Agrobacterium-Mediated Transient Gene Expression and Silencing: A Rapid Tool for Functional Gene Assay in Potato

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Abstract

Potato (Solanum tuberosum) is the third most important food crop in the world, next only to rice and wheat. However, genetic and genomic research of potato has lagged behind other major crops due to the autopolyplody and highly heterozygous nature associated with the potato genome. Reliable and technically undemanding techniques are not available for functional gene assays in potato. Here we report the development of a transient gene expression and silencing system in potato. Gene expression or RNAi-based gene silencing constructs were delivered into potato leaf cells using Agrobacterium-mediated infiltration. Agroinfiltration of various gene constructs consistently resulted in potato cell transformation and spread of the transgenic cells around infiltration zones. The efficiency of agroinfiltration was affected by potato genotypes, concentration of Agrobacterium, and plant growth conditions. We demonstrated that the agroinfiltration-based transient gene expression can be used to detect potato proteins in sub-cellular compartments in living cells. We established a double agroinfiltration procedure that allows to test whether a specific gene is associated with potato late blight resistance pathway mediated by the resistance gene R8. This procedure provides a powerful approach for high throughput functional assay for a large number of candidate genes in potato late blight resistance.

Introduction

Potato (Solanum tuberosum) is the third most important food crop in the world, next only to rice and wheat. However, genetic and genomic research of potato has lagged behind most major crops. Functional discovery of genes in potato is still a lengthy process and is often hampered by the complex characteristics associated with the potato genome, including autotetraploidy, self-incompatibility, and high heterozygosity. Although several gene discovery tools have been used in potato research, including transposon-based insertional mutagenesis [1,2], gene activation-tagging [3], and map-based cloning [4,5], applications of these techniques were time-consuming, resource-intensive, and technically challenging. RNA interference (RNAi)-based potato gene silencing has recently been reported by several laboratories [6,7,8,9,10,11,12,13]. However, the RNAi technique relies on the traditional transformation procedure and is a low throughput methodology. It takes on average six months to develop a transgenic potato line using RNAi constructs. Therefore, this technique can only be used to target a limited number of potato genes.

Transient gene assays are convenient alternatives to stable transformation because such techniques allow a rapid analysis of gene function. Early successful transient gene assay in potato was demonstrated using a microprojectile bombardment-based approach [14]. Virus-induced gene silencing (VIGS) has been successfully used in several plant species, including potato [15,16]. However, VIGS has not yet been proven to be a straightforward technique that can be readily adapted in different potato laboratories. As a similar approach to VIGS, transient gene expression was also accomplished by infection of an Agrobacterium tumefaciens strain carrying a potato virus X (PVX)-based binary vector [17]. This approach was successfully used in high-throughput screening for specific recognition of INF elicitors of Phytophthora infestans in different Solanum species [17]. Leaf infiltration of Agrobacterium is another popular method for transient gene functional assay. The agroinfiltration has been best used in Nicotiana benthamiana [18], although it has also been successfully applied to several other plant species, including Arabidopsis thaliana [19], tobacco [20,21], tomato [22], lettuce [19,23], and grapevine [24,25]. To our knowledge, Agrobacterium-mediated infiltration for rapid functional gene assays without involving a viral based system has not been reported in potato.

An international Potato Genome Sequencing Consortium (PGSC) has been established (http://potatogenome.net) and is expected to fully sequence the 850 Mb potato genome by the end of 2010. This soon available genome sequence will dramatically change the genetic and genomic research of potato. One of the most urgently needed tools is a reliable, efficient, and high throughput technique for discovery and characterization of
We have developed an Agrobacterium-mediated infiltration procedure in potato. Gene expression or RNAi-based gene silencing constructs can be delivered into potato leaf cells using agroinfiltration. We demonstrated that the agroinfiltration technique can be used as a rapid gene assay tool to localize protein expression in sub-cellular compartments and to determine the role of candidate genes in R-gene mediated potato late blight resistance.

