

# Use of Multicopy Transposons Bearing Unfitness Genes in Weed Control: Four Example Scenarios

Jonathan Gressel\* and Avraham A. Levy

Plant Sciences, Weizmann Institute of Science, Rehovot 76100, Israel

We speculate that multicopy transposons, carrying both fitness and unfitness genes, can provide new positive and negative selection options to intractable weed problems. Multicopy transposons rapidly disseminate through populations, appearing in approximately 100% of progeny, unlike nuclear transgenes, which appear in a proportion of segregating populations. Different unfitness transgenes and modes of propagation will be appropriate for different cases: (1) outcrossing *Amaranthus* spp. (that evolved resistances to major herbicides); (2) *Lolium* spp., important pasture grasses, yet herbicide-resistant weeds in crops; (3) rice (*Oryza sativa*), often infested with feral weedy rice, which interbreeds with the crop; and (4) self-compatible sorghum (*Sorghum bicolor*), which readily crosses with conspecific shattercane and with allotetraploid johnsongrass (*Sorghum halepense*). The speculated outcome of these scenarios is to generate weed populations that contain the unfitness gene and thus are easily controllable. Unfitness genes can be under chemically or environmentally inducible promoters, activated after gene dissemination, or under constitutive promoters where the gene function is utilized only at special times (e.g. sensitivity to an herbicide). The transposons can be vectored to the weeds by introgression from the crop (in rice, sorghum, and *Lolium* spp.) or from planted engineered weed (*Amaranthus* spp.) using a gene conferring the degradation of a no longer widely used herbicide, especially in tandem with an herbicide-resistant gene that kills all nonhybrids, facilitating the rapid dissemination of the multicopy transposons in a weedy population.

### INTRACTABLE WEED PROBLEMS AND THE PROPOSED USE OF MULTICOPY TRANSPOSONS IN PEST CONTROL

Most of the intractable weed problems at present can be classified into two general groups: weeds that are closely related to crops, for which there are no selective herbicides, and weeds that are rapidly evolving multiple resistances to herbicides and/or to new habitats. New solutions must be found for these problems, as the chemical solutions, if even available, are not sustainable in the long term. This includes such unsustainable solutions as using transgenic herbicide resistance. The transgene in the first case can rapidly introgress into the related weed, and in the second case resistance evolves quickly. Solutions have been proposed and tested on how to mitigate gene flow from crop to weed by tandemly attaching to the herbicide resistance transgenes a second gene that is neutral to the crop but confers unfitness to the weeds (Gressel, 1999; Al-Ahmad et al., 2005; Rose et al., 2009). Nonchemical solutions that can either partially replace or augment the chemical solutions are desirable to ensure the sustainability of all solutions in the longer term. It may be hard to find solutions if we incorrectly consider (as some do) weeds as wild species whose biodiversity must be protected. The typically intractable weed species are (inadvertently) human domesticated species and thrive only in human disturbed habitats. They codomesticated and coevolved with crops, such that they are typically poor at competing in the wild, just as major crops cannot

compete outside of coddled agroecosystems. When one compares the traits of weeds with those of closely related wild species, one sees that there is a syndrome of traits that relates to weeds and another for wild species, of course with some overlap (Warwick and Stewart, 2005).

The use of transposons to disseminate unfitness genes has been proposed for insect control. Here, we describe modifications of the insect paradigm that might be used in controlling weeds, discussing four disparate weed/crop systems where transposons might be used. We previously discussed in a very preliminary manner how such a technology might be used to deal with the parasitic *Striga hermonthica* (Gressel and Levy, 2000) but did not extrapolate to other weed systems, and we thought that the utility would be limited solely to obligately outcrossing species. This may not necessarily be the case in some instances, as will be discussed in the case of rice (*Oryza sativa*).

The original transposons with armed cassette-targeted insect control strategy (TAC-TICS) concept was to engineer a lethal gene under the control of an inducible promoter into a transposon and then engineer the transposon in multiple copies into the insect pest (Pfeifer and Grigliatti, 1996; Grigliatti et al., 2001) or *Striga hermonthica* (Gressel and Levy, 2000) and release individuals into the field. These transposons introgress into multiple loci on many chromosomes, which results in their dispersal in all progeny. The advantage of multicopy transposons is that all or almost all progeny of matings and their further backcrosses as well as selfed progeny contain the transposon and not just a fraction of the population, 75% by selfing and only 50% by outcrossing, as with single-copy Mendelian genes. In one laboratory

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\* Address correspondence to jonathan.gressel@weizmann.ac.il.  
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experiment, 90% of the insect population contained the inactive, potentially lethal transposon-carried gene, six generations after introduction of a few multicopy transposon-carrying insects into the population (Grigliatti et al., 2001). The gene can be chemically activated at some point after dissemination, decimating the pest population. Sadly, the initial proponents of TAC-TICS and the entomology community have not gone beyond the laboratory experiments cited, due to lack of support.

A typical example of spread through a population is the *Drosophila* spp. P element (Houck et al., 1991). Genetic stocks from multiple collections, together with sequencing data, indicate that the P element is a recent arrival to the *Drosophila* spp. genome. It appeared approximately 40 years ago, and since has invaded most if not all the wild populations of *Drosophila* spp. There are many examples in plants of active transposons that can proliferate while jumping. These include DNA transposons that jump preferentially during DNA replication, such as *Activator/Dissociation (Ac/Ds)*; Greenblatt, 1984) and *Mutator* (Walbot and Warren, 1988), and as a result can increase in copy number. This also includes retroelements that transpose via a copy-paste mechanism through reverse transcription of an RNA intermediate, such as for the rice active retroelements (Hirochika et al., 1996; Naito et al., 2009). Transposons have also been engineered to carry genes, such as antibiotic or herbicide resistance for positive selection, or reporter genes for monitoring and use in promoter and enhancer trapping (Meissner et al., 1997). Usually, the engineered transposon is nonautonomous (i.e. it does not catalyze its own transposition but is dependent on a transposase-encoding gene provided in trans). Positive selection for the presence of transposons has been used in tagging experiments, where the goal is to promote transposition in order to mutate genes and then to monitor the presence of the transposon (Sundaresan, 1996).

