GENETIC ENGINEERING STUDIES ON SUNFLOWER

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ABSTRACT

Domestication of sunflowers (*Helianthus annuus*) by humans for particular structures that are desirable to humans in a relatively benign environmental conditions and stresses have forced these plants to undergo evolutionary increases in the yield, but at the cost of reduced defense mechanisms against biotic and abiotic stresses and diseases. A multitude of factors such as insects and diseases reduce the sunflower yield, and research to develop pest resistance, herbicide resistance, increasing oil per acre yield of sunflower holds indispensable. Wild species of sunflower contain rich source of useful genes, which needs to be transferred to cultivated ones. Though improved transformed techniques have been reported, more efficient transformation protocol needs to be explored. There are some studies involving transgenic sunflower plants to increase yield, oil content, insect/fungal resistance, stress tolerance and production of biopharmaceutical proteins. Studies involving ecological impact of *Bt* sunflowers with regards to "gene flow" remains controversial. Stable transformation is relatively time-consuming with low regeneration rate and has left sunflower transformation recalcitrant. For this reason, transient expression in sunflowers have gained attention in studies of function of promoters, regulation of gene, subcellular localization of proteins, protein stability, protein-protein interactions and small RNA function. In this presentation, we will attempt to give an overview of the genetic engineering studies in sunflower with main challenges, achievements and future prospects.

Key words: Transgenic sunflower, Transient expression, Stress resistance, Insect resistance, Oil yield, Biopharming.

INTRODUCTION

Cultivated sunflower (*Helianthus annuus* L.) has its origin from North America and is one of the few major food crops in the world (Harter *et al*., 2004; Blackman *et al*., 2011). Domestication of sunflowers by humans for particular structures that are desirable to humans in a relatively benign environmental conditions and stresses have forced these plants to undergo evolutionary increases in the yield, but at the cost of reduced defense mechanisms against biotic and abiotic stresses and diseases (Mayrose *et al*., 2011). Mayrose *et al*. (2011) studied growth traits of sunflower under benign environmental condition which they found to be greater for the domesticated genotype population than that for the wild population, but with a drop in defense response in domesticated sunflowers when exposed to biotic and abiotic stresses. Additionally, it was found that lepidopteran pests preferred domesticated sunflowers more than the wild sunflowers in experimental agriculture fields (Chen and Welter, 2002). It was also revealed that

Botrytis cinerea and drought had more negative consequences on domesticated sunflowers than the native plants (Mayrose *et al*., 2010). A multitude of factors such as insects and diseases reduce the sunflower yield, and molecular biology research with a focus on transgenic sunflowers to develop pest resistance, herbicide resistance, increasing oil per acre yield of sunflower holds indispensable as well as its study on ecological impact is pressing.

VARIOUS TECHNIQUES IN GENETIC ENGINEERING OF SUNFLOWERS

Various approaches in production of transgenic plants have been used, but with low efficiencies in transformation: Polyethylene glycol (PEG)-induced vector uptake of pCAMVNEO into protoplasts isolated from sunflower seedling hypocotyls (Moyne *et al*., 1988), microprojectile bombardment (Knittel *et al*., 1994; Laparra *et al*., 1995; Hunold *et al*., 1995) and electroporation (Kirches *et al*., 1991). PEG-induced vector uptake turned out to be mainly labor intensive and some other protocols with *Agrobacterium*-mediated transformation of sunflower plants have been used (Bidney *et al*., 1992; Laparra *et al*., 1995; Rao *et al*., 1999; Weber *et al.*, 2003; Ikeda *et al*., 2005; Mohamed *et al*., 2006).

One possibility as to the reason for less studies with stable transformation experiments with sunflowers could be because of no efficient and reproducible protocol for sunflower transformation (Radonic *et al*., 2008). Selection of transformants, tissue regeneration, long life cycle of sunflower plants, time-consuming homozygous lines generation mainly as compared to the time required to obtain homozygous transgenic *Arabidopsis* plants have possibly made stable transformation of sunflower to be used to elucidate metabolic or signal pathways of sunflowers recalcitrant. This has led to some studies to choose *Arabidopsis* or tobacco heterologous system or transient expression in sunflower leaves to further unravel gene functions in sunflowers (Manavella and Chan, 2009; Cabello *et al*., 2012; Cabello *et al.,* 2016; Tata *et al*., 2016).

THE DEVELOPMENT OF INSECT TOLERANT TRANSGENIC SUNFLOWERS

A polyphagous insect *Helicoverpa armigera* (Noctuidae; Lepidoptera) is reported to cause 20-25% yield losses in sunflowerss and sometimes upto 40-70% in severe conditions (Ranasingh and Mahalik, 2008). Westdal (1975) found that the sunflower beetle *Zygogramma exclamationis* (25 larvae per plant) reduced seed production in sunflower as much as 30%.

