DEVELOPMENT OF GENOME EDITING CONSTRUCT TARGETING PHYTIC ACID AND RAFFINOSE FAMILY OLIGOSACCHARIDES IN *GLYCINE MAX*...

INTRODUCTION:-

- Soybean (*Glycine max*) is a versatile and globally significant legume valued for its high protein content and numerous applications in food, feed, and industrial products.

- Despite its nutritional benefits, soybean contains certain antinutritional factors, such as phytic acid and raffinose family oligosaccharides (RFOs).

- Phytic acid can hinder mineral absorption, while RFOs can cause digestive discomfort.

- Understanding and regulating the genes involved in the production of these antinutritional factor is crucial for improving the nutritional quality and digestibility of soybean-based products.

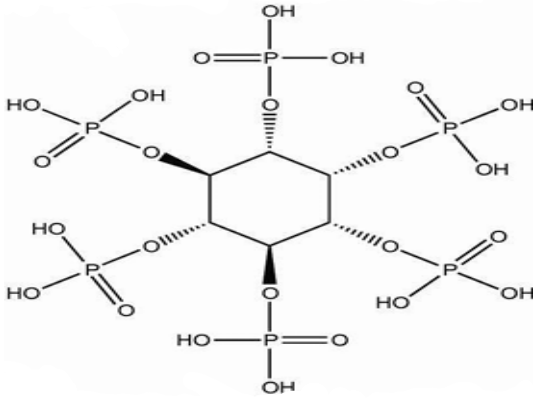
OBJECTIVES

- **To familiarize about gene responsible for Phytic acid and Raffinose family oligosaccharide in Soybean(*Glycine Max*).**
- **To design SgRNA targeting GmIPK1a, GmIPK1b, GmIPK1c and GmGOLS1A, GmGOLS1B.**
- **Development of construct targeting genes responsible for phytic acid and raffinose family oligosaccharide.**

IMPORTANCE AND NEED

- **Enhancing Nutritional Value: Reducing phytic acid and raffinose family oligosaccharides (RFOs) improves mineral absorption and protein digestibility, making soybeans more nutritious (Lin et al.,2024)**
- **Improving Digestibility: Lowering RFOs reduces digestive discomfort, making soy products more acceptable and easier to digest. RFOs can cause gastrointestinal issues due to their fermentation by gut bacteria(James W Anderson et al., 2009)**
- **Supporting Human Health: Reducing phytic acid enhances mineral uptake, preventing deficiencies and promoting better health(Anupam Roy et al., 2023)**
- **Facilitating Food Processing: Fewer antinutritional factors simplify processing, reduce costs, and improve product quality.**
- **Advancing Genetic Research: Understanding these genes aids in crop science advancements and can be applied to other plants for broader agricultural benefits.**

REVIEW OF LITERATURE



- In soybean seeds, PA can count for 4–5% of the seed dry weight, and 50% of the seed total phosphorus content (Oatway et al. 2001). Having six phosphate groups, PA belongs to a class of metal-chelating compounds that may bind to iron, zinc, calcium, magnesium, and potassium, among other metal cations (Raboy 2009).
- During seed germination, the PA complex releases metal cations and provides energy to support seedling growth. However, PA is considered a non-negligible anti-nutritional factor. PA lowers the absorption and utilization of minerals (e.g., iron, zinc, calcium) and negatively affects human health for people subsisting on grain-based diets, mainly in developing countries (Zhou and Erdman 1995).
- The major genes responsible for the low phytic acid germplasm include the inositol pentose-phosphate kinase gene (GmIPK1a) in PA biosynthesis (Yuan et al. 2012), and the vital multi-drug-resistant protein 5 gene (GmMRP5a) involved in PA transport (Gillman et al. 2009).