#### USE OF HERBICIDE SENSITIVITIES AS A KEVORKIAN GENE

A novel addition to the TAC-TICS concept is to use herbicide susceptibility in the transposon as the *kev* (for Kevorkian [chemically induced suicide]) gene in crop varieties or to directly disseminate it separately in weedy rice or shattercane (*Sorghum bicolor*). Genes have been pinpointed that naturally degrade herbicides in specific plant species, providing the herbicide selectivity in those species (Table I). Silencing such genes provides a high level of sensitivity to the herbicide, often greater than that in typically sensitive species, because the sensitive species often have some basal ability to degrade herbicides but not enough to remain alive. Assuming that the genes of choice for crop resistance are to glyphosate, glufosinate, or a protoporphyrinogen oxidase inhibitor that controls grasses as well as broadleaf weeds, the herbicide susceptibility on the transposon should be to a commonly used inexpensive herbicide such as 2,4-dichlorophenoxyacetic acid (2,4-D) that has been supplanted by the latter herbicides (Table I). This is

because 2,4-D is no longer needed in rotations when the above are used, as they control the same weeds. Nuclear introgression of 2,4-D susceptibility would pass resistance to a fraction of the progeny of any hybrids and their backcrosses, but 2,4-D sensitivity in transposons will be conferred upon most progeny. Thus, at any stage of the rotation when it is necessary to use 2,4-D (because weeds have introgressed or evolved incipient resistant populations to the transgenic resistances in rice), 2,4-D can be used. 2,4-D will not only control the crop × weed hybrids that introgressed herbicide resistance, it will control almost all of the weedy backcrosses in the population, due to multicopy transposon spread to all progeny.

#### FOUR EXAMPLES OF INTRACTABLE WEEDS NEEDING POTENTIALLY DIFFERENT SOLUTIONS

Four case studies are presented below depicting how the transposon technology might be utilized in four different weed/crop systems. The differences in crops and weeds necessitate a separate discussion of each.

##### Major Outcrossing Weeds (e.g. *Amaranthus* spp.)

Two *Amaranthus* spp. appeared among the top 15 of the *World's Worst Weeds* (Holm et al., 1977) when that iconic book appeared 37 years ago. Even though the Amaranthaceae are all New World species, they are distributed worldwide, and they have great genetic diversity far from their origin (Ray and Roy, 2009). *Amaranthus* spp. would now rank much higher in such ratings in much of the world, but especially in the U.S. corn belt. Their huge seed output, which provides evolution with many choices, has facilitated their rapid evolution of herbicide resistance to many different modes of action (Legleiter and Bradley, 2008; McMullan and Green, 2011; Nandula et al., 2012; Bell et al., 2013; Heap, 2014). This rapid evolution is further exacerbated by the facts that *Amaranthus* spp. are wind pollinated, many of the species are obligate outcrossers, and many species hybridize intragenerically, thus often resulting in their being allotetraploids (Greizerstein and Poggio, 1995). This polyploidy facilitates rapid evolution, because gene duplication through whole-genome duplication leads to the buffering of mutations due to the redundancy of each gene. Slightly unfit mutations such as those conferring herbicide resistance are not eliminated from the population because of competition when the particular herbicide is not used, due to the presence of the duplicate unmutated gene. This, in turn, allows the accumulation of genes with new functions (Byrne and Wolfe, 2007; Doyle et al., 2008), as they are not deleted by intraspecific competition. Allotetraploids such as *Amaranthus* spp., therefore, have more chances to evolve herbicide resistance than diploids. One species, the wetlands *Amaranthus rudis* = *Amaranthus tuberculatus*, was hardly known in agricultural settings when *World's Worst Weeds* was written but has since emerged from the swamp to become a major weed with multiple herbicide resistances, some possibly due to hybrids with

**Table 1.** Potential *kev* genes that could be used in transposon constructs and dispersed in weeds: They can be engineered under constitutive or inducible promoters, depending on the context

Genes related to primary metabolism that are lethal when introduced in antisense, RNAi, or microRNA with an inducible promoter can also serve as *kev* genes in transposon constructs. CYP, Cytochrome P450; GST, glutathione *S*-transferase; *Δgai*, truncated gibberellic acid acceptor; *SH1*, shattering1.

