Chapter 4
Balancing Trait Improvement with Tradeoff Side-Effects Using Genome Editing Technology

Julia Johanna Wind

Abstract Improving traits by breeding involves compromising between desired traits and possible undesired side effects. Often encountered examples include pathogen resistance versus yield, shelf life time versus fruit quality, and seed number versus seed weight. Genome editing can be used to reduce the effect of some of these tradeoffs. Different genetic reasons underlying a tradeoff require different approaches: important to note is whether a detrimental effect is caused by a unique gene, or several analogs/ homologs, because the strategy needs to be adopted accordingly. Unique genes, for example, can be substituted by analogs, and homologs have the advantage that, amongst the available options, the gene causing the fewest pleiotropic effects can be altered in its activity. When the detrimental effect of a tradeoff is caused by two genetically linked genes, this can lead to linkage drag. To break this type of tradeoff genome editing can be used to force a crossover event. Overcoming a tradeoff can generate a new one, but can nevertheless result in an improved crop variety.

1 Different Genetic Reasons Underlying a Tradeoff

The first plant breeding technique was the selection and propagation of plants with improved heritable traits, which were based on the cumulative net positive outcome of genetic changes. In more modern breeding, Quantitative Trait Loci (QTL) studies have led to the discovery of alleles responsible for the improvement of traits. Molecular breeding was then developed to cross such alleles into the crop variety of interest, thereby increasing speed and accuracy of the breeding process. Studying the effects that these alleles had on a trait made apparent how common pleiotropic effects are due to the introduction of an allele from a wild donor to a receiving elite line: often the improvement of one trait goes hand in hand with negative effects on other traits. The association between breeding values of linked traits can be positive

J. J. Wind (✉)
Keygene N.V., Wageningen, Gelderland, Netherlands
e-mail: julia.wind@keygene.com

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or negative so that if one trait changes, other traits will change as well. A positive value indicates a win-win situation. For example, shorter plants have been shown to be easier for the mechanical harvest and also lead to a yield gain because plants put more energy into seed production. A negative value indicates a tradeoff: for example, an average increase in seed number often leads to a decrease in average seed weight. Genome wide association study (GWAS) experiments are very effective in indicating which genes or alleles are important for a given trait. Trait correlations are often caused by a set of genes that form the connection between two traits. However, some alleles have been discovered which cause breaks in trait associations. They can be the key to overcoming tradeoffs. However, such findings are rare and can often not be translated to crop plants. Transgenic approaches can be used to introduce these alleles. Alternative approaches to influence trait associations include selection of novel alleles from mutant populations or targeted mutagenesis with genome editing. Mutant populations have been created by random mutagenesis with a mutagen such as ethyl methanesulfonate (EMS), and such populations are very expensive to make because they require thousands of chemically treated individuals that are grown for multiple generations. Therefore, targeted mutagenesis is attractive as it can make targeted mutations in the gene or promoter of interest, so that only a few plants need to be edited instead of a large population. A few types of editing are discussed here: editing to create genetic variation including knockouts, editing to alter expression of target genes and cutting DNA to induce crossovers in recombination cold spots. These techniques are discussed in the context of a few well-known tradeoffs.

2 Unique Genes

During plant evolution, two important whole genome duplications have occurred. First, in the ancestral seed plant whole genome duplication has led to the divergence of the seed plants. Second, whole genome duplication in the ancestral angiosperm plant has led to the angiosperm radiation. These whole genome duplications lead to gene duplication. Changes accumulate in the gene copies over time, because the alleles are under different selective pressure. This can result in divergence of gene function. It was estimated that 65% of genes have at least one duplicated copy. Genes are often part of gene families, but this does not mean that they can always be substituted by a homolog or an ortholog: this is clear when a knockout or an allele of the gene results in a phenotype. Such genes can be important regulators of plant growth and development, and often have pleiotropic effects when they are modified. Therefore, reducing the negative effects of a tradeoff is most difficult for these types of genes. Most of the time, finding another gene that affects the trait in a similar way, but with fewer pleiotropic effects would be preferable. Three examples of tradeoffs caused by unique genes are discussed in the next sections.

