

‘Fukusensor:’ a genetically engineered plant for reporting DNA damage in response to gamma radiation

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Received 25 January 2014;

revised 3 July 2014;

accepted 27 July 2014.

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Keywords: transgenic plants, radiation biosensor, green fluorescence protein, DNA repair mutant, *Arabidopsis thaliana*.

Summary

Transgenic plants can be designed to be ‘phytosensors’ for detection of environmental contaminants and pathogens. In this study, we describe the design and testing of a radiation phytosensor in the form of green fluorescence protein (GFP)-transgenic *Arabidopsis* plant utilizing a DNA repair deficiency mutant background as a host. Mutant lines of *Arabidopsis AtATM* (At3g48190), which are hypersensitive to gamma irradiation, were used to generate stable GFP transgenic plants in which a *gfp* gene was under the control of a strong constitutive CaMV 35S promoter. Mutant and nonmutant genetic background transgenic plants were treated with 0, 1, 5, 10 and 100 Gy radiation doses, respectively, using a Co-60 source. After 1 week, the GFP expression levels were drastically reduced in young leaves of mutant background plants (treated by 10 and 100 Gy), whereas there were scant visible differences in the fluorescence of the nonmutant background plants. These early results indicate that transgenic plants could serve in a relevant sensor system to report radiation dose and the biological effects to organisms in response to radionuclide contamination.

Introduction

Nuclear power is a sustainable energy source that reduces carbon emissions and provides a near-inexhaustible source of economical power. Currently, ~13%–14% of the world’s electricity is generated by nuclear power plants, with the US, France, and Japan together accounting for over 50% of the worldwide production. In the US, the licences of almost half of the reactors have been extended to 60 years, and plans to build another dozen are under serious consideration. The 2011 Fukushima Daiichi nuclear disaster prompted some countries to rethink their nuclear energy policies. Although research into safety improvements of nuclear power plants is continuing and new nuclear fusion technologies might be used in the future, there still is an ongoing debate about the role and scope of nuclear energy, particularly with regards to safety. Nuclear power plant accidents that result in radioactive contamination could impact the environment and human health. Full recovery could take several decades and people might wish to use biologically-relevant monitoring systems.

Although radiation dose and radionuclide contamination can be monitored and measured around polluted sites with emission instruments, the internal uptake dose and the biological effects to organisms is more complicated and difficult to measure. A whole organism-based sensing system would allow the evaluation of toxicity and potential mutagenicity of polluted sites and act as real-time biological sentinels. Plants are ethically more acceptable and aesthetically more appealing than animals as sensors of environmental radionuclide contamination (Kovalchuk and Kovalchuk, 2008). Plant sentinels, or ‘phytosensors’, have the

potential for tremendous utility as wide-area detectors for biosurveillance of contamination by chemical or biological agents including plant pathogens and abiotic environmental contaminants (Kovalchuk and Kovalchuk, 2008; Liu *et al.*, 2013; Mazarei *et al.*, 2008).

One of the direct biological effects of radiation to any organism is DNA damage (Ward, 1988). Indeed, DNA mutation rates in plants have been surveyed and plant systems have been used to monitor the genotoxicity of drinking water from the inhabited areas of the Ukraine affected by the Chernobyl accident (Kovalchuk *et al.*, 2000, 2003). The next logical step is the design of a transgenic plant-based phytosensor that is intentionally altered to be more sensitive to radiation exposure and to report the exposure. These plants could be deployed in a contained system at polluted, potentially dangerous sites and therefore amenable as unmanned sentinels.

Higher organisms, including plants, have a complicated DNA repair system (Friedberg, 2003). It has been shown that DNA repair deficiency mutants are more sensitive to radiation exposure (Garcia *et al.*, 2003). We posit that transgenic plants expressing a marker gene, for example, one encoding a fluorescent protein, which is harboured in a DNA repair defective mutant genetic background would have a much higher chance of accumulating mutations in the marker gene relative to engineered plants in a nonmutant background. We propose these mutant background transgenic plants would have decreased fluorescence under radiation stress because of attenuated DNA repair efficiency (Figure 1a). In the described study we developed an initial set of ‘Fukusensor’ transgenic plants to test the loss-of-function strategy using gamma radiation doses.