**Results and Discussion**

I. Transient gene expression mediated by agroinfiltration

Infiltration and plant material optimization. The efficiency and versatility of the agroinfiltration technique in *N. benthamiana* prompted us to test the possibility to adapt a similar approach in potato. Our initial experiments using previously established protocols in *N. benthamiana* only resulted in limited success with a low transformation efficiency. To optimize the procedure in potato, Katahdin, a cultivar highly amenable to whole plant transformation, was used to infiltrate the leaves at various stages of growth. A potato RAR1:: GFP (Required for *Mla12* Resistance, Green Fluorescent Protein) construct, which also contained the *DsRED1* (Red Fluorescent Protein) reporter, was used to optimize all the infiltration conditions. The presence and spread of transgenic cells around the infiltration zones were identified based on red fluorescence under an epifluorescence microscope (Fig. 1). In contrast, no *DsRED1* fluorescence was observed from un-infiltrated potato leaves.

We tested the potato leaves at various growth ages and found that the infiltration was consistently most efficient when using terminal leaflets from 5–6 week-old potato plants. We noticed that the leaflets from middle or lower positioned leaves with less pubescence were easier to infiltrate. The efficiency of infiltration became significantly lower when leaves from 3–4 week old plants were used in the experiments. We then investigated whether the concentration of the *Agrobacterium* cultures had any effect on the outcome of infiltration. Cultures resuspended to OD600 = 0.2–0.5 resulted in the best transient gene expression activity. In order to suppress the silencing of the transgene, we co-infiltrated the silencing suppressor P19 [27] together with the transgene. However, the introduction of P19 appeared to have no effect on the expression of transgenes. We used two different *Agrobacterium* strains, GV3101 and LBA4404, for infiltration. GV3101 showed a higher efficiency than LBA4404 in the experiments, which confirms the high efficiency of GV3101 reported in *N. benthamiana* [21,26].

Evaluation of transient expression using different potato genotypes. We infiltrated leaves from potato cultivars Katahdin, Atlantic, Megachip, USW1, and a wild diploid species *Solanum bulbocastanum* in order to investigate the influence of potato genotypes on the efficiency of transient expression. We observed a considerable variation for the intensity of the *DsRED1* fluorescence from different potato genotypes.

![Figure 1. Red fluorescence derived from *DsRED1* six days after agroinfiltration into potato leaves.](https://example.com/figure1.png)

(A) Red fluorescence from a single infiltration site on Katahdin. (B) The same infiltration site as (A) under bright field. (C) Red fluorescence from a single infiltration site on Atlantic. (D) The same infiltration site as (C) under bright field. (E) Red fluorescence from a single infiltration site on USW1. No transgenic cells were detected on this image. The strong red fluorescence signals in this infiltration site were derived from autofluorescence associated with the necrotic tissue. (F) The same infiltration site as (E) under bright field. All bars are 10 mm. doi:10.1371/journal.pone.0005812.g001
The St invertase (V-Inv) protein, which is required for the initiation of legume nodulation, has previously been shown to be localized in the nucleus [32,33]. We expressed the MDME3::GFP fusion protein into the potato cells. The GFP signals were predominantly localized in the nucleus in most of the transgenic cells (Fig. 2F). These results show that the agroinfiltration-based technique can be used to study both native and heterologous proteins in potato.

II. RNAi-based transient gene silencing mediated by agroinfiltration

Optimization of the transient silencing procedure. We initially used the established optimal agroinfiltration procedure described above for RNAi-based transient gene silencing experiments. However, we observed that young and fully expanded leaves from 3–5 week-old plants were the best plant materials for transient gene silencing. Maximum silencing levels were obtained when Agrobacterium inoculum was diluted to an OD600 value of 0.3–0.7. A low concentration (OD600<0.1) of bacterial suspension resulted in poor transformation thus leading to lower silencing levels, while high concentration (OD600>1.0) occasionally produced necrotic spots around the infiltration zones. We used a previously developed Rar1-RNAi construct [9] in our initial transient gene silencing experiments. This construct was agroinfiltrated into Katahdin leaves. Semi-quantitative RT-PCR was used to confirm the suppression of the potato Rar1 gene in the leaf tissues around the infiltration zone. The Rar1 transcript was significantly reduced compared with the controls (Fig. 3). Reduction of the Rar1 transcript was generally observed 5 and 6 days post infiltration (dpi) and persisted until 8 dpi. However, partial reduction of the Rar1 transcripts started as early as 3–4 dpi in 20–30% of the leaves analyzed. The reduction of the Rar1 transcript compared to the control leaves was as much as 90–99% in different experiments (Fig. 3). Our results are in accordance with the previous finding in N. benthamiana that the production of siRNAs for the target gene in the infiltrated zones started as early as 2 days post infiltration and reached a peak abundance by day 5 [34].

