Controlling the interplay between *Agrobacterium tumefaciens* and plants during the transient expression of proteins

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In May 2012, the first plant-derived biopharmaceutical protein received full regulatory approval for therapeutic use in humans. Although plant-based expression systems have many advantages, they can suffer from low expression levels and, depending on the species, the presence of potentially toxic secondary metabolites. Transient expression mediated by *Agrobacterium tumefaciens* can be used to increase product yields but may also increase the concentration of secondary metabolites generated by plant defense responses. We have recently investigated the sequence of defense responses triggered by *A. tumefaciens* in tobacco plants and considered how these can be modulated by the transient expression of type III effectors from *Pseudomonas syringae*. Here we discuss the limitations of this approach, potential solutions and additional issues concerning transient expression in plants that should be investigated in greater detail.

*Agrobacterium tumefaciens*-mediated transient expression was the first method used to successfully produce recombinant proteins in plants.1 The scaled-up production of proteins by transient expression can be achieved less than 8 weeks after the corresponding DNA sequence is made available.2 Transient expression is therefore ideal for the provision of emergency biopharmaceuticals, e.g. ZMapp antibodies in response to the epidemic of Ebola virus disease.3

Despite these advantages, transient expression often suffers from batch-to-batch variations in yield,4 potentially conflicting with the requirements of good manufacturing practice.5 These inconsistencies have recently been attributed to variations in the harvest time, plant age and environmental temperature.6,7 In addition to these technical parameters, we have speculated that *A. tumefaciens* may trigger the onset of plant defense responses following injection or infiltration into plant tissues, potentially limiting the efficiency of transient expression and causing the synthesis of unwanted secondary metabolites.8 We found that levels of metabolites such as nicotine, chlorogenic acid and rutin increased by up to 4-fold in plants infiltrated with *A. tumefaciens* compared to controls. Furthermore, infiltration resulted in a >20-fold increase in the number of callose deposits in tobacco leaves.

The defense reactions described above can be triggered by many pathogens.9 However, some pathogens have developed countermeasures to suppress plant defenses at two stages, known as pathogen triggered immunity and effector triggered immunity.10,11 Suppression is mediated by proteins known as type III effectors (TTEs) that are synthesized by the bacterial pathogens and injected into plant cells via type III secretion systems. The TTEs target endogenous plant proteins such as mitogen-activated protein kinases (MPK4), defense-related proteins (RIN4) and heat shock proteins (Hsp70).12

We cloned 5 TTEs (Table 1), transferred the sequences to plant expression vectors and investigated their potential to suppress plant defense responses during transient expression alone, in combination or when paired with target proteins such as a monoclonal antibody or a fluorescent marker protein.8 Even though some TTEs reduced the number of callose deposits and the induction of secondary metabolites in leaves infiltrated with...
A. tumefaciens, they did not increase the concentrations of co-expressed target proteins.

One potential explanation for the limited success of this co-expression strategy is that only 5 TTEs were tested, whereas P. syringae typically produces more than 30 TTEs acting on diverse plant cell proteins.10 Furthermore, TTEs are transferred by P. syringae as proteins and can thus act immediately, whereas their introduction by T-DNA transfer prior to transient expression requires transcription and translation in the plant cell, providing the plant with an opportunity to mount defense responses before the TTEs can affect their cellular targets.12 In future experiments, this drawback could be addressed by expressing TTEs in A. tumefaciens prior to infiltration into plants and genetically fusing the effector proteins to a type IV secretion signal that is recognized by the T-DNA transfer apparatus of the bacteria.13 Thereby, the TTEs would be co-transferred with the T-DNA and made available in the plant cells immediately after infiltration, potentially preventing the onset of plant defense responses. Alternatively, transgenic plants harboring several TTEs under the control of an inducible promoter14 could be used to express the effectors shortly before infiltration with A. tumefaciens. However, the generation of such transgenic plants would be laborious and time consuming because the optimal number, type and combination of TTEs for the suppression of plant defense responses would need to be tested using large panels of transgenic lines. Nevertheless, both strategies would ensure that the TTEs do not accumulate in the plant tissue over time, resulting in the induction of necrosis at 2 d post infiltration as we have previously observed, e.g., necrotic lesions of leaf tissue.8 The concentration of the effectors would be limited by the short interval between their introduction or synthesis and the infiltration process.

