Review Article

OVERVIEW AND APPLICATIONS OF RNA SILENCING IN FRUIT CROPS

ABSTRACT

RNA silencing has recently gain momentum in the scientific world mainly due to its sequencing-specific gene inactivation that is conserved in among various organism including animals and plants. In fruit crops, various mechanism such as virus-induced gene silencing (VIGS), DNA methylation, Ribonucleic acid interference (RNAi) and Anti-sense mediated gene silencing has been reported. These epigenetic regulatory mechanisms are highly useful in fruit crops as it suppresses or silences gene responsible for undesirable morpho-agronomic characters.

Key words: Fruit crops, RNA silencing, Methylation, VIGS

1. INTRODUCTION

RNA silencing or gene silencing can be described as a molecular process involved in the down regulation of specific genes and probably evolved as a genetic defense system against viruses and invading nucleic acids (Brigneti *et al*., 1998; Voinnet *et al*., 2000; Waterhouse *et al*., 2001 and Wassenegger, 2002) . Gene silencing defines the epigenetic regulation of a gene at the level of transcription or translation to suppress gene expression. Gene silencing is similar to gene ‘knock-down’ but different from gene ‘knock-out’. When a gene is silenced, its expression is reduced or masked but in the flower colour experiment in petunia, the silencing affected both the transgene and any endogenous genes with a similar sequence *i.e.* there was co-ordinate suppression or co-suppression due to the presence of homologous transgene. The resulting plant produced white flowers because neither the transgene nor the endogenous gene was adequately effective to assist pigment production (Jorgensen, 1995).

Gene silencing can be executed at transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) (Gura, 2000). At present, there are many ways of RNA silencing reported in plants such as RNA interference (RNAi) (Vaucheret *et al*., 2001; Hoffmann *et al*., 2006), virus-induced gene silencing (VIGS) (Lu *et al*., 2003; Burch-Smith *et al*., 2004; Jaakola *et al*., 2010 and Chai *et al*., 2011), DNA methylation (Trusov and Botella, 2006), antisense-mediated gene silencing (Pose *et al*., 2013) and microRNA silencing (Bartel, 2004).

Gene silencing in fruit crops has recently gain momentum. Gene silencing work in fruit crops has been reported in *Citrus aurantifolia* (Christ.) Swing (Domínguez *et al*., 2002 and Fagoaga *et al*., 2006), *Prunus* sp. (Ravelonandro *et al*., 2000; Scorza *et al*., 2001; Hily *et al*., 2004; Di Nicola-Negri *et al*., 2005 and Zhang *et al*., 2006), *Malus domestica* Borkh (Ko *et al*., 1998; Viss *et al*., 2003; Dandekar *et al*., 2004;

Broothaerts *et al*., 2004; Gilissen *et al*., 2005 and Teo *et al*., 2006), *Juglans regia* L. (Escobar *et al*., 2002), strawberry (Hoffmann *et al*., 2006 and Chai *et al*., 2011), pineapple (Trusov and Botella, 2006) and bilberry (Jaakola *et al*., 2010).

Grafting is a technique in which scions and rootstocks with different genomes are joined, has been used in horticulture for thousands of years, and is commonly employed for crops like tomatoes, cucurbits, and fruit trees (Melnyk and Meyerowitz, 2015). One compelling strategy would be to use transgenic rootstocks expressing small RNAs to trigger RNA silencing in non-transgenic scions. Recently, this approach was used to transmit virus resistance in *Nicotiana benthamiana* (Ali *et al*., 2013). This strategy would most easily be applied to improve plants that are widely cultivated using grafting, such as fruit trees (Gohlke and Mosher, 2015). Although transmission of RNA silencing was not observed in non-transgenic scions in apple (Flachowsky *et al*., 2012), a more recent study in cherry trees demonstrates that transgene-derived small RNAs can indeed be transported into non-transgenic scions (Zhao and Song, 2014).