Cry1F-transgenic sunflowers were obtained with a *Cry1F* gene ("Bt" gene) isolated from *Bacillus thuringiensis* which conferred resistance against *Spilosoma virginicia* and *Rachiplusia nu*. Compared to the control, increased tolerance of transgenic plants against larvae at the seedling and preflowering stages were found during the feeding assay with transgenic leaf discs. *Cry1AC* gene was used to develop a transgenic line of *Bt* sunflowers by Pioneer Hi-Bred and Dow AgroSciences which produce CrylAc protein that is lethal to *Lepidopteran* (moth) larvae (Snow *et al*., 2003). Snow *et al.* (2003) reported that the transgenic plants yielded considerably more inflorescences with more mature seeds in more inflorescences and higher number of viable seeds per plant as compared to non-transgenic controls. They observed that the transgenic plants in a greenhouse experiment even without the insect pests produced no difference in the seeds or inflorescences. The study concluded that the transgene itself didn't actually cause the benefit in these transgenics, but the protection from lepidopeterian resulted in the gain of fecundity in transgenics. They suggested that the wild sunflowers and weedy populations near to the cultivated transgenic sunflowers would render recurring events of "gene flow" from the transgenics and it could have detrimental effects on the native lepidopteran herbivores and other populations of coleopteran and dipteran herbivores.

THE DEVELOPMENT OF FUNGAL RESISTANT TRANSGENIC SUNFLOWERS

Charcoal rot disease caused by *Macrophomina phaseolina* in sunflower causes losses on more than 500 cultivated and wild plant species (Khan, 2007). *Alternaria* blight caused by *Alternaria helianthi* is reported to reduce seed and oil yield by 27-80% and 17-33% respectively (Reviewed by Mukhtar, 2009). *Sclerotinia* has been reported to cause damage upto 50% in sunflower in UK (Tu, 1989). Fungal pathogen *Plasmopara halstedii* causes Downy mildew and can lead to more than 50% yield loss (Hvarleva *et al*., 2009).

Oxo-transgenic sunflower plants were obtained by introducing wheat germin gf2.8 *OXO* gene to confer resistance against fungal disease *Sclerotinia* head rot (Lane *et al*., 1991; Lu *et al*., 2000; Hu *et al*., 2003). However, it has been of concern if OXO enzyme could be a human allergen (Jensen-Jarolim *et al*., 2002). The probability of transgenic wild plants being a worse weed is scarce as *OXO* transgene will diffuse neutrally on its escape because the transgenic wild plants do not produce ample number of seeds than the wild population (Burke and Rieseberg, 2003). Chapman and Burke (2006) also ruled out the possibility of "gene flow" concluding that the natural selection is the key in spread of favorable transgene alleles. Human lysozyme gene under CaMV 35S promoter and *Nos* terminator in a binary vector containing *NPTII* and *GUS* marker genes was incorporated in sunflowers using hypocotyl explants with *Agrobacterium*-mediated transformation conferred resistance against mold disease causing *Sclerotinia sclerotiorum* (Sawahel and Hagran, 2006). Lectin or proteinase inhibitor genes have been used to engineer sunflower with insect resistance (Schuler *et al.*, 1998).

THE DEVELOPMENT OF ABIOTIC STRESS TOLERANT TRANSGENIC SUNFLOWERS

Yeast metallothionein gene (*CUP1*) from yeast was incorporated into sunflower to evaluate tolerance of transgenic plants to heavy metals at the callus stage and selected heavy metal-tolerant lines of the transgenic sunflower calli. The results showed use of transgenics to obtain abiotic stress tolerance in sunflowers (Watanabe *et al*., 2005). LBA4404 strain harboring T-DNA containing dsRNA-suppressor of proline dehydrogenase gene, produced based on the *ProDH1* gene of Arabidopsis, was integrated into the genome of sunflower plants transformed *in vitro* and *in planta* to increase sunflower tolerance level to water deficiency and salinity (Tishchenko *et al*., 2014**)**.

MOLECULAR PHARMING IN SUNFLOWERS

Plants have the ability to bring about protein stability and bioactivity by glycosylation and posttranslational modification, and plant and animal cell protein synthesis pathway are alike; it is estimated that to synthesize pharmaceutical proteins in plants is highly economical than using fermentation techniques and mammalian cell cultures (Rybicki *et al*., 2010; Ma *et al*., 2003). Guan and Wang (2014) successfully expressed CTB-LK (Cholera toxin B subunit-Lumbrokinase), peeled seeds of which if administered orally to rats and mice had significant antithrombotic effect, in sunflower seeds using *Agrobacterium* mediated transformation. This study also concluded that the CTB-LK expression in sunflower seeds eradicated the requirement for protein downstream processing. Similarly, the use of *Agrobacterium rhizogenes*-mediated transformation of topinambour in sunflower plants, callus and "hairy" root cultures proved sunflower plants to be a good source of recombinant interferon alpha 2b protein. The plasmid vectors with interferon gene fused with *Nicotiana plumbaginifolia* L. calreticulin apoplast

targeting signal driven by *35S* CaMV promoter or root-specific *Mll* promoter to obtain transgenic *H. tuberosus* cultures with high antiviral activity (Maistrenko *et al*., 2015).

