Induction of Agrobacterium tumefaciens vir genes by the green alga, Chlamydomonas reinhardii

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We have recently reported the development of Agrobacterium-mediated transformation of unicellular green alga, Chlamydomonas reinhardii. We have observed that Chlamydomonas cells could be transformed without the addition of acetylsyringone. Here we report the ability of Chlamydomonas cells to induce Agrobacterium processes—vir gene induction and bacterial attachment, which are essential for T-DNA transfer. These results indicate that Chlamydomonas and the related algal species may be in the natural host range of Agrobacterium.

Keywords: Acetylsyringone, Agrobacterium tumefaciens, Chlamydomonas reinhardii, phenolic compounds, T-DNA, vir genes.

Agrobacterium tumefaciens has been an invaluable system in studying the fundamental biology of host-pathogen interaction and plant biotechnology due to its unique ability to transfer DNA into the plant genome. The process of T-DNA transfer is initiated by the induction of bacterial virulence (vir) genes by the phenolic compounds produced and released by the wounded plant cells through the VirA–VirG two-component signal transduction system. It has been shown that majority of the dicotyledonous plants produce such phenolic compounds and they have been routinely transformed by Agrobacterium. Monocotyledons, which generally do not produce vir-inducing compounds, could be transformed by the exogenous addition of such molecules like acetylsyringone (AS). Interestingly, Agrobacterium gene transfer has also been extended to fungi and human (HeLa) cells.

Recently, we have reported an Agrobacterium-mediated genetic transformation system for the unicellular green alga, Chlamydomonas reinhardii. We have observed that Chlamydomonas cells could be transformed by Agrobacterium without the use of AS. This has raised the possibilities that Chlamydomonas and probably other related algal systems could be capable of inducing the processes in Agrobacterium leading to T-DNA transfer. Therefore, we undertook experiments using a VirE : lacZ fusion system to address the potential induction of Agrobacterium virulence system by cells or cell products of Chlamydomonas, as well as examined the possibility of physical interaction between the algal cells and Agrobacterium.

We have performed experiments to confirm the transformation of Chlamydomonas cells in the absence of AS using transient GUS reporter assay and hygromycin resistance. Co-cultivation of Chlamydomonas strain CC-124 with A. tumefaciens strain LBA4404 (pCAMBIA1304) was performed according to the earlier method. Co-cultivation in the absence of AS consistently produced reporter gene-expressing and hygromycin-resistant cells. Transient transformation frequency of 8–10% of the analysed cells after co-cultivation was observed, which was based on GUS activity. Stable transformation frequency-based hygromycin resistance phenotype has been in the range of 5–8 transformants per million cells plated (Table 1). We have shown earlier that though the addition of AS significantly enhanced transformation frequency many folds (~50-fold), it was not essential for transformation. This indicates the potential of Chlamydomonas in inducing Agrobacterium vir genes and consequent T-DNA transfer. Further experiments were conducted to unravel these possibilities.

In the preliminary experiments to test the ability of Chlamydomonas to induce Agrobacterium vir genes, Chlamydomonas cultures of different ages were co-incubated with Agrobacterium strain A348 (pSM358) having a virE : lacZ fusion for 24 h, and bacterial cells were used for β-galactosidase activity after algal cells were removed by low-speed centrifugation (100 g for 2 min) following vigorous vortexing. All the cultures tested showed β-galactosidase activity, indicating vir gene induction. Chlamydomonas cultures have been reported to secrete several small molecules, including phenolics into the culture medium during culture. It is likely that some of these molecules are capable of inducing the Agrobacterium vir genes, explaining the transformation as well as vir gene induction. To substantiate this information, we have also carried out experiments where the spent medium containing the possibly secreted molecules and the cell extracts were used to study vir gene induction.

Spent medium was prepared by removing cells from late log phase cultures by centrifugation at 10,000 g for 10 min at room temperature. The pH of the resulting supernatant was adjusted to both 7.0 or 5.6 and was further sterilized by passing through a 0.22 μm membrane filter.

