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Agroinfiltration of Strawberry Fruit — A Powerful Transient Expression System for Gene Validation☆

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ABSTRACT

Agrobacterium-infiltration (agroinfiltration) transient expression systems facilitate rapid primary characterization of candidate gene function, protein localization, protein interacting partners, or other information from a delivered construct. Most of these characterizations have been performed in *Nicotiana benthamiana* leaves, which offer a limited set of cell types, metabolites, and developmental plasticity. As efforts to characterize genes associated with fruit and vegetable quality increase, the strawberry fruit has emerged as a useful tool to study the effects of transient gene expression. Introduction of active *Agrobacterium* cultures bearing loss- or gain-of-function constructs into developing fruits can provide robust gene silencing or modest overexpression within days of treatment. Fruits can then be assessed for transgene effects on endogenous gene expression, metabolism, physiology or development. The approach also allows introduction of VIGS-mediated silencing constructs as well as the assessment of promoter activity in fruits. This review details how the strawberry agroinfiltration system has been used as an efficient system to characterize an increasing number of genes associated with fruit development, physiology and metabolism.

Contents

1. Introduction 20
   1.1. Strawberry (*Fragaria* spp.) 20
2. Overexpression and hpRNAi gene silencing 20
   2.1. Manipulation of metabolic pathways 26
      2.1.1. Biosynthetic genes 26
      2.1.2. Transcriptional regulators 28

Abreviations: AAT, alcohol acyltransferase 2; ABA, Abscisic Acid; ABI, ABA Insensitive; ANR, Anthocyanin Reductase; ANS, Anthocyanin Synthase; APG, Acylphloroglucinol; ASR, ABA-stress Ripening; BG, β-Glucosidase; bHLH, Basic-helix-loop-helix; BR, Brassinosteroid; BRI, BR Receptor; CAD, Cinnamyl Alcohol Dehydrogenase; CaMV, Cauliflower Mosaic Virus; CCR, Cinnamoyl-CoA Reductase; CHI, Chalcone Isomerase; CHLH/ABAR, plastid/chloroplast ABA receptor, magnesium chelatase H subunit; CHS, Chalcone Synthase; CTR, Constitutive Triple Response; DAA, Days After Anthesis; DFR, Dihydroflavonol 4-Reductase; DMMF, 2,5-dimethyl-4-methoxy-3(2H)-furanone; dsRNA, double-stranded RNA; EG, Endo-β-1,4-Glucanase; EGS, Eugenol Synthase; EOB, Emission of Benzoid; EXP, Expansin; F3′H, Flavonoid 3′-Hydroxylase; F3H, Flavanone 3-Hydroxylase; Fa, *Fragaria x ananassa*; FGT, Anthocyanidin Glucosyltransferase; FLS, Flavonol Synthase; Fra a, Homolog of the major birch pollen allergen Bet v 1; Fv, *Fragaria x vesca*; GA, Gibberellins/Gibberellic Acid; GAL, β −galactosidase; GalUR, d-Galacturonate Reductase; GAMYB, MYB transcription factor up-regulated by GA; GT, Glycosyltransferase; GUS, β-glucuronidase; HDMF, 4-hydroxy-2,5-dimethyl-3(2H)-furanone; hpRNAi, hairpin RNAi; IGS, Isoeugenol Synthase; ihpRNAi, Intron hpRNAi; JA, Jasmonate/Jasmonic Acid; LAR, Leucoanthocyanidin Reductase; LUC, Luciferase; MBL, Mannose-binding Lectin; MSC, Multiple Cloning Site; MYB, Myeloblastosis; NCED, 9-*cis*-epoxycarotenoid dioxygenase; NIP, Nodulin 26-like Intrinsic Protein; Ob, *Ocimum basilicum*; OST, Open Stomata; PAL, Phenylalanine Ammonia Lyase; PDS, Phytoene Desaturase; PE, Pectin Methyltransferase; PG, Polygalacturonase; Ph, *Petunia hybrid*; PHOT, Phototropin; PL, Pectate Lyase; POD, Peroxidase; PYR, Pyrabactin Resistance; QR, Quinone Oxiredutase; QTL, Quantitative Trait Locus; RG, Rhamnogalacturonan; RNAi, RNA interference; SAMS, S-adenosylmethionine Synthetase; SCL, Scarecrow-like; SHP, Shaterproof-like; SnRK, Sucrose Nonfermenting-related Protein Kinase; SPT, Spatula; SUT, Sucrose Transporter; TF, Transcription Factor; TRV, Tobacco Rattle Virus; UDP, Uridine diphosphate; UGT, UDP-dependent Glycosyltransferase; VIGS, Virus-Induced Gene Silencing; VPS, Valerophenone Synthase; XYL, β-xylosidase.

