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Efficacy of strategies for biological containment of transgenic crops

A literature review

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Preface

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Samenvatting

Biologische inperking van transgene planten en transgenen is het voorkomen of verminderen van de verspreiding van transgene planten of de transgenen die zij bevatten, buiten de gebieden en de plantensoorten waarvoor ze bedoeld waren door middel van het gebruik van of veranderen van de planteigenschappen, in het bijzonder van de voortplanting. Zulke inperkingstrategieën kunnen andere inperkingsmaatregelen, zoals fysische inperking, vervangen of aanvullen. Met een paar uitzonderingen zijn biologische inperkingsstrategieën alle gericht op de sexuele voortplanting van de plant op het niveau van bloei, stuifmeelproductie, zaadproductie of vruchtbaarheid, of een combinatie van deze. Talloze literatuuroverzichten die mogelijke biologische inperkingsstrategieën beschrijven zijn beschikbaar. Veel van die strategieën zijn alleen nog in de conceptuele fase, of de werkzaamheid is aangetoond in plantensoorten met weinig of geen toepassing als gewas. Dit rapport geeft een overzicht van de vele strategieën die zijn voorgesteld of bestudeerd en richt zich vooral op studies over de werkzaamheid of efficiëntie in een of meer plantensoorten, als die informatie voorhanden is. Inperkingsstrategieën, welke werden bekeken, en een conclusie over hun bruikbaarheid en efficiëntie worden hieronder besproken.

Auxotrofie
Auxotrofie is onvermogen van een organisme (plant) om een specifieke, voor de groei essentiële organische stof te maken. Toevoeging van die stof van buitenaf kan dan de normale groei herstellen. Deze methode is de enige in dit rapport die geschikt zou zijn voor het blokkeren van de verspreiding van transgenen door vegetatieve vermeerdering of –voorplanting. Er zijn rapporten van in planten geïntroduceerde auxotrofie, en ook enkele van complementatie van die auxotrofie door het toevoegen van de ontbrekende stof, maar voorbeelden van toepassing in het veld ontbreken. Het wordt niet erg waarschijnlijk geacht dat grootschalige applicatie, bijvoorbeeld door sproeien in het veld betaalbaar zou zijn, of gewenst vanuit milieuoogpunt.

Blokkeren van bloei en volledige steriliteit
Compleet en permanent blokkeren van de bloei lijkt een effectieve strategie voor biologische inperking van transgene gewassen die alleen voor hun vegetatieve delen worden gekweekt en die ook eenvoudig vegetatief vermeerderd kunnen worden. Deze toepassing zou verspreiding van transgenen door stuifmeel of zaad voorkomen. Bomen en grassen, die hun stuifmeel en zaad over grote afstand en gedurende een lange levensduur kunnen verplaatsen zouden vooral doelwit voor deze strategie zijn. Er zijn enkele voorbeelden van succesvolle blokkade van bloei beschikbaar en enkele veldproeven zijn in uitvoering, maar tot nu toe zijn er weinig gegevens over de efficiëntie en stabiliteit op lange termijn van deze benaderingen. Mogelijke effecten van het blokkeren van de bloei op de vegetatieve reproductie van de planten verdient meer aandacht.

Cleistogamie
Cleistogamie is het verschijnsel dat alle, of een deel van de bloemen van een plant niet opengaan en alleen zichzelf bestuiven, waardoor de verspreiding van transgenen door stuifmeel verminderd of voorkomen wordt. Er is geen effect op de verspreiding via zaden. Cleistogamie komt wijdverbreid voor, maar is relatief zeldzaam bij landbouwgewassen, met granen als haver en rijst als voorbeelden van natuurlijk voorkomen. Cleistogamie werd ook verkregen door mutagenese in rijst en koolzaad, maar er zijn geen gegevens over het effect van deze mutaties op daadwerkelijke transgenverspreiding via stuifmeel beschikbaar. Cleistogamie blijft een veelbelovende strategie voor inperking van zelfbestuivende gewassen, maar lijkt nog niet toepasbaar in de nabije toekomst. Positieve resultaten met cleistogamie in koolzaad en rijst geven aan dat deze twee gewassen daarop een uitzondering zouden kunnen vormen.

Transgenexcisie
Deze techniek behelst het verwijderen, geheel of gedeeltelijk, van het transgene construct uit het genoom van de plant, door een enzym (recombinase) dat werkt op twee doelwitsequenties aan weerszijden van het te verwijderen DNA. Het enzym knipt het DNA en brengt de twee herkenningsplaatsen samen, waarbij het tussenliggende DNA verwijderd wordt. Afhankelijk van het ontwikkelingsstadium en het orgaan van de plant waar excisie plaatsvindt, kan deze methode de verspreiding van het transgen via stuifmeel of zaad beperken. De meeste bekende studies richten

zich slechts op de verwijdering van het selectiegen (meestal een antibioticumresistentiegen) uit het construct, meestal samen met het gen dat codeert voor het recombinase (auto-excisie). Niettemin kunnen deze resultaten worden geëxtrapoleerd naar methoden voor de verwijdering van het gehele construct uit het genoom voor of tijdens stuifmeel of zaadproductie. In een commerciële toepassing zou de aanwezigheid van het transgen nodig zijn tijdens het grootste deel van of de gehele groeiperiode. Daarom zou excisie op een goed controleerbare manier moeten plaatsvinden.

Chloroplasttransformatie

Het genoom van planten bestaat niet alleen uit kern-DNA, maar bevat ook onafhankelijk replicerende chromosomen in plastiden en mitochondria. Transformatie van chloroplasten kan twee belangrijke voordelen boven transformatie van kernen hebben:

- Transgenen kunnen een hoger expressieniveau hebben omdat elke cel tot 10.000 plastidegenomen kan bevatten, tegen één kerngenoom
- Maternale (van de moederplant) overerving van chloroplast-DNA is de meest voorkomende vorm van overerving in angiospermen (bedektzadigen, bloeiende planten). Als plastidegenen vooral maternaal overerven, dan zal de transmissie van transgenen via pollen van zulke 'transplastome' planten naar niet-transgene planten significant verminderd of helemaal voorkomen worden.

Er zijn drie mogelijke scenario's volgens welke transmissie van transgenen uit transplastome planten toch zou kunnen plaatsvinden, waarvan sommige experimenteel gekwantificeerd zijn, hoewel tot nu toe alleen in tabak en koolzaad.

1. Het is meerdere keren aangetoond dat transmissie van plastide-DNA door pollen van een transgene plant met een (zeer) lage frequentie toch kan plaatsvinden, zelfs als de gangbare wijze van overerving van het plastid-genoom maternaal is. Slechts in een klein aantal studies is de frequentie van die overdracht betrouwbaar gemeten. Een studie met transplastome tabak maakt melding van een overdrachtsfrequentie naar zaailingen van 10^{-4} tot 10^{-5}, een andere meldde overdracht specifiek naar het apicale meristeem van zaailingen met een frequentie van 2.9×10^{-6} (fractie van transplastome zaailingen geproduceerd door 100% kruisbestuiving) onder ideale omstandigheden. De overdrachtsfrequentie wordt voorspeld (veel) lager te zijn in het veld.
2. Het transplastome genoom kan worden overgedragen naar wilde verwanten wanneer de transplastome plant bevrucht wordt door pollen van wilde verwanten en de hybride zaden zich verspreiden in het milieu. Herhaalde terugkruisingen met wilde verwanten als mannelijke ouders kan resulteren in transplastome planten die sterk lijken op het wilde familielid.
3. Chloroplast-DNA kan met lage frequentie naar de kern migreren en worden geïntegreerd in het nucleaire genoom, waarna het biparentaal (via beide ouders) zal worden overgedragen en dus niet worden ingeperkt door uitsluitend maternale overdracht. Het is niet duidelijk hoeveel van deze migraties stabiel geïntegreerd worden op de lange termijn. Geïntegreerde genen zullen normaal gesproken niet actief zijn in de kern en worden in tabak met slechts lage frequentie geactiveerd. Daarom is, in vergelijking met de overdracht van chloroplasten rechtstreeks via stuifmeel dit traject alleen significant als transgen aanwezigheid per se wordt beschouwd. Daar activering van het transgen veel minder waarschijnlijk is, is dit traject niet significant uit het oogpunt van veiligheid voor het milieu.

In het algemeen is chloroplasttransformatie naar verwachting een redelijk efficiënte inperkingmethode voor de meeste gewassen, die hoofdzakelijk maternale chloroplastovererving hebben.

Mannelijke steriliteit

Mannelijke steriliteit bij planten wordt gedefinieerd als de afwezigheid van functioneel stuifmeel. Dit voorkomt de overdracht van transgenen via pollen. Natuurlijke mutaties die leiden tot mannelijke steriliteit zijn er in vele plantensoorten. Vermeldenswaardig is de cytoplasmatische mannelijke steriliteit (CMS), die gebruikt wordt in verschillende gewassen, zoals maïs. Genetische modificatie, in het bijzonder door expressie van een ribonuclease (meestal barnase) in bloemweefsels, is gebruikt om mannelijke steriliteit te verkrijgen in gewassen waar geen bruikbare bron van natuurlijke mannelijke steriliteit beschikbaar is. Zowel de natuurlijke CMS als transgene mannelijke steriliteit hebben een variabele stabiliteit, afhankelijk van de milieuomstandigheden en de genetische achtergrond, maar voor beide soorten kunnen stabiele lijnen worden geselecteerd. Dus, mits goed geselecteerd en toegepast, kan

transgenoverdracht door pollen effectief worden geremd. Echter, als zaad moet worden geproduceerd in het veld, zullen niet-transgene planten als bestuiver gebruikt moeten worden om voor voldoende bestuiving zorgen. Dit zal niet voor alle gewassen haalbaar zijn.

Parthenocarpie

Parthenocarpie is de vorming van vruchten zonder bevruchting van eicellen. Het kan worden verkregen via klassieke selectie op basis van veredeling, maar ook door middel van genetische modificatie. Zowel natuurlijke parthenocarpen als door modificatie verkregen parthenocarpe planten zijn niet volledig zaadloos onder omstandigheden die gunstig zijn voor de bevruchting. Om parthenocarpie bruikbaar te maken voor biologische inperking op het niveau van zaad, althans in zelfbestuivende planten, zou het moeten worden gecombineerd met mannelijke steriliteit, met als bijkomend voordeel dat dit ook transgenoverbrenging via stuifmeel beperkt. Dergelijke planten worden momenteel ontwikkeld, maar er zijn geen gegevens over de efficiëntie van de biologische inperking.

Apomixis

Apomixis in planten is het proces van ongeslachtelijke voortplanting via zaad. Afgezien van de vele grote agronomische voordelen, kan vermeerdering door apomixie een aantrekkelijk alternatief zijn voor het behouden van zuivere (transgene) plantenlijnen. Wanneer de overdracht van transgenen via stuifmeel kan worden beperkt, bijvoorbeeld door mannelijke steriliteit, chloroplasttransformatie, of pollenspecifieke transgenexcisie, kan apomixie een bijdrage leveren aan biologische inperking op het niveau van pollen, omdat het bevruchting door pollen overbodig maakt. Zo is apomixie op zichzelf dus geen inperkingsstrategie, hoewel in de natuur apomictische planten vaak ook mannelijk-steriel zijn. Apomixis is zeldzaam in de meeste geteelde gewassen en wanneer apomixis beschikbaar is in wilde verwanten van het gewas, is het moeilijk gebleken om de eigenschap door introgressie in het gewas te krijgen. De componenten van apomixis worden Mendeliaans overgeërfd, maar de verantwoordelijke genen zijn niet geïdentificeerd. Apomixis zou kunnen worden verkregen door modificatie van genen die een belangrijke rol spelen in de seksuele voortplanting van planten, maar onderzoek op dit gebied is ook nog in een vroeg stadium. Samen maken deze knelpunten het onwaarschijnlijk dat apomixie in de nabije toekomst zal worden toegepast in de belangrijkste gewassen.

Verminderde zaadverstrooiing

Zaadverstrooiing is een natuurlijk verspreidingmechanisme, waartegen in de meeste gedomesticeerde gewassen is geselecteerd om oogstverliezen te beperken. Verstrooiing bevordert ook verspreiding via transgene zaden die in het veld achterblijven en kan aanleiding geven tot 'opslag' in de volgende jaren. Preventie van verstrooiing zou bijdragen aan de afname van transgenverspreiding via zaden. Koolzaad, bijvoorbeeld, is een relatief recent gedomesticeerd gewas en kampt nog met aanzienlijke verliezen door verstrooiing, wat bijdraagt aan transgenverspreiding. Verschillende strategieën, die zijn gebaseerd op modelplantstudies, om het openen van de hauw in koolzaad te remmen zijn voorgesteld en een ervan werkt in een verwante *Brassica*-soort, maar er zijn tot nu toe geen praktische toepassingen in koolzaad. Het potentieel van deze aanpak is dus hoog, maar nog niet bewezen.