Gene	Trait	System Appropriate	Reference
Enhanced herbicide susceptibility: genes in antisense, or as RNAi, or as microRNA			
<i>CYP72A31</i>	Bispyribac detoxification	<i>indica</i> rice/weedy rice	Saika et al. (2014)
Many unknown	Detoxification in rice	Rice/weedy rice	Usui (2001)
<i>CYP81A6</i>	Bentazon and some sulfonylurea detoxification	Rice/weedy rice	Pan et al. (2006)
Carboxylesterase	2,4-D detoxification 2,4-D and clodinafop detoxification	Rice/weedy rice	Gershater et al. (2006)
Unknown CYP	Prosulfuron detoxification	Sorghum/weedy sorghums	Moreland et al. (1996)
Unknown CYP	Metolachlor detoxification	Sorghum/weedy sorghums	Moreland and Corbin (1991)
Unknown CYP	Bentazon detoxification	Sorghum/weedy sorghums	Burton and Maness (1992); Hinz et al. (1997)
GST	Atrazine detoxification	Sorghum/weedy sorghums	Frear and Swanson (1970)
GST(s)	Propachlor detoxification	Sorghum/weedy sorghums	Lamoureux et al. (1971)
GST B1/B2	Metolachlor	Sorghum/weedy sorghums	Gronwald and Plaisance (1998)
GST(s)	Alachlor/atrazine	Sorghum/weedy sorghums	Hatton et al. (1996)
Unknown	Mesotrione detoxification	Sorghum/weedy sorghums	Joy et al. (2009)
Unknown CYP	Thiazopyr	Sorghum/weedy sorghums <i>Amaranthus/Amaranthus</i> spp.	Feng et al. (1995)
Unknown CYP	Simazine detoxification	<i>Lolium/Lolium</i> spp.	Burnet et al. (1993)
Unknown CYP	Mesotrione detoxification	<i>Amaranthus/Amaranthus</i> spp.	Ma et al. (2013)
Unknown GST	Atrazine detoxification	<i>Amaranthus/Amaranthus</i> spp.	Ma et al. (2013)
Unfit mitigating genes			
<i>Δgai</i>	Dwarfing	Rice, sorghum, <i>Lolium</i> , <i>Amaranthus</i> spp.	Al-Ahmad et al. (2006)
<i>SH1</i>	(Anti)shattering	Sorghum, rice (?)	Lin et al. (2012)
RNA silencing (many)	Male and female sterility	Inducible	Liu and Singer (2013)
Many	Male sterility	Inducible	Kausch et al. (2013)

other *Amaranthus* spp. (Trucco et al., 2009). This species is the first known to agriculture and medicine in which a resistance has evolved due to the loss of a whole codon (Patzoldt et al., 2006).

### Species That Readily Introgess with Crops (e.g. Weedy and Wild *Sorghum* spp.)

Cultivated grain sorghum (*Sorghum bicolor* subsp. *bicolor*) is conspecific with the pasture-grass sudangrass as well as with the major weed shattercane, which is a feral form of the crop that shatters its seed and has secondary dormancy. Shattercane is naturally resistant to selective herbicides used in maize (*Zea mays*) and sorghum and has evolved resistance to most other herbicides used for its control (Heap, 2014).

While the various *Sorghum* spp. predominantly self-pollinate, the crop naturally 10% to 20% outcrosses with its (crop) self (Riccelli-Mattei, 1968) and with shattercane (4%–16% in close proximity and as much as 2.6% in a single panicle 200 m downwind; Schmidt et al., 2013). *Sorghum* 0.1% to 13% outcrosses with sudangrass when cultivated in its proximity (Pedersen et al., 1998). Stratified population structures increase the rate of outcrossing

(Ellstrand and Foster, 1983). Many biotypes of wild *S. bicolor* are indigenous to Africa, the center of origin of cultivated sorghum. These wild species have a 7% to 80% outcrossing rate with cultivated sorghum (Muraya et al., 2011). Thus, transgenes introduced to sorghum would readily introgress into these wild species, which often occur sympatrically with cultivated sorghum in Africa (Mutegi et al., 2010). The possibility of interbreeding with weedy species is increased in hybrid seed production fields, where male sterility is used to obtain hybrids, as the male-sterile plants must receive pollen from whomever is nearby, including from wild or weedy relatives. The initial crop × weed hybrids can continually backcross with the weedy or wild species within breeding distance, transferring any gene that has value to the weedy population.

*Sorghum halepense* (johnsongrass), which reproduces by both seed and underground rhizomes, was considered to be the sixth worst weed in the world (Holm et al., 1977). It might now be considered even worse, as this weed has evolved resistance to all the major selective herbicides that had controlled it in vast areas of transgenic herbicide-resistant soybean (*Glycine max*). This allotetraploid weed shares one of its genomes

with seed sorghum, and the other genome is closely related. A breeder was able to obtain one viable seed from pollination of 36,000 florets of a male-sterile seed sorghum with *S. halepense* pollen (Dweikat, 2005). Such crosses by breeders to obtain *S. halepense* genes in sorghum are much easier when special tetraploid strains of seed sorghum are used (Piper and Kulakow, 1994). There seems to be much greater success when seed sorghum is used as the pollen parent. Crop-specific alleles occurred in more than 30% of *S. halepense* growing in close proximity to long-term seed sorghum cultivation, suggesting introgression and retention (Morrell et al., 2005). As there is ample evidence that seed sorghum genes enter the genome of its weedy relatives, there is a good chance that the crop can be used as a vector to introduce weed-specific unfitness genes into the weeds. Such a technology would require special regulatory scrutiny in Africa, where wild sorghums grow in close proximity to the crop as well as among the crop (where they would act as weeds). Genes that directly confer unfitness may be contraindicated if dispersed into truly wild situations, but probably are not. When a rare pollen grain from a transgenic crop or weed bearing an unfitness trait crosses into the wild (where it is close enough to do so), it must compete with fresher wild-type nearby pollen for fertilization. If the pollen survives and a seed is produced, most of its progeny will be eliminated by the vaster numbers of its more fit siblings, by competition during germination and self-thinning, for many generations to come. Genes that must be chemically activated or that cause herbicide sensitivity probably would be of little consequence even if they possessed neutral fitness, should they disseminate into wild African populations, where they would not come into contact with such chemicals.

