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Monitoring the presence and expression of transgenes in living plants

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A range of bio- and nanotechnologies have been developed that could be adapted towards monitoring the presence and expression of transgenes, in real time and in the field, in plants of agronomic and ecological importance. Transgene escape from crop hosts to wild relatives or landraces is one example in which monitoring might be useful, depending on the ecological impact of the transgene. In addition, there might be non-biosafety-related practical reasons to monitor transgene expression. Transgenes can be tagged with green fluorescent protein and imaged or measured using instruments designed to detect fluorescence signals on the plant. In addition, nanotechnologies using aptamers, quantum dots and molecular beacons are rapidly evolving and could also be used for *post hoc* (after transformation) *in vivo* monitoring. These nanotechnologies have the benefit of being useful on a *post hoc* basis.

Why monitor transgenes?

In many cases, it is desirable to know about the expression of transgenes in plants growing in the field. Regulators, consumer groups and farmers, among others, would like access to real-time data on the presence and expression of transgenes in crops and volunteers, and in wild relatives if transgenes are able to introgress into their genomes. This would apply not only to plant incorporated pesticides (PIPs) and other transgenically produced proteins for agronomic uses (e.g. herbicide tolerance and disease resistance) but also to recombinant plant-produced pharmaceutical and industrial proteins. Although much emphasis has been placed on postharvest monitoring (e.g. food on supermarket shelves), there are compelling reasons for preharvest monitoring of transgenes in living plants by, for example, performing on-the-plant detection in the field and possibly using an identity tracking system.

It is desirable to assess the expression of a PIP in the field for managing *Bt* resistance (i.e. insect resistance to crops genetically modified to express δ-endotoxins of *Bacillus thuringiensis*) as well as assuring agronomic performance. People would like to measure transgene expression quantities and compare the real measurement with the expected value in agricultural fields. Gene expression is known to be perturbed by environmental conditions [1,2]; if a PIP transgene were underexpressed, not only might production of the regulatory-mandated high dose of PIP be unrealized but also certain target and sub-target insects might not be killed upon ingestion of plant tissue. Therefore, the assumptions for *Bt* resistance management strategies might be violated, leading to unwanted outcomes, namely the premature evolution of *Bt* resistance in insects [3,4]. In the case of proteins intended for pharmaceutical and industrial uses, growers could desire knowledge about protein production in living plants before harvest to maximize recombinant protein yield. Regulators could expect benefits from readily estimating or measuring transgene expression in certain circumstances before harvest.

Transgene escape from crops to wild relatives is a recognized risk inherent to certain plant species [5]. In addition, transgenic crop volunteers can also persist in subsequent years in fields containing the same or different species. It is known that certain crops such as sorghum, canola (*Brassica napus*), sugar beet, alfalfa and sunflower easily hybridize with wild relatives [5]. Repeated backcrossing is known to occur in these species and transgenes might be introgressed into wild relatives [5]. Thus, it is foreseeable that transgenes could be harbored in unintended wild and weedy hosts. In addition, the adventitious presence of transgenes in crop landraces has also been a concern, which is another reason to monitor for early transgene escape. A herbicide tolerance transgene has already escaped from cultivation in Canada and its discovery was fortuitous [6]. Transgene stacking has also been documented in Canada [7,8]. Resistance management plans could be subverted if a PIP was hybridized into a nontransgenic refuge. Such a case has been reported in corn, in which a portion of kernels in non-*Bt* plots of corn were transgenic, presumably because of hybridization with *Bt* corn in adjacent *Bt* plots [9]. For several reasons, plant breeders, seed producers, and ultimately farmers, could benefit from a living plant assay for the adventitious presence of transgenes, for example, seed certification and intellectual property protection. Once again, the most desirable systematic assay would occur in real time and on-the-plant for monitoring transgene presence and expression in an era of post-commercialization. Although many scientists (including myself) and regulators make the case that the

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currently commercialized transgenic plants are safe [10], one could envisage innumerable situations where the implementation of new technology for monitoring in living plants could be desirable.