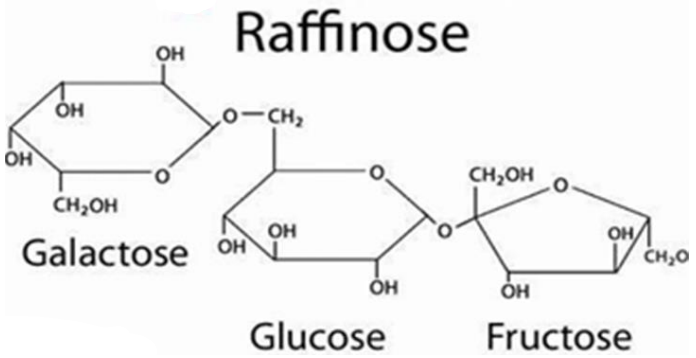
REVIEW OF LITERATURE

- **Studies explored the use of CRISPR/Cas9 technology for gene editing in soybean, specifically targeting two genes involved in the phytic acid synthesis pathway: GmIPK1 and GmIPK2(Carrijo et al., 2021).**
- **GmIPK1 and GmIPK2 were chosen as target genes due to their roles in phytic acid biosynthesis in soybean seeds. GmIPK1 is involved in inositol-pentakisphosphate 2-kinase activity, while GmIPK2 likely contributes to the biosynthesis of phytic acid (Carrijo et al., 2021).**
- **The successful application of CRISPR/Cas9 technology in editing genes related to phytic acid biosynthesis in soybean highlights the potential for targeted crop improvement and trait modification through genetic engineering (Carrijo et al., 2021).**

REVIEW OF LITERATURE

- **Results in studies showed that higher-order mutant lines with multiplex mutations had significantly decreased seed PA content. However, these mutants exhibited poor agronomic performance(Lin et al., 2023).**
- **In contrast, two lines with single mutations in IPK1b and IPK1c showed moderately reduced PA content and regular agronomic performance compared to the wild type (Lin et al., 2023).**
- **This suggests that moderately decreasing PA by targeting single GmIPK1 genes, rather than multiplex mutagenesis for ultra-low PA, is an optimal strategy for low-PA soybean with minimal trade-off in yield performance (Lin et al., 2023).**

REVIEW OF LITERATURE



- The raffinose family oligosaccharides (RFOs) are a major class of water-soluble carbohydrates present in soybean seeds. Soybean RFOs include raffinose, stachyose and verbascose which, respectively, are mono-, di-, and tri-galactosides of sucrose (Kennedy et al., 1985; Hou et al., 2009), with stachyose being the most prominent RFO in mature seeds.
- GOLS catalyzes the first step in the RFO biosynthetic pathway (Nishizawa et al., 2008). Therefore, study hypothesized that knock-out mutations in the *GmGOLS* genes, specifically in *GmGOLS1A* that encodes the major GOLS expressed in developing soybean seeds, would affect the final sugar composition in mature seeds.
- RFOs were proposed to be ubiquitous storage products and factors of desiccation tolerance in soybean seeds, with a potential role in other stress responses (Taji et al., 2002; Wang et al., 2009; Loedolff, 2015; de Souza Vidigal et al., 2016). However, previous studies of soybean seed lines with low stachyose and/or raffinose showed no significant differences in stress tolerance or germination compared to wild-type (Neus et al., 2005; Valentine et al., 2017). RFOs are indigestible by human and other monogastric animals that do not produce α-galactosidase to break the α-1,6-glycosidic bonds in RFOs.

REVIEW OF LITERATURE

- Studies aimed to investigate the impact of knocking out the GmGOLS1A (Glyma.03G222000) and GmGOLS1B (Glyma.19G219100) genes, which encode Galactinol Synthase, on Raffinose Family Oligosaccharide biosynthesis in soybean seeds(Le et al., 2020).

- The researchers employed a CRISPR/Cas9 system with dual guide RNAs to induce targeted knockouts in the GmGOLS1A and GmGOLS1B genes. Genotyping of T0 plants confirmed efficient disruption of the target sites.(Le et al., 2020)

- The knockout of GmGOLS1A and GmGOLS1B genes led to a significant decrease in the total Raffinose Family Oligosaccharide content in soybean seeds compared to wild-type seeds.(Le et al., 2020)

MATERIALS AND METHODS

***In Silico* work:-**

- **Designing of desired sgRNA with the use of sgRNA designing tools such as CRISPR-P & CRISPR-GE.**
- **Sequence of plasmid pDIRECT22_A (from Addgene).**
- **Using sequence in SNAP GENE tool.**
- **Annealing Oligos and Cloning by inserting fragments of vector(AaR1) and Annealed Oligose.**
- ***In silico* PCR for cloned DNA fragment with Primers (gRNA and NB463 sequences)**