2. APPLICATIONS IN FRUIT CROPS

2.1 VIRUS-INDUCED GENE SILENCING (VIGS)

Virus-induced gene silencing is a technology that employs an RNA-mediated antiviral defense mechanism. When a plant virus infects a host cell, it stimulates an RNA-based defense that is targeted against the viral genome (Ratcliff *et al*., 1995). This system involves processing of double-stranded RNA (dsRNA) into short interfering RNA (siRNA) (Zamore, 2001). An RNAse complex is then steered by base pairing of the siRNA so that it precisely targets single-stranded RNA (ssRNA) that is identical to the dsRNA. VIGS can be defined as the silencing of endogenous plant genes initiated by recombinant viral factors (Ruiz *et al*., 1998). It is designed to suppress gene expression and study gene function in plants (Robertson, 2004).

Bilberry (*Vaccinium myrtillus*) is one of the incomparable sources of health promoting phytochemicals such as anthocyanins, the ripe fruit typically contains 29 mg/g of dry weight (Latti *et al*., 2008). In many fruits, these coloured compounds accumulate only in the skin but in bilberry, they occur throughout the fruit flesh. A SQUAMOSA-class MADS box transcription factor, *VmTDR4* is associated with anthocyanin biosynthesis in bilberry (Jaakola *et al*., 2011). *Vm*TDR4 is a bilberry homologue of the tomato *TDR4* and *Arabidopsis FUL* genes. By using VIGS, the expression of *VmTDR4* was suppressed resulting in substantial reduction in anthocyanin level in fully ripe bilberry fruits. Thus, it was found that *VmTDR4* plays a crucial role in the accumulation of anthocyanin during ripening in bilberry (Jaakola *et al*., 2011). Therefore, application of VIGS in other anthocyanin generating fruits such as black currant (*Ribes nigrum*), blackberry (*Rubus fruticosus*) and strawberry (*Fragaria* spp.) will be helpful to study anthocyanin-related gene function in these plants (Jaakola *et al.,* 2011).

The plant hormone abscisic acid (ABA) plays an important role in the regulation of non-climacteric fruit ripening. Strawberry, a non-climacteric fruit do not exhibit a peak in respiration and ethylene production during ripening and the application of ethylene to green strawberry fruits does not affect the rate of ripening (Knee *et al*., 1977; Given *et al*., 1988 and Abeles and Takeda, 1990). However, the ABA content gradually accumulates and promotes fruit ripening during the later stage of strawberry fruit development (Kano and Asahira, 1981; Manning, 1994; Perkins-Veazie, 1995 and Jiang and Joyce, 2003). The molecular or genetic evidence for this phenomenon is lacking. *Fa*PYR1, a strawberry gene homologous to the *Arabidopsis* ABA receptor gene PYR1, is associated with delayed ripening (Chai *et al*., 2011). By using tobacco rattle virus (TRV) induced VIGS, the *FaPYR1* gene can be silenced in strawberry fruit (Chai *et al*., 2011). TRV-mediated VIGS is also a potential tool in studying tomato fruit development and ripening (Fu *et al*., 2005).

For silencing the *Fa*PYR1 gene in strawberry fruit development, a mixture of *Agrobacterium* strain GV3101 cultures containing pTRV1 and pTRV2 carrying a 424 bp fragment of the *FaPYR1* gene in a 1:1 ratio was syringe infiltrated into 2 weeks old BG (big green) fruits and control fruits were infiltrated only with TRV alone (Fig. 1). Two weeks after infiltration, control fruits turned fully red while RNAi (Ribonucleic acid interference) fruits produced various chimeric symptoms which were concomitant with variations in the decrease of transcripts of *FaPYR1*. These findings showed that *FaPYR1* plays an important role in the regulation of strawberry fruit ripening (Chai *et al*., 2011).