Rapid late blight resistance assay using RNAi-based transient silencing. Agroinfiltration assay was successfully used for screening candidate signaling components required for the activation of R-gene mediated disease resistance in N. benthamiana and tomato [26,35]. We intended to develop a similar technique to screen the candidate genes required for the late blight resistance mediated by the RB gene. Gene RB carries a broad-spectrum resistance against the late blight pathogen Phytophthora infestans [5] and recognizes the P. infestans effector, IpiO1 [28]. In our procedure an RNI construct developed against a candidate gene was first introduced into a RB-containing potato plant by agroinfiltration, which will silence the target gene. Four days after agroinfiltration, the second construct containing the IpiO1 gene was agroinfiltrated at the same site. This double infiltration would result in a hypersensitive response (HR) phenotype if the candidate gene is not involved in the RB-mediated resistance, because the silencing of this candidate gene will not affect the resistance. However, no HR would be observed if the candidate gene is involved in RB-mediated resistance.

A transgenic Katahdin line SP925, which contains the RB gene, was used in the double agroinfiltration experiments. The Agrobacterium strain GV3101 produced few or no necrotic spots on RB-Katahdin leaves 7–12 dpi (Fig. 4). In contrast, infiltration with effector IpiO1 in the same plant resulted in a confluent necrosis response starting at 3–5 dpi (Fig. 4). Infiltrations with silencing construct alone or mock infiltration did not produce any...
| GFP signals | Plastids | Bright field | Merged |
|------------|----------|--------------|--------|
| A Empty vector | ![Image](image1) | ![Image](image2) | ![Image](image3) |
| B 35S::GFP | ![Image](image4) | ![Image](image5) | ![Image](image6) |
| C RAR1::GFP | ![Image](image7) | ![Image](image8) | ![Image](image9) |
| D GS2::GFP | ![Image](image10) | ![Image](image11) | ![Image](image12) |
| E V-INV::GFP | ![Image](image13) | ![Image](image14) | ![Image](image15) |
| F M/DM13::GFP | ![Image](image16) | ![Image](image17) | ![Image](image18) |
background effect on potato leaves observed until 10 dpi, although few necrotic spots emerged 10 dpi even in the control experiments, which may be caused by the natural senescence of the tissues around the site of infiltration.

The Rar1 and Sgt1 genes have been extensively studied for their roles in the regulation of disease resistance genes [36]. We have previously demonstrated that SGT1, but not RAR1, is essential for the RB-mediated late blight resistance in potato [9]. We performed double agroinfiltration using Rar1-RNAi and Sgt1-RNAi constructs together with the IpiO1 gene construct. Eight days after the first infiltration, a clear HR response was observed around the infiltrated sites on potato leaves double infiltrated with Rar1-RNAi constructs and IpiO1 (Fig. 4). In contrast, no HR was observed around the infiltrated sites of Sgt1-RNAi and IpiO1 (Fig. 4). These results showed that RB activation by IpiO1 depends on SGT1 but not RAR1, which is consistent with our whole plant stable transformation results [9].

Late blight is the most devastating potato disease worldwide and is also the most extensively studied potato disease [37]. Several late blight resistance genes, including both race-specific and race-non-specific genes, have been cloned in recent years [4,5,38,39,40,41]. However, very limited effort so far has been devoted to understand the resistance pathways mediated by any of these genes. This is at least partially due to the lack of tools in potato for rapid analysis of candidate genes associated with resistance or signaling pathway. The double agroinfiltration technique developed in this study will provide a powerful tool to fill this need in the future.