Blokkeren van zaadkieming

Transgenverspreiding door zaden kan worden beperkt met behulp van strategieën die de opslag door kieming van gemorst zaad, opgeslagen zaden, of gewas/gewas- en gewas/wilde verwant-hybride zaden voorkomt. Alle gepubliceerde strategieën zijn gebaseerd op de embryospecifieke expressie van een cytotoxisch genproduct om zaadletaliteit te bereiken, maar verschillen verder in hun aanpak. Er is slechts beperkt informatie gepubliceerd over de efficiëntie van de verschillende strategieën. Het originele concept van wat bekend is geworden als 'terminator-technologie', richt zich op induceerbare expressie van zaadletaliteit, maar is niet in de praktijk getest. Onvolledige inductie van zaadletaliteit kan leiden tot inefficiënte biologische inperking. Echter, in een omgekeerde strategie zoals bij 'Recoverable Block of Function', komt embryonale letaliteit standaard tot expressie en kan die worden omgekeerd door induceerbare expressie van een herstelgen in te bouwen. Deze aanpak is van nature efficiënter voor biologische inperking. Beide onderdelen van deze strategie zijn effectief in het laboratorium in tabak, maar geen diepgaande, grootschalige studies in tabak of experimenten in andere plantensoorten zijn gemeld.

Tegengaan van kiemrust
Kiemrust van transgene zaden na verspreiding of verlies tijdens en voor de oogst kan leiden tot 'opslag' van transgene planten in de daaropvolgende jaren en daarmee transgenverspreiding via het zaad. De remming van kiemrust kan bijdragen aan de afname van transgenverspreiding via zaden. Landbouwkundige technieken spelen een belangrijke rol in het percentage zaad dat overleeft in de grond en kunnen, wanneer zorgvuldig gekozen, bijdragen aan aanzienlijke vermindering van transgenverspreiding langs deze weg. Genetische variatie voor secundaire kiemrust in koolzaad suggereert dat er perspectief is voor de selectie tegen secundaire kiemrust in de veredeling, hoewel dit niet veel aandacht heeft gekregen tot nu toe. Een directe correlatie tussen het niveau van kiemrust en de omvang van de verspreiding van (transgene) zaden is niet aangetoond, hoewel een hoog aanwezigheidsniveau van transgene opslagplanten in jaren na de teelt van transgeen koolzaad suggereert dat kiemrust een belangrijke bijdrage vormt aan de verspreiding van zaden.

Transgene mitigatie
Transgene mitigatie kan als een strategie op zich niet voorkomen dat transgenverspreiding uit transgene gewassen naar niet-transgene gewassen en wilde verwanten plaatsvindt, maar 'verzacht' de gevolgen van deze verspreiding, dat wil zeggen het doel is om de stabiele integratie van het transgen in de populatie te voorkomen. Zo vermindert het meestal het gevolg van, maar voorkomt niet, de verspreiding van transgenen zowel door stuifmeel als door zaden. In deze strategie, is het gewenste transgen (coderend voor herbicide resistentie of andere gewenste eigenschappen) genetisch nauw verbonden aan een gen dat concurrentienadeel oplevert voor hybriden of opslagplanten, respectievelijk in het natuurlijke milieu van wilde verwanten of in agrarische gebieden met niet-transgene gewassen. De strategie vereist dat de twee transgenen zo nauw verbonden zijn dat ze niet zullen gescheiden kunnen worden tijdens de meiose. Veel mitigerende genen zijn voorgesteld, maar slechts één gen is getest in de praktijk, zowel in tabak als in koolzaad. Korte-termijn concurrentie-experimenten suggereren dat transgene mitigatie kan werken, wat in strijd is met pessimistischer scenario's uit bestudering van modellen. De korte-termijn experimenten zullen moeten worden aangevuld (zoals de meeste andere strategieën voor inperking) met lange-termijn experimenten onder meer realistische omstandigheden, om te bepalen of de strategie werkt in real-life omstandigheden.

Inteins
Inteins zijn cis-of trans-splicing elementen die het mogelijk maken twee inactieve helften van een eiwit in een compleet en dus actief eiwit te fuseren in de plantencel. In theorie zal de expressie van twee inactieve delen in verschillende ouders of in twee verschillende genomen (chloroplast- en kerngenoom) de overdracht van actieve transgenen door pollen of zaad naar verwante gewassen of wilde verwanten voorkomen of vertragen. Het belet niet de overdracht van transgenen per se, omdat de onderdelen nog kunnen worden doorgegeven via de moederplant of via beider ouderlijnen. Hoewel is aangetoond dat delen van het intein-concept werken in het laboratorium, is het nut voor de biologische inperking in de praktijk nog niet aangetoond.

Algemene conclusies
Er is een grote hoeveelheid literatuur die methoden voor de preventie van transgenverspreiding beschrijft. Echter, slechts enkele studies zijn veel verder dan het 'proof of principle' stadium gekomen en weinig strategieën zijn getest in meer dan enkele modelplanten, laat staan in werkelijke gewassen. Remming van de bloei, cleistogamie, chloroplasttransformatie en mannelijke steriliteit zijn meer uitgebreid getest, ook in sommige gewassen en in veldexperimenten. Hoewel de resultaten van modelplanten enige voorspellende waarde voor andere soorten zullen hebben, kan de effectiviteit van een strategie afhankelijk zijn van de soort. De meeste strategieën richten zich op de verspreiding van transgenen via pollen of zaden, of beide. Slechts één methode (auxotrofie) kan de verspreiding door vegetatieve voortplanting voorkomen. Verder richt geen van de methoden zich op alle mogelijke wegen van transgenverspreiding. Daarom zullen, afhankelijk van de specifieke plantensoorten en hun ecologie, en van het niveau van transgeninperking dat nodig is, twee of meer strategieën moeten worden gecombineerd.

Summary

Biological containment (or biocontainment) of transgenic plants and transgenes is the prevention or reduction of the spread of transgenic plants or the transgenes they contain outside the areas or species of their intended use by using and/or modifying the plant's innate characteristics, particularly its reproductive characteristics. Such containment strategies may complement or replace other measures for transgene containment such as physical barriers and harvesting and processing practices. Biological containment strategies, barring a few exceptions, target the plant's sexual reproduction at the level of flowering, pollen production, seed production or fertility, or a combination thereof. Numerous literature reviews are available describing possible biological containment strategies, many of which are still in their theoretical conception phase or only have a proof of concept in a model plant species of little or no value as a crop. This report reviews the various biological containment strategies proposed or studied and focuses on the discussion of reports of their efficacy in one or more plants species, if such information is available. Containment strategies that were reviewed and the conclusion about their utility and efficiency are listed here.

Auxotrophy
Auxotrophy is the inability of an organism (plant) to synthesize a particular organic compound required for normal growth. External application of the required compound may restore normal growth. This method is the only in this report that would be suitable to prevent transgene spread by vegetative reproduction. Reports of engineered auxotrophy in plants exist, as well as in some cases the complementation by application of the missing compound, but no examples in field situations have been described. It is not very likely that large scale spraying of plants in the environment would be cost effective, or desirable.

Inhibition of flowering and complete sterility
Complete and permanent blocking of flowering is an attractive transgene containment strategy for crops that are harvested for their vegetative parts and that can be readily vegetatively propagated. The application would prevent transgene spread by pollen as well as by seeds. Trees and grasses, which are able to spread seeds and pollen over large distances and during a long period, are particular targets for this approach. Several examples of successful inhibition of flowering exist and several field trials are ongoing, but so far no information on the efficacy or the long-term stability of the strategies is available. Potential effects of flowering inhibition on vegetative reproduction of transgenic plants may require more study.

Cleistogamy
Cleistogamy is the phenomenon in which all or a portion of the flowers are permanently closed and self-pollinated, thus decreasing transmission of any transgenes through pollen. It would not prevent transgene spread through seed. Cleistogamy is wide-spread among plants, but rare among crop species, with cereals like barley and rice as examples of natural occurrence. Cleistogamy was obtained by mutagenesis in oilseed rape and in rice (in addition to already available natural cleistogamy), but no reports concerning the effects of those mutations on actual transgene transmission by pollen were found. Cleistogamy remains a promising strategy for transgene containment in autogamous (self-fertilizing) crops, but will not be applicable in the near future. Promising results with engineered cleistogamy in oilseed rape and rice indicate that these may be an exception of that conclusion.

Transgene excision
Transgene excision is the removal of (part of) the transgene construct from the plant cell's genome by an enzyme (recombinase) acting on two target sites flanking the DNA that is to be excised. The enzyme cuts the DNA and splices the two target sites together, thereby eliminating the transgene DNA in between. Depending on the stage of plant development where transgene excision takes place, the method may limit transgene spread by either pollen or seed. Most experiments reported in literature only aimed to remove the selectable marker (mostly antibiotic-resistance) from the construct, usually together with the gene encoding the recombinase (auto-excision). Nonetheless, the results from these experiments may be extrapolated to methods aimed at removing the entire construct from the genome before or during pollen or seed production. In a commercial setting the transgene's

presence is required during most if not all of the plant's growth period, therefore excision has to occur in a controllable manner.

Chloroplast transformation

Plant genomes not only consist of nuclear DNA, but also contain an independently replicating chromosome in their plastids and mitochondria. Chloroplast transformation is thought to have two major advantages over nuclear transformation for the generation of transgenic plants:

- Transgenes can be expressed at a higher level due the higher number of plastid genomes per cell (up to 10,000 plastids versus one nucleus).
- Maternal inheritance of chloroplast DNA is the predominant mode of inheritance in angiosperms. When plastid genes are (largely) maternally inherited then transgene flow through pollen from 'transplastomic' plants to non-transgenic plants can be significantly reduced or avoided.

There are three possible scenarios for transgene transmission from transplastomic plants, some of which were experimentally measured, but so far only in tobacco and oilseed rape.

1. It has been generally shown that transmission of plastid DNA through the pollen of a transplastomic crop may occur at a (very) low frequency if the predominant mode of inheritance is maternal, although only a few examples exist where the transmission frequency of the plastid genome was reliably measured. One study on transplastomic tobacco reported a transmission frequency to seedlings between 10^{-4} and 10^{-5}, another reported transfer specifically to the apical meristem of seedlings at a frequency of 2.9×10^{-6} (fraction of transplastomic seedlings produced by 100% cross fertilization) under ideal conditions. The transmission frequency is predicted to be (much) lower in the field.
2. The transplastomic genome may be transferred to wild relatives when the transplastomic plant is fertilized by pollen from these wild relatives and the hybrid seeds disperse in the environment. Repeated back crossings with wild relatives as male parents could result in transplastomic plants that are highly similar to the wild relative.
3. Chloroplast DNA may move at a low frequency to the nucleus and be integrated in the nuclear genome, after which it will be inherited biparentally, i.e. not be contained by maternal transmission. It is not clear how many of the transposition events will be stably inherited in the long term. Transposed genes will not normally be active in the nucleus and only attain activity in tobacco at a low frequency. Therefore, compared to direct transfer of chloroplasts through pollen, this pathway is significant if transgene presence per se is considered. Since activation of the transgene is much less likely to occur, this pathway is not significant from and environmental safety point of view.

Overall chloroplast transformation is expected to be a reasonably efficient containment method for most crops, which have predominantly maternal chloroplast inheritance.

Male sterility

Male sterility in plants is defined as the absence of functional pollen. This precludes the transmission of transgenes through the pollen. Natural mutations leading to male sterility exist in many plant species. Of particular note is the cytoplasmic male-sterility (CMS) type, which has found it is way into application for several crops, e.g. maize. Genetic modification, particularly by expression of a ribonuclease (mostly barnase) in floral tissues, has been used to engineer male sterility in crops where no useful type of natural male sterility is available. Both the natural CMS types as well as engineered male sterility have variable stability, depending on environmental conditions and the genetic background but for both types stable lines can be selected. Thus, when properly selected and applied, transgene transmission by pollen can be effectively inhibited. However, when seed is to be produced in the field, non-transgenic pollinator plants will have to be used to ensure sufficient pollination. This will not be feasible for all crops.

Parthenocarpy

Parthenocarpy is formation of fruit without ovule fertilization. It can be obtained through classical selection-based breeding, as well as through genetic engineering. Neither bred nor engineered parthenocarpic plants are completely seedless under conditions favorable for fertilization. Thus, in order for parthenocarpy to be of use for biological containment at the seed level, at least in autogamous plants, it would have to be combined with male sterility, with

the added benefit that this would also decrease transgene transmission via pollen. Such plants are currently being developed, but there are no data on the efficiency of biological containment.

Apomixis

Apomixis in plants is the process of asexual reproduction through seed. Apart from many highly-valued agronomic benefits, apomictic reproduction can be an attractive alternative for maintenance of pure (transgenic) plant lines when transmission of the transgene through pollen can be limited, such as by male sterility, chloroplast transformation, or pollen-specific transgene excision. Thus, the apomictic trait by itself is not a containment strategy, although in nature apomictic plants are often also male-sterile. Apomixis is rare in most cultivated crops and when available in wild crop relatives, has proved difficult to introgress by breeding. The components of apomixis are inherited in a Mendelian fashion; however the loci underlying apomictic development have not been identified. Apomixis could be obtained by modifying key reproductive genes from sexual plants; however research in this area is also at an early stage. Together, these bottlenecks make it unlikely that the apomictic trait will be introduced into major crop plants in the near future.