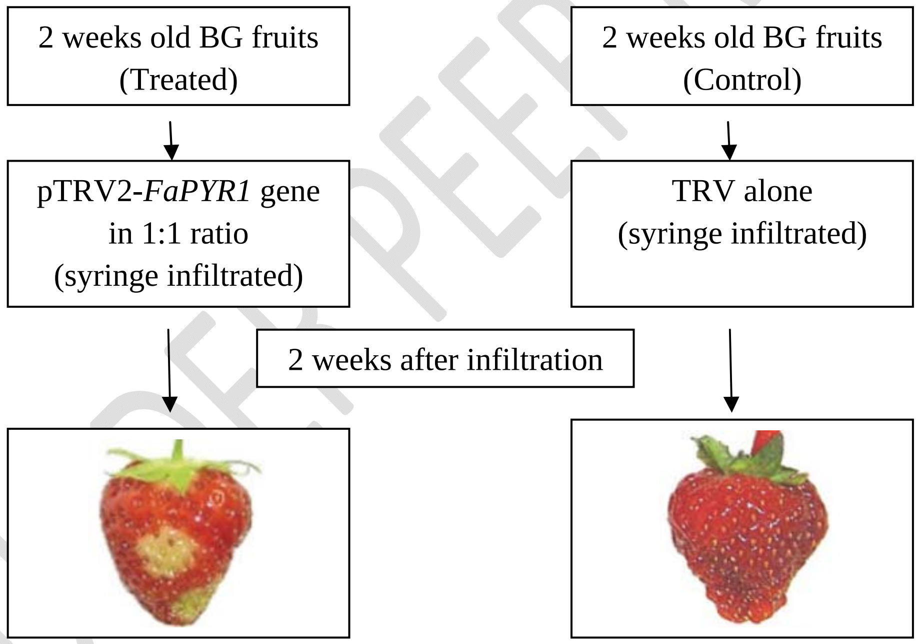

Fig. 1: Silencing of *Fa*PYR1 by VIGS.

2.2 DNA METHYLATION

DNA methylation is a major source of transcriptional gene silencing (TGS), blocking gene expression (Waterhouse *et al*., 2001). In TGS, silenced transgenes coding regions and promoters are densely methylated (Kooter *et al*., 1999). It is also suggested that the increase in DNA methylation induces formation of heterochromatin, which is associated to TGS (Ye and Signer, 1996; Wassenegger and Pelissier, 1998 and Wassenegger, 2000). RNA-dependent DNA methylation stimulates protein binding that recognizes methylated cytosine leading to chromatin remodeling (Alberts *et al*., 2002), thus avoiding the binding of transcription factors (Kooter *et al*., 1999). In pineapple, flowering is one of the most important processes in plant ontogeny. Several factors from environmental to chemical can trigger flowering in pineapple. Due to the dependence of fruit ripening on flowering time and non-climacteric nature of pineapple, synchronization of flowering of plants in the field is a critical importance for the pineapple growers (Trusov and Botella, 2006). To synchronize flowering, growers generally select planting material by size or weight (Reinhardt and Medina, 1992) and once plants reach maturity, treat them with a number of flowering-inducing agents such as ethylene (Bartholomew, 1977 and Reid and Wu, 1991). But still, a fraction of the crop (ranging from 5 % to 30 % and reaching up to 70 % under certain conditions) manages to flower ahead of schedule, a phenomenon known as 'natural flowering' or 'environmental induction'. This phenomenon is a highly undesirable characteristic of pineapples causing interruption in harvest scheduling, market supply and increasing harvest costs (multiple harvest of the same field) resulting in heavy harvest losses (Min and Bartholomew, 1996). A 1-amino-cyclopropane-1-carboxylate synthase (ACC synthase) gene is responsible for triggering 'natural flowering' in pineapple (Trusov and Botella, 2006). To silence *ACACS2* gene, two transgenic pineapple lines is produced containing co-suppression constructs designed to suppress the expression of the *ACACS2* gene (Trusov and Botella, 2006). Northern blot hybridization revealed that the *ACACS2* gene is silenced in a number of transgenic plants in both lines containing co-suppression construct. Southern blot hybridization cleared the differences in the methylation status of a silenced versus non-silenced plants by the inability of a methylation sensitive enzymes such as *Bst*UI to digest within the *ACACS2* DNA extracted from silenced plants indicating that methylation is the cause of the observed co-suppression of the *ACACS2* gene (Trusov and Botella, 2006).

