Identification of a *NLR* disease resistance gene involved in *Nicotiana* hybrid lethality

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Summary

- *Nicotiana tabacum* maternal haploids can be produced using an interspecific cross with a distant relative, *N. africana*
- The cross between *N. tabacum* and *N. africana* results in lethality at the cotyledonary stage ("hybrid lethality")
- A *gfp* reporter system was developed to help distinguish haploids and hybrids
- Transposon-tagging identified a nucleotide-binding site leucine-rich repeat (*NLR*) in *N. tabacum* as the candidate gene causing the hybrid lethality
- *NLR*s are the major class of *R* genes for disease resistance in plants
- Current work using CRISPR/*Cas9* and agroinfiltration as a reverse genetics tool to confirm the role of this gene in hybrid lethality

Hybrid lethality as a biotech tool and research model

Identifying genes involved in hybrid lethality could:

- Allow the development of hybrid lethality as a phenotypic trait and marker; this could be useful in breeding applications such as doubled haploid production or in systems to restrict gene flow
- Improve the understanding of proteins involved in plant-microbe interactions, which are likely involved in hybrid lethality; this could lead to breeding and biotech applications
- Help develop methods to overcome intra- and interspecific reproductive barriers that restrict access to wider germplasm in plant breeding
- Advance understanding of mechanisms involved in reproductive isolation and speciation, which are poorly understood by evolutionary biologists

Objectives and Key Steps

The overall objective is to identify the genes involved in hybrid lethality. For simplicity, the genes have been designated Hl_T and Hl_A representing the *N. tabacum* and *N. africana* alleles, respectively. Key steps in this goal involve:

- Developing a population for transposon-tagging of the hybrid lethality gene
- Screening plants with the desired phenotype and genotype
- Identifying candidate genes
- Verifying candidate genes using reverse genetics (CRISPR/Cas9 knockouts)

In order to efficiently screen plants, preliminary objectives were also met:

- Development of green fluorescent protein (*gfp*) as a dominant seedling marker to distinguish hybrids from haploids
- Mapping the hybrid lethality locus using SSRs; this was possible due to the observation of chromosomal breakage in hybrids, resulting in non-lethal surviving plants

Use of *gfp* as a dominant seedling marker for haploid selection

Use of *gfp* as a dominant seedling marker

- Transformation of *N. africana* with a *gfp* construct (*35S:mgfp-ER*) allowed for discrimination of haploid (non-GFP) and hybrid plants (GFP) under a UV light (Fig 1B). The effect is particularly clear in the stem and veins (Fig 1C).

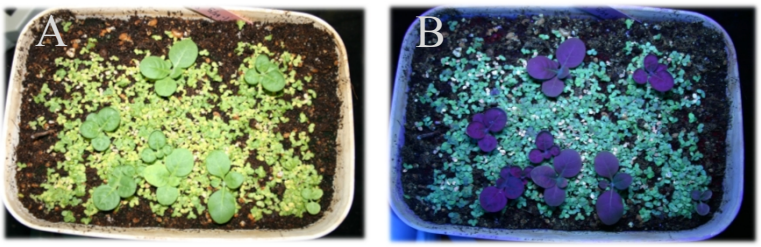
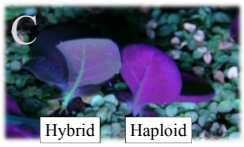


Fig 1. F_1 Progeny of *N. tabacum* (non-GFP, Hl_THl_T) x *N. africana* (GFP, Hl_AHl_A)

Corresponding phenotype-genotype association

- Most hybrids: necrosis (Hl_THl_A), GFP
- Haploids (maternal): viable (Hl_T), non-GFP
- Hybrids missing lethality factor: viable (Hl_T- or Hl_A-), GFP



Identifying an NLR as a candidate gene for Hl_T

Transposon-tagging

- *N. tabacum* transformed with an engineered *Ac/Ds* maize transposon was used in a system to tag Hl_T

N. tabacum (*Ac−; Ds:Su/su;* $P_TP_THl_THl_T$*;* non-GFP) x *N. africana* ($P_AP_AHl_AHl_A$*;* GFP)

Plants representing candidates are characterized by:

- Hybrid state (GFP)
- Intact chromosome H (PT30342, P_TP_A)
- *Ds* present and tagging Hl_T (*Ds:*Hl_T)
- *Ac* absent (stably-tagged)

BLAST identifies a defense signaling gene as the best candidate

- Several flanking sequences were obtained by hiTAIL-PCR and aligned to the *N. tabacum* genome
- Using a series of BLAST searches, a sequence was putatively identified as an *R* gene (*NLR*)
- *NLR* proteins are key signaling components of plant hypersensitive response to pathogen infection

N. sylvestris NLR homolog	Coverage (%)	Identity (%)
R homolog 1	100	90
R homolog 2	80	95
R homolog 3	70	93

Alignment of homologs to candidate Hl_T

Mapping of the hybrid lethality locus

Screening surviving hybrids mapped *Hl* to a nearby SSR (PT30342) on Chromosome H (Table 1). The candidate gene (*R* homolog) identified by transposon-tagging associated closely with PT30342 (Table 2).

Table 1. Counts of plants by genotypic class and ploidy. 96% of diploids monoallelic for either PT30342 allele were viable; necrotic plants showed both *N. tabacum* and *N. africana* alleles (P_TP_A)

PT30342 SSR	Diploid	Haploid
P_T—	31	10
P_TP_A	3	0
—P_A	45	0

Table 2. Counts of diploid plants by genotypic class. The PT30342 *N. tabacum* allele (P_T) segregates nearly perfectly with the R_T candidate gene

PT30342 SSR	R_T (present)	R_T (absent)
P_T—	29	1
P_TP_A	1	1
—P_A	0	45

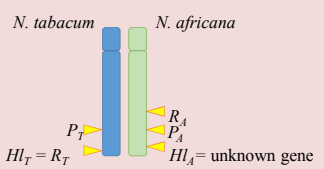


Fig 2. Possible arrangement of relevant markers. PT30342 is near the end of chromosome H in *N. tabacum* suggesting viable hybrids, and the loss of Hl_T or Hl_A, are a result of chromosome breakage or aneuploidy

Hl: hybrid lethality gene
R: *NLR* homolog identified by transposon-tagging
P: PT30342 SSR
T subscript: *N. tabacum* allele/variant
A subscript: *N. africana* allele/variant

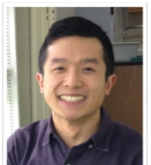
Discussion

Hybrid lethality has been characterized by the hyperactivation of plant immunity

- In several other species, the causal proteins resulting in hybrid lethality have been cloned; in all cases, these involve at least one pathogen-signaling gene; most commonly, these are NLRs, the most common class of *R* genes in plants
- The exact protein interactions resulting in hybrid lethality are not understood, but similar phenotypes are observed in mutants and lesion mimics resulting in constitutive expression
- The *R* gene is a member of a gene family with dozens of homologs
- The fast evolving nature (e.g. intergenic recombination, gene conversion, duplication) of the *R* gene family may explain the evolution of hybrid lethality in *Nicotiana*

Further characterization of the *Hl* locus and its corresponding gene

- Hybrid lethality is observed in other *Nicotiana* interspecific crosses – is the same locus or gene involved in all of these crosses?
- What is the interacting homolog in *N. africana*?
- What is the molecular structure of the Hl_T allele, and how does it interact with the Hl_A variant to produce hybrid lethality?
- What evolutionary steps resulted in hybrid lethality in *Nicotiana*?



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