Developing Multi-Virus Resistant Stone Fruit Cultivars

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Stone fruit species are susceptible to a number of viruses, which can cause serious production losses and decreases in product quality. *Prunus* necrotic ringspot virus (PNRSV), tomato ringspot virus (ToRSV), and prune dwarf virus (PDV) are widespread in production areas worldwide and are very destructive for peach, plum, cherry, and apricot production (Gilmer *et al*., 1976; Ogawa *et al*., 1995). Plum pox virus (PPV), one of the most serious diseases for stone fruit, has devastated stone fruit production in Europe (Kegler *et al*., 1998; Ravelonandro *et al*., 2000). Discovery of PPV in the U.S., Canada, and Chile presents a potential threat that PPV may spread widely throughout North and South America and raises a serious concern about containing and eradicating the virus. Hence, development of new cultivars with resistance to multiple virus pathogens is highly desired by growers. Although breeding for virus-resistant varieties has been pursued for decades, progress remains slow because of the inherent long juvenility of tree species, the lack of resistant genotypes, and the genetic complexity of resistance. To date, no stone fruit germplasm resistant to multiple viruses has been reported. In this regard, biotechnology provides an advantage over conventional breeding and can meet industry's and growers' needs in a more timely manner. For example, a new PPV resistant cultivar has been developed in plum via genetic transformation (Scorza et al., 2001). More importantly, biotechnology makes it possible to manipulate and improve plant resistance to multiple viruses, which is especially important for stone fruit.

Concerns regarding pollen and seed-mediated gene flow and potential food safety of transgenic crops are growing and spurring intense debates. These concerns significantly impact the confidence of both farmers and consumers in adopting and accepting transgenic crops and foods. It is generally feared that copious pollen derived from genetically modified (GM) crops could pollinate and hybridize with their wild relatives, leading to the introgression of superior engineered traits into wild species, thus boosting hybrid fitness through selectively gained advantages (Ellstrand et al., 1999; Ellstrand, 2001; Mikkelsen et al., 1996; Snow & Palma, 1997). It is also feared that seeds released into the environment from transgenic crops could form unwanted, 'weedy' plants in competition with unmodified crops of subsequent years. These volunteer populations could naturalize and persist as feral weed populations and serve as reservoirs from which a transgene could be passed into the genome of a wild relative (Snow, 2002). These prospects raise concerns regarding the creation of super weeds with acquired superior attributes (Hall et al., 2000), which have the potential to add management burdens to farmers, and may result in further invasion of natural habitats, compromising the biodiversity of these habitats (Dale et al., 2002). In addition, the concerns on potential food toxicity and allergenicity derived from transgenic crops have also been raised.

We are fully aware of the serious virus threat challenging stone fruit production and the public concerns on potential biological risk of transgenic plants, and we are responding by developing a comprehensive strategy to address those problems and concerns. Here we present our current research strategy, progress and results in the course of developing new stone fruit cultivars.

Developing virus resistant stone fruit cultivars through conventional breeding has proven to be difficult. Biotechnology and molecular biology provide an alternative approach that can facilitate the improvement of virus resistance in stone fruits to meet industry's and growers' needs in a more timely manner. Biotechnology makes it possible to manipulate and improve plant resistance to multiple viruses, which is especially important for stone fruit.

Engineering of Multi-Virus Resistance for Stone Fruit

Gene silencing, or RNA silencing mechanisms, provide an efficient approach for engineering virus resistance in plants. The resistance is based on the expression of small interfering RNA (siRNA) species, ranging from 22 to 25 nucleotides in length, that are produced by the degradation of double-stranded RNA (dsRNA) species formed from the transcripts of the introduced virus gene fragment in transgenic plants. The siRNA binds viral RNA and initiates RNA degradation by a host-specific enzyme complex, thus mounting a robust defensive mechanism against virus infection. We are utilizing the RNA silencing principle to engineer virus resistance against six major stone fruit viruses including plum pox virus (PPV), tomato ringspot virus (ToRSV), *Prunus* dwarf virus (PDV), *Prunus* necrotic ringspot virus (PNRSV), peach mosaic virus (PMV) and American plum line pattern virus (APLPV) by creating a chimeric artificial gene, *PTRAP6*,

that is composed of gene fragments from each of the six stone fruit viruses. The *PTRAP6*
gene was used to construct a dsRNA silencing-competent carrier, *PTRAP6i*, that is expected to confer resistance to all six viruses targeted by expressing viral gene-specific siRNA in transgenic plants. *PTRAP6i* was first introduced into *Nicotiana benthamiana* to test if it was able to confer a strong resistance to the multiple viruses targeted. Characterization of transgenic *Nicotiana* plants showed that the majority of transgenic lines carrying *PTRAP6i* underwent gene silencing as evidenced by DNA methylation of the inserted genes and siRNA production. Virus inoculation studies showed that the gene silencing lines were resistant to multiple virus infections. The present study shows evidence that it is feasible to engineer multi-virus resistance in herbaceous plants; hence, this approach can be directly applied to engineering multi-virus resistance in stone fruit in the future.

