PlantEd
COST Action CA18111

2nd PlantEd conference Plant genome editing – the wide range of applications.
20 – 22 September 2021 - Lecce, Italy

Genome editing of *Ocimum basilicum* L. through CRISPR / Cas9 to induce resistance to the pathogen *Peronospora belbahrii*

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Introduction

Downy mildew disease of sweet basil (*Ocimum basilicum* L.) is caused by the obligate biotrophic oomycete *P. belbahrii*, currently the most destructive disease on this crop (Fig. 1A-B-C). Control strategies based on chemicals have limited results on basil and negative effects on the environment, demanding sustainable and greener agricultural solutions. For these reasons, conferring resistance to pathogens is a priority in the genetic improvement programs of plant species.

With the recent development of genome editing technologies, in particular the CRISPR/Cas9 system, it is possible modifying characters of agricultural interest, such as turning off genes that make basil vulnerable to pathogens. Of particular interest is the susceptibility gene *DMR6* (*Downy Mildew Resistance 6*) whose mutation has been shown to confer resistance to oomycetes in several species. This work describes the isolation of the entire orthologous sequence of *DMR6* (*ObDMR6*, 1260bp), in *O. basilicum* cv. 'FT Italiko' and the application of genome editing using CRISPR/Cas9 technology.

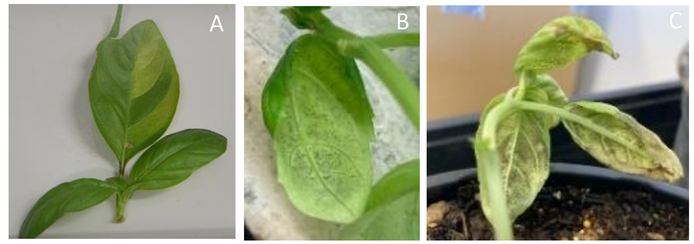
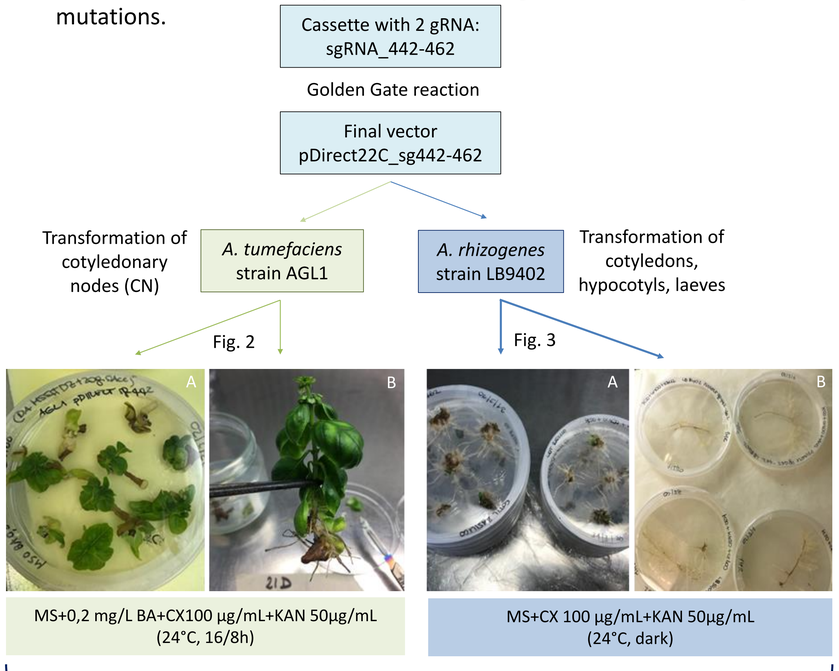


Fig. 1 - Symptoms affect the entire leaf system, with chlorosis and lesions of the leaf near the central vein (A) and the appearance, on the underside, of an abundant sporulation (B). Finally, the leaves gradually become necrotic and fall off, necrotrophic phase (C).