#### **Weeds That Poorly Introgress with Crops (e.g. Weedy [Red, Feral] Rice with Rice)**

Weedy (often termed feral or red) rice is a bane to rice production (Valverde, 2005; Gressel and Valverde, 2009a; Sun et al., 2013). It had been kept under a modicum of control by transplanting selected, 1-month-old rice seedlings into a just-cultivated, then flooded, weed-free field. This gives rice a head start to close the canopy, and the flooding delays the germination of weedy rice and other weeds. Transplanting is being supplanted by direct seeding, as labor is becoming unavailable to perform this backbreaking task (Kumar and Ladha, 2011). Direct-seeded cultivated rice does not have the head start over weedy rice and other weeds. The other weeds can be controlled by rice-selective herbicides, but not weedy rice. Weedy rice became the major weed throughout the world soon after going from transplanting to direct seeding, first in Europe (Busconi et al., 2012), then the Americas, then Thailand as it industrialized, followed by other Asian countries (Gressel and Valverde, 2009a), including China (Sun et al., 2013). This follows the order of massive labor movement from field to factory, leaving no choice but to direct seed

rice. Direct-seeded rice and weedy rice germinate together, and the more vigorous and taller feral rice outcompetes the semidwarf or dwarf Green Revolution rice varieties. It had been nearly impossible to chemically control weedy rice with a selective herbicide because weedy rice and cultivated rice are usually conspecific rice.

The first selective chemical solution was to cultivate rice having a mutation in the *ACETOLACTATE SYNTHASE (ALS)* gene conferring target-site resistance to the imidazolinone group of herbicides (Clearfield rice). This gave excellent control of weedy rice, and there was little fear that the gene would cross into weedy rice because rice is cleistogamous, the anthers self-pollinate the stigma before the flowers even open. Cleistogamy is not complete, and there is typically less than 0.1% outcrossing (Gealy, 2005). That small amount of outcrossing proved sufficient to allow rapid dissemination of the resistance gene in weedy rice. This is due to the subsequent selection pressure exerted by the herbicide, which facilitates the dispersal of resistant hybrid populations and their progeny by destroying plants that did not introgress the gene. The rate of resistance appearing by outcrossing from rice to weedy rice was much higher than the mutation to resistance in weedy rice (Goulart et al., 2012), despite the many possible mutations in the *ALS* gene responsible for this resistance. The Clearfield technology was lost in a few seasons in Italy (Busconi et al., 2012), Brazil (Marchesan et al., 2011), and Central America and some other areas, sometimes exacerbated by using low-quality (often contaminated with weedy rice) seed for planting (Gressel and Valverde, 2009a).

The major weedy rice management strategy suggested by the herbicide manufacturer is to rotate the imidazolinone-resistant rice with other varieties or other crops so that any weedy rice hybrids or backcrosses will be depleted. This strategy allows weedy rice to emerge and backcross during the nonimidazolinone seasons. Growers could use expensive cultural practices such as deep plowing (Chauhan, 2012) or rotate rice with a crop that has an herbicide available that will selectively control weedy rice, but farmers are loathe to grow other crops in expensive rice paddies. But let us assume they would. Can crop rotation fulfill the function? By analogy, crop rotation had been thought to be the solution to corn rootworm (*Diabrotica barberi*), but rootworm evolved an extended diapause that correlated with the length of the rotation (Levine et al., 1992). Weed scientists know that seed dormancy (the weed equivalent of diapause) can vary, and different populations of the same species have different levels of dormancy as a function of selection pressure favoring short- or long-dormancy strains.

Based on the above, it is a foregone conclusion that any mutated or engineered herbicide resistance introduced into rice will rather rapidly introgress into weedy rice, as it has with the imidazolinone resistance. Gene flow from hybrid rice may be even quicker to weedy rice, as the anthers and stigma protrude from

hybrid rice, shedding far more pollen than conventional rice. This would increase the chance of pollen from rice fertilizing weedy rice and pollen from the taller weedy rice pollinating male-sterile rice inbreds. Must we go back to backbreaking and expensive hand transplanting or just expensive machine planting?

Mitigation strategies were proposed to prevent the spread of introgressed genes (Gressel, 1999; Lin et al., 2008; Gressel and Valverde, 2009b; Liu et al., 2012). They rely on tandemly coupling a gene that confers unfitness in the weed (but not in the crop) with the herbicide resistance gene. Thus, whenever the weed introgresses the herbicide resistance gene, it also introgresses the unfitness gene. Two types of unfitness gene have been proposed: genes for biological traits (Gressel, 1999; Gressel and Valverde, 2009b) and genes for biochemical traits (Lin et al., 2008; Gressel and Valverde, 2009b; Liu et al., 2012). The biological traits proposed are genes such as dwarfing genes, which will not affect already dwarfed cultivars but will render the weedy rice less competitive with its weedy cohorts and lower it to the same playing field as the cultivars. Dwarfing has already been utilized in a model system (tobacco [*Nicotiana tabacum*]; Al-Ahmad et al., 2004, 2005) as well as with oilseed rape (*Brassica napus*) in confinement (Al-Ahmad et al., 2006; Al-Ahmad and Gressel, 2006) and in the field (Rose et al., 2009). An even more striking mitigation gene to use would be the various antiseed shatter genes (Thurber et al., 2010; Akasaka et al., 2011; Zhu et al., 2012), which would prevent the weed from reseeding itself. A crop-related weed with an antiseed shatter gene growing in the crop will be harvested with the crop, and will not reseed itself, if the farmer uses weed-free certified seed in the next season. The gene that confers a feral trait may not be the same gene that was selected for during domestication; dedomestication shattering is not controlled by the same gene that mutated to nonshattering during domestication (Zhu et al., 2012). Transgenically suppressing the yet unknown gene(s) controlling secondary dormancy also could be used as mitigation. If secondary dormancy is abolished, the weedy rice can be eliminated prior to planting rice.