2.3 RIBONUCLEIC ACID INTERFERENCE (RNAi)

Ribonucleic acid interference (RNAi) of genes, initiated by dsRNA is an important tool to study gene function (Waterhouse and Helliwell, 2003). The dsRNA is regarded by the Dicer enzyme, a member of the RNase III family of nuclease that specifically cleaves dsRNA (Bernstein, 2001). This enzyme cleaves the dsRNA into shorter RNA duplexes of 21 to 28 nucleotides, which have 5' phosphate and 2-nucleotide overhangs (Bernstein, 2001; Hamilton and Baulcombe, 1999; Elbashir *et al*., 2001 and Meister and Tuschl, 2004). These short RNA duplexes are known as short interfering RNA (siRNA) (Baulcombe, 2004), which execute RNAi-mediated gene silencing. This technique of gene silencing is also used in strawberry (*Fragaria* × *ananassa* cv. Elsanta) fruits to suppress ripening-related chalcone synthase (CHS) gene (Hoffmann *et al*., 2006). The expression of the CHS gene in fruit tissue is developmentally regulated and associated with fruit colouring (Aharoni and O'Connell, 2004). By using a construct (ihpRNA) containing the partial sense and corresponding antisense sequences of CHS separated by an intron obtained from a strawberry quinine oxidoreductase gene (Hoffmann *et al*., 2006). This technique in combination with metabolic profiling analysis will be useful for studying the function of unknown genes during the development and ripening of strawberry fruit (Hoffmann *et al*., 2006). Härtl *et al*. (2016) describe the downregulation and the spread of silencing of two endogenous strawberry genes *Fragaria x ananassa* chalcone synthase (*FaCHS*; Lunkenbein *et al*., 2006) and *F. x ananassa* O-methyltransferase (*FaOMT*; Wein *et al*., 2002) by ihp (inverted hairpin construct) constructs and transitive RNAi vectors. Another important application of RNAi-induced silencing is the production of seedless fruits or parthenocarpic fruits in tomato by suppressing chalcone synthase (CHS) gene in the flavonoid biosynthetic pathway (Schijlen *et al*., 2007). Post-transcriptional gene silencing is also exploited to confer resistance to viruses in transgenic Mexican lime (Fagoaga *et al*., 2006) and *Prunus* sp. (Liu *et al*., 2007).

2.4 ANISENSE-MEDIATED GENE SILENCING

Antisense-mediated gene silencing refers to the post-transcriptional silencing of genes using small sequence specific (antisense) molecules that through complementary base pairing suppress translation or direct degradation of specific target mRNAs. This technique of gene silencing is a convenient alternative to reduce gene expression to different levels and to silence multigene families (Kuhlmann *et al*., 2006). Antisense technology is used in some fruit crops such as strawberry (Posé *et al*., 2013) and apple (Atkinson *et al*., 2012) to extend the post-harvest shelf life and to increase fruit firmness. Antisense-mediated down-regulation of polygalacturonase (PG) gene, *FaPG1*, in strawberry resulted in reduce fruit softening and extended post-harvest shelf life which is attributed to a reduced cell wall disassembly due to *FaPG1* silencing (Posé *et al*., 2013). *FaPG1* silencing significantly reduced strawberry fruit softening without affecting other ripening-related traits such as colour, weight, or soluble solids (Quesada *et al.,* 2009)**.** The increase firmness of transgenic antisense *FaPG1* strawberry fruits are predominantly due to decrease in pectin solubilization (Quesada *et al*., 2009) and depolymerization that correlates with more tightly attached cell wall bound pectins (Posé *et al*., 2013).

3. CONCLUSION

RNA silencing is a unique, powerful and yet simple molecular mechanisms to down regulate or suppress gene. This article demonstrates the use of gene silencing technique to study gene-related functions such as pigment production in bilberry and counter undesirable gene-related characteristics of fruit crops such as asynchronous flowering in pineapple. Gene silencing can be used to know the functions of unknown genes. Recently, the work on gene silencing has gained momentum but this mechanism is yet to be fully utilized in fruit crops to know its full potential effect in regulating genes responsible for susceptibility to various biotic and abiotic stresses.

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