Reduced shattering

Shattering is a natural seed dispersal mechanism, which in most domesticated crops has been selected against in order to reduce yield losses during harvesting. Shattering also promotes transgene spread through seeds which remain in the field and can give rise to volunteers in the following years. Thus, prevention of shattering would contribute to the decrease of transgene spread through seeds. Oilseed rape, for example, is a newly domesticated crop and still suffers from considerable seed losses by shattering, which contributes to transgene spread. Several strategies based on model plant studies to inhibit pod dehiscence (opening) in oilseed rape have been suggested and one works in a related *Brassica* species, but there are no practical applications in oilseed rape so far. Thus the potential for this approach is high, but as yet unproven.

Blocking seed germination

Transgene flow through seeds may be contained using strategies that prevent germination of volunteer seeds, saved seeds, or crop/crop and crop/wild relative hybrid seeds. All published strategies are based on embryo-specific expression of a cytotoxic or cell-lethal gene product to achieve seed lethality, but further differ in their approach. Only limited information has been published on the efficiency of the different strategies. The original concept of what has been become known as 'terminator technology' proposes inducible expression of a seed-lethal gene, but has not been demonstrated in practice. Incomplete induction of seed lethality may lead to inefficient biological containment. However in a reverse strategy such as 'Recoverable Block of Function', embryo lethality is expressed by default and may be recovered by inducible expression of a recovery construct. This approach is inherently more efficient for biological containment. Both components of the strategy have been shown to be effective on a laboratory scale in tobacco, but no in-depth, large-scale studies in tobacco or studies in other plant species have been reported.

Inhibiting seed dormancy

Dormancy of transgenic crop seeds after spilling or shattering may lead to volunteer emergence in subsequent years and to transgene flow through the seed. Thus, inhibition of seed dormancy may contribute to the decrease of transgene spread through seeds. Agronomic practices play an important role in the seed survival rate and may, when carefully chosen, contribute significantly to diminishing gene flow in this manner. Genetic variation for secondary dormancy in oilseed rape suggests that there is perspective for selection against secondary dormancy in breeding, although this has not received attention so far. A direct correlation between levels of dormancy and the extent of transgene flow through seeds has not been shown, although high levels of transgenic volunteers in years following the cultivation of transgenic oilseed suggest that it is an important contributor.

Transgenic mitigation

Transgenic mitigation as a strategy does not by itself prevent transgene flow from transgenic crops to non-transgenic crops or wild relatives, but 'mitigates' the effects of such gene flow, i.e. its goal is to prevent the establishment of the transgene in volunteer populations or in populations of wild relatives if hybridization can occur. Thus it mostly mitigates the effect of, not blocks, transgene escape both by pollen as well as by seeds. In this

strategy, the 'trait' gene (such as herbicide resistance or other desired traits) is closely linked to a gene that confers competitive disadvantage to hybrids or volunteers, in natural stands of wild relatives or agricultural fields with non-transgenic crops, respectively. The strategy depends on the two transgenes being so closely linked that they will not segregate during meiosis. Many mitigating genes have been suggested, but only one has been tested in practice, in both tobacco and oilseed rape. Short term competition experiments suggest that transgenic mitigation can work, thus contradicting more pessimistic scenario's from modeling studies, but these experiments will need to be complemented (like most other strategies for transgene containment) with long-term competition experiments under more realistic conditions to determine if the strategy holds up in real life situations.

Inteins

Inteins are cis- or trans-splicing elements that allow the splicing together of two inactive halves of a protein into a complete and therefore, active protein within the plant cell. In theory, the expression of two inactive parts in different parents or in two different genomes (chloroplast and nuclear) will prevent and delay the transmission of active transgenes by pollen or seeds to related crops or wild relatives. It does not prevent the transmission of transgenes *per se*, since the components can still be transmitted through the maternal or both maternal and paternal lines. Although parts of the intein concept have been shown to work in the laboratory, it's usefulness for biological containment has not yet been demonstrated.

Overall conclusions

There is a large amount of literature describing proposed methods for preventing transgene spread. However, few have gone far beyond the 'proof of principle' stage and few have been tested in more then a few model plants, not in actual crop plants. Inhibition of flowering, cleistogamy, chloroplast transformation and male sterility have been more extensively tested, also in some actual crops and in field experiments. Although results from model species will have some predictive power for other species, the efficacy of a strategy may differ from one species to another. Most strategies address the spread of transgene through pollen or seeds, or both. Only one method (auxotrophy) might prevent the spread through vegetative reproduction. Additionally none of the methods targets all possible avenues of transgene spread. Therefore, depending on the particular plant species and its ecology, and on the level of trans-gene containment that is required, two or more strategies might have to be combined.

1 Introduction

1.1 Biological containment

Biological containment (or biocontainment) of transgenic plants and transgenes is the prevention or reduction of the spread of transgenic plants or the transgenes they contain outside the areas or species of their intended use and is obtained by exploiting and/or modifying the plant's biological characteristics, particularly its reproductive characteristics.

The spread of transgenes or transgenic plants outside the areas of their intended use may be undesirable when it concerns plants that have not been approved for release into the environment or in the case of approved plants, if their spread is undesirable from a safety point-of-view (such as for plants producing pharmaceuticals) or from a consumer choice point-of-view. The European Commission has recommended EU Member States to take measures to ensure co-existence of genetically modified crops, conventional crops and organic crops.

From the above it may be apparent that the measure of success of a particular (biological) containment strategy depends as much on the efficacy of the biological containment strategy as on the level of containment required. The containment level may need to be higher, close to 100%, for safety purposes when using plant-produced pharmaceuticals or industrial chemicals, while much lower containment levels may already contribute significantly to reaching co-existence goals. Although co-existence deals only with transgene transmission to non-transgenic plants from the same species, transgenes may also be transmitted to wild or feral relatives of the crop plant if they are able to sexually hybridize. Transgenes can become introgressed and fixed in wild populations if such hybrids are fertile. The potential risks and consequences of such out-crossing have been reviewed elsewhere (Haygood *et al.*, 2003; Stewart Jr *et al.*, 2003; Haygood *et al.*, 2004; Chapman and Burke, 2006; Chandler and Dunwell, 2008; Warwick *et al.*, 2009)

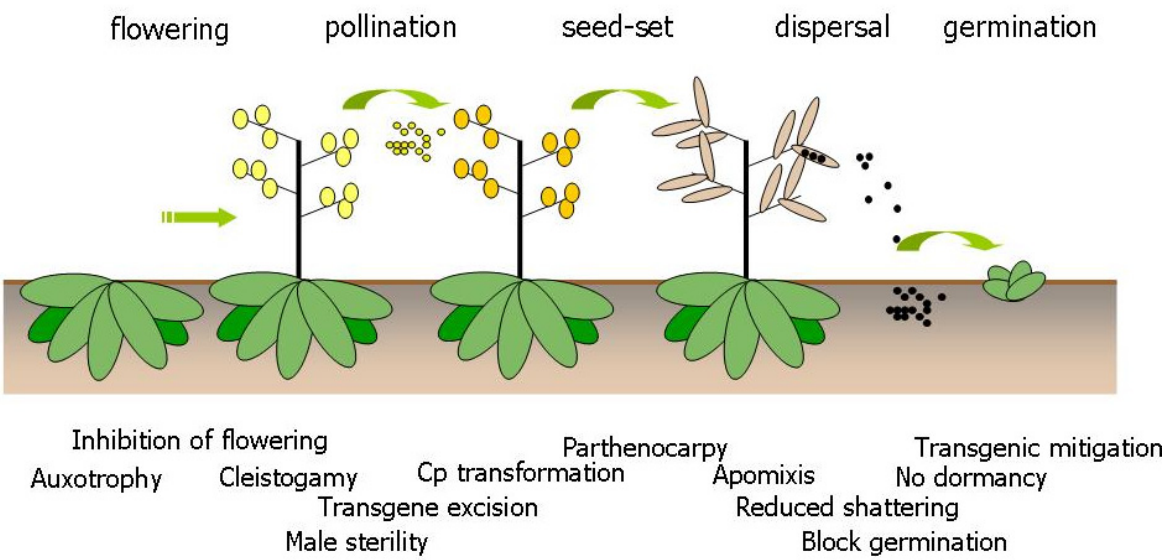

Figure 1. Biological containment strategies and the relative position in the plant life cycle where they act.

Transgenes or transgenic plants may be transmitted in various ways and biological containment strategies aim at blocking these transmission avenues (Fig. 1). Most biological containment strategies consider transmission via pollen or seeds. However, some plants readily reproduce vegetatively, albeit not usually over large distances, by

production of rhizomes, stolons, runners, adventitious buds, bulbs or tubers. Only a few biological containment strategies addressing this type of reproduction could be found in the literature and when described, they usually require additional agronomic practices to work. For example, a crop may be rendered auxotrophic, i.e. not able to grow without the addition of an essential metabolite, and thus growing such a crop requires application of the metabolite in the field (see in the section 'Auxotrophy' of this report). Another reported strategy is to use genetic modification to render a crop plant, in this case rice, sensitive to a herbicide to which it is normally tolerant. Spraying with this herbicide would then selectively kill escaped feral plants, volunteers or progeny of hybridization with wild relatives (Lin *et al.*, 2008). All other biological containment strategies aim at modifying the sexual reproduction of the crop plant to inhibit transmission through pollen or seed. Transmission by either of these avenues may be inhibited by prevention of flowering, which is only possible for crops that are harvested for their vegetative parts. Transmission of transgenes through pollen may be inhibited by cleistogamy, excision of transgenes from pollen DNA, by male sterility or by inserting the transgene in the chloroplast genome, which is not normally transmitted by pollen. Such strategies may be combined with apomixis, which is the production of seed without fertilization. Transmission by seed may be further prevented by parthenocarpy (fruit production without the necessity for seed formation), by reduction of shattering during harvest and transport of the seeds, or by blocking the germination of transgenic seeds. If seed spilling nonetheless occurs, then inhibition of secondary dormancy may prevent the long term survival of transgenic seeds in the seed bank and, in combination with agronomic practices, will lead to fatal germination. Finally, even if initial transgene transmission to wild relatives occurs, the introgression and fixation of that gene in the wild population may be inhibited or strongly delayed by transgenic mitigation.

This literature review focuses on the efficacy of various biological containment strategies published in peer-reviewed literature. There is a large amount of literature describing strategies for biological containment, which have been also reviewed elsewhere (Daniell, 2002; Gressel and Al-Ahmad, 2005a; Chapman and Burke, 2006; Lee and Natesan, 2006; Hills *et al.*, 2007; Murphy, 2007; Bock and Timmis, 2008). For detailed descriptions of the strategies and the experimental approaches we refer you to the original publications and reviews cited in this report. Rather, this report focuses on what has been published about the efficacy of such strategies. It appears that many proposed strategies have not yet moved beyond a theoretical concept or a proof of principle in model plants with no or limited value as a crop (*Arabidopsis*, tobacco), as only a few strategies have been applied in actual crop species. On the other hand, where strategies that are useful for containment have been applied in crop plants, they were often intended for other purposes, such as to facilitate breeding or for hybrid seed production. Therefore, even when applied, containment strategies were not studied with their use in biological containment in mind.

1.2 Transgenic crops already released and currently under development for release in the European Union

Crops that have an EU market authorization (food&feed; import; cultivation or a combination of these) are maize, cotton, carnation, soybean, rape seed, and sugar beet. Approved traits are insect resistance and herbicide tolerance. Approved for cultivation are only maize varieties. Additionally, applications for approval have been filed for potato and rice. An overview of the applications and approvals, as well as their current status in Europe, can be found on the website of GMO Compass (http://www.gmo-compass.org/eng/gmo/db/). Similarly for world-wide approvals, there is the Agbios GMO-database (http://www.agbios.com/dbase.php). An overview of which plants and traits are under development can be obtained by reviewing the authorized field trials in the European Union from the website of GMO Compass (http://www.gmo-compass.org/eng/agri_biotechnology/field_trials/228.summary_gmo_field_trials_eu_year_crop_trait.html) as well as on the website of the European Union's Joint Research Centre (http://gmoinfo.jrc.ec.europa.eu/). The before mentioned site shows that a total of 66 plant species have been used or are currently being used in field trials somewhere in Europe. The most important species for field trials in Europe were (in order of prevalence): maize, rapeseed, beet, potato, tomato, cotton, chicory, tobacco, rice, and wheat.

1.3 Current research in the subject area

A number of national and international research programs have studied biological containment methods, often together with other aspects of co-existence. The European Commission financed three related project in the Framework 6 program.

