RSR number 22-161-01rsr

RSR Request for Petunia Developed Using Genetic Engineering for Luciferin Monooxygenation and Antibiotic Resistance

Submitted by:

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1. Background

Following demonstration of the first bioluminescent plants over 35 years ago (Ow, *et al*., 1986), the public has been enthusiastically fascinated by the prospect of auto-luminescent ornamental plants. Responding to this, we have created auto-luminescent petunia by incorporating genes derived from naturally bioluminescent mushrooms. These genes provide a metabolic cycle to produce light via luciferin monooxygenation. Green light (530 nm) is emitted continuously by the plants at a low level, most prominently from young flowers (Figure 1). In all other aspects, these genetically modified petunia ("GM petunias") appear indistinguishable from unmodified petunias.

Bioluminescent plants have been created previously utilizing genes derived from fireflies or marine bacteria. In several instances, the Animal and Plant Health Inspection Service (APHIS) was petitioned to determine whether these genetically modified plants were subject to 7 CFR part 340 (Table 2). In each case, APHIS concluded that it "has no reason to believe" the modified plants are plant pests and thus are not regulated under 7 CFR part 340. Notably, stated in their response dated 2016, "APHIS also has no reason to believe that introduction of the auto-luminescent trait to these three plant species [*N. tabacum*, *Petunia x hybrid* and *A. thaliana*] will increase their weediness or invasiveness." As a result of these conclusions by APHIS, autoluminescent plants have been made commercially available in the United States.

The mechanism of action (MOA) in these previous bioluminescent plants is also based on monooxygenation of a luciferin. Notably, the luciferin in each case is not normally present in plants. This differs from mushroom bioluminescence, where light emission is achieved by repurposing metabolites which are native to plants. Light is generated through a two-step oxidation of hispidin (hydroxylation followed by monooxygenation). Following light emission, the product molecule is then hydrolyzed to pyruvate and caffeic acid, which is recycled to make new hispidin. Hispidin is found in many plants, such as horsetail (*Equisetum arvense*), piper (*Piper methysticum*), pistachio (*Pistacia atlantica*), and others. Even though petunias are not used for food or feed, hispidin has been demonstrated to have very low toxicity for human consumption (Palkina, *et al*., 2021). Furthermore, while many bioluminescent mushrooms are not edible, the bioluminescent components apparently are not themselves toxic (Harmon, 2009; Miller, *et al*., 2006).

To generate the auto-luminescent petunias, a gene encoding neomycin phosphotransferase II (NPTII) was also incorporated as a selectable marker. NPTII confers resistance to the antibiotics kanamycin and neomycin. The resulting combination of plant/trait/MOA for NPTII has previously been determined by APHIS to not require regulation under 7 CFR part 340 (A1-DFR petunias; APHIS, 2020-1). Furthermore, NPTII is found in many crops currently approved for commercial production (e.g., corn, potato, oilseeds, tomato, papaya, petunia, rose, flax, and chicory). Its environmental safety has been reviewed by regulatory authorities of several countries with no reports of adverse effects on humans or the environment.

Petunias are used primarily as bedding plants (small plants for transplanting into the ground, such as in flower beds), and for potting and hanging baskets. Commercial production is generally done in horticultural greenhouses, primarily through propagation of cuttings. They are then used by consumers in small volumes, typically on balconies and isolated beds. Petunias are predominantly grown as annuals, but are tender perennials in hardiness zones 9 to 11. As GM petunias are handled in the same manner as non-GM petunias (e.g., A1-DFR petunias), no changes are needed in agricultural or cultivation practices. Several cut flower varieties developed using genetic engineering are also presently produced commercially, including carnation, rose, and baby's breath.

There are no petunia species that are native to the United States, although there are several introduced (naturalized) species. Nonetheless, petunias are not listed as invasive plants and not known for invasiveness even in the southeastern United States where the climate is most similar to the habitat of their parental lineages (*P. axillaris* and *P. integrifolia*). No plants among the petunia genera are on the Federal noxious weed list nor are they listed as invasive by any state. Petunia do not intercross with other genera (e.g., *Calibrachoa parviflora*, a.k.a. *Petunia parviflora*).

The total planted area for petunia in the United States is estimated to be fairly small, less than 10,000 acres (APHIS, 2020-2). Moreover, the circumstances of their cultivation mitigate conceivable effects on non-target organisms. Petunias are normally grown in human-created environments where people live and work daily, such as homes, businesses, or botanical gardens. Typically in these environments, nighttime illumination due to artificial lighting far exceeds light emission from the auto-luminescent petunias. Greenhouse practices routinely include pest and disease control, and no pest targeting petunia are covered by Plant Protection and Quarantine (PPQ). The introduced genes have no apparent influence on plant vigor and are not expected to impart any new plant pest or disease risk.

Figure 1. Petunia developed using genetic engineering for luciferin monooxygenation and antibiotic resistance, shown under external illumination (left) and in the dark (right).

Table 1. Prior inquiries to APHIS on the regulatory status of transgenic bioluminescent plants.