Biochemical mitigation is based on introducing a gene that prevents the degradation of an herbicide occasionally used in rice (Gressel and Valverde, 2009b; Liu et al., 2012). The antisense or RNA interference (RNAi) construct is engineered in tandem with a gene for resistance to a previously nonselective herbicide (Lin et al., 2008). Transgenic rice containing the tandem construct is rotated with wild-type rice, where the herbicide that controls only the weedy rice is used. This concept was further refined into having a series of rice varieties, each having transgenic metabolic resistance to one herbicide and sensitivity to two others. By using the appropriate herbicide with each variety, native weedy rice and weedy rice that introgressed the previous varieties' transgenes are controlled (Gressel and Valverde, 2009b). We discuss in a later section how this could be made more effective by using multicopy transposons

### Weeds That Are Also Crops (*Lolium* spp.)

*Lolium* spp. are important pasture grasses in part of a pasture/crop rotation, typically with a grain such as wheat (*Triticum aestivum*) in dryland agriculture. The various perennial and annual *Lolium* spp. are considered to be a single botanical species, as they interbreed among themselves, despite the different phenologies, and also cross with *Festuca* spp. (Charmet et al., 1996). The annual *Lolium rigidum* germinates quickly after the first rains, supplying fresh fodder to livestock just when needed in many subtropical areas, especially Australia and Spain. The seedbank remaining after pasture is rotated into wheat results in *L. rigidum*'s becoming a pernicious weed that has evolved resistance to virtually all herbicides used in the wheat phase of a rotation, often with multiple resistances within the same individuals in field populations. Widespread multiple-herbicide-resistant *L. rigidum* is ubiquitous in Australian wheat (Yu et al., 2009; Broster et al., 2011; Boutsalis et al., 2012).

The only adequate control measure at present works where multiple resistance is not rampant and requires early-season herbicide use coupled with harvesting weed seed. A trailer-mounted cage mill having a separate diesel engine grinds weed seeds, chaff, and straw coming from the combine harvester (Walsh et al., 2013). Their data show excellent reduction of the weed *Lolium* spp. seedbank and very low reinfestation rates with the coupled system. No data were presented for fields where there is multiple-herbicide-resistant *Lolium* spp., where the herbicide would not lower the infestation, resulting in a much larger seed output to grind. It can be predicted from models that the seed-grinding system, which has a 95% efficiency of killing *Lolium* spp. seeds where there is little or no herbicide resistance, will not be effective when there is not an initial herbicide lowering of the infestation. A 95% reduction of seeds returned to the seedbank would only reduce the weed density by 50% in 3 years with a weed having very high fecundity (such as multiple-herbicide-resistant *Lolium* spp.), where it would remain at a steady state, based on models for another fecund weed (Smith et al., 1993), nor is it clear that the cage mill would be as efficient with such a high infestation as it was with the infestation levels reported by Walsh et al. (2013).

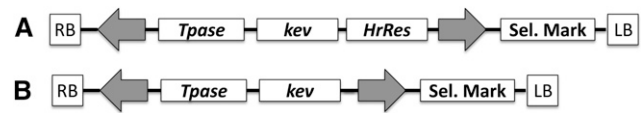
It is thus imperative to find a way to control the multiple-herbicide-resistant weeds such as *Lolium* spp. by lowering their fitness and rendering them susceptible to an herbicide. This is where the transgenic pasturegrass *Lolium* spp. is speculated to be useful. Transgenic efforts are being made to improve the pasture value of *Lolium* spp. by modifying lignin to increase digestibility as well as to increase cellulose and sugars (Spangenberg et al., 2008), along with delaying the growth-stopping flowering that toughens the tissues (Ong et al., 2012). The transgenes, if inserted with others in multicopy transposons, might be a way to deal with multiple-herbicide-resistant *Lolium* spp.

## DIFFERENT TRANSPOSON SOLUTIONS FOR DIFFERENT WEED TYPES

In general, we suggest that the concept proposed previously (Pfeifer and Grigliatti, 1996; Grigliatti et al., 2001) be seriously considered for controlling weeds (i.e. to insert and disseminate transposons containing potentially deleterious genes), but how this will be done is a different question. The British expression “horses for courses” explains that different types of horses are appropriate for different racecourses and is appropriate here, as different solutions are needed for different intractable weed problems. This is due to the very different biologies of the systems. Thus, the disparate possible solutions are discussed separately, but the reader is encouraged to mix and extrapolate potential solutions to other weed species, as appropriate.

### *Amaranthus* spp., a Case Where Modified Traditional TAC-TICS May Work

In the case of *Amaranthus* spp., there is no concern of the escape of genes from the crop to the weed but of gain of resistance in the weed. In such a case, we propose using transgenic *Amaranthus* spp. that would contain, on a transposon, herbicide resistance (to be able to exist among resistant cohorts) in tandem with a negative selection *kev* gene and a transposase gene to catalyze transposition (Fig. 1A). The rapid spread of the *kev* gene will be ensured by the positive herbicide resistance, if the herbicide is used in the cropping cycle together with the proliferation via transposition. If such herbicides provide 90% to 95% control, the remaining 5% to 10% can cross with the transposon-bearing weeds, facilitating more rapid transposon dispersal. Such a weed population would then be easy to control, as it contains also the Trojan horse, namely the negative selection *kev* gene. Despite the evolution of multiple resistances in the same field biotypes, there are herbicides that still provide a modicum of control, albeit often expensively. The initial *Amaranthus* spp. plants carrying the transposon should multiply, due to the presence of the transposase, thus effectively spreading herbicide resistance throughout the field. Crop combine harvesters are very effective at uniformly spreading weed seeds from isolated foci when they separate crop from weed seed and blow the weed seed in the trash as they pass through fields. Thus, a *kev*-bearing transposon can rapidly disperse through the population. The *kev* can be classical and encode an antisense or RNAi to a gene of primary metabolism, and it can be under the control of an inducible promoter to be used after dispersal is nearly complete (Table I). It can also be neoclassical and constitutively encode an antisense or RNAi that suppresses the expression of a gene conferring herbicide resistance, resulting in hypersensitivity to that herbicide. Thus, after dispersal, this other herbicide can be used without an inducer. It is also possible to introduce the debilitating transposon into the background of an herbicide-resistant weed, in which case there is no need for the herbicide resistance inside the transposon to promote