Table 1. Transformation frequencies of Agrobacterium-mediated transformation of Chlamydomonas without acetylsyringone

<table>
<thead>
<tr>
<th>Cells plated (× 10⁶)</th>
<th>Hygromycin resistance frequency (× 10⁻⁶ ± SD)</th>
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<tbody>
<tr>
<td>1.85</td>
<td>5.58 ± 1.19</td>
</tr>
<tr>
<td>2.2</td>
<td>6.36 ± 1.68</td>
</tr>
<tr>
<td>1.3</td>
<td>8.2 ± 2.76</td>
</tr>
</tbody>
</table>

Note: Transformation frequency of Agrobacterium-mediated transformation of Chlamydomonas with acetylsyringone was 311 ± 10.4 (×10⁻⁶ ± SD), which is about 50-fold higher than the transformation frequency without acetylsyringone.

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and used in the induction experiments as described above. Medium pH of 5.6 was chosen, as acidic pH is optimum for *Agrobacterium* vir gene induction. The induction cultures were buffered with 12.5 mM sodium-phosphate buffer of respective pH. β-Galactosidase assay following 24 h induction showed that spent media at both pH 7.0 and 5.6 induced *Agrobacterium* vir genes, with the acidic induction medium giving higher level of induction than the former (Table 2). These results support the possibility of secreted molecules from the *Chlamydomonas* cells to the medium in inducing *Agrobacterium* virulence.

We have also tested the *Chlamydomonas* cell extracts in inducing expression of vir genes. Briefly, 100 ml log phase cultures were pelleted and cells were re-suspended in 4 ml fresh medium and lysed by sonication. Cells debris were removed by centrifugation at 10,000 g for 10 min. The extract was made up to original volume and used for induction after adjusting the pH as earlier. β-Galactosidase assay showed that cell extracts at both pH conditions showed only marginal induction contrary to the spent medium (Table 2). This again indicates that the molecules that are secreted from *Chlamydomonas* cells are responsible for induction. This warrants detailed analysis of the secreted molecules by *Chlamydomonas* cells.

Physical interaction between the host cells and agrobacteria is crucial in DNA transfer, which is initiated by phenolic signalling, vir gene induction and consequent chemotaxis. *Agrobacterium* attachment on *Chlamydomonas* cells was examined using scanning electron microscopy (SEM). Samples collected from the co-cultivation mixture after 48 h were fixed in Karnovsky’s fixative and analysed using a LEO 435 VP SEM. These observations revealed the propensity of *Agrobacterium* cells to attach with *Chlamydomonas* cells. We have found intense bacterial colonization around and close attachment with the algal cells (Figure 1a-d). An equivalent control experiment performed using an unrelated bacteria (Escherichia coli) has shown no evidence of such specific association, indicating that the bacterial attachment on *Chlamydomonas* cells is specific to the transformation process. Moreover, bacterial cells were attached to the algal cells in a polar manner (Figure 1c, d), which has been shown to be a characteristic feature of *Agrobacterium* attachment.

Our results suggest that *Chlamydomonas* cells release compounds that have potential to initiate the virulence and DNA transfer processes in *Agrobacterium*. Therefore, *Chlamydomonas* and may be other related algae could be within the natural host range of *Agrobacterium*. In fact, it was shown that *Chlamydomonas* cells release phenolic compounds into the culture media. It is worth noticing that a chimeric gene (nos :: npt) flanked by the transcriptional regulatory elements, nopaline synthase promoter and polyadenylation sequences obtained from the Ti plasmid of *Agrobacterium* have been functional in *Chlamydomonas* cells. However, further work is needed to throw more light into the interaction between algal cells and agrobacteria. The findings of this study may be useful in refining the recently developed *Agrobacterium* mediated genetic transformation method for *Chlamydomonas* and also in extending the same to other algal systems.

### Table 2. Vir gene induction by spent media and cell extracts of *Chlamydomonas*

<table>
<thead>
<tr>
<th>Inducer</th>
<th>β-Galactosidase activity (Miller units ± SD)</th>
<th>pH = 7</th>
<th>pH = 5.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAP</td>
<td>54.91 ± 1.90</td>
<td>62.55 ± 3.99</td>
<td></td>
</tr>
<tr>
<td>TAP + 2 μM AS</td>
<td>317.75 ± 28.02</td>
<td>476.77 ± 42.74</td>
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<tr>
<td>Spent medium</td>
<td>103.28 ± 6.05</td>
<td>158.23 ± 10.03</td>
<td></td>
</tr>
<tr>
<td>Cell extract</td>
<td>64.84 ± 3.64</td>
<td>81.68 ± 7.71</td>
<td></td>
</tr>
<tr>
<td>Tobacco control</td>
<td>ND</td>
<td>2575 ± 65.50</td>
<td></td>
</tr>
<tr>
<td>(fresh leaf explants)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eggplant control</td>
<td>ND</td>
<td>155 ± 8.60</td>
<td></td>
</tr>
<tr>
<td>(24 h pre-cultured leaf explants)</td>
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ND, Not done.