Sigmea (2004-2007; http://www.inra.fr/sigmea), which was focused on the study of gene flow and ecological and economical impacts of GM crops. The final activity report of the project can be found here: http://www.inra.fr/sigmea/content/download/2810/28252/version/2/file/Final-publishablesummary_v2.pdf

Co-Extra (2006-2009; http://www.coextra.eu), which dealt with different aspects of co-existence between GM- and non-GM or organic crops in Europe. Most of its work was directed at analysis of supply chains, GMO detection, and communication, with Work Package (1): Biological Approaches For Gene Flow Mitigation, focusing on the use and utility of biological containment strategies. This included field trials to test the efficacy of maize with non-GM cytoplasmic male sterility and oilseed rape with non-GM cleistogamous flowers for biological containment.
A scientific report was still forthcoming at the time of preparation of this report. Individual deliverable reports are available at http://www.coextra.eu/library/deliverables.html (Note: these are not peer-reviewed publications). These include preliminary reports on the efficiency of cleistogamy, of CMS lines and the Hybrid Plus system, state-of-the-art of chloroplast transformation, and recommendations for further use of biological containment strategies (http://www.coextra.eu/deliverables/deliverable1249.pdf), Manuscripts about Maize CMS field trials (Weider *et al.*, 2009) and efficiency of cleistogamy have been published. Reports on specific subjects will be referred to in the respective chapters below.

The EU FW6 project Transcontainer (2006-2009; www.transcontainer.org), for which the authors of this report were the coordinators, aims at the development of biological containment strategies as well as the socioeconomic and environmental assessment of the impact of such strategies. Technical workpackages included those for chloroplast transformation, controllable flowering and controllable fertility (including male sterility, parthenocarpy, apomixis, and recoverable block of function). The website also contains fact sheets describing the state-of-the-art of the different technologies and the objectives of the project regarding those (http://www.transcontainer.wur.nl/UK/Fact+sheets/). The scientific report of the project was in preparation at the time of writing of this report

2 Auxotrophy

2.1 Conclusions

Auxotrophy is the inability of an organism (plant) to synthesize a particular organic compound required for normal growth. External application of the required compound may restore normal growth. This method is the only in this report that would be suitable to prevent transgene spread by vegetative reproduction. Reports of engineered auxotrophy in plants exist, as well as in some cases the complementation by application of the missing compound, but no examples in field situations have been described. It is not very likely that large scale spraying of plants in the environment would be cost effective, or desirable.

2.2 Background

Auxotrophy is defined as the inability of an organism to synthesize a particular organic compound required for its growth, and is widely used in microbiology for selection and containment of organisms within the laboratory environment. Several examples exist of engineered auxotrophy in plants for methionine, biotin, and auxin, and in some cases these could be complemented with externally added compounds (Kim and Leustek, 2000).

2.3 Experimental evidence for efficacy of the technology for transgene containment

No literature was found that deals with experiments on the efficacy of containment by auxotrophy. In theory, efficacy would depend, in cases where auxotrophy was engineered by RNAi, on the stability of transgene expression and the penetration of the phenotype in the population. No examples were found for studies of complementation of auxotrophy in the field.

3 Inhibition of flowering and complete sterility

3.1 Conclusions

Complete and permanent blocking of flowering is an attractive transgene containment strategy for crops that are harvested for their vegetative parts and that can be readily vegetatively propagated. The application would prevent transgene spread by pollen as well as by seeds. Trees and grasses, which are able to spread seeds and pollen over large distances and during a long period, are particular targets for this approach. Several examples of successful inhibition of flowering exist and several field trials are ongoing, but so far no information on the efficacy or the long-term stability of the strategies is available. Potential effects of flowering inhibition on vegetative reproduction of transgenic plants may require more study.

3.2 Background

Complete prevention of flowering or bisexual sterility are considered a realistic strategy for containment of transgenic pollen and seeds in crops that are mostly or exclusively grown for their vegetative parts and for which vegetative propagation is economically feasible. These approaches are particularly attractive for perennial trees and grasses, which reproduce over a long period, produce large amounts of wind borne pollen and produce large amounts of seeds, which in some species are also wind borne. Prevention of flowering or bisexual sterility is also a valuable strategy to prevent transgene spread in annual or biannual crops that are usually harvested before flowering, such as sugar beet (*Beta vulgaris*). In general two strategies to prevent flowering or induce bisexual sterility can be distinguished:

1. Expression of cell-lethal (cell ablation) genes under control of plant promoters that are specific for flowering stages or floral organs
2. Interference with the expression or activity of endogenous genes that normally stimulate flowering (by down-regulation of expression or expression of dominant-negative forms) or of genes that normally inhibit flowering (by ectopic expression).

Most of these approaches are reviewed in detail elsewhere (Brunner *et al.*, 2004; Brunner *et al.*, 2007; Meilan *et al.*, 2007).

3.3 Experimental evidence for efficacy of the technology for transgene containment

Expression of cell-lethal genes

In this approach, cells in the inflorescence or floral organs are killed (ablated) by the expression of a cytotoxic protein. Specific applications aimed at achieving unisexual sterility, e.g. male sterility is discussed below. The most widely used cytotoxic protein is the bacterial RNAse 'barnase', however, other proteins and cytotoxic mechanisms are also used (Day *et al.*, 1997). One of the challenges of this approach is to find promoters that are specific enough to ablate generative tissues without producing side effects on vegetative growth. This is illustrated in early studies in birch (*Betula pendula*), where a birch *MADS1*-promoter/*BARNASE*-construct successfully inhibited flowering through ablation of floral structures, but also had a negative effect on vegetative growth (Lemmetyinen *et al.*, 2004). A birch *FULL1*-promoter/*BARNASE*-construct was used to inhibit flowering, although some normal appearing plants were produced (Lännenpää *et al.*, 2005). A poplar (*Populus trichocarpa*) PTD-promoter/DTA (Diphtheria Toxin A)-construct prevents flowering, but in contrast to the above-mentioned examples has no effect on vegetative growth (Skinner *et al.*, 2003). Side effects of low level expression of barnase expression in vegetative tissues may also be mitigated by expressing an inhibitor of barnase, called barstar, in non-target tissues (Kobayashi *et al.*, 2006). This approach has been shown to work for a poplar *LEAFY*-promoter/barnase-construct in poplar. The same study

showed that the ratio of barnase to barstar is important criterion for success, but that this ratio may change during field trials (Wei *et al.*, 2007). All the studies mentioned above produced considerable numbers of transgenic trees with complete flowering inhibition, however only a small number of plants per transgenic line was examined and none of the studies were carried out over a long period, thus the efficacy of this approach over time remains to be determined (see below). There are no reports of the use of this strategy for complete sterility in grasses.

Flowering inhibition

Flowering in Angiosperms is regulated by a complex network of positive and negative regulators, which are in turn regulated by both intrinsic and extrinsic factors. The genes encoding these regulators and their regulatory networks have been extensively characterized in *Arabidopsis thaliana* (*Arabidopsis*), although similar networks and their components have been identified and well characterized in many other plants (Jaeger *et al.*, 2006; Whipple and Schmidt, 2006; Wilkie *et al.*, 2008; Michaels, 2009). Prevention of flowering by modifying the expression or the activity of endogenous floral regulators was not only proposed as a transgene containment strategy but also, in some cases, as a means to increase resource allocation to vegetative growth. Most functional studies on flowering genes have been performed in model plants; however orthologs from crop plants are continuously being identified and evaluated in the context of inhibition of flowering. Poplar orthologs of *Arabidopsis* flowering inducers such as *FT*, *AGL24*, *AGL20*, and *FPF1*, or repressors such as *TFL1* and *SVP* are being tested in an extensive program involving field trials, but results have not been published yet (Brunner *et al.*, 2004; Brunner *et al.*, 2007), (http://www1.eere.energy.gov/industry/forest/pdfs/strauss.pdf). The only known literature report for inhibition of flowering in grasses involves ectopic expression of a *Lolium perenne TFL1* homolog, which strongly inhibited flowering in transgenic red fescue (*Festuca rubra*) even after natural vernalisation over a two-year period (Jensen *et al.*, 2001; Jensen *et al.*, 2004).

Inhibition of flowering may also have primary economical purposes for plant breeders, since flowering may decrease the vegetative production and/or nutritional quality of crop (for instance in grasses). This could imply that reversely, inhibition of flowering can increase vegetative growth and hence increase the extent of vegetative reproduction of a plant. A clear example of how sterility does not preclude reproduction is the case of Japanese knotweed, a highly invasive species and a pest in Northwestern Europe, which is a single clone and which is male sterile (Bailey *et al.*, 2009). Such potential effects of engineered sterility were not reported in literature, but may need more study.

4 Cleistogamy

4.1 Conclusions

Cleistogamy is the phenomenon in which all or a portion of the flowers are permanently closed and self-pollinated, thus decreasing transmission of any transgenes through pollen. It would not prevent transgene spread through seed. Cleistogamy is wide-spread among plants, but rare among crop species, with cereals like barley and rice as examples of natural occurrence. Cleistogamy was obtained by mutagenesis in oilseed rape and in rice (in addition to already available natural cleistogamy), but no reports concerning the effects of those mutations on actual transgene transmission by pollen were found. Cleistogamy remains a promising strategy for transgene containment in autogamous (self-fertilizing) crops, but will not be applicable in the near future. Promising results with engineered cleistogamy in oilseed rape and rice indicate that these may be an exception of that conclusion.

4.2 Background

Cleistogamy is defined as a breeding system with permanently closed, self-pollinating flowers. Three types of cleistogamy are distinguished: Complete cleistogamy, in which all flowers of the plant stay closed during its entire lifetime, dimorphic cleistogamy, in which both closed as well as open (chasmogamous) flowers are present simultaneously on a plant, and induced cleistogamy, in which the production of cleistogamous flowers is induced by environmental conditions. Cleistogamy is an attractive strategy for transgene containment because of its ability to limit transgene transmission through pollen (Daniell, 2002), as well as its ability to prevent pollination of transgenic crops by non-transgenic or wild relatives, thereby preventing transgene spread by hybridization. Cleistogamy is wide-spread throughout the plant kingdom, but rather rare in crops species (Culley and Klooster, 2007). Examples of crop species that have some degree of cleistogamy are barley (Honda *et al.*, 2005) and rice (Maeng *et al.*, 2006). The molecular basis for the cleistogamous trait has been identified in two crop species. INRA in France used chemical mutagenesis to generate cleistogamous mutants of oilseed rape (*Brassica napus*; Renard and Tanguy, 1997). In rice, mutation of a class-B MADS-box gene, *SUPERWOMAN-1* altered lodicule identity, preventing flower opening (Yoshida *et al.*, 2007). A similar engineered approach, for example using RNA interference to knock-down *SUPERWOMAN1* expression, might well work in other cereals.

4.3 Experimental evidence for efficacy of the technology for transgene containment

Few examples exist of research on cleistogamy in crops, neither on the stability of the trait under different conditions or in different varieties, nor on its effect on pollen dispersion and hence, possible transgene transmission by pollen. In two cleistogamous lines derived from the INRA oilseed rape mutant, studied in two seasons, flowers opened to some degree, from 0 to 94% depending on year, time of year, and genotype, showing that the trait currently is too unstable. Nonetheless, cleistogamous lines emitted ten times less pollen then open-flowered varieties (Fargue *et al.*, 2006). New lines bred for stable cleistogamy were studied and one of these lines showed significantly better stability in a three-year study in different environments, with 95% of the flowers remaining closed in 61% of the sampled plots (Leflon *et al.*, 2009). This data suggests that cleistogamous oilseed rape could contribute to a reduction in transgene transmission by pollen; however the actual amount of dispersed pollen or the frequency of transgene transmission was not reported in this study.

5 Transgene excision

5.1 Conclusions

Transgene excision is the removal of (part of) the transgene construct from the plant cell's genome by an enzyme (recombinase) acting on two target sites flanking the DNA that is to be excised. The enzyme cuts the DNA and splices the two target sites together, thereby eliminating the transgene DNA in between. Depending on the stage of plant development where transgene excision takes place, the method may limit transgene spread by either pollen or seed. Most experiments reported in literature only aimed to remove the selectable marker (mostly antibiotic-resistance) from the construct, usually together with the gene encoding the recombinase (auto-excision). Nonetheless, the results from these experiments may be extrapolated to methods aimed at removing the entire construct from the genome before or during pollen or seed production. In a commercial setting the transgene's presence is required during most if not all of the plant's growth period, therefore excision has to occur in a controllable manner.

Recombinase activity can be induced (physico-) chemically by heat or chemical treatments, or by the use of a developmentally regulated promoter. Chemical- or heat-induced excision works well for lab-based selection of marker-free plants, however the low efficiency of transgene excision, combined with the problems of current field-level induction strategies suggest that it will not contribute significantly to transgene containment at this time.