**Figure 1.** Constructs appropriate for direct dissemination of *kev* genes via outcrossing weeds such as *Amaranthus* spp. A, When the weed does not possess herbicide resistance, an herbicide resistance gene (*HrRes*) is added to promote dissemination of the transposon. B, If the weed to be transformed is already resistant to an herbicide in the field, then there is no need to add herbicide resistance. In the example shown here, the construct is cloned between the right (RB) and left (LB) borders of a transfer DNA cassette and transformed into *Amaranthus* spp. plants, whose seed is then disseminated into weedy fields. The thick arrows represent the terminal inverted repeats that flank the transposon (e.g. an *Ac/Ds*-like element) that are needed for transposition through cleavage by the transposase protein (*Tpase*). A *kev* gene or genes is/are added to reduce the fitness of the recipient plants. The *kev* gene(s) can be constitutive or inducible (see examples in Table I). An appropriate selectable marker is used for selection during transformation (Sel. Mark).

the spread of the *kev* gene (Fig. 1B). Transposon jumping might result in genetic defects in some individuals but would have little impact on a population of amaranths, as thousands of seeds are produced that replace a single individual, and the small proportion of defective plants will be removed from the population due to competition. The introduction of the transposons into the weedy *Amaranthus* spp. should be relatively straightforward, as four cultivated *Amaranthus* spp. have already been transformed (Pal et al., 2013).

A science fiction scenario can be considered that will enhance the rate of transposon dispersal while (possibly) lowering the seed output of the weed. This would require at least two more genes in tandem with the *kev* gene. One gene, under the control of a pollen-specific promoter, will encode the secretion of an allelochemical from the pollen, which weakens or kills other pollen, and a second gene conferring resistance to the allelochemicals. If this could be done, pollen not containing the transposon would be weakened or killed, resulting in a higher proportion of the seeds bearing the *kev*-bearing transposons.

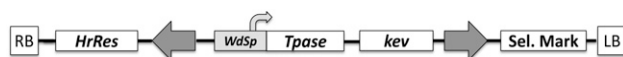
### Dispersing Transposons to Weed Species That Introgress with Crops (e.g. Rice and Sorghum)

It is not necessary to seed a field with *kev* transposon-bearing weed seeds, as outlined for *Amaranthus* spp., if crop-weed hybrids are formed. The *Amaranthus* spp.-type preemptive transposon-based insurance strategy to deal with transgene-introgressed herbicide-resistant weedy rice or sorghum is biologically feasible. One would directly engineer weedy rice or shattercane with transposons containing an herbicide sensitivity gene, as shown in Figure 1A, and release it in a field population. This may be inconceivable in the present regulatory climate, and the biosafety would have to be carefully evaluated.

It should be an easier and more efficient strategy to introduce the *kev*-bearing transposons directly into the crop, along with crop-favorable genes such as herbicide resistance (Fig. 2). This strategy combines both classical mitigation, where the crop is transformed with a construct bearing a gene of choice along with a gene that renders the weed but not the crop unfit, together with the debilitating transposons. This, as in the TAC-TICS described for *Amaranthus* spp., promotes the spread of the *kev* gene. In this case, the herbicide resistance need not be inside the transposon. Upon hybridization with a related weed, the F<sub>1</sub> plant would become herbicide resistant and would also contain a jumping debilitating transposon. The transposon would proliferate through activation of the transposase in the weed.

We propose here to express the transposase under the control of a promoter (natural or synthetic) that would be inactive in the crop but activated in the weed. This could be achieved either by identifying a promoter that is the target of a weed-specific transcription factor (as shown in Fig. 2) or through the use of an inducible promoter. Several domestication genes are transcription factors that were mutated or deleted in the crop. Such loss of transcription factors or having different transcription factors has been seen in the domestication of wheat (Sakuma et al., 2011), rice (Li et al., 2006), sorghum (Tang et al., 2013), soybean (Dong et al., 2014), and *Brassica* spp. (Lenser and Theissen, 2013), suggesting that this is indeed a feasible approach to differentiate between crops and their related weeds.

The *kev* gene inside the transposon can be expressed under the control of an inducible promoter. It can also be an herbicide-susceptible gene, as discussed earlier. This strategy may sound contraindicated, but by doing so, most of the weeds without the transposon are killed, increasing the rate of dissemination of the transposon into the small minority of weeds that did not succumb to the herbicide treatment. Thus, the time will be shortened to when the *kev* gene can be activated. The scenarios of activation will be different for rice, which is typically a monoculture crop, and sorghum, which is a rotational crop.



**Figure 2.** A construct appropriate for the dissemination of *kev* genes in row crops that outcross to related weeds such as rice and sorghum. In the example shown here, the construct is cloned between the right (RB) and left (LB) borders of a transfer DNA cassette and transformed into the rice or sorghum crop in order to confer herbicide resistance to the crop (*HrRes*). In the case of escape to the weedy relatives, use of the herbicide will kill most weeds except those that contain the transgene. In the weedy background, the debilitating transposon would be activated and increase in copy number. This could happen through the use of a promoter that would be active only in the weed, a weed-specific promoter (*WdSp*), or through an inducible promoter (not shown). The combined action of the herbicide resistance and of the multicopy transposon would ensure the rapid spread of the *kev* gene in the population and thus would enable efficient control of the weeds and of the escapees.