Technology for monitoring transgenes in living plants

Transgene and transgene product detection technology continues to advance. Some postharvest techniques such as PCR and ELISA (for the detection of specific nucleic acid sequences and proteins, respectively) can be easily adapted for living plant applications. However, these require sampling and sometimes expensive laboratory testing. New test strips for *Bt* and herbicide resistance proteins are often easy to use but give qualitative, not quantitative, data on transgene expression; there are tradeoffs between ease of use and accuracy, which can lead sometimes to ambiguous data interpretation. For example, sophisticated PCR techniques allowed researchers at the University of California-Berkeley (USA) to assay for transgene promoters in Mexican landrace corn [11], but the sensitivity and complications of the assays led to the unsupported conclusion that transgenes were present and moving around within genomes of landrace corn. The data and methods were highly criticized and the editor of *Nature* eventually distanced the journal from this research [12].

This article reviews various technologies for transgene monitoring in living plants that are based on optical or fluorescent markers in various stages of development – a mix of what is and what could be. Interestingly, there are ongoing parallel discussions about noninvasive monitoring of human gene therapy using many of the same tools [13]. One technology is based on transgenic synthesis of a marker molecule such as the green fluorescent protein (GFP). Such a platform could be used for the real-time monitoring of transgene movement and expression on a large geographic scale [14–16]. Essentially, most of the components are already developed for this application. Other technologies are transgene-independent and might be applied on a *post hoc* basis (after a transformation event has been created) using nanotechnology as well as custom identifiers that could be used to track individual transgenic events. The goal of this paper is to identify existing technological strengths and, thus, indicate where additional data and development are needed for potential implementation (Table 1). It is not designed as a treatise on regulatory needs or the economics of monitoring schemes. I will refrain from prescribing technologies to heal perceived risks. One reason for this is that at this early stage of technological development many cures might seem worse than the disease.

Monitoring transgene presence and expression with GFP

GFP from the jellyfish *Aequorea victoria* is well characterized, has been modified for increased expression and optical properties, and has been used in transgenic plants for nearly 10 years. The protein has the unique ability to transduce UV or blue light to green light (507 nm). Therefore, one can see GFP fluorescence in transgenic plants merely by shining a bright ultraviolet light on leaves in an otherwise darkened location [15] (Figure 1). For instance, the variant mGFP5 (discussed in [15,17]) has undergone site-directed mutagenesis to enable dual UV and blue light excitation and better heat tolerance. GFP does not require cofactors or substrates for fluorescence. Either constitutive or inducible expression of GFP can be detected in intact plant organs (Figure 1). There is no measurable cost to plants expressing GFP [18], and GFP has been shown to be non-toxic to rats when ingested in purified form or when synthesized in transgenic plants [19]. These extraordinary properties suggest that GFP is not toxic to mammals and is ecologically neutral, which makes it an attractive reporter gene in transgenic plants. Although GFP expression is not part of any potential commercial monitoring scheme to date (it has not been encoded for in any commercially released

Table 1. Intrinsic properties of various potential preharvest, *in vivo* transgene monitoring systems

System	Description	Target(s)	Strengths	Weaknesses
GFP tagging	Genetically encoded fluorescent marker	Protein	Most technologically advanced Monitoring large areas Amenable to remote sensing New fluorescent protein colors	Requires investment up front when producing transgenic plants GFP suboptimal wavelength
Aptamer–quantum dots	Nucleic acid aptamer tagged with a quantum dot	Protein, mRNA	Aptamers can be designed for many targets Quantum dots are bright	Requires stringent hybridization conditions to remove unbound probe Speculative Introduction into tissues
Molecular beacons	RNA tagged with fluorophore and quencher	mRNA	Shown to work *in vivo* Fluorescence quenched when not hybridized Specific to mRNA sequences	Sequence availability might be limited Somewhat speculative Introduction into tissues
Split GFP	Protein fusion with small half of GFP complemented by exogenously added large half	Protein	More appropriate tag than whole GFP	Requires investment up front when producing transgenic plants GFP suboptimal wavelength Introduction into tissues
DNA barcodes	Specific DNA sequences tagging transgenic events	DNA	Ready for implementation	Not *in vivo* No information about gene expression

Figure 1. Nontransgenic *Brassica napus* plant (left) and a plant constitutively expressing GFP (right) under UV light.

genome), it has been used for several years for transgenic monitoring in research settings [20,21].