Developmentally regulated excision in pollen can be highly efficient, but in the few reports of seed-specific excision are the excision rates are not high enough to contribute significantly to transgene containment. The use of other promoters might raise the efficacy of this approach. Some points to note when considering this option are:

- Experimental data are limited mainly to tobacco (*Nicotiana tabacum*) and *Arabidopsis*. The utility of the transgene excision approach in other crops, which will mainly depend on the activity of the promoter controlling the recombinase activity, needs to be established;
- A strategy based on developmentally regulated excision makes it difficult to maintain breeding lines in crops that depend on seed propagation; and
- The progeny of plants obtained using current excision methods will retain a single target site as a byproduct or 'footprint'

5.2 Background

Site-specific recombination as a means to excise transgenes from the genome of the transgenic plant has been demonstrated using a number of prokaryotic or lower eukaryotic integrases, consisting of a recombinase protein and its corresponding recognition sites in the DNA flanking the transgene to be excised (mechanisms reviewed in Hare and Chua, 2002). Examples of such recombinase/target sites two-component systems shown to work in plants are Cre/*lox*, Flp/*FRT*, R/*RS* and int/*attP*, with most plant applications using the Cre/*lox* system (reviewed in (Ow, 2007; Gidoni *et al.*, 2008). One of the most frequent applications has been the removal of selectable marker genes from the transgenic plant genome when the presence of the marker is considered undesirable, such as antibiotic resistance markers. In such cases, excision of the marker may be achieved by introduction of a site-specific recombinase after the initial transformation event either by crossing the transgenic line with a recombinase-expressing plant, by retransforming the plant with a recombinase construct, or by transiently expressing the recombinase using either Agrobacterium (*Agrobacterium tumefaciens*) infiltration or infection with a recombinant virus expressing the recombinase. An example of antibiotic (*nptII*) selectable marker removal that was achieved by crossing with a recombinase-expressing line is the lysine-enhanced maize line LY308, marketed as Mavera™, which received regulatory approval in the US and Canada in 2005/2006. Alternatively, the initially-introduced construct may already contain the recombinase gene, but under control of an inducible promoter. Induction of the promoter and thus, recombinase activity and marker excision, may be achieved chemically (e.g. ethanol or steroid-based induction systems), by heat shock or may be induced as a consequence of the plant's own development by using

promoters that are active in the floral meristem, male or female germline, or embryo, resulting in marker free pollen and ovules, pollen or ovules, and embryos, respectively.

Site-specific recombination has also been used for marker excision from transformed plastids (Corneille *et al.*, 2001). An additional use of site-specific recombination may be to resolve undesired multi-copy tandem insertions of the transgene construct. Incorporation of recombinase recognition sites into the construct, followed by induction of recombinase activity would excise any superfluous tandem insertions, leaving a single copy insertion.

The same approach might be used to excise a whole transgene construct from germ line cells or embryos by including all components of an insert between recombination sites (Keenan and Stemmer, 2002). Chemical or developmental stage-specific induction of the recombinase would then result in the elimination of the entire insert from pollen, ovules or embryos. It should be noted that the recombination systems used so far leave a single recombinase recognition site footprint (32 bp for *loxP*) and the resulting plants might still be considered transgenic and could be identified as such. Recombinases that recognize native plant sequences could be engineered (Thyagarajan *et al.*, 2000; Buchholz and Stewart, 2001; Sclimenti *et al.*, 2001), but have not yet been demonstrated to work in plants.

5.3 Experimental evidence for efficacy of the technology for transgene containment

Most studies on experimentally confirmed transgene excision were specifically targeted to removal of a marker gene rather than to excision of the entire construct and as such were intended for biological containment of the marker gene and not of the whole construct. These studies along with their reported success rates are effectively summarized and reviewed by Gidoni *et al.* (2008). Most known examples of either chemically- or heat-shock-induced activation of recombinase activity were performed *in vitro* on explants from different plant organs (callus, embryos, seeds or seedlings), and to a less extent on whole plants. Nonetheless the overview by Gidoni *et al.* shows that even under the relatively controlled conditions of *in vitro* experiments the excision rate ranges from 5 to almost 100%. Only the most significant examples summarized by Gidoni *et al.* will be discussed here. In many cases excision of a marker is not only monitored through the plant's loss of antibiotic resistance on a growth medium, but also by the appearance of β-glucuronidase activity from a *GUS* gene that is only actively transcribed from a promoter when the interrupting fragment between the promoter and the *GUS* gene is excised.

In one example using field-grown plants, *GUS* and *nptII* transgene excision was achieved in transgenic tobacco plants using an *Arabidopsis* heat shock promoter driving *Cre* expression. The authors suggested that an 18 day period with temperatures above 30 °C induced the heat shock promoter and thus Cre-based gene excision in field grown plants. 112 out of the 117 leaves tested showed GUS activity and kanamycin sensitivity indicating a 95.7% excision rate on a total leaf basis (Liu *et al.*, 2005). Transgene transmission to the next generation was not reported. In maize, heat shock-inducible autoexcision was compared side-by-side with excision obtained by crossing with a recombinase-expressing line. Both approaches were effective in inducing excision. Heat-shocked induced excision in calli was approximately 67% based on the number of antibiotic-resistant and/or *nptII*-expressing regenerants (found in 4 out of 12 heat-shocked calli). Excision upon crossing with *Cre*-expressing plants was deemed efficient, but no absolute frequencies were calculated (Zhang *et al.*, 2003). In another example heat shock promoter-induced recombinase activity in tobacco resulted in a 47%-79% excision frequency (Wang *et al.*, 2005). Excision of an *FRT*-flanked marker by FLP under control of an oxidative stress-inducible promoter in tobacco occurred on hydrogen peroxide-containing medium at 13-41% (Woo *et al.*, 2009). Summarizing the above, all examples of inducible recombinase activity are efficient enough for production of transgene- or marker-free plants *in vitro* if followed by selection, but seem not to be efficient enough to contribute significantly to field-level transgene containment.

An alternative to chemically induced recombinase activation is developmentally regulated activation, where the recombinase is transcribed under the control of a developmental stage-, organ- and/or tissue-specific promoter. The promoters used are usually germ line cell (generally pollen) or embryo/seed-specific, although there is one example where a floral meristem-specific promoter was used (Gidoni *et al.*, 2008). Transgene presence and expression is

usually required during some point in vegetative growth because it confers the trait for which the plant is marketed, thus excision should occur late in development during flowering or seed development, when the trait is no longer required. However, excision from ovules, embryos or seeds is not an option when the added trait needs to be expressed in the seed. It should also be noted that excision from the germline produces an additional level of complexity as maintenance of production lines in seed propagated crops becomes more difficult.

Few studies have addressed the option of transgene excision during seed or embryo-development. Using the embryo-specific napin gene promoter to drive seed-specific *Cre* expression in oilseed rape (*B. napus*) rates of excision of a herbicide resistance marker were between 13 and 81% (Kopertekh *et al.*, 2009). Li *et al* (2007) used an embryo-specific *Arabidopsis* promoter to driving *Cre* expression in soybean, and showed 25% and 31% complete and chimeric excision events respectively in regenerated plants from somatic embryos.

Efficient excision of transgenes from *Arabidopsis* and tobacco pollen was achieved using the tobacco *NTM19* microspore-specific promoter. Relatively small scale seed germination assays (100 per line) from backcrosses of single-event transgenic plants to wild type plants did not yield any kanamycin-resistant plants, in tobacco or *Arabidopsis,* indicating 100% transgene excision. Large scale assays on back-crossed tobacco lines yielded two non-excised plants out of a total of 16,800 analyzed, corresponding to a failure rate of only 0.024% (Mlynárová *et al.*, 2006). In a similar study using two pollen- and one pollen plus seed-specific promoter, excision by Cre/*lox* alone was found to range anywhere from 0-100%, but a combination of Cre/*lox* and FLP/*FRT* in a single construct gave 100% excision in approximately 25,000 progeny of backcrosses to wild-type for 6 lines tested, which corresponds to less then 0.008% failure rate in tobacco (Luo *et al.*, 2007). Transgene excision in rice reached 37% excision efficiency when a flower-specific promoter that induced excision in both male and female germ-lines was used (Bai *et al.*, 2008). Use of another flower-specific promoter, as well as a germ-line specific promoter resulted in excision in all transgenic *Arabidopsis* lines, but the efficiency per line was not reported (Verweire *et al.*, 2007).

6 Chloroplast transformation

6.1 Conclusions

Plant genomes not only consist of nuclear DNA, but also contain an independently replicating chromosome in their plastids and mitochondria. Chloroplast transformation is thought to have two major advantages over nuclear transformation for the generation of transgenic plants:

- Transgenes can be expressed at a higher level due the higher number of plastids per cell (up to 10,000 plastids versus one nucleus).
- Maternal inheritance of chloroplast DNA is the predominant mode of inheritance in angiosperms. When plastid genes are (largely) maternally inherited then transgene flow through pollen from 'transplastomic' plants to non-transgenic plants can be significantly reduced or avoided.

Possible scenarios for transgene transmission from transplastomic plants:

1. It has been generally shown that transmission of plastid DNA through the pollen of a transplastomic crop may still occur, although at a (very) low frequency, if the predominant mode of inheritance is maternal. Only a few examples exist where the transmission frequency of the plastid genome was reliably measured. One study on transplastomic tobacco reported a transmission frequency to seedlings between 10^{-4} and 10^{-5}, another reported transfer specifically to the apical meristem of seedlings at a frequency of $2.9x10^{-6}$ (fraction of transplastomic seedlings produced by 100% cross fertilization) under ideal conditions. The transmission frequency is predicted to be (much) lower in the field.
2. The transplastomic genome may be transferred to wild relatives when the transplastomic plant is fertilized by pollen from these wild relatives and the hybrid seeds disperse in the environment. Repeated back crossings with wild relatives as male parents could result in transplastomic plants that are highly similar to the wild relative. A recent study reported that chloroplast capture from *B. napus* by wild Brassicas varied between 0.6 and 12% of the plants depending on the relative distance between the populations. The frequency will also depend on the viability of the hybrids and the biology of the crop and its relatives. This aspect should be taken into consideration when chloroplast transformation is not combined with other measures to prevent maternal transmission of the transgene.
3. Chloroplast DNA may move at a low frequency to the nucleus and be integrated in the nuclear genome, after which it will be inherited biparentally, i.e. not be contained by maternal transmission. The frequency of chloroplast-to-nucleus transfer followed by pollen transmission in tobacco ($6\text{-}9x10^{-5}$) is significant compared to direct transfer of chloroplasts through pollen when transgene presence is considered. It is not clear how many of the transposition events will be stably inherited in the long term. Transposed genes will not normally be active in the nucleus and only attain activity in tobacco at a low frequency ($3x10^{-8}$ on a per cell basis; N.B. data derived from a single study). Therefore, compared to direct transfer of chloroplasts through pollen, this pathway is significant if transgene presence per se is considered. Since activation of the transgene is much less likely to occur, this pathway is not significant from and environmental safety point of view.

All data cited here was derived from studies on tobacco (scenarios 1 and 3) or *Brassica* (scenario 2).

6.2 Background

Plastids contain an autonomously replicating single chromosome varying from 120 to 220 kb in size, depending on the species. Plastid genomes (plastomes) are highly conserved between species and contain 120-130 genes involved in photosynthesis and in the general transcription and translation machinery of the plastid. Plastids are attractive candidates for genetic engineering because a typical plant cell contains some 100 plastids, each containing approximately 100 copies of its chromosome, thus bringing the potential number of copies of any inserted gene to at least 10,000. The exceptionally high foreign protein levels that can be achieved using plastid transformation makes it an attractive option for, among others, production of pharmaceutical proteins in transgenic

plants (reviewed in Grevich and Daniell, 2005; Bock, 2007). Plastid transformation was first achieved in *Chlamydomonas* in 1988 (Boynton *et al.*, 1988) using autonomously replicating vectors in suspension cells (Ye *et al.*, 1990) and shortly thereafter in tobacco by stable integration into the chloroplast genome (Svab *et al.*, 1990). Stable integration into the plastid genome is generally achieved using biolistic bombardment and homologous site-specific recombination using vectors containing homologous flanking sequences derived from plastid DNA on both sides of the transgene to be inserted. Selection for transgenic events is mostly done using antibiotic resistance, although herbicide resistance and salt tolerance derived from betaine aldehyde dehydrogenase activity have also been used. Chloroplast transformation has been achieved in a large number of crops, although tobacco remains the species of choice for this technology because of the relative ease of obtaining transplastomic plants. To date, chloroplast transformation has been achieved in the following plants: *Chlamydomonas*, *Arabidopsis, Petunia*, cotton carrot, tomato, potato, soybean, lettuce, *Brassica oleracea* (specifically cauliflower and cabbage), *Lesquerella fendleri*, poplar, sugar beet and rice (reviewed in (Grevich and Daniell, 2005; Bock, 2007; Wang *et al.*, 2009).