In vitro work:-

- **Formation of sgRNA oligoduplex.**
- **Materials which we will need for this reaction will be:-**

Chemicals	Quantity
Forward Primer(100µM)	3µl
Reverse Primer(100µM)	3µl
T4 DNA Ligase Buffer	3µl
T4 Polynucleotide kinase	2µl
MQ water	19µl

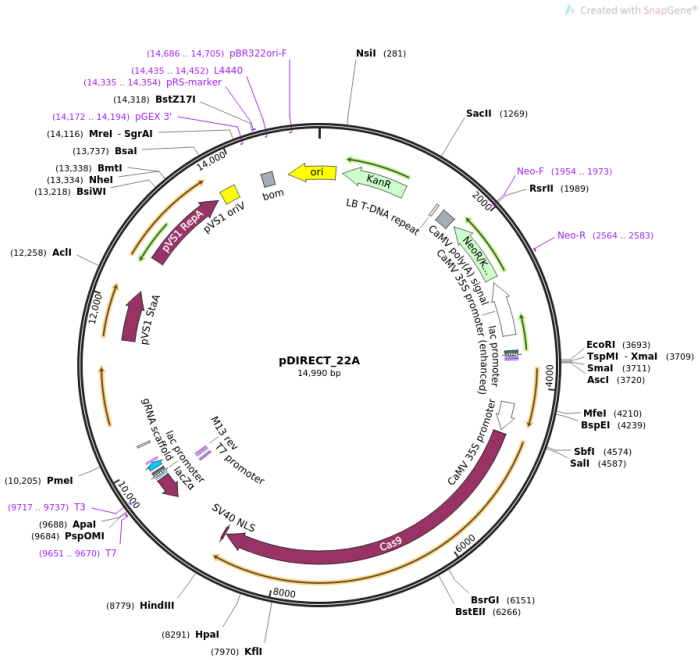
- **Followed by PCR:-**
  37°C (60 min)
  95 °C (5 min)
  25 °C (0.1 °C/sec RAMP mode or water bath)

- **Isolation of Plasmid(pDIRECT22_A).**

GOLDEN GATE REACTION:-

- Involves simultaneously Restriction digestion and Ligation.
- We will need:-

Chemicals	Quantity
pDIRECT_22A	50ng/µl
25x diluted oligo duplex	1µl
Restriction enzyme Aar1	0.5µl
Aar1 Oligonucleotide	0.4-0.5µl
T4 DNA Ligase Buffer	2µl
T4 DNA Ligase	1µl
MQ water	depends
Final volume	20µl



Followed by PCR:- 37°C(5 min), 16°C(10min), 37°C(15 min), 80°C(5 min, for inactivation)

PREPARATION OF ULTRACOMPETENT CELLS

Materials and Reagents:-

1. *E. coli* strain (e.g., DH5α)
2. LB medium (Luria-Bertani)
3. LB plates with the appropriate antibiotic
4. Transformation buffer:
    1. PIPES (1 M, pH 6.7)
    2. $CaCl_2$ (0.1 M)
    3. KCl (1.5 M)
    4. $MnCl_2$ (0.55 M)
5. Sterile distilled water
6. Glycerol (10%)
7. Sterile microcentrifuge tubes
8. Ice-cold 100 mM $CaCl_2$ solution

PROCEDURE OF MAKING COMPETENT CELLS of *E. coli* :-

•Preparation of Inoue Transformation Buffer:-

1. **Dissolve 30.24 g of PIPES in about 80 mL of sterile distilled water.**
2. **Adjust the pH to 6.7 with KOH.**
3. **Add 10.9 g of $CaCl_2 \cdot 2H_2O$, 37.2 g of KCl, and 4.95 g of $MnCl_2 \cdot 4H_2O$.**
4. **Adjust the final volume to 100 mL with sterile distilled water.**
5. **Sterilize the solution by passing it through a 0.22 µm filter.**
6. **Store the buffer at 4°C. Do not autoclave.**

