Step 1: Identify Genes and Homologs

- We selected four target genes for CRISPR application in **Coffea arabica**:
    - **S-adenosylmethionine Synthase (SAMS)**
    - **Putrescine N-Methyltransferase (PMT)**
    - **Methyl Putrescine Oxidase (MPO)**
    - **Quinolinate Synthase (QS)**
- Using BLAST, we identified Coffea arabica homologs for each of these genes to target their promoter regions in order to enhance expression via CRISPRa.

Step 2: Vector Selection and Preparation

- We chose **pCAMBIA1301** as the base vector for the CRISPRa system due to its established use in plant genetic modification and compatibility with CRISPR/Cas9 systems[1].
- **pCAMBIA1301 Key Components**:
    - **CaMV 35S Promoter**: This will drive expression of Cas9 and other necessary components.
    - **NOS Terminator**: Included to ensure effective transcription termination.
- **Modifications Needed**:
    - **Cas9 Integration**: Addition of a Cas9 expression cassette, preferably with a plant-specific promoter.
    - **gRNA Expression Cassette**: Typically driven by a **Pol III promoter like AtU6,** necessary for each gRNA.
    - **Restriction Sites**: Removal of conflicting sites, such as BsaI, for easy gRNA insertion.

Considerations and Potential Adjustments

- **Specificity and Efficiency**: Modify the vector for higher transformation efficiency and use validated gRNA designs for on-target accuracy.
- **Plant-Specific Optimization**: For Coffea arabica, ensure the gRNA design is specific to each gene's promoter to increase CRISPRa efficacy.
- **Remove the GUS Gene**
    - Location: The GUS gene is located between the CaMV 35S promoter and the NOS terminator.
    - Action: Use restriction enzymes like BglII and NcoI to remove the GUS gene.
- **Insert Cas9 Expression Cassette**
    - Components: Cas9 gene with a suitable promoter (e.g., CaMV 35S) and a terminator.
    - Location: Insert in place of the removed GUS gene.
- **Add gRNA Expression Cassette**
    - Components: Pol III promoter (e.g., AtU6), your specific gRNA sequence, and a terminator.

- Location: Insert at a suitable multiple cloning site (MCS) or another available region in the vector.

Supporting Research

- Pol III Promoters for gRNA Expression: Pol III promoters like U6 are commonly used for gRNA expression due to their efficiency in transcribing short RNAs [2][4].

- Cas9 Expression with Pol II Promoters: The CaMV 35S promoter is a strong constitutive promoter often used for Cas9 expression in plants [5][7].

- CRISPR/Cas9 Systems in Plants: Studies have demonstrated successful use of dual promoter systems where Cas9 is driven by a Pol II promoter and gRNAs by a Pol III promoter [2][5].

These modifications will help convert pCAMBIA1301 into a CRISPR/Cas9 vector suitable for plant transformation.

Citations:

[1] https://www.snapgene.com/plasmids/plant_vectors/pCAMBIA1301

[2] https://www.frontiersin.org/journals/genome-editing/articles/10.3389/fgeed.2022.870108/full

[3] https://pmc.ncbi.nlm.nih.gov/articles/PMC8835944/

[4] https://pmc.ncbi.nlm.nih.gov/articles/PMC9845283/

[5] https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2019.01173/full

[6] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9782292/

[7] https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2016.02036/full

Step 3: Vector Selection and Preparation (to be confirmed)

Step 3.1: Define gRNA Target Sites

1. **Promoter Region Identification**: For each target gene (SAMS, PMT, MPO, QS), identify the promoter region upstream of the transcription start site (TSS).
2. **gRNA Binding Site Selection**: Select binding sites within 200–300 bp upstream of the TSS, where gRNAs will recruit the CRISPRa complex effectively.

Step 3.2: Design gRNA Sequences

1. **Target Sequence Criteria**:
    - **PAM Sequence**: Ensure each target site includes the Cas9-specific PAM sequence (NGG for SpCas9).
    - **Length**: Design each gRNA to be 20 nucleotides upstream of the PAM sequence.
2. **Tools for gRNA Design**:
    - Use a gRNA design tool, such as Benchling, CRISPOR, or CHOPCHOP.
    - Input the Coffea arabica promoter region sequences for SAMS, PMT, MPO, and QS, and allow the tool to generate potential gRNAs based on PAM sites, on-target scoring, and off-target effects.

Step 3.3: Evaluate gRNA Candidates

1. **Check Efficiency Scores**: Select gRNAs with high on-target efficiency scores.
2. **Off-Target Analysis**: Ensure the selected gRNAs have minimal off-target binding potential.
3. **Prioritize Specificity**: For CRISPRa, prioritize gRNAs with specific targeting upstream of the target gene to avoid undesired binding to other genomic regions.

Step 3.4: Insert gRNA Sequences into Vector

1. **Generate and Add gRNA Sequence to Vector**:
    - Design each gRNA sequence for insertion into the **pCAMBIA1301** vector at the placeholder site, using the Pol III promoter (e.g., U6).
2. **Confirm Sequence Compatibility**:
    - Ensure each gRNA fits the vector without creating conflicting restriction sites.