**The genomic basis of a trade-off between defense (inducible indolic glucosinolate) and reproductive traits (flower colour related to pollinator preference) in California 'wild' radish *Raphanus sativus* (Brassicaceae)**

**1. Summary of the research plan**

The factors that maintain genetic and phenotypic variation within natural populations are a central problem in evolutionary biology. On the one hand, natural selection removes genetic variation from populations, but on the other, substantial genetic variation, more than can accounted for by mutation-selection or mutation-drift balance, persists in natural populations. This suggests that balancing selection (in the broad sense), is a more important force than previously believed in maintaining variation, through processes that include spatially varying natural selection, temporally fluctuating natural selection, overdominance (heterozygote advantage), conditional neutrality and antagonistic pleiotropy. Balancing selection refers to a number of selective processes by which multiple alleles are actively maintained in the gene pool of a population at frequencies larger than expected from genetic drift alone. Interactions between plants and their biotic interactors (mutualists and antagonists) have played an especially important role in addressing questions regarding maintenance of trait variations. However, the extent to which adaptive evolution is constrained by genetic correlations between defense and reproductive traits affecting plant interactions with pollinators and herbivores is unclear. Colour-polymorphic species are observed across diverse taxa but are relatively rare and younger than monomorphic species. Because, differences between colour morphs should result in the fixation of one of the alleles under natural selection, and it is likely that genetic drift removes alternative morphs from small populations. Therefore, understanding the mechanisms behind the maintenance of genetic variation notwithstanding genetic drift is of crucial importance to decipher the mechanisms of adaptation. Antagonistic pleiotropy is one of the mechanisms by which polymorphism in flowering plants is maintained through balancing selection. Wild radish *Raphanus sativus* (Brassicaceae) in California is colour-polymorphic species that segregates as two petal colour morphs: anthocyanin plus and anthocyanin minus (phenylpropanoids are a class of metabolites) and pollinators prefer the anthocyanin minus morph, which is also more susceptible to herbivores (lower production of inducible indole glucosinolate (GSL) levels). California wild radish is a recently derived population that evolved as a result of a hybridization event between cultivated radish and its wild progenitor, which were both introduced into California. A hybrid swarm has now replaced both of these parental species in the wild. It is now known that the petal colour and anti-herbivore defenses (induction of GSLs), in this system are interdependent as a result of pollinator- and herbivore-mediated selection. Phenylpropanoids and GSLs are two classes of secondary metabolites that are synthesized through apparently independent biosynthetic pathways, but genetic evidence has revealed that the accumulation of some GSL intermediates limits phenylpropanoid production. Here, I will use genomic tools; Genome Wide Association Studies (GWAS) to identify the precise genetic architecture and evolutionary history of a defense-linked colour polymorphism in the *R. sativus.* I will use crossing experiments to test if one or more loci control these two traits separating the effects of loci and will undertake a detailed analysis of the of the locus or loci controlling flower colour and inducible GSL production associated with the colour polymorphism. Taken together, my findings will present novel mechanism by which floral-colour variation is maintained, shed light on the genomic basis of a trade-off between defense and reproductive traits and will illustrate the importance of an inclusive, pluralistic view of selection when investigating the evolution of complex phenotypes. These results will represent exciting areas for my future research career because an integrated view of the evolution of plant reproduction and defense could help to explain the patterns and processes that have shaped these plant traits and how they influence biotic interactions.