Please cite this article as: Peng, Y., Allen, S., Millwood, R.J. and Neal Stewart Jr, C. (2014) ‘Fukusensor:’ a genetically engineered plant for reporting DNA damage in response to gamma radiation. *Plant Biotechnol. J.*, doi: 10.1111/pbi.12247

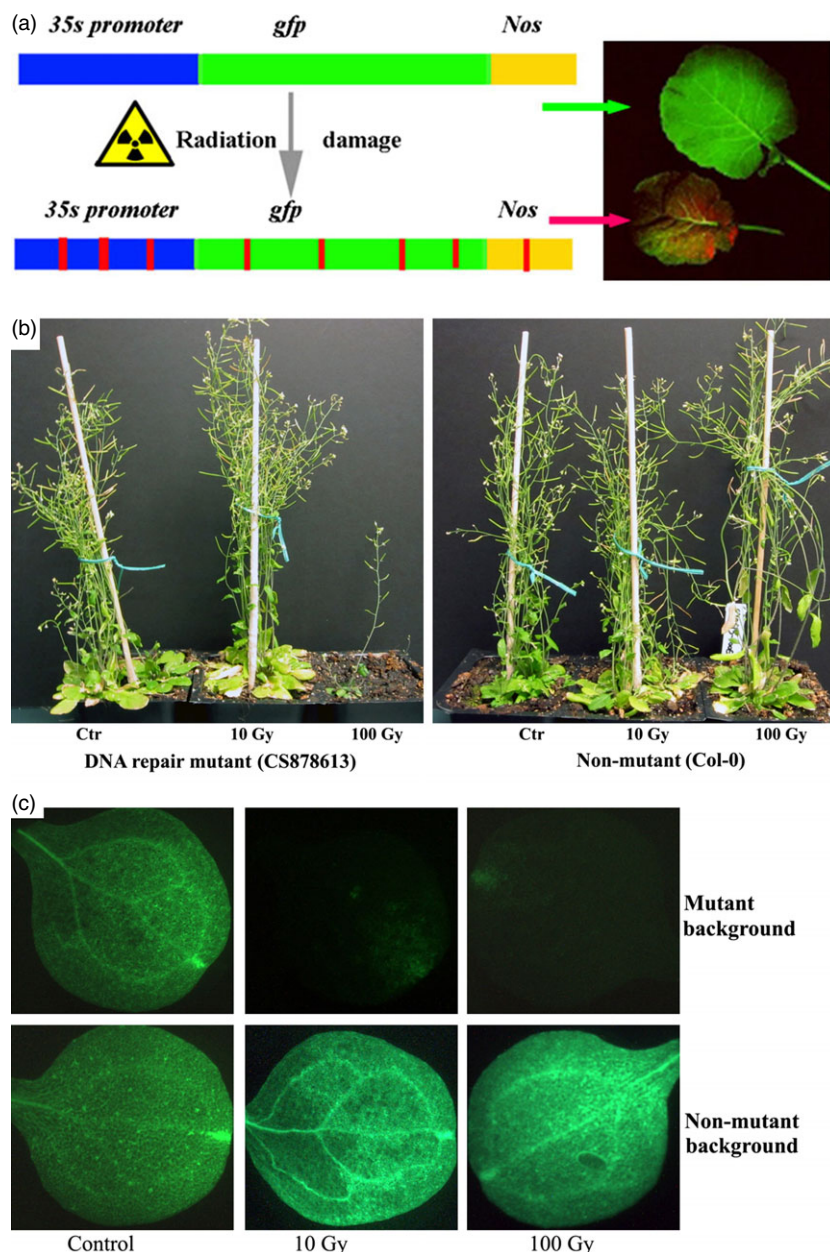


Figure 1 Engineered plant for reporting DNA damage in response to gamma radiation. (a) Pictorial representation of the loss-of-function strategy. Accumulation of DNA damage in transgenic cassettes (promoter, coding region and terminator) results in a nonfunctional *gfp* gene and loss of fluorescence over time, wherein DNA damage accumulates faster in DNA damage repair mutants relative than transgenes in a nonmutant background, as seen by attenuated green fluorescence signals. (b) Response of DNA repair deficiency mutants to radiation exposure. Two-week old young seedling of GFP transgenic Arabidopsis plants (mutant and nonmutant backgrounds) were exposed to gamma radiation (control, 10 Gy and 100 Gy) and grown under standard growth chamber conditions. Growth of mutant plants was affected by the 100 Gy dose (left), whereas there was a lack of apparent phenotypic differences among other plants. Shown here are 8-week old plants at the reproductive growth stage. (c) GFP expression (green fluorescence) in young leaves of mutant and nonmutant genetic background transgenic plants treated with gamma radiation—shown 1 week post-treatment. Fluorescence was significantly reduced in mutant background plants, while, there are no apparent visible differences among nonmutant background plants.

Results and discussion

Building a radiation sensing plant using the loss-of-function strategy

