Manipulating Plant Histone Genes to Improve Agrobacterium- and Direct DNA-mediated Plant Genetic Transformation

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Introduction

Agrobacterium-mediated plant genetic transformation has become a "core technology" for both plant basic research and for agricultural biotechnology applications. Compared to direct DNA uptake techniques, including PEG/electroporation-mediated DNA uptake into protoplasts and particle bombardment, *Agrobacterium*-mediated transformation generally results in a lower integrated copy number of transgenes that show fewer rearrangements. This is important for regulatory purposes for which a complete characterization of foreign DNA is required for field-grown plants and for gene expression purposes: multiple copy transgene integration events have a higher propensity to "silence" than do low or single copy events. However, many plant species, or particular varieties/genotypes of these species, remain highly recalcitrant to *Agrobacterium*-mediated transformation. Improving transformation frequency remains an important aspect of applied *Agrobacterium* research. In addition, improving the "quality" of transgenic events (single-copy transgene integration, no vector "backbone" integration, and predictable and stable transgene expression) forms the basis of next-generation transformation technologies.

Improving *Agrobacterium*-mediated transformation frequency

Scientists have used three major approaches to improve the frequency of *Agrobacterium*-mediated plant transformation. The first involves screening for varieties/genotypes that transform more efficiently and manipulating tissue culture parameters to increase the frequency of transformation. For any given genotype, some tissues may transform more easily than others. However, developing protocols to transform particular tissues must be linked with plant regeneration protocols for these same tissues. This is frequently difficult to achieve, as the most highly transformable tissues or cell types may not be amenable to regeneration, and *vice versa*. Altering tissue culture media during *Agrobacterium* infection may improve transformation. For example, adding anti-oxidants to the medium has improved the frequency of *Agrobacterium*-mediated transformation of corn and soybeans by inhibiting tissue necrosis.[1,2] The phytohormone content of the medium may also influence transformation frequency. For example, the frequency of *Agrobacterium*-mediated transformation of *Arabidopsis* root segments is greatly increased by pre-incubating the segments on medium containing auxins and cytokinins prior to infection (**Fig. 1**), and *Arabidopsis* root transformation and regeneration protocols generally include a phytohormone pretreatment.[3]

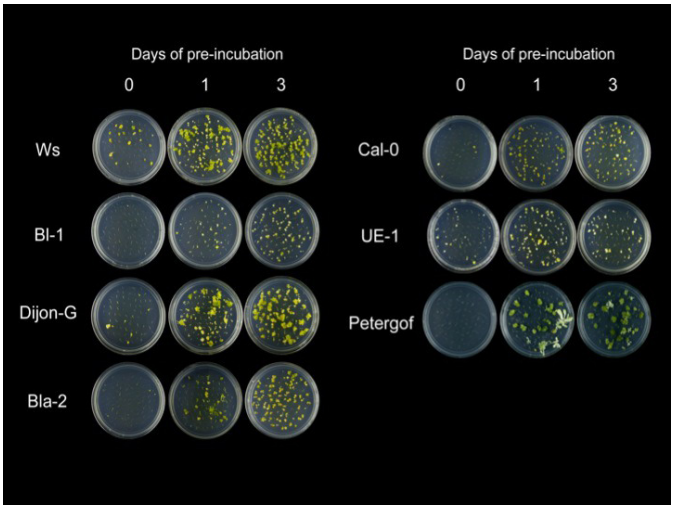

Figure 1. Pretreatment of *Arabidopsis* root segments with phytohormones results in increased susceptibility to *Agrobacterium*-mediated transformation. Roots of various Arabidopsis ecotypes were excised from one month-old plants grown in solidified B5 medium and incubated on agar plates containing auxins and cytokinins for the indicated number of days. The roots were cut into 3-5 mm segments and co-cultivated with the tumorigenic strain *A. tumefaciens* A208 for two days. Root segments were subsequently moved to MS medium lacking phytohormones but containing timentin (to kill the bacteria). The plates were photographed after one month. Note that pre-incubation of roots with phytohormones greatly increased the number of tumors formed, indicating increased transformation. *Courtesy of Drs. Jaime Humara and Ho Chul Yi.*