In general, plastids are considered to be maternally inherited, i.e. plastid DNA (ptDNA) is not normally transmitted to the egg cell via the pollen. There are several known mechanisms underlying this phenomenon (reviewed in (Hagemann and Schröder, 1989; Birky Jr, 1995):

a. During pollen development unequal division of the microspore results in generative cells (forming the sperm cells), which are free of plastids (the *Lycopersicon* type, the most common mechanism)
b. Young generative cells may contain some plastids, but these degenerate during maturation of the sperm cells (the *Solanum* type)
c. In monocotyledonous plants sperm cells contain plastids, but these are degraded before fertilization (the *Triticum* type)
d. In *Chlamydomonas* plastids are transmitted to the zygote, but the paternal ptDNA is degraded soon afterwards by nucleases, while the maternal ptDNA is protected by methylation

While maternal plastid inheritance seems to be the rule in angiosperms, exceptions do exist. In alfalfa (Matsushima *et al.*, 2008) and evening primrose (*Oenothera*) plastids are equally divided over the generative and vegetative cells after the first pollen division, and plastids are transmitted through the sperm cells. Maternal inheritance appears to be the rule throughout the plant kingdom, with the exception of, among gymnosperms, the conifers (Coniferophyta) where paternal plastid inheritance is prevalent (reviewed in (Mogensen, 1996). The evidence for exclusive maternal inheritance is generally derived from cytological observations (as listed above), whereas careful genetic screening of larger progeny populations using unequivocal markers for specific chloroplast DNA transmission would be needed to determine the frequency of rare events. Reports of such studies are rare in literature (see next section).

6.3 Experimental evidence for efficacy of the technology for transgene containment

Transgene transmission from transplastomic plants to non-transgenic plants from the same species or to wild relatives in the field may occur through different mechanisms, which may differ in relevance for the different crops and will depend on the specific conditions (time, geography, etc.) under which the crop is grown. Possible scenarios for transmission are:

1. Straightforward transmission of plastid DNA through pollen from the transplastomic crop may occur at a (very) low frequency
2. Transplastomic plants (the crop or feral populations) may be fertilized by pollen from a wild relative to generate a hybrid transplastomic seed. Depending on the fertility of these hybrids, repeated back crossings with wild relatives as male parents could result in transplastomic plants with more or less all the characteristics of the wild relative.
3. Chloroplast DNA may become integrated into the nuclear genome at very low frequency, after which it will be inherited biparentally

6.3.1 Plastid DNA transmission through pollen

For scenario 1, only a small amount of data was obtained with actual transplastomic plants. This is probably due to the relative novelty of the technique and the fact that plastid transgenesis is only routine in tobacco. Most older reports are of limited use for extrapolation to transplastomic plants as they use plastid-encoded natural resistance- or pigmentation markers to track ptDNA transmission through pollen and in general they do not establish heritability of transmitted ptDNA.

Exceptions have been detected in several plants that were previously thought to exhibit strict maternal inheritance of plastid DNA. These include *Arabidopsis* (Azhagiri and Maliga, 2007), snap dragon (*Antirrhinum majus* (Diers, 1967), *Epilobium hirsutum* (hairy willow-herb) (Schmitz and Kowallik, 1986), *Petunia* (Derepas and Dulieu, 1992) and the cereal crop millet (*Setaria italica*) (Wang *et al.*, 2004).

Medgyesy *et al.* (1986) used alloplasmic (plastids derived from a different species; interspecific hybrids or cybrids) *N. plumbaginifolia* with plastid-derived streptomycin resistance to track transmission of ptDNA via pollen. Transmission was detected by inducing calli from seedlings on streptomycin-containing medium. In *Nicotiana plumbagnifolia* x *N. plumbaginifolia* and the *N. plumbaginifolia* x *N. abacus* crosses 2.5% and 0.07% of the offspring were found to contain paternal (*N. tabacum*) plastids, respectively. It has to be noted that transmission to the next generation through the germ line was not demonstrated. In petunia, which largely shows maternal plastid inheritance, one out of 19 tested genotypes showed up to 2% paternal plastid inheritance (Cornu and Dulieu, 1988).

Two reports specifically addressing the frequency of ptDNA transfer through pollen from *transplastomic* tobacco have been published. Svab and Maliga (2007) crossed alloplasmic cytoplasmic male sterile tobacco plants or normal fertile tobacco as the female parent with a paternal transplastomic line containing the *aadA* spectinomycin/streptomycin-resistance gene. They recorded paternal ptDNA transmission into seedlings at a frequency between 10^{-4} and 10^{-5}. Moreover by analyzing chloroplast DNA using RFLP markers they concluded that the entire ptDNA, not just fragments, is transmitted via pollen. The authors emphasize that retrieval of seedlings resulting from parental ptDNA transmission is greatly facilitated by the use of male sterile maternal lines and very stringent selection on streptomycin, and that under natural conditions the frequency may be lower. In a similar experiment (Ruf *et al.*, 2007) crossed male sterile mother plants of tobacco with transplastomic paternal tobacco containing *aadA* as well as *GFP* (encoding Green Fluorescent Protein), a visual marker. Of seedlings that were selected on antibiotic containing medium, which showed some green sectors as evidence of paternal plastid transfer, 39 (out of a total of 2.1 million seedlings screened) were confirmed as being the result of paternal plastid transmission (frequency 1.6×10^{-5}). Building on their former report (Svab and Maliga, 2007), Svab and Maliga established that a significant number of these seedlings contained paternal plastids in the cotyledons and not in the apical meristem and thus the resistance gene would not be transmitted to the next generation. Transmission frequency into the apical meristem was determined to be 2.9×10^{-6}. Also here the authors emphasize that cross-fertilization, stimulated in these experiments by the use of male-sterile maternal lines, occurs in the field at 10% or less in mixed plots. Minimal physical separation will decrease cross-fertilization even further, resulting in practical transmission frequencies under realistic field conditions of 10^{-8} or lower.

6.3.2 Transmission of chloroplast DNA through hybridization with wild relatives

Detailed studies of chloroplast transmission between crop plants and wild relatives under realistic field conditions have been published for oilseed rape (*Brassica napus*), albeit not transplastomic oilseed rape. Oilseed rape is a so-called allotetraploid plant containing two copies each of the *Brassica* A genome, derived from *B. rapa*, and the C genome, derived from *B. oleracea*. Oilseed rape hybridizes readily with wild *B. rapa* (Metz *et al.*, 1997). Using natural ptDNA markers to track transmission, Scott and Wilkinson (Scott and Wilkinson, 1999) analyzed hybrids collected from sympatric *B. napus* plants, and found no evidence for paternal ptDNA transfer. Taken together with low levels of hybridization under natural conditions the authors conclude that there would be no or negligible transmission of ptDNA through pollen under these conditions (Scott and Wilkinson, 1999). It was also indicated that

feral populations of *B. napus* will rarely survive long enough to form mixed stands, especially when combined with other control practices, but that mixed stands might persist longer at certain locations, such as British riversides. The authors considered that transmission into *B. rapa* by 'chloroplast capture' is a more likely route for chloroplast transfer between the species: *B. rapa* may hybridize as pollen donor with *B. napus* in mixed or sympatric stands and give rise to *B. rapa*-like plants that contain *B. napus*-derived chloroplasts after two generations of repeated back crossing with paternal *B. rapa*. In a subsequent study, out of 14 *B. rapa* populations growing sympatrically (closer then 5m) with *B. napus*, two were found to contain in total 53 plants with *B. napus* chloroplasts, of which 45 lacked the C genome, indicating that they were not F1 hybrids (Haider *et al.*, 2009). In another study only 0.6% of wild *B. oleracea* plants from sympatric stands were found to contain *B. napus* chloroplasts. Chloroplast capture was found to be much more frequent in sympatric riverside *B. rapa* populations (12.1%) then in allopatric populations (0.9%) (Allainguillaume *et al.*, 2009). Results of modeling and preliminary experiments suggested that the relatively high frequency in sympatric populations could be explained by a selective advantage emanating from the presence of the *B. napus* cytoplasm, and further suggests that under specific conditions chloroplast transformation may speed up, rather than slow down transgene spread. These results show that the successful use of chloroplast transmission for biological containment is very dependent on the context and conditions under which it is used.

6.3.3 Transposition of chloroplast DNA to the nuclear genome

Chloroplasts are commonly believed to be the result of a single or multiple endosymbiotic events occurring during evolution between photosynthesizing bacteria and early eukaryotic cells. During evolution to modern day plants, numerous chloroplast genes are thought to have migrated into the nuclear genome (Bock and Timmis, 2008). In an attempt to reconstruct such events, transplastomic plants containing a plastid-active *aadA* gene as well as an *nptII* gene with nuclear-active expression signals (conferring kanamycin resistance) were constructed. Gene transfer to the nucleus was selected for by regeneration from leaf explants on kanamycin and was found to be surprisingly frequent (12 resistant regenerants from 20,000 explants, estimated to represent a frequency of $2x10^{-7}$ at the cellular level) (Stegemann *et al.*, 2003). More relevant may be the frequency of gene transfer in pollen or during pollen formation. Using similar tobacco plants, a frequency of one transposition event per 16,000 pollen ($6.4x10^{-5}$) was observed for transmission of kanamycin resistance by pollen after transfer of the gene to the nucleus (Huang *et al.*, 2003). Transposition was found to occur frequently in somatic cells as well. Transposition was much more frequent during male gametogenesis than during female gametogenesis, possibly due to the availability of plastid DNA for transport to the nucleus following plastid degradation during pollen formation (Sheppard *et al.*, 2008). In this study the transposition frequency was 1 per 11,000 pollen ($9.1x10^{-5}$). The transgene is inherited biparentally upon (stable) transposition of the transgene to the nucleus; therefore this would constitute a breakdown of the containment provided by chloroplast transformation. It is not clear to what extent transposed chloroplast DNA is stably inherited. At the given rate of transposition, the nuclear genome size would constantly increase, and the lack of such increase together with other evidence suggests that transposed ptDNA in the nucleus is not always stably integrated. This was also the case for at least some of the lines described by Sheppard *et al.* (Sheppard and Timmis, 2009).

Normally, chloroplast-derived (trans-) genes equipped only with plastid-specific expression signals would not be active in the nucleus, although they could be activated by endogenous nuclear genomic regions, for example by landing next to a nuclear promoter sequence. Similar events could be reconstructed by selecting for activation of the chloroplast-encoded, but normally nuclear-inactive *aadA* gene on the above-mentioned lines that contained chloroplast-derived DNA in the nuclear genome. Selection of 5564 explants on spectinomycin gave 8 true activation events (estimated frequency $3x10^{-8}$), which were inherited in Mendelian fashion (Stegemann and Bock, 2006). Although significant on an evolutionary time scale, the frequency of the combined product of transposition and activation, is probably insignificant in comparison to paternal transfer of the active transgene through the chloroplast (see above), especially when considering that the event would need to occur in the apical meristem or germ-line cells to ensure transmission to the next generation. Thus, transposition to the nucleus is a significant factor (compared to direct chloroplast transfer via pollen) when transgene presence is absolutely undesirable. However, since activation of the transgene's expression is much less likely to occur, transposition to the nucleus is less significant from an (environmental) safety point of view.

7 Male sterility

7.1 Conclusions

Male sterility in plants is defined as the absence of functional pollen. This precludes the transmission of transgenes through the pollen. Natural mutations leading to male sterility exist in many plant species. Of particular note is the cytoplasmic male-sterility (CMS) type, which has found it is way into application for several crops, e.g. maize. Genetic modification, particularly by expression of a ribonuclease (mostly barnase) in floral tissues, has been used to engineer male sterility in crops where no useful type of natural male sterility is available. Both the natural CMS types as well as engineered male sterility have variable stability, depending on environmental conditions and the genetic background but for both types stable lines can be selected. Thus, when properly selected and applied, transgene transmission by pollen can be effectively inhibited. However, when seed is to be produced in the field, non-transgenic pollinator plants will have to be used to ensure sufficient pollination. This will not be feasible for all crops.

7.2 Background

Male sterility, the absence of functional pollen, is another method to prevent transmission of transgenes through pollen, and may be combined with other strategies such as parthenocarpy to enhance biological containment. More importantly for breeders, male sterility is a valuable tool for the production of hybrid seeds, as it eliminates the need for manual emasculation of female parent plants to prevent self-pollination. However, the way in which male sterility would be used for transgene containment would be fundamentally different from the way in which it used for breeding. In hybrid breeding the female parent is male sterile, however if seeds are the harvested product (such as in hybrid oilseed rape or maize), then male fertility needs to be restored in the F1 hybrid progeny. In contrast, for biological containment the harvested crop variety should be male sterile. This is easily achieved when the harvested product is derived from the vegetative parts, apomictic seeds or parthenocarpic fruits. However, if seeds are the harvested product, then pollination should be achieved with fertile pollen from non-transgenic pollinator plants.

The occurrence of male sterile plants in natural plant populations is wide spread. As many as 7.5% of European Angiosperm species are gynodioecious, meaning that they have both hermaphroditic and female (male sterile) plants. There may be multiple causes for male sterility and the phenotypes may range from complete lack of male floral organs to the inability of pollen to germinate (reviewed in (Budar and Pelletier, 2001). The most common (as well as most practical) form of male sterility is cytoplasmic male-sterility (CMS), which is caused by mitochondrial genome mutations and is maternally inherited. CMS usually has fewer pleiotropic effects than nuclear-encoded sterility. During evolution many species have co-evolved so-called nuclear 'restorer' (*Rf*) genes that restore male sterility when crossed into CMS lines, a prerequisite for a crop from which seeds are the harvested product. Alternatively, restorer lines may be replaced by a small portion of male fertile plants to act as pollinator plants for male sterile F1 hybrid female parent plants. Breeding with CMS lines has been used since 1943 for onion, sugar beet, maize, *Sorghum*, sunflower, rice, oilseed rape, cabbage, tobacco, and carrot (Pelletier and Budar, 2007).