Protein kinase ATM has been shown to play a very important role in DNA repair by activating enzymes that fix the broken strands of DNA (Bakkenist and Kastan, 2003). The T-DNA insertion mutant *AtATM* in Arabidopsis plants was shown to be hypersensitive to radiation exposure (gamma) but not UV-B exposure (Garcia *et al.*, 2003). Therefore, it was chosen as the reporter transgene host designed to serve as a radiation sensor in which radiation would give attenuated GFP fluorescence (Figure 1a). The accumulation of DNA damage in the transgenic GFP cassette (promoter, GFP coding region and terminator) could result in a nonfunctional GFP reporter and loss of fluorescence over time as DNA damage accumulated in the transgenic plant cells. The loss of GFP

fluorescence is the reporter readout of interest. To test our hypothesis, GFP transgenic Arabidopsis plants in *AtATM* mutant and nonmutant background were generated.

Effect of sensor plants to radiation exposure

The development and growth of mutant plants was drastically affected by the 100 Gy dose, whereas there was a lack of phenotypic difference in the other plants (Figure 1b). After one week postgamma exposure, the fluorescence intensity was not changed in low (1, and 5 Gy) doses, but much reduced in mutant background plants for medium and high (10 and 100 Gy) doses, while, there was scant visible differences in the nonmutant background plants and controls (Figures 1c and 2a). These early results indicate that the Fukusensor plants have potential to sense radiation dose and act as an indicator for potential biological effects of radionuclide contamination to organisms. However, the drastic decrease in the amount of detected GFP may be not only