Scientists have also increased the frequency of *Agrobacterium*-mediated transformation by screening for or generating bacterial strains with higher virulence. For example, several "disarmed" (i.e., lacking oncogenes in the T-DNA) *Agrobacterium* strains, such as EHA101,[4]
EHA105,[5] and NT1(pKPSF2)[6] are considered "super-virulent." However, high virulence on one species does not always

indicate a similar level of virulence on other species: The *tumorigenic* parent of *A. tumefaciens* EHA101, A277, does not show hyper-virulence on *Arabidopsis* root segments.[7] Additionally, scientists have increased *Agrobacterium* virulence by generating strains with extra copies of the *vir* gene transcriptional activator *virG*,[8,9] or by using *virG* mutants in which the protein is "locked" in the active conformation.[10]

More recently, scientists have attempted to increase the frequency of *Agrobacterium*-mediated plant transformation by manipulating the plant.[11] This has generally required identifying host genes important for transformation by screening *Arabidopsis* T-DNA insertion mutants to identify lines that are resistant to *Agrobacterium* transformation (*rat* mutants).[12,13] Additionally, scientists have used viral induced gene silencing (VIGS) to identify tomato genes that are important for *Agrobacterium*-mediated transformation of *Nicotiana benthamiana*.[14] In some instances, over-expressing these genes in either their native host or in an alternative host species results in increased transformation.[15]

Plant histones affect *Agrobacterium*-mediated transformation

One of the first characterized *Arabidopsis rat* mutants, *rat5*,[15] contains a T-DNA insertion in the 3' untranslated (UTR) region of the HTA1 gene encoding the histone H2A-1 protein. Genetic and molecular characterization of this mutant revealed that the roots were permissive for *Agrobacterium*-mediated transient transformation, but were blocked in stable transformation because of a deficiency in T-DNA integration into the plant genome.[12] Yi et al. correlated expression of the *HTA1* gene with the region of the *Arabidoposis* root that was most susceptible to transformation.[16] *HTA1* encodes an unusual type of histone, termed a "replacement" histone. Unlike most histone genes whose expression is limited to the S phase of actively dividing cells, replacement histones are expressed at low levels throughout the cell cycle in most cells. *HTA1* is one of a 13-member *HTA* gene family. From among the tested members of this family, only the *HTA1* promoter responded to wounding and *Agrobacterium* infection.[17] Thus, a "special" relationship appears to exist between the *HTA1* gene and *Agrobacterium*-mediated plant transformation. Indeed, over-expression of the *Arabidopsis HTA1* gene could enhance the transformation of *Arabidopsis* root segments,[15] rice calli,[18] and maize and *Brassica napus* tissues (B. Frame, K. Wang, S. Johnson, and S.B. Gelvin, unpublished). To explore the reason why over-expression of *HTA1* increased *Agrobacterium*-mediated plant transformation, we recently conducted a large-scale analysis of the effects of over-expressing various *Arabidopsis* histone genes on transformation.[19]

The *Arabidopsis* genome comprises 47 genes encoding 33 different core histone proteins (H2A, H2B, H3, and H4; several different genes encode histone proteins with the same amino acid sequence). We generated more than 1000 transgenic *Arabidopsis* lines individually containing expression cassettes for one of 20 representative core histone cDNAs. We then tested roots of these transgenic plants for transformation susceptibility, compared to wild-type plants. Our results indicated that over-expression of all seven tested *HTA* cDNAs, the *HFO* (histone H4) cDNA, and one of the four tested *HTR* (histone H3) cDNAs resulted in enhanced transformation susceptibility. However, over-expression of all seven tested *HTB* (histone H2B) and three *HTR* cDNAs did not alter transformation susceptibility. Thus, increasing host transformation susceptibility occurred with numerous, but not all, histone cDNAs.[19]