**2. Research plan**

**2.1. Current state of research in the field:**

Understanding the degree to which species interactions affect the intensity and direction of evolutionary change remains a fundamental challenge in evolutionary ecology. Many plants rely on animal pollinators to vector pollen and reduce inbreeding in their offspring. However, plant defense against herbivores may compromise attraction of mutualist pollinators, yet little is known about the mechanisms underlying such signaling trade-offs. Ecological or pleiotropic (where an allele has multiple phenotypic effects) costs may result in trade‐offs between attraction and defense characters 1. Recently it was shown experimentally that evolutionary trade-offs driven by pollinator behavior and herbivore attack can be observed as quickly as eight generations, suggesting that trade-offs have rapid evolutionary consequences 2. Yet, we do not understand if such trade-offs are due to distinct (modular) evolutionary responses, or genetic constraints, such as pleiotropy at a single locus controlling both traits, or physical linkage between different loci controlling each trait. Understanding this is important for both applied (crop breeding) and basic (evolutionary genetics) perspectives. For example, herbivores discriminate among petal colour morphs in some plant species 3,4. If herbivores prefer some floral colours over others and the floral colour is heritable, there may be selection for colour that is not directly related to pollinator preferences. Consequently, selection on floral colour by a mutualist pollinator may be countered by selection from an antagonist herbivores if both agents prefer the same colour 5. One explanation for the links between defensive compounds and petal colour may be that genes controlling flower colour could directly influence plant resistance to herbivores, if pleiotropic effects exist between the synthesis or regulation of floral pigments and defensive plant compounds 3,6. Alternatively, if petal colour genes and defense genes are tightly physically linked, then selection on one trait may cause correlated changes in values of the other trait. Although, maintenance of defense-related petal colour polymorphism seems to be pervasive in nature, experimental evidences illustrating the genomic basis of such evolutionary trade-off is lacking. We will perform the first evaluation of this hypothesis, using the state-of-the-art genomic analyses to illustrate the evolutionary mechanisms underlying the maintenance of such colour polymorphism.

One example of how non-pollinating agents may affect the frequency of colour morphs can be found in wild radish, *Raphanus sativus* (Brassicaceae). Approximately 100 years ago, two species of radish (*Raphanus raphanistrum* and the cultivated radish *R. sativus*) were introduced into California from Europe and became naturalized. By the early 20th century, a few apparent hybrids were observed in the wild, and by the 1960s some populations were completely composed of morphologically intermediate plants, which came to be known as ‘wild radish’ 7. Presently, wild populations of the two parental species had actually been replaced by a hybrid swarm in California, and there is genomic evidence that the wild *R. sativus* have evolved as a result of bilateral genetic assimilation between *R. sativus* and *R. raphanistrum* 8. Given that California wild radish's evolutionary success most likely resulted from the evolution of a unique combination of traits relative to its naturalized alien progenitors, with a well documented history of introduction, it is a powerful system for studying evolutionary genomics of invasive species. *R. sativus* individuals, in California retain one of four different petal colours: yellow, white, pink or bronze. Petal colour is determined by two independently assorting loci, each with two alleles controlling the expression of carotenoids and anthocyanins 7. Yellow petals are result of the carotenoid pigments production with yellow allele recessive to white (absence of carotenoid). Pink petals, on the other hand, are the result of anthocyanin pigments production with white allele recessive to pink (presence of anthocyanin). Plants with bronze petals express both anthocyanin and carotenoids and thus have at least one dominant allele at the anthocyanin locus and only recessive alleles at the carotenoid locus. 5.

