotechnological approach used by Aresa to produce the landmine detecting plants is legitimate, we feel that their specific implementation of this approach has important shortcomings. The Aresa sensor system could be improved using enhancements, such as selection of xenobiotic-specific promoters, which we discuss in more detail elsewhere.[10] However, the success of other researchers using engineered receptors of TNT has suggested a much more specific sensor system is obtainable.

2.3. Synthetic Receptor

Biology provides thousands of examples of the ability to distinguish between closely related molecules in the environment; even a human nose can discriminate the scent of lemons from the scent of mint, despite their similar chemical structure. The basis of the remarkable specificity of natural biological sensors is the presence of a specific receptor for each of the compounds that a cell might want to detect.

Plant cells are naturally surrounded by thousands of species of small molecules such as nutrients, hormones, and toxins. In order to detect and respond to these molecules, plants have evolved special proteins on the surfaces of their cells, which bind to a specific molecule from the environment, and trigger a series of biochemical events that often leads to a particular gene being activated or deactivated. For example, a plant cell might have on its surface a protein receptor that specifically recognizes a toxin from a particular fungal pathogen. In response to binding this pathogen, this receptor protein activates a series of other proteins that ultimately switch on a gene that produces enzymes to degrade the cell wall of that fungus. Such receptors are the basis of the DARPA

strategy for TNT detection. Often the relationship between a receptor and the compound it detects (i.e. its ligand) is described as a "lock and key"; only ligands with the appropriate chemical structure will fit in the the receptor and thereby activate it.

A TNT-specific receptor would be an ideal component around which to build a landmine-sensing plant. However, such a receptor would not be expected to exist in nature (and indeed one has never been found), thus it would have to be designed *de novo*. The relationship between protein structure and function in general is still poorly understood. Therefore, it is not currently possible to predict a particular protein sequence that would bind specifically to TNT, and in doing so activate a biochemical response. A more practical approach is to take an existing receptor, such as periplasmic binding proteins (PBP), that binds a molecule that is similar in size or shape to TNT, and then modify the binding site of the existing receptor, until it becomes specific for TNT. This is still not a trivial task, but Dr. Hellinga, a member of the DARPA BIOS program,[8] succeeded in producing a synthetic receptor for TNT that exceeded expectations.

Producing a synthetic receptor to bind TNT is a remarkable accomplishment, but it is only part of the solution to the sensor problem. If a receptor simply binds TNT without initiating a biological response, it cannot be used as a sensor. Receptors, to be effective, must act as switches for biological responses.

2.4. Reporter System

Natural chemical receptors, upon binding to their ligand, specifically activate other proteins. This ultimately leads to the activation of a promoter for a specific gene. This is called a *signal transduction pathway*, and typically involves a cascade of phosphorylation, i.e., an activation message, in the form of a phosphate group, being passed from protein to protein. Two notable attempts to tie the signal transduction pathway to an observable physiological change have been reported; one using Green Fluorescent Protein (GFP) genes spliced from jellyfish,[11] and Aresa's approach, which triggers a change in the plant's leaf colour. While these are interesting, we feel any fielded systems based on these approaches may be prone to false-alarms from naturally occuring interference.[10] A more promising route may be the engineered signal transduction being pursued through the DARPA BIOS program.

In order to translate the binding of TNT by the synthetic PBP receptor into a biological response, Dr. Hellinga and colleagues combined parts of related signal transduction pathways into a single chimeric pathway that resulted in activation of a reporter gene of their choice, following the binding of TNT to its synthetic receptor. Bacteria containing the synthetic receptor, the modified signal transduction pathway, and a reporter gene that produced a fluorescent protein could be used as highly specific and sensitive bacterial biosensors for TNT. An obvious next challenge will be to introduce a version of this engineered receptor system into plants.