Understanding the mechanism of histone-mediated transformation enhancement

Histones are chromatin proteins that coat chromosomal DNA. Because disruption of the *HTA1* gene decreases *Agrobacterium*-mediated transformation by interfering with T-DNA integration, we initially considered that over-expressing *HTA1* increased transformation by enhancing T-DNA integration. However, experiments in our laboratory indicated that over-expressing *HTA1* also increased the extent of *Agrobacterium*-mediated transient transformation, a process that does not require T-DNA integration (**Fig. 2**). We therefore hypothesized that histone over-expression may sensitize cells to transformation at a step prior to T-DNA integration. We subsequently adapted a protoplast-based assay to eliminate *Agrobacterium* from the transgene delivery system and to "uncouple" transgene expression from transgene integration. In this assay, we introduced transgene DNA directly into tobacco BY-2 protoplasts by electroporation.

Our initial assays examined transgene expression in the presence and absence of histone over-expression. We co-introduced a plant-active β-glucuronidase (*gusA*) gene with either a histone cDNA expression cassette or an "empty vector" lacking the histone cDNA, and assayed for GUS activity 24 hr later. There was a perfect correlation between those histone cDNAs that increased *Agrobacterium*-mediated transformation and those that increased *gusA* transgene expression.

We next examined which region of the histone H2A-1 protein mediated enhanced transgene expression. Previous work indicated that the N-terminal 37 amino acids of an animal histone H2A protein, when introduced as a complex with a transgene, could enhance transgene transient expression in an animal transfection system.[20] We therefore co-electroporated a cDNA encoding the first 39 amino acids of *Arabidopsis* histone H2A-1 (corresponding to the first 37 amino acids of

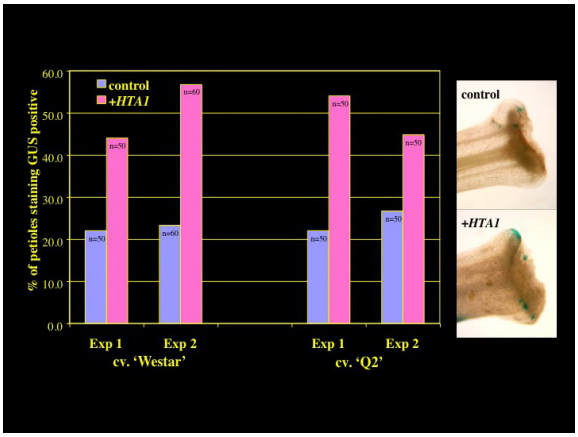

Figure 2. Co-transformation of *Brassica napus* with a *gusA* reporter gene and an *HTA1* expression cassette increases transient transformation. Cotyledonary petioles from cultivars Westar or Q2 were inoculated with *A. tumefaciens* containing T-DNAs harboring *gusA*-intron genes and either an *HTA1* expression cassette or an empty expression vector (control). Petioles were stained with X-gluc after 6 days. Note that co-transformation with the *HTA1* expression cassette increased the percentage of transformed petioles ~2 1/2-fold. Exp = experiment number; n = number of petioles inoculated. *Courtesy of Ms. Susan J. Johnson.*

the animal histone H2A protein) with a *gusA* reporter construction into BY-2 cells and assayed GUS activity after one day. Similar to the situation with the co-transfected histone H2A protein fragment in animal cells, the co-electroporated *Arabidopsis* cDNA encoding the N-terminal fragment of *HTA1* also enhanced reporter gene expression 2- to 4-fold. However, in these experiments we showed that co-transformation of a reporter gene with particular plant histone cDNA expression cassettes could enhance transgene expression. Our experiments differ from those using animal systems in that we did not have to purify histone proteins, interact them with transgene DNA, and use these complexes to enhance transformation.