Pollinator preference for anthocyanin-recessive colour morphs (yellow and white flowers) of wild radish, over anthocyanin-dominant morphs (pink and bronze flowers) is well documented in the literature. In particular, honeybees prefer yellow and white colour morphs over pink and bronze 9. A study by Irwin et al (2003) revealed that herbivores of *R. sativus* generally also preferred leaves from anthocyanin-free morphs over the anthocyanins-producing morphs. Therefore, floral colours in wild radish are associated with herbivore resistance factors in leaves 4, however, no relationship was found between leaf anthocynanin content and herbivore performance, and traits linked closely to petal colour, seem to influence herbivore preference (S. Y. Strauss, unpublished data). A1 colour morphs produce higher concentrations of indole GSLs (a class of GSL compounds) in leaves than A2 morphs, in the presence of herbivore damage. Therefore, herbivores may exhibit lower preference for A1 colour morphs, because they are heavily defended once damaged. Subsequent findings indicated that petal colour variants differed in their induced responses to damage; yellow and white morphs, preferred by the dominant bee pollinators and by herbivores, were generally less inducible than anthocynanin-containing pink and bronze petal morphs. In addition, induction of indole GSL in leaf and petals show that there can be induction in petal tissue in response to leaf damage 5. Results from this study and previous studies 5,9 suggest that in the presence of pollinator discrimination, the colour composition of *Raphanus* populations should be evolving towards increasing frequencies of yellow colour morphs. However, ongoing studies in *R. sativus* populations in California indicate that colour frequencies stay relatively constant over short and long time periods 10. Given that variation in petal colour and inducible indole GSL levels are heritable traits in this species 11,12, conflicting selection pressures exerted by pollinators and herbivores, coupled with pleiotropy, tight linkage, or strong selection (co-selection) at independently segregating loci (long-range linkage disequilibrium) between colour and defense loci, could maintain variation in both traits 5,13. Therefore, pleiotropy, linkage or co-selection between loci may play a role in the maintenance of this polymorphism 5,6,14.

In line with these findings, there is genomic evidence that anthocyanin production may also be pleiotropic with respect to GSL production in Brassicaceae plants. *Arabidopsis thaliana* (Arabidopsis)mutants with altered alkyl GSL biosynthesis simultaneously affected phenylpropanoid metabolism, from which anthocynanin pigments are derived 15. The phenylpropanoid pathway converts amino acid Phe to a number of important secondary metabolites, including flavonoids, the class of metabolites in which anthocyanins belongs. In one hand, downstream metabolic branches from phenylpropanoid pathway lead to production of certain class of secondary metabolite sinapate esters, which are found in Brassicaceae plants, including Arabidopsis 16. On the other hand, Brassicaceae plants synthesize amino acid Met-, Phe-, and Trp-derived GSLs 17. These compounds are nitrogen- and sulfur-containing secondary metabolites, which are substrates for the enzyme myrosinase, which degrades them into biologically active products that are toxic to herbivores. Cytochrome P450- dependent monooxygenase enzymes (P450s) catalyze a number of the reactions in GSL biosynthesis. In wild-type Arabidopsis plants, the primary functions of CYP83A1 and CYP83B1 are in alkyl and indole GSL biosynthesis, respectively 15. Arabidopsis mutants impaired in CYP83B1 production accumulate lower amount of indole-derived GSLs than the wild type18-20. In addition, Arabidopsismutants impaired in CYP83A1 production contain higher levels of indole GSL 21. Recent findings indicate that a loss of alkyl GSL biosynthesis leads to an increase in indole GSL levels. Additionally, these findings suggest that a loss of alkyl GSL biosynthesis (CYP83A1) simultaneously perturbs phenylpropanoid metabolism in these mutants.15. A more recent study revealed that Arabidopsis plants harboring a mutation in CYP83B1 displayed defects in GSL biosynthesis and in phenylpropanoid accumulation in a way that the accumulation of the CYP83B1 substrate indole-3-acetaldoxime (which is a precursor of indole GSLs) negatively influence phenylpropanoid accumulation 22. This further supports a model whereby pleiotropy at a single locus can affect both indole GSL and anthocyanin (via phenylpropanoid) levels. Our aim is to take advantage of these recent genomic findings to evaluate experimentally whether such genomic mechanisms are acting in an ecologically relevant natural system of *R. sativus* and are responsible for maintaining the colour polymorphism.