2.1 Flowering Time vs. Yield

FLOWERING LOCUS T (FT) is a well-known and often studied flowering time regulator that is unique for its central role in the induction of flowering in most plant species [1]. Knockouts of FT exhibit a late flowering phenotype, and overexpression reduces time before flowering. FT also plays a role in control of growth, heterosis, tuberization and the regulation of stomatal opening in a variety of plants [1]. Therefore, FT can have pleiotropic effects on plant growth and development. Natural variation for flowering time in canola is frequently associated with FT alleles and comes with a biomass tradeoff [2]. Swinnen and colleagues have summarized examples from literature of genetic variation in cis-regulatory elements in genes that underly initial domestication of various crops [3]. A large part of these alleles are in the FT promoter, suggesting that altered expression pattern of this gene is of major importance in the domestication of crops, very likely due to its effect on flowering time and flowering synchrony. Pleiotropic effects are also evident in other flowering time regulators, such as TERMINAL FLOWER 1 (TFL1). *Brassica napus* knockouts for BnaC03.TFL1 does not only have reduced flowering time but also reduced plant height, as well as reduced branch number, number of siliques, and seeds per silique [4]. Variation in the FT promoter through genome editing technologies could result in more genetic variation aimed at reducing pleiotropy. Changing expression of FT is also likely to improve synchronous flowering as a positively correlated trait, but it might be hard to limit the negatively correlated traits that result from early maturity, because of the link between early flowering and early maturity. If earlier flowering time is needed, it could be preferred to find genes that can speed the overall growth so that the plant matures faster. A knockout in cpn60β4 in *Arabidopsis thaliana* was shown to accelerate plant development [5]. Using CRISPR, cpn60β4 orthologs could be knocked out in the species for which accelerated flowering is needed, as this gene is conserved in angiosperms [5]. This might lead to a similar effect on reducing flowering time without early maturity.

2.2 Fruit Shelf Life vs. Lycopene Content

The Food and Agriculture Organization from the United Nations has calculated that tomato has become the third most grown vegetable worldwide in the last decade and is therefore considered a very valuable crop [6]. Two properties are indispensable for the success of tomato: firmness and high lycopene content. Delayed ripening improves firmness and reduces the damage during the shipping of tomatoes as well as storability, while high lycopene improves the attractiveness for consumers, the value for the processing industry, as well as its nutritional value. Transcription factors that are important in improving shelf life are NON-RIPENING (NAC-NOR) and RIPENING-INHIBITOR (MADS-RIN). Both proteins are not knockouts but

alleles causing a phenotype: NOR is a partially mistranslated but functional protein and RIN is a fusion between two proteins. They both cause physiological, transcriptional and hormonal changes in ripening tomato fruits. Both of these regulators delay fruit ripening in similar but not identical ways. Crossing the RIN mutation to eight different wild type tomato lines clearly demonstrated that the improvement of shelf life negatively correlates with reddening of the tomato in the F1 progeny, and it was shown that the reduced reddening is due to a twofold reduction of lycopene. Conclusively, RIN and NOR mutations can improve shelf life at the cost of lycopene production. However, transgenic lines aiming to improve astaxanthin content surprisingly accumulated high levels of lycopene and also displayed an improvement in shelf life [7]. The authors explain the phenotype as the result of the extended duration of lycopene synthesis, so lycopene can accumulate, whereas the lack of β-carotene or its metabolic products possibly reduced the feedback inhibition. This phenotype demonstrates that firmness and lycopene content are not necessarily always correlated. The genes that were overexpressed originated from marine bacteria from the *Brevundimonas* genus and these genes are not present in tomato. However, the enzyme that forms β-carotene or its metabolic products are present in the tomato genome and hence could be knocked out. Alternatively, reducing the expression with an RNAi construct of a fruit-specific expressed pectate lyase (Solyc03g111690) reduced the softening of the tomato fruit, showing that it is possible to bypass the overall climacteric ripening program [8]. This could be easily reproduced with a genome edit aiming to knockout or knockdown this gene. A third example that firmness and carotenoid levels are not always correlated is the phenotype of the *hp1* and *hp1-w* mutants. These plants produce tomatoes with delayed ripening, higher levels of carotenoid and other phytonutrients due to altered light transduction [9]. However, the authors point out that this results in undesirable whole plant phenotypic changes and therefore they suggest that the knockdown of the gene responsible for the phenotype, DNA damage-binding protein 1 (*DDB1*), should be occurring only during fruit ripening. This demonstrates that the tradeoff of high lycopene content vs. delayed ripening can be resolved by taking one of several possible approaches that could bypass the tradeoffs: either by rerouting the lycopene pathway, directly targeting the enzymes involved in softening, or by altering the light signaling pathway specifically in the tomato.