*kev* genes that confer herbicide hypersensitivity would be appropriate, for example, where the nontransgenic imidazolinone-resistant rice is cultivated or where selective acetolactate synthase- or protoporphyrinogen oxidase-inhibiting herbicides have supplanted 2,4-D (as an example) in rice or sorghum weed control. In such cases, it would be justified to release weedy rice or sorghum with sensitivity to 2,4-D into the population of weedy rice or sorghum and let the transposon disperse in the population. Other possible herbicide sensitivity genes that might be considered are described in Table I.

Under this scenario, when weedy rice or sorghum is no longer readily controllable by other means, 2,4-D is introduced into the rotation, killing the weedy rice or sorghum bearing the transposon, along with the broadleaf weeds that it normally controls.

#### Dispersing Transposons Where the Crop Is Also a Weed Later in Rotation (e.g. *Lolium* spp.)

As described above, transgenic *Lolium* spp. strains are being generated that enhance its value as a pasture grass: maximum tillering to increase the proportion of edible leaves, lower lignin rendering more cellulose available to ruminant livestock, and later flowering and senescence prolonging the grazing season. These genes of interest can also be considered as *kev* genes, rendering the pasture *Lolium* spp. that may remain in the field as seed, unfit to compete with a rotating wheat crop or with the indigenous weedy *Lolium* spp. These genes of interest/*kev* genes will have little effect on the weed, as the wild-type weeds will outcompete the transgenic material, which will eventually remain at a low proportion in the field. When it was just known that such efforts were being pursued, it was proposed to insert such genes on transposons, instead of by standard nuclear transformation (Gressel, 2002), as a method of dealing with this pernicious weed.

A scheme of how such constructs might be composed is presented in Figure 3. If used, any sown pasture *Lolium* spp. plant that remains as a volunteer in the wheat phase, or that has crossed with weedy *Lolium* spp., is a highly tillering wimp that is cut before it sets seed, when the wheat is harvested due to late *Lolium* spp. flowering. If needed, the debilitating transposon can be activated (in the wheat or the pasture field) through an inducible transposase gene (Fig. 3). The residual pasture *Lolium* spp. and pasture × weed *Lolium* spp. hybrids will then proliferate as less-fit weeds. They would be in much larger numbers than the small residue of weedy *Lolium* spp. remaining in the seedbank after the pasture phase of a few years, as *Lolium* spp. have a short seedbank half-life. Thus, the multicopy transposons can disseminate as a Trojan horse, because they leave much more residual seed in the field than weedy *Lolium* spp., spreading unfitness genes, against a fitness gradient. This could be even more effective if a gene or genes for herbicide sensitivity are included in the same transposon (Fig. 3). Again, a gene preventing 2,4-D degradation would be useful. 2,4-D had been the original modern

herbicide used in wheat over half a century ago. It has mainly been supplanted by herbicides that control both broadleaf and graminaceous weeds, which are also prone to the evolution of resistance. If 2,4-D could control *Lolium* spp. and broadleaf weeds, it would become a useful tool in rotation.

### CAN TRANSPOSON TRANSFORMATION WORK?

Endogenous transposons are typically silenced by cytosine methylation-mediated small interfering RNAs derived from the transposon itself through a complex machinery of RNA polymerases, proteins involved in RNA processing, and methyltransferases (Zemach et al., 2013). However, in transgenic systems, a newly introduced transposon that did not yet proliferate abundantly in the genome, or that is not native to the genome, can pass under the radar, remaining active for a few generations in a way that enables it to accumulate between two and 10 transposable element copies (Yoder, 1990; Meissner et al., 1997). This number is sufficient to ensure that the vast majority of the progeny of such multicopy transposons will transmit one or several copies to the next generation, as required for the TAC-TICS approach. Note also that transposon systems such as the *Ac/Ds* system are widely distributed. For example, the *Ac/Ds* transposon system was active in all (more than 15) of the plant species where it had been tested (Kunze, 1996) as well as in species from other kingdoms, such as yeast (Weil and Kunze, 2000) and zebrafish (Emelyanov et al., 2006). This is taken as an indication that the system is fully autonomous and does not rely on plant-specific host factors. Therefore, it is most likely that the *Ac/Ds* system will also be active in all weeds.

### FINDING THE NEEDED GENES FOR HERBICIDE SENSITIVITY

2,4-D was introduced over 50 years ago, when there were no regulatory requirements to know the mode of degradation and degradation products in crops. The



**Figure 3.** A construct appropriate for the dissemination of *kev* genes in pasture crops that outcross to related weeds such as *Lolium* spp. In the example shown here, the construct is cloned between the right (RB) and left (LB) borders of a transfer DNA cassette and transformed into *Lolium* spp. The gene of interest (*GOI*) introduced here confers desirable traits to *Lolium* spp. as a pasture crop, such as low lignin and high tillering, but can be considered as a *kev* gene, reducing fitness of *Lolium* spp. as a weed in the wheat phase of a rotation. The *GOI/kev*-carrying transposon can be activated through an inducible transposase (*Tpase*) promoter (*Ind-P*) so as to increase its spread among the weed relative, which can then be controlled through the herbicide susceptibility gene (*HrSusc*).