There are two components to using GFP in monitoring: production and detection. On the production side, a *gfp* gene could be linked or fused to a gene of interest. The presence, therefore, of green fluorescence would indicate that the second transgene is present and expressed [15,18,20]; this system is also quantitative [21,22]. It has been shown specifically that genetic linkage of GFP to a *Bt* transgene is sufficient to monitor the expression and protection against herbivory in transgenic canola, crop× wild hybrids, and introgressed wild hybrids of canola [18,21,22]. Thus, it appears that the biotechnology is in place to use GFP to monitor putative transgene escape from a crop host to wild plants or weeds [23].

The expression patterns in plants have also been documented: when the *CaMV 35S* promoter is used to regulate transgene expression, more GFP fluoresces in new leaves, stems and apical meristems than in older leaves. Thus, by using this promoter we know what parts of the plant to monitor for expression [24]. However, promoter dynamics would need to be assessed on a case-by-case basis and fluorescence standards determined for commercial monitoring.

A priori, it would seem that a protein fusion would be better for using GFP to monitor the real protein of interest. Although it has been shown that hundreds of different proteins can either be fused to GFP on the N- or C-termini with no loss of function [25], the new fusion protein would need to be thoroughly characterized. An alternative to protein translational fusion would be transcriptional fusion using an internal ribosomal entry site (IRES) upstream of a second transcript for bicistronic expression [26].

The second component – the use of GFP as a detection device – has lagged behind the biotechnology slightly because this relies on instrumentation specifically designed to detect GFP in plant tissues. Whereas one can simply use a UV spotlight at night to find GFP in plants, more sophisticated techniques are needed for commercial applications. Aside from research-grade large spectrofluorometers that are restricted for laboratory usage, there are few off-the-shelf GFP detection technologies. One recent solution has been the development of a GFP Meter (OptiSciences, http://www.optisciences.com) that uses a fiber-optic-facilitated leaf clip to sample spectra on intact leaves. This portable spectrofluorometer performs in a similar way to laboratory-sited instruments and can take measurements every few seconds [27]. It is envisaged that such an instrument could not only be valuable for research but also for field surveys or mounted as a tractor-driven implement for GPS-interfaced monitoring of commercial fields. A standoff laser-induced fluorescence-imaging device has also been developed and tested in GFP transgenic plants. Like the GFP Meter, it can be used in the daylight but it can interrogate entire plant canopies from standoff mode (meters) (C.N. Stewart Jr *et al.*, unpublished). Unlike the GFP Meter, laser-based instruments are currently expensive and not commercially available. However, these kinds of instruments might be deployable in aircraft or towers to survey large geographical areas relatively quickly. Such applications might also be used in precision agriculture [15,16,28]. In addition, it is possible for pollen grains to be tagged transgenically with GFP to monitor short- and long-distance pollen flow [29]. Just as with constitutive expression, expression in pollen carries no cost to pollen fitness or germination [30]. Thus, there is strong evidence to support the belief that GFP is a neutral marker. Seeds have been tagged with GFP and other fluorescent proteins [31,32] and, therefore, it could be feasible to sort transgenic versus nontransgenic seeds automatically based on a fluorescence signal. Monitoring via GFP need not be limited to crops. Trees and other plants that actively synthesize proteins in accessible green tissue could also be monitored.