Engineered male sterility has been attempted in those crops where CMS is not available, where no restorer lines are available, or where natural CMS has a yield penalty. Many different strategies (and mutations) leading to male sterility have been described, too many to be described in detail here, but reviewed elsewhere (Perez-Prat and van Lookeren Campagne, 2002; Dunwell and Ford, 2005; Chase, 2006). One of the most common problems associated with many of these strategies is the maintenance of the male sterile line, as most of these strategies involve nuclear-encoded male sterility. Different strategies to maintain male-sterile lines are described by Perez-Prat and van Lookeren Campagne (2002). The most extensively used strategy for engineered male sterility is that of expression of a destructive ribonuclease (barnase is mostly used, but a variety of others exist) in the tapetum tissue of the anther during pollen development (Mariani *et al.*, 1990). An added advantage of this system is that fertility can be restored by the introduction of a gene encoding an inhibitor of barnase, barstar (Mariani *et al.*, 1992).

A novel form of engineered cytoplasmic male sterility is the introduction of a gene encoding a bacterial β-ketothiolase in the chloroplast genome. Although no nuclear restorer gene for this form of sterility has been identified so far, exposure to continuous light does result in some male fertile flowers (Ruiz and Daniell, 2005; Chase, 2006).

7.3 Experimental evidence for efficacy of the technology for transgene containment

The multitude of available male sterility strategies suggests that at least some of these will form the basis of viable biological containment strategies in the future. However, as noted above, there are many practical breeding problems associated with male sterility. Moreover, this strategy prevents transgene transmission in only one direction, as pollen from wild relatives or other crops may still fertilize the male-sterile maternal parent. Thus, depending on the harvested product, male-sterility may have to be combined with female sterility (if the vegetative parts of the plant are harvested), parthenocarpy (for seedless fruit production) or apomixis (for seed production without fertilization) to more completely block transgene transmission. One problem in assessing the utility of the strategy is that most reports deal with the use of male-sterile plants as the maternal parent in hybrid seed production, where the produced hybrid seeds have restored fertility, while for biological containment the male-sterile plant should be the final product for use in the field. It is difficult to quantitatively assess the efficiency of transgene containment by various strategies because containment was not the primary goal of most experiments. Male sterility or decrease in pollen viability can be measured at different levels: the total production of pollen without distinction between live and dead pollen (often reported as low or absent in engineered male sterility; the percentage of viable pollen (as determined by the uptake of a vital stain); pollen germination and formation of a pollen tube in a laboratory assay; formation of seeds by self-pollination in an autogamous crop and/or ability to fertilize a wild-type pollen recipient. In many reports the last criterion, failure to form seeds (which can be reversed by hand pollination with wild type pollen) is the only measure used for assessing the sterility of the plant. Many reports are limited to the observation that no pollen is produced.

7.3.1 Cytoplasmic Male Sterility (CMS)

A literature review of reports on the stability of natural cytoplasmic male sterility systems indicated that there is high variability for different genotypes under different environmental conditions and over successive years. This was observed for cotton (*Gossypium* spp (Sarvella, 1966), in two CMS systems of oilseed rape seed (*Brassica napus* L.) (Fan and Stefansson, 1986), maize (Duvick, 1965), onion (Peterson and Foskett, 1953), and sunflower (Hvarleva *et al.*, 2009).

Maize has three types of male-sterile cytoplasm (T, S, and C), which may be restored by nuclear *rf* genes or by specific environmental conditions. In a recent study, twenty-two CMS versions of European maize hybrids were evaluated in 17 environments during two years. Stable as well as unstable male sterility occurred in all three CMS types. T-cytoplasm and C-cytoplasm hybrids were generally more stable, while S-cytoplasm hybrids often showed partial reversion to fertility. Climatic factors, especially air temperature and humidity around anthesis (time of pollen shed) seemed to particularly influence the stability. This study illustrates that T- and C-cytoplasm in particular offer viable possibilities for containing transgenic pollen, especially for Bt-maize (Weider *et al.*, 2009). Unfortunately T-cytoplasm is no longer used for breeding due to its linkage to susceptibility to the fungal pathogen *Bipolaris maydis*.

Thus, it may be concluded that CMS can be a means for limiting transgene transmission via pollen when used in carefully selected genotypes and when thoroughly tested for stability. The Plus-Hybrid System (Feil and Stamp, 2002; Feil *et al.*, 2003) developed at ETH Zürich has been proposed as a strategy for biological containment using CMS lines as the carrier of the transgene in maize. This would require a high level of male sterility. By growing 80:20 mixtures of CMS transgenic hybrids: male-fertile non-transgenic hybrids, with the latter acting as pollen donor for the entire field, normal yields can be obtained. Additionally, appropriate combinations of CMS hybrids and pollinator genotypes could lead to a significant yield gain (Stamp *et al.*, 2000; Weingartner *et al.*, 2002).

7.3.2 Engineered male sterility

7.3.2.1 Ribonuclease/Barnase expression

The stability of the male sterility trait in different transgenic lines was compared in a number of different greenhouse regimes as well as in the field (Denis *et al.*, 1993). In a more detailed study on primary transformants and T1 generation oilseed rape plants expressing either barnase or RNase T1 (Mariani *et al.*, 1990). It was shown that several lines were instable at high temperature in the greenhouse as well as in the field, but nonetheless, that lines were found that were stable under all tested conditions (stability expressed as the lack of anther dehiscence and pollen spread in all of 10-42 plants, depending on the particular experiment). The number of barnase plants was too low to accurately quantify stability (Denis *et al.*, 1993).

In a study on potato lines expressing a maize ribonuclease (b32RIP) or barnase in the tapetum, 4 out 1350 and 1 out of 500 pollen were viable respectively, as evidenced by staining with a vital dye. With the same lines, 5 out of 1110 and 1 out 150, respectively, formed pollen tubes in an *in vitro* assay. Finally, hand pollination of wild type pollen recipients in 10 and 5 separate attempts, respectively, gave no initiation of fruit growth (Green *et al.*, 2005). These numbers indicate that ribonuclease (barnase-) mediated male sterility is highly effective, without being able to quantify the rate of failure accurately.

7.3.2.2 Split barnase

The 'split-barnase' strategy is a special application of barnase expression-mediated male sterility. The strategy is based on the capacity of two inactive parts of the barnase protein to reconstitute non-covalently and produce an active protein. It was shown that two tomato lines, each expressing one inactive part of barnase from a constitutive promoter, were both viable without any developmental side-effects, but that a cross between the two lines failed to yield any viable progeny, presumably because of reconstitution of active barnase in all cells of the progeny. Expressing the two components in the anther tapetum resulted in viable progeny, but they were male-sterile. Of these, 13 progeny did not produce any seed, indicating complete male sterility (Burgess *et al.*, 2002). As the authors note however, the use of a split barnase may render the protein less stabile at higher temperature and this may compromise sterility in the field. Thus more testing on different conditions is required to quantify the rate of failure of this strategy.

7.3.2.3 Intein-spliced barnase

One application of intein-splicing of proteins (see Chapter on Inteins) is a two-component system in which two halves of the barnase protein are expressed in two different parental lines, so that in their progeny the two halves are spliced into an active protein. The benefit of this strategy is that only the hybrids are male sterile and the two parent lines can be easily maintained (Gils *et al.*, 2008). While this strategy is primarily aimed at easier production of uniform male-sterile female parent populations for fertile hybrid seed production, this system could have utility for biological containment if the hybrid seed itself is rendered male-sterile by bringing the two components together one generation later in the field grown plant. However, the strategy depends on efficient splicing of the two components under all environmental conditions, and this has not been tested sufficiently.

8 Parthenocarpy

8.1 Conclusions

Parthenocarpy is formation of fruit without ovule fertilization. It can be obtained through classical selection-based breeding, as well as through genetic engineering. Neither bred nor engineered parthenocarpic plants are completely seedless under conditions favorable for fertilization. Thus, in order for parthenocarpy to be of use for biological containment at the seed level, at least in autogamous plants, it would have to be combined with male sterility, with the added benefit that this would also decrease transgene transmission via pollen. Such plants are currently being developed, but there are no data on the efficiency of biological containment.

8.2 Background

Parthenocarpy is the production of fruit without ovule fertilization. Parthenocarpic fruits are seedless and thus could contribute to transgene containment by blocking transmission of transgenes by seed. Moreover, parthenocarpy could provide both pollen- and seed-based containment when combined with male sterility. Parthenocarpy may be further divided into stimulative parthenocarpy, where pollination or other stimulation is required for fruit-set, as in watermelon. When pollination is not required for fruit development, it is called vegetative parthenocarpy, as in cucumber. Parthenocarpy may be the only way to produce fruit, such as when the plant is sterile (banana, pineapple) or may be facultative such as in tomato mutants, which will readily produce seeds if properly fertilized. Stenospermocarpy, where seed development is aborted after fertilization (such as in seedless watermelons and grapes) (Varoquaux *et al.*, 2000), is not strictly-speaking parthenocarpy, but also leads to seedless fruits. For biological containment issues this distinction is not important, because both parthenocarpy and stenospermocarpy produce less or no viable seeds. Parthenocarpic mutants exist in many more species but are often pleiotropic, which is the reason why they have not been used commercially.

Parthenocarpy can be induced in *Arabidopsis* by expressing a variety of transgenes, and has been engineered for commercial use in tomato (Ficcadenti *et al.*, 1999; Carmi *et al.*, 2003), eggplant (Donzella *et al.*, 2000), strawberry (Mezzetti *et al.*, 2004), raspberry (Mezzetti *et al.*, 2004), melon and chicory (unpublished results referred to in (Rotino *et al.*, 2005)). The strategy that was used in these plants, expression of a *DefH9-iaaM* construct (an ovary/placenta-specific promoter driving an auxin-biosynthetic gene), can presumably be used to engineer parthenocarpy in many other species.

8.3 Experimental evidence for efficacy of the technology for transgene containment

Although parthenocarpy has been mentioned earlier as a strategy for biological containment, no systematic attempt to actually measure seed production in the field or greenhouse could be found in the literature. Parthenocarpic tomato mutants do produce seeds which is not in agreement with the goals of biological containment, but on the other hand low seed production affects commercial seed production (Gorguet *et al.*, 2005). In the most important tomato mutant *pat*, short stamens are a pleiotropic effect of the mutation leading to defective pollination, although female fertility is also compromised as cross-pollination also fails to give seeds. This phenotype is dependent on the season (Mazzucato *et al.*, 1999) and seed can be produced later in the year. Other mutants are much less defective in seed production and would probably be not directly usable for biological containment at the seed level. Tomato and tobacco with engineered parthenocarpy will produce parthenocarpic fruits under adverse conditions for pollination or when emasculated, but will also produce seeds when properly pollinated under the right conditions (Rotino *et al.*, 1997). However, in practice no or only a few seeds are produced per fruit in field trials of tomato, (Rotino *et al.*, 2005). Taken together these results suggest that natural or engineered parthenocarpy may contribute to biological containment to some extent at the seed level, but is unlikely to be sufficient on its own. Parthenocarpy

in combination with male sterility in an autogamous plant like tomato would bring seed production close to zero and would have the additional benefit of limiting transgene transmission through pollen. For open-pollinated species or varieties some form of female sterility would have to be included to prevent fertilization by external pollen donors. Complete seedlessness in tomato and eggplant, by combining engineered parthenocarpy with male sterility, is one of the objectives of the EU FW6 Transcontainer project.

9 Apomixis

9.1 Conclusions

Apomixis in plants is the process of asexual reproduction through seed. Apart from many highly-valued agronomic benefits, apomictic reproduction can be an attractive alternative for maintenance of pure (transgenic) plant lines when transmission of the transgene through pollen can be limited, such as by male sterility, chloroplast transformation, or pollen-specific transgene excision. Thus, the apomictic trait by itself is not a containment strategy, although in nature apomictic plants are often also male-sterile. Apomixis is rare in most cultivated crops and when available in wild crop relatives, has proved difficult to introgress by breeding. The components of apomixis are inherited in a Mendelian fashion; however the loci underlying apomictic development have not been identified. Apomixis could be obtained by modifying key reproductive genes from sexual plants; however research in this area is also at an early stage. Together, these bottlenecks make it unlikely that the apomictic trait will be introduced into major crop plants in the near future.