Conclusions

The soon available potato genome sequence will dramatically accelerate our pace to identify genomically important potato genes. The power of comparative genomics will also allow us to discover potato genes based on the information from other extensively studied model plant species. Thus, a rapid and simple functional gene assay tool is urgently needed for potato genetics and molecular biology research. We demonstrate that Agrobacterium-mediated infiltration, which has been an effective gene delivering technique in several plant species, can be adapted in potato. Katahdin, a potato cultivar that is highly amenable for Agrobacterium-mediated whole plant transformation, showed the highest efficiency for agroinfiltration in our study. However, it will be possible to identify potato cultivars (genotypes) that have greater efficiency for agroinfiltration than Katahdin. Agroinfiltration of GFP-based gene expression constructs into potato leaf cells is a simple and highly efficient approach to examine protein expression in sub-cellular compartments. We also demonstrate that double agroinfiltration of RNAi-based silencing construct and a late blight pathogen effector can be used for screening candidate genes involved in late blight resistance pathway mediated by the corresponding resistance gene. This double agroinfiltration approach is simple and fast compared to the traditional approach consisting of stable transformation followed by disease resistance evaluation [9]. It can be readily adapted to dissect the resistance pathways mediated by a wide range of potato R genes in the future.

Materials and Methods

Plant materials

Potato cultivars Katahdin, Atlantic, Megachip, USW1 (a haploid clone derived from Katahdin), and a diploid wild potato species S. bulbocastanum were used for transient gene expression experiments. Katahdin and a RB-transgenic Katahdin line, SP925 [42] were used for RNAi-based transient silencing assays. All the plants were maintained in the greenhouse facility of the University of Wisconsin-Madison. Growth conditions with 70% humidity, 16-h day/8-h night regime, 19°C/15°C, 500 μmol m⁻² s⁻¹ light were applied.

Plasmid construction and detection

The constructs used for transient gene expression and localization studies were derived from binary vector pK7FWG2, which allows C-terminal fusion of protein of choice with GFP [43]. These vectors were obtained from the Ghent University, Belgium. A modified pK7FWG2-R vector containing an additional DsRED1 marker driven by 35S-UBQ10 [32] was used to evaluate the transformation efficiency in different potato genotypes. The 675-bp CDS of the potato Rar1 gene (TIGR potato EST T121848; http://compbio.dlci.harvard.edu/cgi-bin/tgi/tgi/report.pl?gudb=potato) was amplified without its stop codon using the primers 5′-CACC ATG GAG AGA CTT CGA TGT CAG AGG-3′ (forward) and 5′-GGA CAC TGG GTC AGC GTT GTG C-3′ (reverse). The sequence verified PCR products were cloned into the pENTR/D-TOPO vector using the pENTR Directional TOPO Cloning kit (Invitrogen, Carlsbad, California). The products were then transferred to the pK7FWG2 vector by LR recombination reaction (Invitrogen), resulting in 35S:-RAR1::GFP fusion. The same

Figure 3. RT-PCR analysis of transient silencing of the potato Rar1 gene in two independent potato leaves (A and B). Leaf samples around the infiltrated spots were collected at days 1, 2, 5 and 6 dpi. Lane 1: 100 bp DNA ladder marker; Lane 2: leaf sample from uninfiltrated control; Lane 3: leaf from infiltrated site 1 dpi; Lane 4: leaf from infiltrated site 2 dpi; Lane 5: leaf from infiltrated site 5 dpi; Lane 6: leaf from infiltrated site 6 dpi. Actin was amplified as a control for the amount of template. The amplified Rar1 and Actin transcripts are 339 bp and 360 bp, respectively.