Although GFP has been widely used in plants and other transgenic organisms, it is not the only fluorescent protein available. Fluorescent proteins, mainly from nonbioluminescent Anthozoa, have various excitation and emission wavelengths, providing researchers with a rainbow of colors [33–37], which can also be expressed in plants [32]. Fluorescent proteins with additional colors are necessary for the multiple tagging of different traits (i.e. transgene stacking) and for labeling various plant species. Several of these 'new' fluorescent proteins from Anthozoa, such as the red fluorescent protein (RFP) from the coral *Discosoma* sp. (DsRed), have the disadvantage of not forming monomeric proteins when mature. The formation of tetramers or even dimers makes transgenic protein fusions problematic and can also cause solubility and aggregation problems. However, DsRed has been monomerized and its spectral signature altered [37,38]. In addition to increasing its brightness (once normalized for monomerization), the range of emission of DsRed-type RFP (583 nm) has been extended from yellow (537 nm) to far red (649 nm) [34,36]. RFPs are of special interest in plants because there is not much fluorescence in plants in the red wavelengths when excited by wavelengths of light in the green to red wavelengths, thus RFP should be easier to detect in intact green tissue than GFP is [32]. There are also several other non-GFP-like fluorescent

markers, such as phycobiliproteins and uroporphyinogen III methyltransferase, but these would not be as useful to monitor in plants, reviewed in [25]. However, research is underway to assess the possibilities of using various chromoproteins and even autocatalytic bioluminescence in transgenic plants (C.N. Stewart Jr *et al.*, unpublished). All these molecules and approaches could also be applied for monitoring purposes.

Nanotechnologies for monitoring transgenes

Nanotechnologies for sensing have been developed over the past few years. Although many have not been applied to detect transgenes or their products in plants or other organisms yet, the prospects are clear and early work is promising. For example, it should be possible to detect any transgenic transcript and protein, perhaps on the plant, by designing complementary nucleic acids (for mRNA), antibodies or DNA aptamers (for proteins) along with an appropriate fluorescent reporter molecule. Some of the most-promising technologies will be discussed, but none has been proven effective in living plant cells yet.

Aptamers and quantum dots

DNA aptamers, which are single-stranded pieces of DNA optimized for binding to other specific molecules, are among the most intriguing potential molecules for monitoring purposes because of the wide variety of potential targets. They have been designed to bind ligands such as specific inorganic ions, ATP, antibiotics and proteins using combinatorial approaches [39–42]. Aptamers can be covalently tagged with various small fluorescent molecules such as a bis-pyrenyl fluorophore [41] or quantum dots. Quantum dots, which are nanometer-sized semiconductor crystals of fluorescent metals (e.g. CdSe in the core and surrounded by a shell of CdS) are water soluble and have been used in several biological microscopic applications [43–45]. Fluorescent wavelengths vary as a result of materials and crystal sizing. High extinction coefficients and quantum yields (factors endowing brightness) along with the wide variety of quantum dots should enable increased use of quantum dots in sensing and monitoring applications. Instrumentation [27] designed to measure GFP in plants should be effective in these applications as well. Indeed, because it appears that aptamers can feasibly be designed to bind to practically anything, mating them with quantum dots seems like a useful match. One inherent difficulty with quantum dots is their continual fluorescence (bound or unbound). After binding to their target, there would need to be a stringent wash step to remove any non-bound aptamer–quantum dot probe from the detection area.

Molecular beacons

Molecular beacons are nucleic acid probes that contain a fluorescent molecule at one end and a quencher molecule at the other [46]. Comprising between 15 and 35 nucleotides, molecular beacons have quenched fluorescence when not bound to their targets owing to a hairpin and self-complimentary structure enabling the quencher to come into close proximity with the fluorophore. However, when bound to its complimentary nucleic acid, fluorescence is activated (Figure 2). Although molecular beacons have most often been used in *in vitro* techniques such as quantitative PCR, they seem to be particularly suited for *in vivo* gene expression analysis, particularly for monitoring specific mRNA pools in cells [47,48]. However, molecular beacons have also been used in conjunction with aptamers to report the presence of proteins [48]. Molecular beacons have successfully been used to detect rRNA in bacterial cells [49] and mRNA in mammalian [e.g. 50] and fly [51] cells. In each eukaryotic organism, the molecular beacons were microinjected into cells and cells were visualized under fluorescence microscopy. Several combinations of fluorophore and quencher pairs are available, yielding many available colors and fluorescence resonance energy transfer (FRET) pairs [48], in which excitation light is transduced from one fluorophore to another. Diana Bratu *et al.* [51] provide a particularly sophisticated demonstration of the power of molecular beacons to visualize mRNAs in living cells. They used an altered RNA backbone by substituting an oxymethyl group for the hydrogen atom at the second position of the ribose on each nucleotide, thereby conferring nuclease resistance. RNases could otherwise digest the molecular probes before hybridization. These researchers also used two different fluorophores designed to hybridize head-to-head along an mRNA strand, which yielded a FRET readout. In this case, FRET assures that RNA probes are interacting (if fluorescence is observed) as well as steering clear of cellular autofluorescence. As a result of target hybridization, molecular beacon fluorescence could be detected in 15 min and intracellular transport of mRNA could be visualized. Of all the technologies reviewed, this one seems most appropriate for *in vivo*, *post hoc* monitoring. Although it has not yet been used in plants, it has shown unequivocal results when used to probe living animal cells.