2.3 Seed Number vs. Seed Weight

For many crops, an increase in seed number leads to a reduction in seed weight. Grain Weight 2 (TaGW2-A1), is a well-characterized gene in wheat that has a positive effect on seed weight but reduces the number of seeds. The introduction of the wild emmer allele *GNI-A1* into wheat was demonstrated to break this correlation because this allele could improve seed weight without affecting seed number, as reviewed by [10]. It was demonstrated that a single amino acid substitution is responsible for this (G182R). Alternatively, overexpressing an α-expansin gene in

wheat led to transgenic lines with a higher seed weight, no significant change in seed number, and hence an increase in grain yield of 12.3% [10]. These two examples show that an increase in seed weight does not always lead to a reduced seed number. The opposite was also demonstrated. In rice, elevated *NOG1* expression was shown to increase the number of grains per plant without affecting the grain weight, and this was due to a natural variation of a 12 bp insertion in the promoter [10]. Crossing an allele that improves seed weight to another allele that improves seed number would be a good strategy to improve total yield. The above examples are in wheat and rice, and thus analogs/homologs should be found in other grain crops of interest. A list of candidates that could have a comparable function could be made, and these genes can be edited, either by SNP introduction (to GNI-A1 homologs) or through promoter editing (for α-expansin or NOG1 expression). This could be done simultaneously with genome editing technologies aimed at multiplexing of editing tools. Together these examples show that the genetic link of two traits causing a tradeoff can be broken through introducing/creating/knocking out alleles that do not affect both traits. Different tools can be used to achieve this effect. The detailed molecular studies of tradeoffs allow for the use of targeted mutagenesis. Hence, genome editing can be very helpful when multiple alleles need to be modified or when natural variants are not available in the available germplasm.

3 Making Use of Expression Diversity in Orthologs/Homologs

As mentioned before, many genes are part of gene families. This essentially means that a mutation in such a gene, if is not dominant or dominant-negative, does not lead to a phenotype because the function is compensated by a homolog with a similar function. For breeders this means that for many genes, single knock-outs are not effective for improving traits. However, if a gene that is important for a trait, is part of a gene family, and causes additional negative effects on other traits, there is an opportunity to substitute such a gene with a homolog.

3.1 Fruit Size vs. Inflorescence Branching

Using genome editing technology to modify promoters can be aimed at introducing single nucleotide polymorphisms (SNPs), small and larger deletions and even rearranging the promoter randomly though multiplex editing. Generating a population of plants with variations in the promoter sequence of a single gene can be used for fine-tuning the desired effect. A series of deletions in the promoter of CLAVATA3 in tomato (SlCLV3) was linked to altered expression and it was shown that the altered expression coincided with an altered level of locule number and thus fruit size, though not predictably [11]. Reducing SlCLV3 activity also promoted inflorescence

branching [12], which can lead to excessive number of fruits, dampening the effect that reduced SlCLV3 expression can have on fruits size due to disturbed source to sink transport. It was shown that a null mutant for SlCLV3 in tomato is compensated by elevated expression of the ortholog SlCLE9 [12]. The authors also show that SlCLE9 has a similar function as SlCLV3, though the SlCLE9 knockout has a weak phenotype with only a subtle effect on locule number. This means that an edited promoter population for either SlCLV3 or SlCLE9 could be a valid strategy to obtain a tomato plant with a high number of locules and regular inflorescence branching.

3.2 Shorter Plants vs. Plant Morphology

Mutants in the gibberellic acid biosynthesis and catabolism pathway often result in a dwarf or elongated phenotype respectively, due to modified gibberellic acid levels. However, this often comes with pleiotropic effects such as increased tillering in rice, increased culm bending in sorghum, and in one case can result in complete inhibition of flowering in rice. Tomato *internode elongated-1* (*EI-1*) is a splice-site mutation in the SlGA2ox7 gene. SlGA2ox7 is a gibberellin 2-oxidase that catalyzes the breakdown of certain bioactive gibberellins and *EI-1* results in a dwarf phenotype. *EI-1* leads to an increase in bioactive gibberellins in stems and petioles [13]. However, since SlGA2ox7 is more highly expressed in hypocotyls and internodes than in petioles, the elongated internode mutation phenotype is stronger here. Due to this organ specific expression, *EI-1* exhibits a reduced elongation specific effect only, while its ortholog Solyc10g005360 has a different expression pattern and is therefore expected to have more pleiotropic effects, including in the leaves. Consequently, the former gene would be a preferred candidate for genome editing as higher expression could lead to shorter plants without affecting the morphology of the leaves. Similarly, in pea the mutant *Le-1* has a mutation in a gibberellin 3-oxidase, which results in a shoot specific phenotype, while the roots are unaffected. In summary, to shorten the height of a crop species, unwanted pleiotropic phenotypes could be avoided by learning about the expression patterns in all genes that are affecting this phenotype, and choosing a gene that has a tissue specific expression.