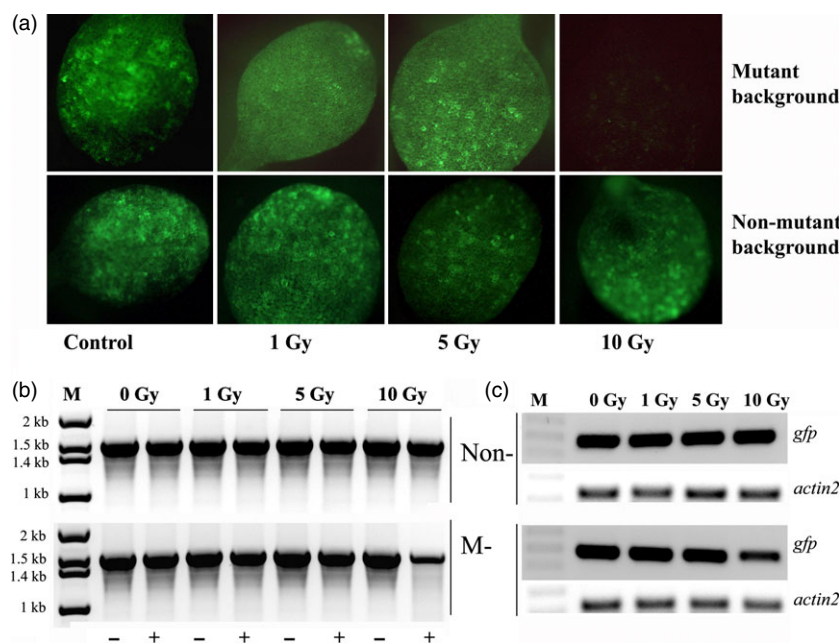


Figure 2 Gene expression and mutation assays under low-level radiation exposure. (a) Green fluorescence assays of plants exposed to low doses of gamma radiation: 0, 1, 5 and 10 Gy corresponding to exposure times of 0, 23 s, 116 s and 3.5 min, respectively ($n = 6$). (b) Surveyor nuclease assays were performed on PCR products from genomic DNA from each sample. Band density is associated negatively with mutations. (c) RT-PCR transcription assays of *gfp* and actin genes. The latter was used as the internal control gene as it is invariant under treatments.

due to massive level of unrepaired breaks altering the GFP gene, as the plants exposed to 10 Gy and under appeared morphologically normal. Actually, the mutation rate of the 35S-*gfp*-nos cassette did not appear to be linear relevant to the radiation dosage, with the breakpoint of detection occurring between 5 and 10 Gy. Only ca. 20% mutation rate was detected in the Surveyor nuclease digestion assay in plants treated with 10 Gy radiation (Figure 2b), whereas *gfp* abundance was reduced by at least half in the same plants (Figure 2c). A second possible mechanism could be transcriptional or post-transcriptional gene silencing. The induction of silencing in damaged *atm* mutants has been observed prior wherein high numbers of small noncoding RNAs were produced in *atm* mutation organisms (d'Adda di Fagagna, 2013; Huen and Chen, 2010; Shanbhag *et al.*, 2010; Wei *et al.*, 2012). These noncoding RNAs might be highly transcribed against the *gfp* gene, resulting in transcriptional silencing.

Fukusensor deployment scenarios

Except for ionizing radiation, many other factors, such as heavy metals, UV light, and other abiotic stresses could also damage DNA in plants (Friedberg, 2003). A successful radiation sensor must be specifically responsive to radionuclide contamination. GFP synthesis in our sensor plants was not affected by salt (50 mM NaCl), cold (4 °C) or heavy metal (1 mM CsCl) treatment (Figure S1). We are testing other factors currently. Moreover, GFP transgenic plants are being generated in six additional genetic backgrounds that have various DNA repair deficiencies, which also be similarly assayed. Although there was a lack of phenotypic difference between mutant and nonmutant backgrounds after a medium dose (10 Gy) treatment, the changes of GFP expression were drastic (Figure 1b,c). One issue raised by these initial experiments is the degree of sensitivity of our sensor plants to radiation exposure. To address this issue, GFP fluorescence in leaves will be quantified using fluorescence spectroscopy following dose range experiments from lower (5–10 Gy) radiation and monitored from the first day post-treatment. In short, to create a self-contained unmanned sensor device using the phytosensors,

multidisciplinary research is necessary. We envisage deploying transgenic plants in enclosed apparatus that would contain lights, spectral filters, and imaging and telecommunication capabilities so that Fukusensor signals could be remotely transmitted for off-site monitoring. The device, replicated many times and placed in strategic locations could be battery and solar-powered to serve as unmanned sensors at various strategic locations in Japan or other places of interest. Aside from the specific Fukusensor application, these plants could also be useful to study the basic biological effects of low-level ionizing radiation.

Experimental procedures

Generation of GFP transgenic plants

Wild type (Col-0) and DNA repair deficiency mutant *Arabidopsis thaliana* seeds (Col-0, CS878613 from SALK) were soaked in water at 4 °C for 2 days before being sown in a 'peat-lite' potting mix. Flats were placed in a growth chamber maintained at 20 to 22 °C; 16 h/8 h day/night photoperiod with $\sim 150 \mu\text{mol}/\text{m}^2/\text{s}$ irradiation, and plants were watered as needed. GFP transgenic *Arabidopsis* plants in the nonmutant and mutant backgrounds were generated using the floral dip method as described in Clough and Bent (1998). The mGFP-5ER construct contains the *mgfp5er* (GFP) mutant driven by the cauliflower mosaic virus 35S promoter and an NOS (nopaline synthase) terminator. T_1 transgenic seeds were surface sterilized, planted and screened on MS media containing 200 mg/L kanamycin in Petri dishes. Resistant plants were transferred to soil and self-pollinated, and T_2 seeds were collected. Expression of GFP as observed by green fluorescence in T_2 plants were tested under blue light. The T_2 plants expressing the *gfp* gene were self-pollinated to produce T_3 homozygous plants which were used for further analysis.