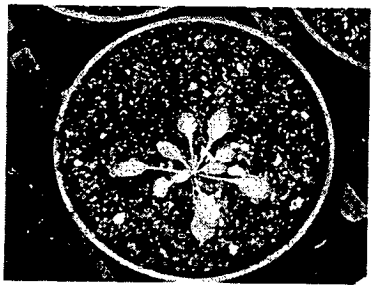
3. ORIGINAL TECHNOLOGY

To address some of the key deficiencies in existing strategies for producing TNT-detecting plants, we completed several research projects. First, we expanded the repertoire of genes that might be induced in the vicinity of landmines by generating full-genome expression profiles for the TNT-derived compounds: 2,4-DNT, 2,6-DNT, and 1,3-DNB. In many field situations, these compounds are more abundant than TNT in the vicinity of a mine. Second, we tested the inducibility and specificity of selected TNT-responsive gene promoters to identify regulatory regions that might provide higher specificity to a biosensor system. We demonstrated that specific plant genes could be induced by compounds such as TNT derivatives, although the system is not yet practical as a landmine detector due to false-negative signals that may be reduced with further refinement. Third, we investigated solutions to the problem of root-to-shoot signal transduction, which is a problem that has been largely ignored by other research groups.

3.1. Explosives-inducible Gene Discovery

Although the results of hundreds of *Arabidopsis* microarray experiments are available in the public domain,[12] none of these experiments have analysed the plant response to those volatiles known to be most abundant in the soils surrounding buried landmines:[3] 2,4-DNT, 2,4,6-TNT, 2-Amino DNT and 4-Amino-DNT. One microarray-like technology, Serial Analysis of Gene Expression (SAGE), has profiled the *Arabidopsis* responses to 2,4,6-TNT,[13]

(a) *Arabidopsis* seedlings germinating on sterile nutrient medium



(b) *Arabidopsis* plant that has been transformed with a putatively TNT-responsive gene

(c) Mature *Arabidopsis*, about eight weeks old

Figure 2. Stages in *Arabidopsis* growth cycle. Fig. (a) shows *Arabidopsis* seedlings germinating on sterile nutrient medium, used to test dose responses in transgenic seedlings. Fig. (b) shows one of the test plants transformed with a putatively TNT-responsive gene, seeds from which were collected to test for 2,4-DNT, 2,6-DNT, and 1,3-DNB inducibility. Fig. (c) shows a mature *Arabidopsis* plants, at about eight weeks old. Although from an unrelated project, one can see why this spindley plant would not make an ideal TNT sensor.

but further research is needed to identify genes that are responsive to the broader range of explosives-associated compounds, and provide insight into the metabolism of such compounds in plants.

Since a typical gene (encoded by DNA) must be converted into an RNA transcript before it can ultimately have any biological activity as a protein, a convenient method of identifying gene activity is by measuring the abundance of its corresponding RNA transcripts. The transcript abundance is, in turn, controlled in large part by the gene's promoter. Thus, if our ultimate objective is the production of explosive-responsive genetic circuits, then an effecient means of identifying explosive-responsive promoters is by treating plants with TNT or its derivatives and then using a technique such as DNA microarray analysis to identify genes whose transcription is induced by the treatment. The principles of DNA microarray analysis is detailed in several reviews.[14]

To expand the public repetoire of genes that are transcriptionally induced by chemicals found in the vicinity of landmines, we treated hydroponic-grown *Arabidopsis* plants separately with 2,4-DNT, 2,6-DNT, or 1,3-DNB. Plants were grown for 14 days in 1/4 X MS medium, and treated by replacing the 1/4 X MS solution with either 15 mg/L 1,3-DNB, or 15 mg/L 2,4-DNT, or 15 mg/L 2,6-DNT in 0.177% DMSO 1/4 X MS, or 0.177% DMSO in 1/4 X MS alone as a mock-treated control. After 24 hours, roots were removed, frozen immediately in liquid nitrogen, and RNA was extracted using a Qiagen RNEasy Mini kit.