Split GFP tagging and detection

A hybrid technology combining the transgenic expression of GFP and nanotechnologies is embodied in split GFP tagging. Indraneel Ghosh *et al.* [52] determined that GFP

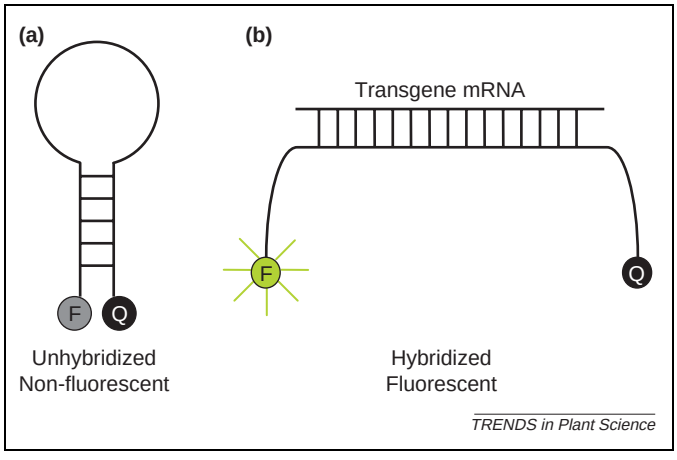

Figure 2. Representation of the mechanism of molecular beacons. **(a)** In their native state with no complementary target available, the fluorophore and quencher are in close proximity and hence no fluorescence is observed. **(b)** When the molecular beacon hybridizes with its specific transgene mRNA target, the fluorophore is sufficiently separated from its quencher to fluoresce.

could be expressed in two halves that are not fluorescent individually but form a normal green-fluorescent molecule when recombined. Split GFP was recently refined to be self-associating and soluble in living systems [53]. Stéphanie Cabantous *et al*. [53] produced a genetically encoded split-GFP fusion protein with several candidate bacteria proteins using a small (16 residues) portion of GFP. When the larger 'half' (214 residues) of GFP was synthesized in the host bacterial cells, fluorescence was recovered within hours because of the self-association of the GFP fragments. None of the target proteins used for fusions had their solubility or functions altered, probably because of the diminutive size of the linker and split GFP additions [53]. It is not a large step to imagine that specific recombinant proteins in plants could be fused with split GFP, with the larger GFP half added to cells exogenously. Like the other technologies, recovered GFP fluorescence could be monitored using existing instrumentation and, like molecular beacons, there would be no concern over non-specific fluorescence.

Keeping track of it all: DNA barcoding

As an increasing number of commercialized transgenic species and transgenes are released, it will become important to monitor the fate of specific transgenic events. One technology suggested has been called 'biobarcode' [54]. When plants are engineered, part of the transferred DNA would include a small piece of non-coding DNA with uniform recognition sites flanking the gene or DNA segment of interest (conserved for a single and universal PCR primer pair amplification) and an assigned variable region in between. Thus, PCR-automated sequencing could be used to determine the origin of transgene escape and could also be useful to answer questions that might occur about liability and potential intellectual property violations. Although this is not an on-the-plant monitoring technology, it should be considered as a component to any transgenic plant scheme for the sake of posterity.