To understand the mechanism by which the N-terminal histone H2A-1 fragment could enhance transgene expression, we generated numerous different amino acid substitutions in this fragment and tested them for their ability to enhance transgene expression. The results of these experiments indicated that amino acids involved in histone-DNA interactions were important for transgene expression enhancement, whereas amino acids involved in histone-histone interactions were not. These results, combined with our findings that many transformation-enhancing mutant H2A-1 N-terminal fragments are localized to the cytoplasm of plant cells (**Fig. 3**), suggested that the mechanism of histone enhancement of transgene expression involved protection of incoming transgene DNA in the cytoplasm, rather than a direct effect on transgene transcription in the nucleus. Consistent with this hypothesis was our finding that over-expression of *HTA1* could not increase expression of a previously integrated *gusA* transgene, nor could it reverse the silencing of a previously integrated but non-active *nptII* (kanamycin-resistance) gene.

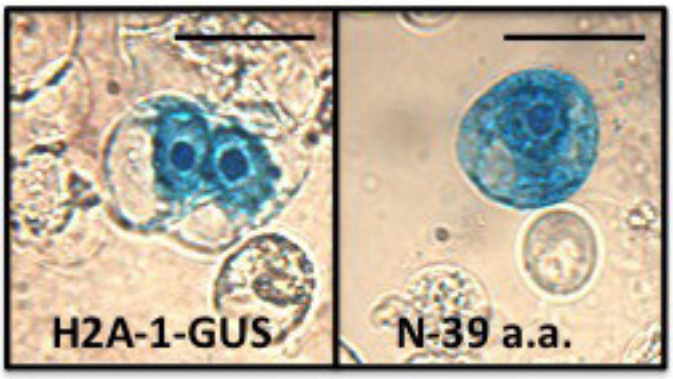

Figure 3. Regions of the H2A-1 protein that effect increased transgene expression do not necessarily localize to the nucleus. Full-length H2A-1-GUS and N-terminal 39 amino acid H2A-1-GUS fusion protein expression cassettes were electroporated into tobacco BY-2 cells. Three days later, the cells were stained with X-gluc for GUS activity. Note that the full-length H2A-1 fusion protein localizes exclusively to the nucleus (the dark circle is the nucleolus), whereas the N-terminal 39 amino acid fragment of H2A-1 is found throughout the cell. Over-expression of both full-length and the N-terminal fragment, however, results in increased transgene expression. Bar indicates 50 mM. *Courtesy of Dr. Lan-Ying Lee.*

To test whether over-expression of various core histone genes could protect incoming transgene DNA, we co-electroporated tobacco BY-2 protoplasts with a gusA transgene and histone cDNA expression cassettes (or an empty expression vector). At various times, we treated the protoplasts with DNAse I (to eliminate DNA sticking to the outside of the protoplasts), isolated DNA from the cells, and quantified the amount of transgene DNA within the cell using DNA PCR. Once again, there was a perfect correlation between those histones whose over-expression enhanced *Agrobacterium*-mediated transformation or transgene expression, and those histones whose over-expression resulted in increased amounts of transgene DNA within the cells. We obtained these same results using single-stranded transgene DNA (the form of T-DNA *Agrobacterium* introduces into plant cells) or double-stranded DNA. Thus, the mechanism by which particular histones enhance both *Agrobacterium*-mediated and naked DNA-mediated transformation appears to be by protecting incoming transgene DNA, most likely in the plant cytoplasm.

Conclusions and future prospects

Our results indicate that co-introduction of expression cassettes for particular histone genes with genes of interest can significantly increase transgene expression and, hence, transformation efficiency. Transformation enhancement may oc-

cur by allowing more transgene DNA to reach the nucleus, thus providing more substrate for selectable marker expression and transgene integration. Although histone-mediated enhancement of transformation and transgene expression has important agricultural biotechnology applications, several questions remain regarding this technology. Why are certain core histones effective, whereas others are not? Do particular modifications mediate the ability of histones to enhance incoming transgene stability? Does histone over-expression increase the copy number of integrated transgenes in plant cells transformed either by Agrobacterium or by naked DNA (an undesirable characteristic for transformed cells)? Additional investigations will be required to answer these important questions.

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