For analysis, we used whole-transcriptome oligonucleotide microarrays from *Arabidopsis*, which were produced at the University of Alberta using an Omnigrid 100 microarrayer and *Arabidopsis* array-ready oligonucleotides from Qiagen-Operon. RNA was reverse transcribed and labeled in pairwise hybridizations using the Genisphere Array 900 Cy3/Cy5 system. Each hybridization included an untreated control and a treated sample, and each RNA aliquot was hybridized to two different slides in a "dye-reversal" design. Spot intensity values were collected using an Applied Precision ArrayWorx scanner. Raw TIFF images were converted to signal intensity values, then normalized and further analyzed with the TIGR TM4 software suite (www.tm4.org), using the Loess normalization algorithm and Student's t-test for statistical significance.

Table 1 shows a selection of genes from *Arabidopsis* that were induced or repressed at least 1.5 fold (0.58_{log2} scale) following hydroponic treatment with one of three TNT derivatives. The study found a total of 74 genes that are candidates for further characterization as components of an explosives-sentinel system.

3.2. Promoter Analysis

We conducted promoter analyses using reporter gene fusions of the beta-glucuronidase reporter (GUS) to upstream genetic fragments of genes that had been previously described as being TNT-responsive in the public

AGI ID	*Annotation*	$\log_2(\frac{Treated}{Control})$	*Raw p value (t-test)*	*Treatment*
At3g10720	pectinesterase, putative	1.74	0.00616	2,4-DNT
At3g62040	haloacid dehalogenase-related hydrolase family	1.66	0.03989	2,4-DNT
A013098_01	Arabidopsis thaliana DNA chromosome 4, BAC clone T5J17 (ESSA project)	1.64	0.00859	1,3-DNB
At4g07820	pathogenesis-related protein, putative	1.6	0.00361	2,4-DNT
At1g49860	glutathione transferase, putative	1.51	0.00129	2,4-DNT
At3g01190	peroxidase, putative	1.48	0.03127	2,4-DNT
A013934_01	Arabidopsis thaliana DNA chromosome 4, BAC clone F25I24 (ESSA project)	1.27	0.01492	2,6-DNT
At5g58820	subtilisin-like serine protease	1.16	0.04143	2,6-DNT
At3g08030	expressed protein	1.11	7.00E-04	2,4-DNT
At2g22170	expressed protein	1.05	0.0408	2,4-DNT
At2g45220	pectinesterase family	-1.45	0.02487	2,4-DNT
At1g80840	WRKY family transcription factor	-1.48	0.014	2,4-DNT
At4g37710	expressed protein	-1.48	0.0263	2,4-DNT
At1g78380	glutathione transferase, putative	-1.52	0.04951	2,6-DNT
At3g47340	glutamine-dependent asparagine synthetase	-1.54	0.01814	2,4-DNT
At4g04840	expressed protein	-1.55	0.02115	2,4-DNT
At4g21840	expressed protein	-1.68	0.02905	2,4-DNT
At4g21850	expressed protein	-1.75	0.02217	2,4-DNT
At3g58210	expressed protein	-1.76	0.01661	2,4-DNT
At2g18210	expressed protein	-1.78	0.03502	2,4-DNT

Table 1. Ten most induced and ten most repressed transcripts in four *Arabidopsis* replicate hybridizations (two dye-reversals for each of two biological replicates) for each of three TNT derivatives: 2,4-DNT, 2,6-DNT, and 1,3-DNB. If the p-value associated with the t-test is small (e.g. < 0.05), there is evidence that the means are significantly different at the significance level reported by the p-value.

scientific literature.[13] We were successful in identifying an *Arabidopsis* gene (At4g01870 or TolB) whose transcript abundance we showed to be induced up to 28-fold by treatment with 2,4-DNT, 2,6-DNT or 1,3-DNB. Transgenic plants with an upstream (500bp) fragment from this intronless gene fused to the GUS reporter showed strong inducibility in response to treatment with 2,4-DNT, 2,6-DNT or 1,3-DNB (Fig. 3). However, the inducibility was masked at many stages of development by background expression of the reporter gene from this 500bp fragment. Closer analysis of the genome in the vicinity of this gene indicates that approximately 200bp or less of upstream DNA is required for proper expression; therefore elimination of approximately 300bp of extra promoter DNA present in our prototype construct would likely reduce background expression while retaining the explosives-inducibility of this promoter.