THE DEVELOPMENT OF SUNFLOWERS WITH INCREASED FATTY ACID OIL CONTENT BY MUTAGENESIS

Modifying oil quality is crucial as it is one of the edible oils worldwide known for its salubrious quality and lipid peroxidation (Moschen *et al*., 2014). Many sunflower lines have been developed with elevated saturated fatty acid content with greater than 25% of fatty acids compared to 12% in normal sunflower using physical or chemical mutagenesis. Osorio *et al*. (1995) developed CAS-3 and CAS-5 mutants with high amount of stearic acid and palmitic acid contents respectively. Fernández-Martínez *et al*. (1997) reported CAS-12 mutants with high palmitic acid and oleic acid contents. Fernández-Moya *et al*. (2002) developed CAS-14 mutants with upto 37% stearic acid content. Velasco *et al*. (2008) used ethylmethane sulfonate as a chemical mutagen and obtained M2 seeds from a single M1 plant with 5-39% palmitic acid content. 10-30% of palmitic acid was obtained from the progenies of all selected M2 seeds.

THE DEVELOPMENT OF TRANSGENIC SUNFLOWERS WITH INCREASED FATTY ACID OIL CONTENT

The Δ9-stearoyl-(acyl carrier protein) desaturase coding sequence from *Ricinus communis* was transferred in sunflower under the control of seed-specific promoter and terminator sequences of *Hads10*. Seed oil composition analysis showed significant decrease in stearic acid content in the seeds obtained from transgenic plants. Some progenies exhibited saturated fatty acid content below 10% whereas other plants had elevated palmitic acid content with reduced stearic acid content (Rousselin et al., 2002). Hydroxymethylglutaryl-CoA (*Hmgr*-CoA) and *Erwinia uredovora* phytoene desaturase (*Crtl*) genes were introduced into sunflower to obtain potential increase in oil quality (Dagustu *et al*., 2008).

OTHER MOLECULAR STUDIES IN TRANSGENIC SUNFLOWERS

Post-transcriptional gene silencing (PTGS) in transgenic sunflower expressing *glucuronidase* (GUS) activity has been performed using grafting procedure. In two weeks silencing was observed and the study showed that the RNA infiltration in sunflower induces transient silencing and is not transmitted to offspring (Hewezi *et al*., 2005). Shulga *et al*. (2015) reported first transgenic sunflower with alteration in *HAM59* expression to study the function of *HAM59* MADS-box gene in sunflower which is involved in formation of reproductive organs of flower.

The elucidation of role of PLFOR48 sequence in resistance to mildew in sunflower was studied assessing loss of function, by expressing antisense cDNA PLFOR48 construct in RHA 266 sunflower line. Transgenic sunflower lines displayed severe developmental abnormalities. The same antisense expression in transgenic tobacco lines resulted in higher susceptibility to *Phytophthora parasitica*. It was reported that TIR-NBSLRR R genes in sunflower and tobacco have a dual role in plant development and fungal resistance (Hewezi *et al*., 2006).

CURRENT STIUATION AND CHALLENGES

A progress is still being made in efficiently transforming sunflower crops but stable transformation of sunflower plants is just yet time-consuming in generating homozygous lines and in regeneration of tissue. Also, sunflower has a long life cycle and transient expression of genes can be an alternative method in elucidating molecular mechanisms such as function of promoters, regulation of gene, subcellular localization of proteins, protein stability, protein-protein interactions and small RNA function (Manavella and Chan, 2009). Despite of this constraint, several studies by developing transgenic sunflowers are still being conducted.

Wild sunflower species provide greater contribution as a rich source of genes in crop improvement to bring about economic viability in cultivates species as major oilseed global crop (Seiler and Fredrick, 2011). As having a narrow background in domesticated sunflowers with deficient genes, discovery of unique genes from wild sunflower plants is indispensable and is still underway. This could help in developing transgenic sunflowers with desired traits from wild population.

Clearfield and Express Sun technologies saw restriction on growing of genetically modified crops for "not being biotech product" (Reviewed by Kaya, 2015). Genetically modified crops have always been a matter of debate and public acceptance regarding this remains divided with some people being reluctant on the use of biotechnology in crop amelioration.

FUTURE DIRECTIONS IN GENETIC ENGINEERING OF SUNFLOWER

Transgenic technology holds imperative role in sunflower breeding and exerts strong promises to increase yield, oil content, insect/fungal resistance, stress tolerance and production of biopharmaceutical proteins. Albeit having improved transformed techniques in sunflower, more efficient transformation protocol needs to be explored for generating increased success rates in obtaining transgenic sunflowers as well as search for candidate genes with elite traits in developing transgenic crops does remain apparent.

Traits that are being studied in sunflower for environment release is sparse. Sunflower is known to have a high exposure to gene flow ultimately generating continuous variability. Strict environmental monitoring is inevitable to preclude undesired outcomes (Cantamutto and Poverene, 2007).

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