Radiation dose and environmental stress experiments

A Co-60 radiation source at Oak Ridge National Laboratory (ORNL) was used to carry out two sets of radiation dose assays. A wide range of dosing was performed first (0, 10 and 100 Gy), followed by a lower dose experiment (0, 1, 5 and 10 Gy). Two-

week old GFP transgenic plants in sterile Petri dishes on MS media were transported to ORNL for radiation treatments, and then returned to a growth chamber at the University of Tennessee the next day.

One week after dosing, GFP expression in young leaves of six individual plants for each treatment were assayed as described in Liu *et al.* (2013) using an epifluorescent microscope (Olympus stereo microscope model SZX12, Olympus America, Center Valley, PA) using a GFP filter set (emission 509 nm) and QCapture 2.56 imaging software, and an Olympus Q-colour 5.

Reverse transcriptase PCR experiments were performed to assay for transcriptional changes of the reporter gene. Total RNA was isolated from leaves of treated samples, utilizing TriReagent according to manufacturer's protocol (MRC, Cincinnati, OH). The residual genomic DNA in the extract was removed by treatments with RNase-free DNase I (Invitrogen, Carlsbad, CA). First strand cDNA was synthesized using: ~2 µg of total RNA, 0.5 µg oligo(dT)₁₈ and SuperScript[®] III reverse transcriptase, according to the manufacturer's instructions (Invitrogen, USA). RT-PCR was carried out in an Eppendorf mastercycler (Hamburg, Germany) using GoTaq[®] Green Master Mix (Promega, Madison, WI), which was programmed as follows: 2 min at 95 °C for predenature; 25–40 cycles of 15 s at 94 °C, 15 s at 55 °C and 20 s at 72 °C for each gene. *AtActin2*, was selected as reference gene. The oligonucleotide primers (Table S1) were designed with the Primer Express 2.0 software (Applied Biosystems-Perkin-Elmer, Foster City, CA). All primers were synthesized by Integrated DNA Technologies (IDT, Iowa City, IA).

A Surveyor nuclease mutation assay (Qiu *et al.*, 2004) was also performed to better understand mutations. In brief, genomic DNA was isolated from each treated samples and used as templates for amplification of the 35S-mgfp-nos cassette using high fidelity TaKaRa Ex Taq. One hundred nanograms of PCR product from each sample was subjected to Surveyor digestion (-/+ with/without Surveyor nuclease S) by first melting and randomly reannealing the amplicons (95 °C for 5 min, 95–85 °C at -2 °C/s, 85–25 °C at -0.1 °C/s), which converts any mutations into mismatched duplex DNA. We then add 1 µL of Surveyor nuclease S to the test sample, but not to the negative control (incubate at 42 °C for 20 min in a thermocycler). The products were separated on 2% agarose gels.

The seedlings were transferred to pots in potting media for observation during their life cycle to assess any phenotypic effects of radiation exposure. Plants were also exposed for 1 week to a suite of environmental stresses. Seeds of GFP transgenic plants were surface sterilized and planted on MS media for stresses treatments (50 mM NaCl salt stress, 1 mM CsCl heavy metal stress, and 4 °C chilling stress). GFP fluorescence was assayed 1 week after stress.

Acknowledgements

Thanks to Larry Avens, Denise Lee and Nan Zhao at ORNL for assistance in radiation assays of the transgenic plants. All

biosafety regulations were adhered to in this research. We appreciate funding from the National Academies Keck Futures Initiative, Oak Ridge National Laboratory and the University of Tennessee.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 GFP expression in mutant and nonmutant background transgenic plants was not effected by other factors.

Table S1 Primer sequences used for PCR.