Our results demonstrate the feasibility of isolating natural DNA sequences that are inducible by TNT and its derivatives, as depicted in Fig. 1(a). However, natural sensors are expected to lack specificity for artificial compounds. Indeed, an analysis of public *Arabidopsis* microarray expression databases[15] shows that induction of the gene used in our promoter analysis (At4g01870) is also highly induced by naturally occurring stresses including salinity, and chilling, and the endogenous plant hormone salicylic acid. Furthermore, these databases indicate that nitrate reductases as described in Aresa's patent (At1g37130, At1g77760), are inducible by environmental factors such as light quality and bacterial pathogens. Therefore, promoter fusions using a single promoter are not likely to provide sufficient specificity as sensors of explosives in field applications.

The limited specificity inherent in natural promoters may be overcome by combining multiple independent reporters through a biological implementation of a logical AND gate. For example, starting with a comprehensive list of explosive-induced genes (such as we have generated by microarray analysis), a subset of genes with

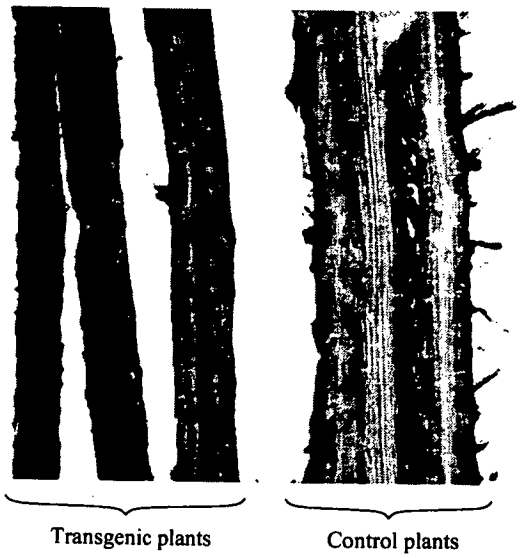


Figure 3. Novel transgenic *Arabidopsis thaliana* plants responsive to the TNT-derivative 1,3-dinitrobenze were produced. In the left half of the photo, the core of the roots from 1,3-DNB treated plants (15mg/L) appear dark; in the right half of the image, roots from mock treated (control) plants of the same line appear much lighter in colour. (original image edited for clarity)

minimally-overlapping responses to natural phenomena could be selected by comparison to public microarray expression resources.[15] Selected promoters, could each be used to direct transcription of a different necessary component of a reporter system, such as a series of genes in a pigment biosynthetic pathway. In this way, the human-readable reporter system would be activated only when all of the promoters were stimulated by TNT, and not by any one set of natural environmental factors,

3.3. Root-to-Shoot Signalling

Both of the sensor-reporter systems described in previous sections of this report (i.e. the Aresa strategy and the DARPA strategy) are cell-autonomous, that is, they are designed so that perception of the TNT signal and induction of a visible reporter must occur within the same cell. Thus, in application, some TNT (or NO_2 in the case of Aresa) would need to be transported from the soil along with water, through the roots, and up the shoot to each cell of the leaves, where the synthetic or endogenous receptors can induce reporter genes to cause a change in pigment. The low solubility of TNT in water is likely to limit the efficiency of this transport process, as is the possible requirement for TNT to cross several membranes in entering and exiting the transpiration.

Whatever the efficiency of TNT transport from root to shoot may be, it is certain to attenuate the signal and therefore reduce the overall sensitivity of the system. To maximize sensitivity, it will therefore be necessary to spatially separate the sensor system and the reporter system in the plant, and connect these two components with a third component for long-distance signal transduction.

Long-distance signalling between different tissues in plants has long been known to involve the action of plant hormones, such as *auxin* and *cytokinin*.[16,17] More recently, other types of molecules have also been implicated in signalling between roots and shoots, including the control of shoot development based on the amount of nitrogen or other nutrients in the soil.[18 20] Further, it is planned to consider importing genes from animals such as *glucocorticoid, oxytocin* or *vanilloid* receptors. One could extend this concept even further: Volatile messenger compounds could be released into the air,[21] triggering the signalling response in neighbouring plants of the same species. This inter-plant communication would further amplify the overall response to the initially sensed explosive compounds resulting in an increased probability of detection.