few published studies date back more than three decades (Feung et al., 1976; Scheel and Sandermann, 1981), years before the modern metabolomic procedures were invented. The primary degradation products of 2,4-D in situ were consistent with products expected of cytochrome P450 monooxygenases in all the grasses that were then studied (Frear, 1995), but peculiarly, there have been no publications that could be located via major databases on the in situ metabolism of 2,4-D in rice, despite the widespread use of this inexpensive herbicide in rice. Many other possible genes/herbicides that might be effective are summarized in Table I. There is an endogenous cytochrome P450, CYP72A21, whose expression in rice seedlings increases following treatment with 2,4-D (Hirose et al., 2007), but no evidence was presented that this enzyme can actually oxidize 2,4-D. The situation for rice is indeed murky, as there is information published that callus cultures derived from rice roots primarily catabolize the herbicide to carboxylic glycoside esters of 2,4-D and not P450-hydroxylated metabolites (Feung et al., 1976). The relevance of root-derived calli to what occurs in rice stems and leaves to foliar-sprayed 2,4-D in the field is unclear. Still, if transgenic suppression of CYP72A21 results in sensitivity to 2,4-D, it could be used. If it does not, a considerable amount of preliminary basic research on 2,4-D metabolism in rice must be performed to elucidate the gene(s) that would have to be suppressed.

The situation is far clearer for the more expensive and thus less used broadleaf weed-controlling herbicide bentazon. A cytochrome P450 monooxygenase, CYP81A6, is responsible for degrading bentazon (and some sulfonylurea herbicides) in rice, and when transformed in antisense it renders rice sensitive to bentazon (Pan et al., 2006). This antisense construct was used as the biochemical mitigator in a tandem construct with a *5-enolpyruvylshikimate-3-phosphate synthase* conferring resistance to the herbicide glyphosate (Lin et al., 2008).

### ECOLOGICAL BIOSAFETY CONSIDERATIONS

The most widely distributed and pernicious weed species evolved as a result of human activity, exist only in agricultural and ruderal ecosystems, and often are quite distinct from their progenitors that exist in wild (pristine) ecosystems. Such weeds can often be considered as introduced species, and there would be few regrets should they be rendered extinct. That possibility is exceedingly unlikely with the weeds that are conspecific with crops, as they continuously evolve by back mutations to domesticated feral forms. Thus, the best that can be hoped for is to keep crop-related weeds at a very low frequency in the population such that they do little economic damage to the cultivated crop. We speculate that continuously dispersing transposons and the occasional use of the relevant herbicide should perform this function.

There is an issue, though, that must be addressed, which is the possibility that the transposon will disperse



to the fully interbreeding progenitors where they still exist near the cultivated crop. This could happen between rice and the perennial *Oryza rufipogon* and the annual *Oryza nivara* in parts of Asia, with sorghum and its relatives in Africa, and with sunflower (*Helianthus annuus*) with its weedy progenitor in North America. The first question to be asked is whether the transposon and the gene it carries would have any effect on wild populations. There is the possibility that suppressing the specific cytochrome P450 that degrades 2,4-D (or whatever gene is used) will have untoward secondary effects on these wild species, which would render them unfit to compete with the majority of wild-type individuals. This unfitness may be inconsequential if, as discussed above, it would only introgress rarely and would be competed away. It is clear that the sensitivity to 2,4-D itself should be inconsequential, as 2,4-D is not used in natural ecosystems where the wild progenitors are found. An additional mitigating *kev* gene can be introduced in tandem, encoding such traits as dwarfing, high tillering, or reduced lignin, should there be ecological or regulatory issues with having herbicide sensitivity as the sole *kev* gene. These would prevent the transposon-containing weeds from establishing outside of cultivated areas.

If there is some untoward effect of the transposon on the wild progenitors, and they are found within pollination distance from the cultivated crop, the next question would be whether these populations are or are not near the center of origin. One might consider the ecological risk too great near the center of origin. In other parts of the world, the progenitors are introduced species: either escapes from breeding programs, where they were used to access disease resistances and other traits, or contaminants from when the crop was introduced into a new region. In the case of rice, some *O. nivara* may even be weedy rice that dedomesticated to a point where it is hard to distinguish it from its progenitor. Where the progenitors were introduced and have been naturalized, there is justification, at least for those who are xenophobic to introduced species, to have their proportion of natural populations decrease.

## CONCLUSION

In this article, we have speculatively extended the TAC-TICS to the control of weeds that might have become herbicide resistant as a result of natural mutations or of transgene escape. We take the counter-intuitive approach of promoting the dispersion of the herbicide resistance into the weed population to achieve rapid dispersal of *kev* genes that can be used later to control the weeds. This is achieved through the combined use of herbicide resistance together with proliferating transposons bearing genes that confer unfitness on weeds. This unconventional tool could indeed be useful to deal with pernicious, intractable weeds. Dissemination of the transposons will always be faster if resistance to the most commonly used herbicides for the particular crop is part of the construct, as

this will hasten dissemination by limiting transposon dispersal to a smaller subpopulation of weeds that have already evolved resistance to the herbicide. While we have only solved the problem on paper, we believe that we have provided some guidelines and accentuated the missing information/genes/constructs needed to test whether the speculated concept works. Disseminating genes encoding sensitivity to crop-selective herbicides in populations of crop-related weeds, or in weeds in general, might well be a successful method of augmenting present (and failing) weed-control methods. By having such transposons bearing sensitivity disperse through the populations, there will be another way to control weeds in the future.

The initial dispersal of the transposon can be performed by introducing it into the crop where there are introgressing weeds or directly into the weeds. In most cases, except possibly in the center of origin of the crop, at present there seem to be no perceived bio-safety risks from this methodology.

We are currently developing simulation models to predict how quickly the transposons will disseminate under different agronomic scenarios, while considering the reproductive biology of each crop, as well as other parameters, such as fitness, dormancy, weed seedbank size, and longevity.

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