Vast potential for using the piggyBac transposon to engineer transgenic plants at specific genomic locations

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ABSTRACT
The acceptance of bioengineered plants by some nations is hampered by a number of factors, including the random insertion of a transgene into the host genome. Emerging technologies, such as site-specific nucleases, are enabling plant scientists to promote recombination or mutations at specific plant loci. Off target activity of these nucleases may limit widespread use. Insertion of transgenes by transposases engineered with a specific DNA binding domain has been accomplished in a number of organisms, but not in plants. The piggyBac transposon system, originally isolated from an insect, has been utilized to transform a variety of organisms. The piggyBac transposase is amendable to structural modifications, and was able to insert a transgene at a specific human locus through fusion of a DNA binding domain to its N-terminus. Recent developments demonstrating the activity of piggyBac transposase in plants is an important first step toward the potential use of engineered versions of piggyBac transposase for site-specific transgene insertion in plants.

KEYWORDS
chimeric transposase; insect transposon; plants; site-specific insertion; transformation

The creation of bioengineered or transgenic plants has revolutionized agriculture in many parts of the world. Recent studies indicate the cultivation of transgenic plants benefits both farmers as well as the environment\textsuperscript{1,2}. Many bioengineered plants are created through random insertion of transgenes that include a marker gene for selection of transgenic cells from non-transformed tissues. Some regions of the world are resistant to growing bioengineered plants for a number of reasons. For example, the European Union views genetically modified plants as flawed, in part, due to the random insertion of the transgene\textsuperscript{3}.

In the last few years, new emerging technologies in genetic modification may soon enable targeting of transgenes to user defined locations in bioengineered plants. In addition, changes to existing genes can be completed to improve agronomic qualities or enhance resistance to biotic or abiotic stress. Gene editing and targeted transgene insertion are now possible with the advent of user-defined site specific nucleases including meganucleases, zinc finger nucleases, TALENs and the CRISPR/Cas9 system\textsuperscript{4}. If mutations are desired at the target gene, nonhomologous end joining creates insertions or deletions at the site of the double strand break mediated by the site specific nuclease. Alternatively, insertion of a transgene can be mediated through the designer nuclease double strand break and subsequent homologous recombination with the additionally supplied DNA transgene\textsuperscript{4}. The only drawback to utilizing these site-specific nucleases is that breaks may occur at unintended locations in the genome, necessitating the need to check the bioengineered organism for deleterious mutations, which can be time-consuming and expensive.

Transposons are mobile segments of DNA that can move from one location to another by a “cut and paste” mechanism\textsuperscript{5}. These reactions are catalyzed by a protein, transposase, which recognizes inverted repeat sequences at the termini of the transposon, cut the transposon from the donor location, and insert the transposon to a new location. These transposases generally insert the transposon at random locations, but do have biases for certain genomic regions\textsuperscript{6}. Any segment of DNA placed between the transposon inverted repeat sequences can be moved from a donor to a target location by the transposase, although there may be
a size constraint on the length of the DNA segment. The transposase is usually encoded on the transposon itself, but the placement of the transposase gene outside of the terminal inverted repeat sequences creates a non-autonomous transposon that can be moved and form stable insertions once the source of transposase is removed.

Transposons and their transposases have been utilized in genetic studies for several years. For example, transposases have been used in generating plant mutants that enable better characterization of genes or biochemical pathways. The insertion specificity of some DNA transposons has been modified by fusing specific DNA binding domains to the transposase protein (or by tethering the transposon DNA to specific targets using bifunctional DNA binding proteins). Most of these experiments were completed in organisms besides plants. However, recent developments with an insect DNA transposase, piggyBac, suggest that engineered versions of piggyBac transposase could one day insert transgenes into plant chromosomes with high specificity. The possibility of producing an engineered piggyBac transposase for targeted insertion of genes into plants is the main focus of this commentary.

The piggyBac transposon was first isolated from the genome of the moth *Trichoplusia ni*. A study examining a limited number of eukaryotic genomes indicated that piggyBac was an ancient transposon, but remained active only in *T. ni*. Laboratories involved in insect research found that piggyBac was amendable to transformation of a wide range of insects. Surprisingly, piggyBac could also transform other organisms such as *Plasmodium falciparum*, zebrafish embryos, mammalian cell lines, fission yeast and mice. More recently, piggyBac was found to be active in tobacco and rice. Only 2 insertion events were detected in tobacco but reinserter following excision was more common in rice, up to 40%. The piggyBac transposon is amendable to a number of modifications that improve or alter its activity. Seven amino acid substitutions in the transposase resulted in dramatic improvements in both excision and integration activity. This hyperactive piggyBac transposase was active in rice. Additional studies demonstrated that fusion of a specific DNA binding domain to either the N- or C-terminus of piggyBac transposase did not alter transposition activity. The fusion of an engineered transcription activator like effector domain to the N-terminus of piggyBac transposase resulted in targeted insertion of a transgene in human embryonic kidney cells. Although targeting efficiencies were low in the human cell system, the insertion of transgenes to noncritical positions within the human genome is an important advancement. We believe a fusion transposase with a targeted DNA binding domain is more advantageous for plant or mammalian transformation than using a nuclease with a targeted DNA binding domain because off target sites should contain the inserted transposon and could be easily detected by Southern blot analysis or quantitative PCR-based copy number assays; however, off target nuclease-targeted DNA binding domain activity could cause mutations induced by double strand breaks that may not be easily detected without sequencing of the entire genome of the modified organism.

A number of different piggyBac targeting plasmids were developed by Owens et al., but the simplest plasmid design (hG3-TALC1) resulted in at least 5 independent insertion events. Plasmid hG3-TALC1 contained both the coding sequence for the piggyBac fusion transposase expressed by a constitutive promoter as well as the transgene bordered by the inverted terminal repeats (Fig. 1A). One of the terminal inverted repeats was embedded in the piggyBac transposase coding sequence, which ensured that excision of the transposon abolished activity of the piggyBac fusion transposase. The transposon was inserted near to the TALE recognition sequence by the piggyBac fusion transposase (Fig. 1B). We envision a similar plasmid could be utilized for plant transformation, although a negative selectable marker will likely need to be added to the targeting plasmid as plants can incorporate exogenous DNA through nonhomologous recombination. A negative selectable marker gene expresses a protein that is deleterious to cell survival if the targeting plasmid is inadvertently added to the genome. The piggyBac targeting plasmid could be introduced into plant nuclei using bombardment procedures or by *Agrobacterium tumefaciens* infection. Transient expression of the piggyBac fusion transposase in the nucleus would excise the transposon from the targeting plasmid and insert it to a TTAA site near the targeting site.

We predict the targeted insertion frequency of the transgene would be low, especially with a negative selection marker. In order to be competitive with
nuclease mediated gene insertion in plants,\textsuperscript{24,25} at least 2–5% of the embryogenic transgenic events should display the targeted insertion mediated by the piggy-\textit{Bac} fusion transposase. The advantage to using piggy-\textit{Bac} fusion transposase over a nuclease for gene insertion is that the transgenic plant would not need to be sequenced for unintended genetic changes that could occur with nucleases. It may be possible in the future to further improve the efficiency of targeted insertion by evolving \textit{piggyBac} transposase in the laboratory to only bind at desired genome locations. We anticipate there are a number of plant science laboratories willing to investigate \textit{piggyBac} mediated targeted insertion of transgenes.

**Disclosure of potential conflicts of interest**

No potential conflict of interest was disclosed.

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