(a) Apex of one seedling grafted to basal region of another

(b) Epifluorescent illumination of graft region

(c) Limited GFP signature observed at a distance from the graft region

Figure 4. The left panel shows a seedling in which the apex (cotyledons, leaves and upper hypocotyl) of a GFP expressing transgenic seedling has been grafted to the basal region (lower hypocotyls and roots) of a non-transgenic seedling. The Tygon collar surrounding the graft junction is visible in the center of the image. The central panel shows the same view under epifluorescent illumination. The graft junction is visible as a distinct border between the fluorescent and non-fluorescent cells. The right pane shows the maximum GFP signal observed at a distance from a graft junction in non-transgenic tissue; this root tissue shows limited GFP expression.

There are two long-distance transport systems within land plants: xylem and phloem. Xylem transports water and dissolved mineral from the soil upwards, while phloem transports the products of photosynthesis from sources such as leaves, to sinks such as storage organs and actively growing tissues. Either one of these tissues may serve as a target for an artificial long-distance signalling to link, TNT perception by roots with a human-readable reporter system in shoots. Xylem is thought to be the natural route of trafficking of some hormones (such as cytokinin and abscisic acid), and phloem has recently been shown to transport informational complex molecules such as nucleic acids through the plant.

To model the potential for transmission of artificial substances from roots to shoots, we took advantage of existing transgenic Arabidopsis plants that expressed green fluorescent protein (GFP) within regions of their vascular tissue as enhancer traps (courtesy J. Haseloff, Cambridge, UK). We grafted roots from ten different lines of these transgenic plants to shoots of non-transgenic plants to see whether we could observe movement of GFP into the shoot. We were able to detect only limited movement of the protein across the graft, and only from shoots to roots. We were not able to detect GFP movement from root to shoot with this model system, due in part to the localization of the GFP to vascular tissues with limited shootward flux, and due to limited sensitivity of detection of GFP in the shoots. We conclude that a large polypeptide signal such as GFP is likely not to be the best type of molecule for root to shoot signalling, despite other recent reports about phloem-borne protein signals.

4. CONCLUSION

It is our objective to leverage advances in genetic engineering and biotechnology to design new biological-based explosives sensors. After development costs, the low technology system proposed in this paper will be inexpensive and very scalable, even in countries that have very basic infrastructure. The specific plant selection and development could be sponsored by an international demining agency and the starter seed delivered to the afflicted area for cultivation. Low cost production facilities could then be set-up and the required seed quantities produced by local concerns for delivery throughout the area. Using this system to accurately demarcate the

boundaries of mine affected areas, truly contaminated areas can be identified for clearing, greatly accelerating the return of land to productive use.

We have identified the following characteristics of a practical plant-based TNT sensor:

- The system must be responsive to a wide spectrum of TNT-derivatives that may accumulate in the vicinity of a landmine (i.e. 2,4-ADNT, 2,6-DNT, etc. in addition to TNT). Responsiveness to additional explosives, such as RDX, would be beneficial.
- The system must be specific for explosive-derived compounds, and not other naturally occurring nitrogenous compounds (e.g. NO_2).
- The system must produce an unambiguous, human-readable display that is distinct from natural responses, and which does not inhibit the ecological competitiveness of the plant. Existing strategies rely on induction of stress-responsive anthocyanin pigments, or bleaching of leaves resulting in immediate plant death.
- The system must be transferable to a variety of plants suited to different environments, including grasses, woody, and herbaceous species.
- The system must be implemented in plants with sufficient stature to be easily visible at distance, and in plants that will not be obscured by larger neighbours. The current implementations of sensor plants have been produced in the diminutive weed, *Arabidopsis*.
- Transgenic plants must include safeguards to prevent adverse effects on the natural environment.
- The system must have sufficient sensitivity to be able to detect TNT and its derivatives in concentrations expected to be present in soil. We envision a number of simple adaptations for the amplification and transduction of these signals.

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