The control of secondary metabolite accumulation may not require the sophisticated expression and interplay of different TTEs, but might be achieved instead by changing process conditions such as the lighting used during plant cultivation. As we recently demonstrated, the cultivation of tobacco plants under light emitting diodes (LEDs) in a phytotron reduced the concentration of various secondary metabolites to ~1% of the levels found in plants grown under ambient light and quicksilver or sodium-vapor lamps in a greenhouse, based on the UV signal observed during fast performance liquid chromatography.8 Reduced concentrations of secondary metabolites in the plant biomass will help to ensure these potentially toxic compounds15 are completely removed during downstream processing16 thus ensuring regulatory compliance.5

Table 1. P. syringae type III effectors used for transient co-expression experiments in tobacco

| Type III effector | Activity                        | Cellular target   | Reference |
|-------------------|--------------------------------|-------------------|-----------|
| AvrPtoB           | Ubiquitin ligase               | PAMP receptor     | 22        |
| AvrRpt2           | Cysteine protease              | RIN4              | 23        |
| HopAO1            | Protein tyrosine phosphatase   | Unknown (MAP kinase) | 24    |
| HopF2             | Mono-ADP-ribosyltransferase    | MKK5              | 25        |
| HopI1             | ATPase control                 | Hsp70             | 26        |

Figure 1. Indirect localization of A. tumefaciens in infiltrated tobacco leaves. Sporadic callose deposits (blue) are observed in non-infiltrated tobacco leaves (A, B). More callose spots are observed in tobacco leaves 2 d post infiltration with A. tumefaciens (C, D). These spots appeared predominantly in the vicinity of leaf veins (red). Pseudo-color representation of veins and callose deposits based on light microscopy images (LEICA DRM) with 50× magnification.
Recombinant protein expression levels of 10g L$^{-1}$ have been reported for processes based on mammalian cell cultures, which is much higher than the 0.05g kg$^{-1}$ typically achieved in plants even though Agrobacterium-mediated transient expression can increase yields to 1.0g kg$^{-1}$ in the best cases. In our latest research, we found that bacterial cells infiltrated into tobacco leaves were distributed heterogeneously in the leaf tissue, as indicated by the appearance of callose deposits, a plant defense reaction against penetration attempts by pathogens. We compared the pattern of callose spots with tissue morphology, revealing that the system of leaf veins appeared to exert a sieving effect on the infiltrated bacteria, resulting in their accumulation near the veins (Fig. 1). We therefore speculate that infiltration procedures achieving a more even distribution of bacteria throughout the leaf could increase the yield of recombinant proteins produced by transient expression because the capacity of plant cells for protein biosynthesis would be used more effectively. Several attempts to optimize infiltration procedures have already been described, including the variation of vacuum conditions, changes in the concentration of the infiltrated bacteria and the inclusion of additives (e.g. Silvet L-77) in the infiltration medium. Interestingly, if the concentration of bacteria increased above $1 \times 10^9$ CFU mL$^{-1}$ then the recombinant protein yield declined. This may indicate that bacterial cells block the tissue structures responsible for the sieving effect described above, thereby limiting the distribution of bacteria in the leaves and thus reducing T-DNA transfer and ultimately recombinant protein synthesis.

Ultimately, a better understanding of the mechanisms of transient protein expression in plants will help to enhance product yields and reduce production costs. It will also improve process control, facilitating regulatory acceptance and improving the economic competitiveness of this production platform.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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