doi:10.1371/journal.pone.0005812.g003
products were also transferred to pK7FWG2-R modified binary vector. A 1642 bp CDS of the potato Glutathione Synthetase gene (GenBank Accession, AF017984) without a stop codon was amplified using the primers 5'-CACC ATG GGC AGC GGC TGT TCT TCT CCA-3' (forward) and 5'-AAC CAA GTA TAT ACT GTC CAA AA-3' (reverse), transferred into binary vector pK7FWG2 to obtain 35S::StGS2::GFP fusion. Similarly, a 2,030-bp CDS of the potato Vacuolar Invertase gene (TIGR Accession ID, TC132799) was amplified without a stop codon using the primers 5'-CACC ATG GCC ACC CAG TAC CAT TCC AGT-3' (reverse), transferred into binary vector pK7FWG2 to obtain 35S::StV-INV::GFP fusion. A MtDMI3::GFP fusion construct was described previously [32,33]. All PCR amplifications were performed using Platinum Taq DNA polymerase (Invitrogen) and leaf cDNA from Katahdin was used in all amplifications. Electro-competent cells of A. tumefaciens strain GV3101 were prepared and transformed as described previously [44]. Agrobacterium was also transformed with empty vectors pK7FWG2 and pK7FWG2-R to generate control plasmids. Transformation was achieved by using 2 μl of purified construct DNA per 30 μl of Agrobacterium competent cells (GV3101) shocked at 2.2 kV voltage and 25 μF capacitance using an electroporator. After two hours of incubation at 28°C, the cells were plated on selective media (spectinomycin, gentamycin, or rifamycin) and grown for 1–3 days at 28°C. For transient gene silencing assay, Agrobacterium strain LBA4404 harboring either Rar1-RNAi or Sgt1-RNAi construct [9] was used as previously described for transient silencing experiments. An empty vector construct was obtained by transforming Agrobacterium strain LBA4404 with the pHellsgate-8 silencing plasmid [45]. The same electroporation method was followed described above for obtaining fusions. Agrobacterium strain, GV3101 harboring pGR106-IpiO1 (effector) was described previously [28].

**Agroinfiltration and imaging**

A single colony of recombinant Agrobacterium strain of GV3101 was cultured in 5 ml of LB culture containing antibiotics spectinomycin (50 mg/ml) and rifamycin (25 mg/ml) and grown overnight (28°C at 225 rpm). A large LB media suspension was then inoculated with the overnight culture and grown at 28°C to an OD600 of ~1.0. cells. The cells were harvested by centrifugation at 5500 rpm for 2 min
and resuspended in 1 ml of infiltration buffer (10 mM MgCl₂ and 100 μM acetylsyringone). This step was repeated at least once and the concentration of bacterial suspension was measured by spectrophotometry (OD₆₀₀) and adjusted to a final desired concentration with the infiltration buffer and left at room temperature for 1–2 h. An OD₆₀₀ of 0.2–0.5 was adjusted for transient expression studies. The bacterial suspension was taken in a syringe and infiltrated through the abaxial surface of the leaf. Before infiltration, a small incision was made at the site of infiltration using a sterile toothpick to enhance the efficiency of infiltration. Five to six days after infiltration, the localization of GFP-fused proteins was observed using Zeiss LSM 510 meta inverted confocal laser microscope. A 1 x 1 cm² leaf section was taken 3–5 cm away from the infiltration zone for GFP signal analysis. The DRED fluorescence was observed by separating infiltrated leaf from the plant, 6 days post infiltration, and observed under Leica MZ16-F epifluorescence microscope under a DRED filter set. The same procedure described above for transient expression assays with only minor modifications. A. tumefaciens cells were harvested by centrifuging at 5500 rpm for 20 min and resuspended in 5 ml infiltration buffer containing 10 mM MES (pH 5.7), 10 mM MgCl₂ and 100 μM acetylsyringone. An OD₆₀₀ of 0.3–0.7 was adjusted for transient silencing studies and suspensions were left at room temperature for 3 hrs. For double agroinfiltration experiments, leaves from a RB-containing transgenic Katabehin line (SP295) were infiltrated with a mixture strain carrying either Rar1-RNAi or Sgfl-RNAi construct [9] with an OD₆₀₀ of 0.3–0.7. Four days later, the same infiltration sites were challenged with an A. tumefaciens strain carrying the pGR106-Ipio1 plasmid. A similar OD₆₀₀ of 0.5–0.7 was adjusted for these experiments.

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**Acknowledgments**

We are grateful to Dr. Sophien Kamoun for providing the pGR106-Ipio1 plasmid, which was the key resource for the double infiltration experiments.

**Author Contributions**

Conceived and designed the experiments: PBB JJ. Performed the experiments: PBB MV LW. Analyzed the data: PBB MV LW JJ. Contributed reagents/materials/analysis tools: JMA. Wrote the paper: PBB JJ.
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