• Growth of Bacterial Culture:-

1. **Inoculate a single colony of *E. coli* into 50 mL of LB medium.**
2. **Incubate the culture overnight at 18-22°C with shaking at 250-300 rpm.**
3. **The next day, dilute the overnight culture 1:100 into fresh LB medium.**
4. **Incubate at 18-22°C with shaking until the OD_{600} reaches 0.55-0.6 (mid-log phase).**

•**Preparation of Competent Cells**:-

1. **Chill the culture on ice for 10 minutes.**
2. **Transfer the culture into pre-chilled sterile centrifuge bottles.**
3. **Centrifuge at 2500 x g for 10 minutes at 4°C.**
4. **Discard the supernatant and gently resuspend the pellet in 80 mL of ice-cold Inoue transformation buffer.**
5. **Incubate on ice for 10 minutes.**
6. **Centrifuge again at 2500 x g for 10 minutes at 4°C.**
7. **Discard the supernatant and gently resuspend the pellet in 20 mL of ice-cold Inoue transformation buffer**.

•**Addition of Glycerol**:-

1. **Add 1.5 mL of sterile 100% glycerol to the cell suspension to a final concentration of 7%.**
2. **Mix gently by swirling.**

•**Aliquoting and Storage**:-

1. **Aliquot 200 µL of the competent cell suspension into pre-chilled sterile microcentrifuge tubes.**
2. **Flash freeze the aliquots in liquid nitrogen.**
3. **Store the frozen aliquots at -80°C.**

***E. Coli* Transformation procedure:-**

1. **Keep the prepared competent cells in ice until Thaw.**
2. **Add 250ng of complete GOLDEN GATE REACTION product.**
3. **Tap Gently.**
4. **Keep competent cells in ice for 15 min.**
5. **Transfer vial 42°C in water bath/ heating block for 90 secs.**
6. **Transfer vial to ice.**
7. **Add 1ml of LB Broth to vial.**
8. **Incubate the vial culture at 37°C with shaking for 1.5 hrs.**
9. **After incubation centrifuge the culture at 5000 rpm for 5 minutes.**
10. **Discard the supernatant (precaution; let some LB broth be inside).**
11. **Resuspend the cell pellet with LB broth (100µl)**
12. **Spread the culture on selection plate.**
13. **Spread the aliquot on petri plate until it become completely dry(LB plates containing X Gal, IPTG and Kanamycin).**
14. **Incubate the plate at 37 °C for 12 hrs.**
15. **Select white colonies (Blue white screening method) and do Colony PCR Forward Primer(sgRNA) and Reverse Primer(NB463).**
16. **If we get 429bp amplicon as a result then we will send it for sequencing for further confirmation.**

PLAN OF WORK

- Identification of target sequence
- sgRNA designing
- Primer designing and Oligoes designing
- *In silico* development of CRISPR construct
- Isolation of Plasmid
- DNA oligo duplex preparation
- Cloning (Golden Gate Reaction)
- Competent cell preparation(E Coli DH5α)
- *E Coli* Transformation(Heat and Shock)
- Confirmation and Validation of plasmid(PCR)
- Agrobacterium Transformation(Freeze Thaw method)

IMPLICATIONS

- **Knowledge of CRISPR Cas9, gRNA design, competent cell preparation, cloning, Transformation.**
- **Proficiency in tools like CRISPR-P, CRISPR-GE, CHOP-CHOP, Soykb, Phytozome, Soybase.**
- **Proficiency in softwares like SNAPGENE for designing, analysis, visualization.**
- **Crop improvement, sustainable agriculture, ethical implications.**
- **Competent cell preparation, cloning and transformation, gRNA design knowledge.**
- **Gene editing knowledge will help in doing something beneficial for mankind.**

ACTIVITIES PROPOSED AND WORK SCHEDULE

Task	Duration
Identification of desired genes and gene IDs	3 days
Valid gRNA design	3 days
Primers/Oligoduplex in silico formation	1 day
Snapgene	1 week
DNA oligoduplex formation (in vitro)	1 day
Golden Gate reaction (Digestion and Ligation)	1 day
Preparation of competent cells	2 days
E. coli Transformation	1 day
Sequencing and Validation	2 weeks
Agrobacterium Transformation	1 day
Validation of Transformation	1 day

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