Materials and methods

- Cloning of *ObDMR6*, ortholog of *AtDMR6*. The entire CDS of *ObDMR6* (1008bp) is made up of 4 exons and encodes a 336aa protein.
- Two target sites (gRNA) on *ObDMR6* exon 2 were identified by CRISPRdirect tool (Naito *et al.*, 2015) and used for the creation of a cloning cassette in the binary vector pDirect_22c (Cermak *et al.*, 2017) for the simultaneous expression of the 2 gRNAs, to obtain targeted mutations.



Verification by PCR of the integration of Cas9 in 26 clones of regenerated plants (Fig. 2A-B) and 30 hairy roots HR (Fig. 3A-B). In order to evaluate the editing, a 400bp fragment of the *ObDMR6* gene, including the editing target sites, was amplified with PCR, cloned and sequenced.

Results

The transformation of 150 cotyledonary nodes (CN) with *A. tumefaciens* led to 130 regenerated shoots, by direct organogenesis. 26 shoots (of 130) were selected and propagated *in vitro*; each shoot gave rise to a distinct clonal line, with 82% of regeneration efficiency (Fig. 2A-B). 84.6% of the clones were positive for the integration of Cas9 and 82.3% of which resulted edited (Tab. 1).

Two weeks after *A. rhizogenes* transformation, all explants formed callus and generated several HRs which were excised and cultured *in vitro* separately as independent clonal lines (Fig. 3A-B). 29 out of 30 HRs tested were positive for Cas9 integration, confirming a high rate of co-transformation (96%).

AGL1_pDirect_22C_sg442-462	
% CN regeneration*	82%
regenerated shoots · CN^{-1}	2.6
Cas9+	84.6%
Edited shoots	82.3%

Tab. 1: Regeneration efficiency and molecular evaluation related to AGL1_pDirect_22C_sg442-462 genetic transformation. (*n° of regenerated CNs/total number of CNs)

Conclusion and perspectives

We propose a highly efficient CRISPR/Cas9 mediated gene editing system for targeted mutagenesis of ObDMR6 gene (1260bp) for sweet basil cv. 'FT Italiko' (the elite cultivar used to produce "Pesto Genovese DOP") using *Agrobacterium* transformation.

The resistance to the pathogen *P. belbahrii* of the edited clones of basil will has to be evaluated by *in vitro* infection assays.

The homozygous mutated T2 *ObDMR6* lines will be tested for the removal of Cas9.

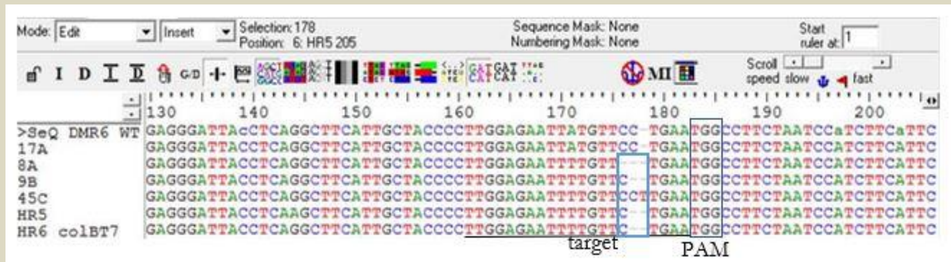


Fig. 4 Sequences of the different analyzed samples were aligned with the Bioedit software and compared against the wild type sequence, to observe the changes occurred at the editing site level. PAM sequence and indel mutations are boxed and target site underlined.

A high frequency of insertion/deletion type mutations, with consequent replacement of amino acids in the ObDMR6 protein or with complete *ObDMR6* knockout, was detected through molecular analysis of the regenerated plants (Fig. 4). This demonstrates the effectiveness of the methodology adopted and pDirect22C_sg442-462 construct.

In vitro regenerated transgenic plants (T1) were successfully transferred and acclimatized in environmental controlled conditions for flowering and seed production (Fig. 5); several abnormal phenotypic traits were observed, such as fusion of two leaves together and red stripes on the leaves (Fig. 6 A-B). Seeds from self pollination were collected into selfing bags and sown (Fig. 7), in order to obtain T2 seedlings.

Fig. 5 Regenerated edited clone, with self bags.

Fig. 6 Fusion of two leaves together (A) and red stripes on the leaves (B).

Fig. 7 Germination of T2 seeds.