Species	**Trait**	**MOA**
CBI (transgenic plant)	CBI (auto-luminescence)	CBI (bacterial bioluminescence)
BioGlow LLC (September 20, 2012) www.aphis.usda.gov/biotechnology/downloads/reg_loi/APHIS_response_BioGlow_092012.pdf		
CBI (two ornamental plants)	CBI (auto-luminescence)	CBI (bacterial bioluminescence)
BioGlow LLC (March 21, 2013) www.aphis.usda.gov/biotechnology/downloads/reg_loi/aphis_response_bioglow_032113.pdf		
Arabidopsis thaliana	bioluminescence	Firefly luciferase
Glowing Plant, Inc. (December 23, 2014) www.aphis.usda.gov/biotechnology/downloads/reg_loi/brs_air_response_glowing_plant_bioluminescent_a_thaliana.pdf		
Petunia x hybrid *Nicotiana tabacum* *Arabidopsis thaliana*	auto-luminescence	bacterial bioluminescence (from *Vibrio fischeri*)
Glowing Plant, Inc. (December 19, 2016) www.aphis.usda.gov/biotechnology/downloads/reg_loi/15-264-01_air_response_signed.pdf		
Nicotiana tabacum	bioluminescence	Firefly luciferase
Glowing Plant, Inc. (December 19, 2016) www.aphis.usda.gov/biotechnology/downloads/reg_loi/15-296-01_air_response_signed.pdf		

2. Information about requestor

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3. Does the request contain Confidential Business Information (CBI)?

This RSR request contains Confidential Business Information. General information on the use of fungal bioluminescence genes/enzymes to provide an auto-luminescence phenotype is not confidential. However, the identity of specific genes/enzymes, their modifications, and configurations is proprietary to Light Bio and subject to pending patents.

4. Description of the comparator plant

Petunia hybrida

5. Genotype of the modified plant

The auto-luminescent phenotype in *Petunia hybrida* is provided by two independent insertions of genetic material. Both comprise linear DNA inserted into the chromosomal DNA of the host (fasta files provided).

Insertion 1 **[ ]**: **CBI**

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Insertion 2 **[ ]**: **CBI**

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Table 2. Annotation of Inserted Genetic Material (insertion 1, **[ ]**). Enzymes comprising the **CBI** MOA for auto-luminescence are shown in bold. Fasta files provided for all inserted components.

	Nucleotide position	**Name of inserted component**	**Construct component donor**	**Function**	
[[[[					] **CBI**] **CBI**] **CBI**] **CBI**
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Table 3. Annotation of Inserted Genetic Material (insertion 2, **[ ]**). Enzymes comprising the MOA for auto-luminescence are shown in bold. Fasta files provided for all inserted components. **CBI**

	Nucleotide position	**Name of inserted component**	**Construct component donor**	**Function**	
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6. Description of new trait

Intended trait:

Auto-luminescence

Auxiliary trait:

Resistance to aminoglycoside antibiotics

Intended phenotype:

Sustained, low-level auto-luminescence (peak wavelength, 530 nm)

Auxiliary phenotype:

Resistance to up to 50 mg/L of aminoglycoside antibiotics (e.g. kanamycin, geneticin or neomycin) in growing medium

Description of the MOA:

The MOA for producing visible light in the modified petunias is monooxygenation of a luciferin. This is essentially the same mechanism as in previously deregulated plants having analogous phenotypes (i.e., auto-luminescence or bioluminescence). The chemical structures of luciferins differ depending on the source of the inserted DNA. The luciferin utilized in bioluminescent mushrooms is 3-hydroxyhispidin.

The inserted genetic material also contains a gene encoding phosphopantetheinyl transferase (npgA). This provides a prosthetic group (phosphopantetheine) required for hispidin synthase (HispS) activity. Analogous phosphopantetheinyl transferases are naturally present in unmodified plants.

All substrates and products of the MOA for auto-luminescence have been described in unmodified plants except oxyluciferin. Oxyluciferin is hydrolyzed by the action of caffeoyl pyruvate hydrolase (CPH) to yield caffeic acid and pyruvate.

The MOA for aminoglycoside antibiotic resistance in the modified petunias is phosphorylation of the given antibiotic molecule leading to its inactivation. This process is catalyzed by neomycin phosphotransferase II (NPTII).

Table 4. MOA for the low-level auto-luminescence phenotype.

Enzyme	Substrates	Products
hispidin synthase (HispS)	caffeic acid malonyl-CoA	hispidin
hispidin-3-hydroxylase (H3H)	hispidin	3-hydroxyhispidin (luciferin)
Luciferase (luz)	3-hydroxyhispidin (luciferin)	caffeoyl pyruvate (oxyluciferin)
caffeoyl pyruvate hydrolase (CPH)	caffeoyl pyruvate (oxyluciferin)	caffeic acid pyruvate

Table 5. MOA for the aminoglycoside antibiotic resistance phenotype.

Enzyme	Substrates	Products
neomycin phosphotransferase II	aminoglycoside antibiotic (e.g., kanamycin, geneticin, neomycin)	phosphorylated aminoglycoside antibiotic

The MOA and associated phenotype have no apparent influence on the morphology or vigor of the plants. The modified petunias exhibit normal growth characteristics and appearance compared to unmodified comparator plants.

No component of the MOA has been associated with an increased plant pest risk. Because the MOA imparts no discernable effects, apart from auto-luminescence and resistance to aminoglycoside antibiotics (absent in natural environments), no influence on distribution or density of the plants is expected.

7. Technical resources

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Kotlobay, Alexey A., *et al*. (2018). Genetically encodable bioluminescent system from fungi. Proceedings of the National Academy of Sciences. 115 (50): 12728-12732. doi.org/10.1073/pnas.1803615115

8. References

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Ow DW, De Wet JR, Helinski DR, Howell SH, Wood KV, Deluca M. (1986) Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. Science. 14;234(4778):856-9. doi: 10.1126/science.234.4778.856.

Attachment 1: Nucleotide Sequence (CBI)

Attachment 2: Nucleotide Sequence (CBI)