The current state-of-the-art of the field can be summarized: 1) both pollinators and chewing herbivores simultaneously exert selection pressure on colour morph frequencies in wild radish; 2) evolutionary trade-offs driven by pollinator behavior and herbivore attack can be rapidly observed, with evolutionary evidence after only eight generations in mustard plants, suggesting that trade-offs have large evolutionary consequences; 3) pleiotropic interactions between genes affect both the GSL and anthocyanin pathways in mustards. This project will advance this cutting-edge question to understand if such trade-offs are due to distinct (modular) evolutionary responses, or due to genetic constraints, such as pleiotropy at a single locus controlling both traits or physical linkage between different loci controlling each trait. In addition, it will enrich our understanding of whether the loci controlling these traits are maintained by balancing selection for a long time similar to disease resistance genes (R genes) in plants23

**2.3. Detailed research plan**

**Objectives and research questions**: This project is organized in two **work packages (WPs)** that include three research questions. The key objective of this project is to investigate the genomic basis of a trade-off between defense (inducible indolic glucosinolate levels related to chewing herbivore defense) and reproductive traits (flower colour related to pollinator preference) in California 'wild' radish.

**Question 1: Is the variation in pollinator vs defense traits maintained by balancing selection?** Flower colour in *R. sativus* is associated with consistent differences in secondary chemical variation, implying that herbivores could act as selective agents on this flower colour polymorphism. Additionally, consistent preference by pollinators for particular colour morphs, suggests that pollinators could also act to maintain such variation in anti-herbivore defensive chemistry; indicating the conflicting selection pressures imposed by both agents4,5,10. To understand whether this conflicting selection maintains variation among genotypes of wild radish by means of balancing selection, we will perform a field experiment to determine whether balancing selection operates on flower locus by understanding the gene frequency changes as an indication of balancing selection.

**WP 1: Convergent Selection experiment to understand whether differences among petal morphs in leaf defensive chemistry is maintained by balancing selection.** We will perform a “convergence” experiment in the first year of the fellowship in which we establish experimental populations, originating from seeds from 200 families collected from one field population in California, will be grown as a base generation. We will then document flower colour and induced indole GLS co-variation in these plants over one generation to determine whether their frequencies tend to converge toward typical, intermediate values, as would be expected if balancing selection was operating. In order to characterize the GLS compounds present in each plant, we will use the high performance liquid chromatography coupled to mass spectrometry (HPLC-MS/MS).

**Question 2: Are the two traits controlled by the same gene network (pleiotropy); are the two traits controlled by a different genetic architecture or are the loci linked physically (or are in linkage disequilibrium)?** One explanation for the links between inducible indolic GSL production and petal colour may be that pleiotropy exists between the synthesis or regulation of floral pigments and induction of indole GSLs. Alternatively, if petal colour genes and defence genes are tightly linked; then selection on one trait may cause correlated changes in values of the other trait—and variation in the two traits act as a supergene. In order to understand the genetic correlations between GSL and anthocyanin production, we will first perform a genetic correlation study followed by Genome Wide Association Studies (GWAS).

**WP 2: Genetic correlation analysis and GWAS to identify the genomic architecture of the two traits. WP 2.1**: we will first perform a **genetic correlation study** using the plants of base generation from WP 1, in which each base generation plant will be crossed to each of three other plants, and each plant will also act as a father to three mothers. The full-sib/half-sib progeny will be grown to test for genetic correlation between the two traits. This will quantify the degree of pleiotropy and/or linkage operating with respect to variation in the two traits. **WP 2.2**: In parallel, we will perform **Genome Wide Association Studies (GWAS)** on the base generation plants for these traits to identify the loci controlling the variation. Here, we will use genomic and transcriptomic tools to identify the precise genetic architecture and evolutionary history of a defense-linked colour polymorphism in the *Raphanus sativus.* We will undertake a detailed analysis of the region associated with the colour polymorphism and indole GSLs production using genomic and transcriptomic tools, and identify the genomic location of the locus as a putative regulatory region.

**Question 3: Is there genomic evidence for balancing selection at loci associated with low and high indolic glucosinolate and presence or absence of anthocyanin?** Using the data from the Convergent Selection experiment (**WP 1)** coupled withGWAS data obtained from (**WP 2.2**), we will be able to confirm whether loci associated with low and high indolic GSLs are experiencing balancing selection with presence or absence of anthocyanin.

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