Barriers to implementation

There are several impediments to implementing the above technologies for monitoring. The GFP-based technologies (GFP and split GFP), which are the most advanced, have several areas that need further research. First, in the same way that many antibiotic resistance markers have now been widely accepted for use and regarded as safe (although see Refs [55,56], which discuss underestimation of horizontal gene flow and possible exacerbated problems with antibiotic resistance), GFP and other fluorescent proteins need to be assessed for their universal safety. Although all the available evidence indicates that GFP is safe for the environment and in food and feed [15,19], more research is needed – particularly on the food safety side. To use GFP-monitoring, companies would have to create protein fusions or otherwise re-engineer plants for the purpose of monitoring, which would be costly. In addition, GFP detection instruments also require refinement. I doubt companies would approach re-engineering willingly, but only under regulatory coercion. It is doubtful that there is sufficient motivation to make such a paradigm shift yet, but there could be any number of future products bearing known risks that could benefit from an up-front engineering scheme to enable large-scale monitoring.

Nanotechnology-based detection systems, including aptamers, quantum dots and molecular beacons, require more basic and applied research to demonstrate that they can feasibly be used in living plants in field-based assays for transgene expression. Even though these could be used on only one plant at a time, virtually any sort of transgene or metabolite could be monitored and existing fluorescence detection devices could be adapted. Thus, such a *post hoc* scheme would be more easily implemented compared with GFP systems. However, it is not clear how nanomaterials could be introduced into plant tissues. Microinjection will not be effective at the tissue level but perhaps micro-projectile bombardment would be effective [57]. In any case, additional instrumentation for cellular introduction, hybridization and detection would need to be modified for this specific purpose and commercial monitoring.

DNA barcoding, like the GFP system, would require genetic engineers to introduce novel DNA into transgenic constructs. Unlike GFP, these DNA sequences would probably not require special regulatory approval.

Perspectives

Rudimentary systems for commercial transgene monitoring currently exist but require more research to implement. Paradoxically, the regulatory barriers are probably greater than the technical challenges (would regulators give *carte blanche* approval to any transgene like GFP?). The very regulatory schemes that mandate monitoring, such as those in Europe [58], require streamlined transgenic constructs, which might preclude using an encoded marker such as GFP, even if it is safe. GFP-monitoring has not been mandated by US regulatory officials because monitoring transgenes has not been viewed as absolutely necessary [59]. But perhaps the greatest hurdle is public acceptance [10]. Would the public accept GFP in food if it were used for large-scale transgene monitoring and proven to be safe? People who would probably accept GFP in food would probably also not care if GFP-monitoring were implemented. Those who are in favor of monitoring would probably also be opposed to GFP in food. However, it would be interesting to survey naturally fluorescent 'junk food' by testing it with a UV light as a baseline of 'naturally' glowing edibles. And perhaps the greatest question is how much post-commercial monitoring is really necessary and in what forms?

The US Environmental Protection Agency held a workshop on monitoring plant incorporated protectants in 2004, presumably to identify current and future products and applications potentially in need of monitoring (http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=84389). By identifying the ideal monitoring situation and publicizing it to interested parties, scientists and companies can devise technological solutions, and then hope for the best in the public acceptance arena. I think that future monitoring requirements will be greater for plant-produced pharmaceuticals than for transgenic crops for consumption. The scope of future monitoring will be commensurate with risk. Kent Bradford *et al*. [60] argue against 'one-size-fits-all' regulatory scrutiny based on

transgenesis alone and urge regulators to scale regulations according to risk categories. They also argue that certain innocuous transgenes such as GFP should not be regulated. If regulators took their recommendations to heart, then a GFP-based monitoring scheme could be useful. For the time being at least, the combination of ever-increasing progress in nanotechnologies and the increased palatability of *post hoc* versus genetically encoded GFP-based tools should result in rapid developments in technologies such as adapting molecular beacons for monitoring assays for transgenic plants.

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