Development of the Tissue-Specific Transgene Removal and Containment System

To address consumer concerns on the safety of transgenic plants, we proposed the Tissue-specific transgene REmoval and Containment System (TRECS). The TRECS is principally based on the execution of two events in a temporal manner: excision of the transgene during the early stage of reproduction and further containment of the transgene by selectively eliminating the tissues that fail in gene excision during later stages of reproduction. The ideal TRECS should carry a single transgene that contains all the components necessary for transformation selection, agronomical improvement, and its excision and containment. The TRECS transgene should be bracketed by specific DNA fragments that can be recognized and cleaved by molecular scissors and can be efficiently excised in targeted tissues. Ideally, the molecular scissors must be highly efficient to ensure that the transgene is excised in all targeted cells or tissues, and the excision events should take place early in the stage of floral meristem initiation to ensure that the derived pollen and gynoecia are free of the transgene. Any pollen and fruit, which fail in gene excision, are subsequently eliminated by activating the gene whose product is able to ablate or arrest or abscise the targeted tissues. Apparently, the entire TRECS transgene can not be inherited through sexual reproduction due to its nature of suicide action in pollen and fruit tissues; therefore, it must be stably maintained through asexual propagation. The TRECS transgene must also be maintained in a single copy, hemizygous state in transgenic plants for maximal excision efficiency. Stone and other fruit crops are routinely maintained, regardless of their hemizygosity or homozygosity state, from generation to generation through vegetative propagation, without altering their genetic makeup. Therefore, fruit crops, by any standards, satisfy all requirements imposed by the TRECS.

We have made a series of TRECS constructs by assembling a molecular scissors gene, a selection marker gene, a suicidal gene and a fuid (GUS) gene into a roughly 18 kb TRECS transgene fragment that is flanked by two copies of Target sites in a tandem repeat. The Target sites are recognized and cleaved by the molecular scissor gene expressed in early floral meristem tissue by a tissue-specific promoter. As a result, the entire 18 kb TRECS transgene is completely excised in the floral meristem tissue. Hence, the 18 kb TRECS transgene serves as a gene excision initiator and target. The suicidal gene is only activated in pollen and stigma tissues under a specific promoter so that any pollen and fruit derived from floral meristem tissue carrying the unexcized TRECS transgene will be automatically eliminated by the suicidal gene action. Thus, gene excision efficiency can be directly analyzed and compared by evaluating fruit setting, viable pollen, as well as loss of GUS expression in these tissues. We have introduced the TRECS constructs into *Arabidopsis* and found that some of the transgenic lines were able to efficiently excise the entire integrated TRECS transgene, as evidenced by production of viable transgene-free pollen, seed, and fruit or silique. Further molecular and genetic characterization of transgene excision is in progress.

Future Research

The TRECS technology directly addresses the consumer and scientific concerns on biological risk of transgenic crops and will potentially strengthen the confidence of consumers and growers on the acceptance and adoption of transgenic food and crops. Our long term goal is to develop the reliable and efficient TRECS and multi-virus resistance approaches and ultimately integrate them together into a breeding program - developing biologically risk-free, strong multi-virus resistant, and consumer-accepted stone fruit cultivars.

Conclusions

The development of new stone fruit cultivars that are resistant to virus diseases is highly desired by breeders and growers but encompasses enormous genetic and biological challenges for conventional stone fruit breeders. Biotechnology and molecular biology provide an alternative approach that can facilitate the improvement of virus resistance in stone fruits and other woody plants without the lengthy genetic crosses. However, the concerns about food safety and pollen and seed-mediated gene flow of transgenic crops are growing and spurring intense debates, which are deteriorating the confidence of growers and consumers of transgenic crops. We are currently developing a new approach to engineer multi-virus resistance in stone fruit to address the serious virus problems encountered in agricultural practice. We are also developing and testing a tissue-specific transgene excision system in transgenic plants to address the concern about food and environmental safety of transgenic plants. Ultimately, we wish to integrate the two approaches to produce multi-virus resistant, biologically risk-free stone fruit cultivars in the future.

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