3.3 Vitamin C vs. Growth

Ascorbate peroxidases (APX) catalyze the H_2O_2-dependent oxidation of ascorbic acid (vitamin C) in plants, and can therefore reduce ascorbic acid levels. In Arabidopsis, a knocked out major cytosolic isoform of APX led to severe growth retardation. In contrast, in tomato there are nine homologs that encode for APX enzymes that catalyze the breakdown of ascorbate, with one being highest expressed

in red ripe tomato fruits. Specific mutation of *SlAPX4* by genome editing led to an increase of ascorbic acid in fruit with no detected growth impairment [14]. Hence, this approach might be easily copied to other crops for the biofortification of fruits, in case their genomes contain a family of *APX* homologs.

3.4 Blast Resistance vs. Yield

Rice blast is a disease caused by the fungal pathogen *Magnaporthe oryzae* and is a huge problem in rice cultivation. Resistance to this disease has a tradeoff with yield. *Pigm* is a locus that has been used in breeding for durable and broad-spectrum resistance to rice blast. The locus consists of 13 homologs coding for NLR receptors and two of these, *PigmR* and *PigmS* are separated by just two genes. While *PigmR* confers blast resistance but at a yield cost, *PigmS* attenuates the blast resistance, and therefore counteracts the yield cost by promoting seed set [15]. Interestingly, increased expression by transgenic overexpression of both loci, overcomes this tradeoff. Hence, the *Pigm* locus effect on yield could be improved by editing their promoters for improved expression of both.

4 Overcoming Linkage Drag

Sometimes a tradeoff is caused by two genes that are in close proximity to each other on a chromosome. Such genes might genetically be linked if recombination between the genes is low or absent. This would make separation of these genes difficult or even impossible. If one of the two genes has a positive effect on a trait, but the other one has a negative effect on the same or another important trait, this presents a tradeoff known as linkage drag. Linkage drag examples include virus resistance vs. yield in tobacco, viral resistance vs. bacterial resistance in tomato, abiotic stress resistance vs. yield and quality traits in sunflower, and heading date vs. root biomass in wheat. In a series of near isogenic lines, the precise site was determined where recombination could break the tradeoff between viral resistance and bacterial resistance in tomato [16]. Recombination of the *I-3* gene out of the donor *Solanum pennelli* introgression at the end of the chromosome would remove the unknown gene from *S. pennelli* that causes bacterial spot susceptibility. The technology to induce precise crossover events is still in development, but some progress has been made. Controlling the recombination event has been shown in yeast. In plants linkage drag could be broken by swopping chromosome arms between linked loci [17]. The authors also demonstrated how two chromosome arms were exchanged in the *ALS2* locus for the *Solanum pennellii* and *Solanum lycopersicum cv. M82*. This shows that performing controlled recombination to remove a gene with a deleterious effect while maintaining the novel introgressed allele in plants is a promising new breeding tool.

5 Concluding Remarks and Future Perspectives

Applying genome editing technology to reduce tradeoff side-effects has potential as a tool in plant breeding. Especially knockout editing or promoter editing has been proposed due to their broad applicability. More precise edits can result in altered protein function, and to obtain this, different editing technologies are being developed [18]. Due to the nature of DNA editing enzymes and the properties of chromosomes in alive cells, not every nucleotide can be edited in such a controlled manner. Also, off-target effects are a problem for genome editing success. Solving this remains a challenge for the future. It should be considered that not all crops can be edited yet. For example, incomplete sequenced genomes, difficulty in editing itself, resistance to regeneration or obligatory outcrossing are hurdles to overcome. Some tradeoffs were broken through an allele that was discovered with a QTL experiment. Other tradeoffs can be broken through editing (promoters) of genes, especially if tradeoff-breaking alleles have not been discovered in the crop of interest. Both approaches can be useful additions to the breeders' toolbox. Breaking tradeoffs is not easy. In fact, breaking one tradeoff could create a novel one. An example is the GNP1^{TQ} allele in rice which has broken the seeds number vs. seed weight tradeoff. When this allele was introduced in the Lemont background, this did not result in a higher yield because the variety could not meet the increased sink capacity needed for the filling of the grains [10]. So next, the grain number vs. sink capacity tradeoff needs to be addressed. In the end, by balancing tradeoff side-effects with trait improvement, new crop varieties can be bred that meet novel breeding demands.

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