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2.1.3. Novel functions and regulators 29
2.2. Fruit development and ripening 29
2.2.1. Transcriptional regulators 29
2.2.2. Crosstalk between signaling pathways 30
2.2.3. Cell wall structure 32
2.3. Response to stress 32
3. RNAi virus-induced gene silencing 33
4. Promoter analyses 35
5. Conclusions 35
Funding 36
References 36

1. Introduction

While whole genome and transcriptome data continue to accumulate, the process of developing relationships between sequence and function remains a central goal of modern biology. Transient expression of genic sequences in plants has been a valuable tool to infer function, speed gene characterization, study protein localization, or even manufacture industrial-scale protein products. Most reports describe using the leaves of *Nicotiana benthamiana*. A transformed *Agrobacterium tumefaciens* culture is pressed into the leaf, and allowed to colonize. Transgenes are transiently, or perhaps stably, expressed within several days after introduction. The technique has many advantages over other transient expression methods, such as particle bombardment, microinjection or protoplast expression.

But a leaf only offers one set of cell types, substrates and developmental processes, and may not be as useful in functionalizing the majority of plant transcripts. For instance, the genes that control fruit development, quality, post-harvest performance, consumer liking, nutrient accumulation, and many other attributes have been the center of intense study. At the same time, the plants that produce fruits and vegetable products oftentimes have long generation times, substantial juvenility periods, or are sometimes nearly impossible to stably transform. Over the last decade a number of laboratories have explored and developed agroinfiltration of strawberry fruits as a means to test loss- and gain-of-function effects of candidate transcripts. The fruits are abundant on relatively small, herbaceous plants. Transcriptome analyses have detected over 15,000 transcripts in the fleshy receptacle and achenes [1], and the "fruits" present a variety of tissues and a diverse suite of metabolites. Many research groups have exploited this system to test hypotheses concerning flavors, aromas, firmness, ripening, and other aspects of fruit biology.

1.1. *Strawberry (*Fragaria *spp.)*

The species of the genus *Fragaria* are commonly referred to as strawberries, and represent twenty-one species ranging over many levels of ploidy. Over evolutionary time several of the diploid wild species have undergone polyploidization, leading to the emergence of auto- and allopolyploid genotypes. Human intervention improved the natural germplasm and ultimately merged South and North American genetics together to create the modern commercial strawberry, *Fragaria* × *ananassa*. Strawberry is a valuable commercial crop with a value of $3 B worldwide.

But strawberry also has great value in the laboratory as a functional genomics system. Strawberry is a member of the Rosaceae family, a collection of crop species including apples, peaches, cherries, almonds, roses and blackberries. Unlike its rosaceous crop relatives, the small herbaceous strawberry grows rapidly and produces fruit quickly. It can be easily propagated clonally by runners or by splitting off vegetative propagules called branch crowns. All of these attributes make strawberry a useful surrogate for studies of genes isolated from other crops with large spatial demands and long juvenility periods.

The woodland strawberry (*F. vesca*) is commonly used as a model for gene function studies. An ancestor of this extant species is known to be a constituent of the commercial strawberry's subgenome, it is easily transformable, and a full genome sequence is available. These aspects render diploid strawberry as a useful model for some aspects of gene discovery and functional validation.

However, the diploid strawberry is insufficient to fully examine fruit characteristics, as the fruits are small and fragile, and do not match the commercial varieties in size, firmness, post-harvest quality or metabolism. Stable transformation of octoploid varieties can however be laborious and time consuming, and transient transformation is faster and may be a best first approach for gene functional studies.

The purpose of this review is to examine the state of the art for using the octoploid *F.* × *ananassa* strawberry as a model for exploring gene function. The current literature concerning various transient expression and silencing methods is reviewed, and describes the utility of the strawberry as a means to establish at least preliminary validation of gene function. Previous reviews have provided (short/general) overviews of transient expression in strawberry [2,3]. Here we present a more extensive approach on this subject, focusing on technical details, including cloning vectors, *Agrobacterium* strains, strawberry cultivars, timing of infiltration and harvesting, as well as a synopsis of the knowledge generated from these assays. Outcomes are discussed from analysis of genes associated with metabolic pathways responsible for fruit flavor and aroma to the mechanisms underlying fruit development and ripening.

2. Overexpression and hpRNAi gene silencing

Characterization of gene function can be achieved by overexpressing or downregulating the gene of interest, and then examining the resulting physiological and molecular outcomes. Overexpression involves cloning of a gene under a strong promoter, usually the CaMV 35S promoter, and introduction of the vector into the biological study system. Downregulation can be achieved by post-transcriptional gene silencing, such as RNA interference (RNAi). RNAi relies on the formation of double-stranded RNAs (dsRNAs), which are synthesized with specific sequences complementary to a gene of interest and introduced into a biological system. Double-stranded RNAs are then recognized as exogenous genetic material, leading to activation of the RNA silencing pathway. There are two major RNAi cloning approaches. The intron hairpin RNAi (ihRNAi) involves cloning of fragments of the target gene in sense and antisense orientation in the same construct and separated by an intron sequence. Fragments vary roughly from 300 to 600 bp. The intron sequence is derived from a variety of sources depending on the vector used. When the sense and anti-