### Figure 1. a-d. Colonization and attachment of *Agrobacterium tumefaciens* to *Chlamydomonas* cells. Cells after co-cultivation were fixed in Karnovsky’s fixative and analysed by scanning electron microscopy. Bar = 1 μm.


Pre-breeding efforts to utilize two wild *Morus* species

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Among the four species of mulberry available in India, *Morus laevigata* and *M. serrata* are wild and possess unique features of bigger leaf size, higher leaf moisture and moisture retention, higher protein and carbohydrate with greater adaptability to adverse climatic condition. In an effort to transfer these traits to cultivated species, inter-specific hybridization was effected between *M. indica* (var. Kanva-2) and *M. laevigata* as well as *M. indica* (var. Kajli) and *M. serrata*. The present communication reports the successful hybridization of mulberry involving wild and cultivated species. *M. laevigata* was collected from natural populations of Andaman Islands and *M. serrata* from northwestern Himalayan belt. After repeated trial of hybridization, successful F₁ seeds were obtained in both the crosses. The F₁ seeds were sown for seeding and behaviour studies. The F₁ plant (*M. indica × M. laevigata*) showed better performance than the female parent in most of the characters, while it was better than male parent for a few characters. In another cross, the F₁ plant (*M. indica × M. serrata*) showed better performance than both parents for most of the characters. The crosses are expected to carry some genetic load, as the wild species were genetically and geographically distant and carry valuable genes.

Keywords: Hybridization, *Morus* sp., pre-breeding, wild species.

Mulberry is the sole food plant of silkworm. The quality and quantity of cocoon production depend on the quality leaf of mulberry. Among the four species of mulberry reported in India, the present cultivated form of mulberry belongs to *Morus alba* and *M. indica*. Propagation of mulberry through stem cuttings of a particular variety/cultivar makes the plantation almost homogenous. *M. laevigata* collected from Andaman Islands represents the wild species from the mainland, which is found in diploid to tetraploid (2n = 4x = 56) forms. *M. serrata* is another wild species found in India, which is endemic to northwestern Himalayas. In general, mulberry is diploid (2n = 2x = 28), but in natural population of *M. serrata*, the ploidy level varies from diploid to hexaploid (2n = 6x = 84). Both *M. laevigata* and *M. serrata* trees grow in forest areas and are used for a variety of purposes other than sericulture. Till date, these species have not been used for mulberry crop improvement due to their non-availability or suitability for the silkworm industry. The cultivated species exhibits considerable genetic diversity, but the diploid mulberry showed narrow genetic base and threat to genetic erosion.

In order to broaden the genetic base, new gene pools have to be incorporated into those of the cultivated forms. *M. serrata* and *M. laevigata* possess several agronomically important traits, including resistance to abiotic stresses like drought and frost. Earlier attempts of interspecific crosses involving *M. laevigata* and *M. serrata* with cultivated mulberry species showed a reproductive barrier. Some researchers studied the crossability among different *Morus* species and their inheritance pattern. All the reports are preliminary in nature. This study is an attempt to obtain successful hybrids of wild species of *M. laevigata* and *M. serrata* with cultivated species.

The study was undertaken to assess the performance of F₁ hybrids obtained from crosses of wild species, *M. laevigata* and *M. serrata* with *M. indica*, the cultivated form, in an attempt to transfer desirable traits from wild to cultivated species. A comparison between parents and hybrids in respect of morphological, anatomical, reproductive and growth traits was made.

*Morus* species, viz. *M. indica* (var. Kanva-2), *M. indica* (var. Kajli), *M. laevigata* and *M. serrata* maintained in the field gene bank at Central Sericultural Germplasm Resources Centre, Hosur were used for this study. The