9.2 Background

Sexual propagation in flowering plants is characterized by double fertilization, in which fertilization of the egg produces the embryo, while fertilization of the central cell leads to formation of the endosperm, which provides nutrients to the embryo or seedling. Apomixis is the process of asexual reproduction through seeds where either the embryo develops without fertilization (pseudogamous) and fertilization remains necessary for endosperm development, or where both embryo as well as endosperm development progress without fertilization (autonomous) (Spillane *et al.*, 2001; Spillane *et al.*, 2004). Apomixis in crop plants is highly sought after as it would provide several major agronomic benefits:

- Fixation of hybrid vigor
- Propagation of the products of wide crossings that normally would not propagate sexually
- True seed production for crops, which are now propagated vegetatively
- Increasing speed of breeding programs because of immediate conversion of beneficial genotypes into cultivars, enabling faster response to changing needs for a crop

A potential negative aspect is that apomictic plants may replace sexually reproducing plants of the same species, although in many cases apomictic and sexually reproducing plants of the same species in a natural population stably exist next to each other. This possibility would have to be assessed for new apomictic crop plants. Apomixis is widely found in Angiosperms, but is rather rare in the gene pools of crop plants, with the exception of some cereals like maize and wheat, turf grasses, citrus, apple, mango, and orchids (Spillane *et al.*, 2001). Backcrossing apomixis into commercial varieties is difficult and was only successful so far for Kentucky bluegrass. Much hope is put on the introduction of 'apomixis' transgenes from a list of candidate genes identified by mutations in model species like *Arabidopsis* (Spillane *et al.*, 2001).

9.3 Experimental evidence for efficacy of the technology for transgene containment

Apomictic seed development in itself is not a means for biological containment of transgenes, unless it is accompanied by, combined with or complements other strategies for prevention of transmission of the transgenes. Transgene containment by male sterility or transgene excision (see elsewhere) brings with it the problem of maintaining the transgene in the crop population. This problem would be solved if the transgenic crop is propagated apomictically. Most apomictic species in nature are only facultative apomicts and also reproduce sexually in the same seed or plant. This implies that viable pollen is produced and that transgene transmission through pollen is possible. Likewise, apomictic or sexual egg-cells could produce transgenic seed through hybridization with pollen

from wild relatives, although in the case of the former there may be ploidy barriers due to the production of 2n egg cells. Pollen transmission could then be mediated by introducing male sterility or, in the case of self-pollination being required for endosperm development, by chloroplast transformation or pollen-specific transgene excision. Natural apomicts that are triploid are often, but not always (such as in Dandelions), male-sterile, and produce autonomous endosperm. On the other hand when fertilization of the endosperm is required for normal seed development, pollen from other plants would have to supplement the male sterility of such apomictic plants.

Many examples of production of transgenic citrus trees were found in literature, but none specifically addressed the combination with reduced pollen fertility. Citrus species do produce pollen, so the level of transgene containment there is unknown. There are some more recent reports on the production of transgenic apomictic turf grasses, mainly in Bahia grass *Paspalum notatum* (Agharkar *et al.*, 2007; Curtis and Grossniklaus, 2008; Sandhu and Altpeter, 2008) and one in the tropical forage grass *Dichanthium annulatum* (Dalton *et al.*, 2003). The latter species is facultative apomictic and limited analysis of progeny from transformed plants suggested that some progeny arose from sexual reproduction, implying production of viable pollen. Herbicide-resistance gene transfer by pollen from a transgenic, apomictic tetraploid plant to diploid non-transgenic pollen receptors synchronously flowering under greenhouse conditions was 0.16% (Sandhu *et al.*, 2009).

10 Reduced shattering

10.1 Conclusions

Shattering is a natural seed dispersal mechanism, which in most domesticated crops has been selected against in order to reduce yield losses during harvesting. Shattering also promotes transgene spread through seeds which remain in the field and can give rise to volunteers in the following years. Thus, prevention of shattering would contribute to the decrease of transgene spread through seeds. Oilseed rape is a newly domesticated crop and still suffers from considerable seed losses by shattering, which contributes to transgene spread. Several strategies based on model plant studies to inhibit pod dehiscence (opening) in oilseed rape have been suggested and one works in a related *Brassica* species, but there are no practical applications in oilseed rape so far. Thus the potential for this approach is high, but as yet unproven.

10.2 Background

Dispersal of seeds by shattering is an ecologically important process for plant species to ensure reproduction success. In agriculture, precocious shattering leads to yield losses during harvesting and hence was selected against as a trait during the long-term domestication of crops, particularly in cereals. Seed shattering, in particular in combination with long-term survival of seeds in the soil through secondary dormancy, can lead to transgene escape via seeds if the shattered seeds produce volunteers in the fields in the first years after growing the transgenic crop. Seed shattering has been substantially reduced in most crops harvested for their seeds (e.g. cereals) as compared to the wild ancestral species. An example where reduced shattering would have benefits for containement is oilseed rape, a relatively (very) late, not-yet-fully domesticated crop, which shows considerable shattering during harvest, leading to up to 11 to 25% yield loss (Price *et al.*, 1996). Thus there is considerable interest in reducing shattering for both agronomical reasons, as well as for transgene containment. Attempts to breed oilseed rape for reduced shattering are hampered by the low amount of genetic variation for shattering in the currently used cultivars (Morgan *et al.*, 2000). Therefore, transgenic strategies based on knowledge about regulation of shattering in the model plant *Arabidopsis*, which like oilseed rape is a cruciferous plant, have been considered.

In both *Arabidopsis* as well as in oilseed rape, seed shattering requires opening (dehiscence) of the fruit, the silique The silique consist of two valves separated on two sides by dehiscence zones, which rupture at the completion of pod maturation and drying, leading to opening of the pod and exposure of the seeds. In *Arabidopsis* double mutants of two very similar genes, *SHATTERPROOF1* (*SHP1*) and *SHATTERPROOF2* (*SHP2*), have no dehiscence zones so the mature fruits fail to break open (Liljegren *et al.*, 2000). Another gene, *FRUITFULL* (*FUL*), interacts antagonistically with the *SHP* genes during development of the valve margin, and thus overexpression of *FUL* has similar results to the double mutation of the *SHP* genes (Ferrandiz *et al.*, 2000). Early on it was recognized that knocking out *SHP* expression, or overexpressing *FUL*, could result in shatter-resistant oilseed rape plants. Moreover, besides the reduced transgene spread through seed dispersal, the reduced shattering would reduce the fitness of hybrids formed with wild relatives and decrease the likelihood of introgression of transgenes in the wild population if the reduced shattering is a dominant trait (see chapter on Transgenic Mitigation). Besides *SHP* and *FUL* genes, subsequently discovered transcription factors involved in dehiscence zone formation, such as *ALCATRAZ* (Rajani and Sundaresan, 2001) and *INDEHISCENT* (Liljegren *et al.*, 2004) have been suggested as targets for knock-down or mutation. Other suggested targets are effector genes, such as those coding for cell wall hydrolyzing enzymes involved in the actual cell separation process during dehiscence. Alternatively, cells in the dehiscence zone could be targeted for ablation using a cytotoxic enzyme encoding gene under control of dehiscence zone-specific promoters (Roberts *et al.*, 2000). None of these latter approaches have been demonstrated successfully so far. A common problem may be that few or none of the effector genes or their promoters are truly specific for the pod dehiscence zone only, but are also active in dehiscence zones of anthers.

10.3 Experimental evidence for efficacy of the technology for transgene containment

Although there are many patents describing the above described transgenic approaches to inhibition of pod dehiscence, there are no published examples where these approaches have been applied to crop plants like oilseed rape. The only exception is the report of the expression of the *Arabidopsis FRUITFULL* gene in transgenic Indian mustard (*B. juncea*). The resulting pods, as predicted, showed no separation of the valves. In fact, the transgenic fruit was too tightly closed for thrashing in a combine harvester, indicating that such strategies can significantly limit transgene spread by seed shattering, but weaker phenotypes are necessary for practical use in agriculture (Østergaard *et al.*, 2006).

11 Blocking seed germination

11.1 Conclusions

Transgene flow through seeds may be contained using strategies that prevent germination of volunteer seeds, saved seeds, or crop/crop and crop/wild relative hybrid seeds. All published strategies are based on embryo-specific expression of a cytotoxic or cell-lethal gene product to achieve seed lethality, but further differ in their approach. Only limited information has been published on the efficiency of the different strategies. The original concept of what has been become known as 'terminator technology' proposes inducible expression of a seed-lethal gene, but has not been demonstrated in practice. Incomplete induction of seed lethality may lead to inefficient biological containment, however in a reverse strategy such as 'Recoverable Block of Function', embryo lethality is expressed by default and may be recovered by inducible expression of a recovery construct. This approach is inherently more efficient for biological containment. Both components of the strategy have been shown to be effective on a laboratory scale in tobacco, but no in-depth, large-scale studies in tobacco or studies in other plant species have been reported.

11.2 Background

When seeds are the final product of a crop, transgene flow via seeds may be prevented either by seed-specific excision of the transgene (see chapter on Transgene excision) or by preventing seed germination and the production of a viable seedling. The latter strategy is specifically linked to the term Genetic Use Restriction Technologies (GURTs), because it was proposed as a means for variety protection by seed producers by preventing seed saving by farmers. More specifically it is considered a V-GURT (variety-level GURT, because it altogether prevents the propagation of the specific variety) as opposed to T-GURTs (trait-specific GURTs). In T-GURTS the expression or the transmission of the transgenic trait is regulated. For instance, expression of the transgene may be made inducible, for example by chemicals activating a promoter, or expression may selectively be switched off, by activating post-transcriptional gene silencing (also called RNA interference – RNAi). Alternatively, without affecting the viability of the seed, the transgene can be made excisable in an inducer-dependent manner. This approach is described in Chapter 5 'Transgene excision' of this report.

In a broader sense all strategies described in this report which interfere with transmission of the transgene to the next generation, are in fact GURTs. In the particular case of the original patent describing a strategy for preventing seed germination, the strategy was named 'terminator technology' by opposing NGOs.

The original concepts of modifying seed viability are described in a series of patents, of which the details have been described (Dunwell and Ford, 2005; Hills *et al.*, 2007). The method described in the original patent (Fig. 2) requires three components: 1) a gene encoding a toxin or cell-lethal product under control of a tissue-specific promoter (such as a late-embryo specific promoter), with promoter and gene separated by a blocking sequence flanked on both sides by excision sequences; 2) a gene encoding a recombinase that is specific for the excision sequences and which is placed under control of a repressible promoter; and 3) a gene encoding the repressor specific for the repressible promoter (see (Hills *et al.*, 2007). Addition of a chemical compound binding to the repressor molecule would activate the promoter driving recombinase expression, which in turn would excise the blocking sequence and activate expression of the lethal gene.

An alternative scenario has been described and demonstrated, in which a seed-lethal gene under control of a repressible promoter, linked with the trait of interest is expressed in one parent plant, while a specific repressor is expressed in the other parent plant. Crossing results, among others, in plants containing both the lethal gene as well as the repressor, allowing initial seed production in an agricultural setting. Hybridization of crop plants hemizygous for both genes with non-transgenic or wild relatives would result in segregation of the lethal gene/trait combination and the repressor gene, in which case embryos receiving only the lethal gene/trait-combination would die

(Schernthaner *et al.*, 2003). Although in the ideal case (the two inserts are on the exact same location on both parental chromosomes) the two inserts cannot be co-inherited, this obviously prevents only transmission of the lethal gene/trait-insert, not that of the repressor construct.

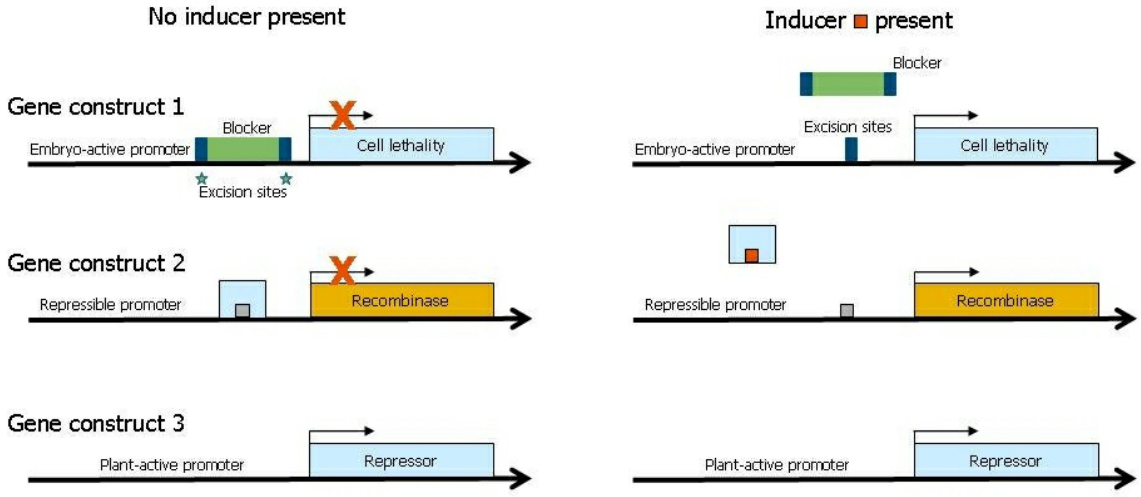

Figure 2. Principle of V-GURT or 'Terminator Technology'.

A strategy that switches from induced activation of the lethal gene in the embryo to induced block of lethality in the embryo is called Recoverable Block of Function (RBF). In this strategy the trait of interest is linked to a lethal gene under control of an embryo-specific promoter (the blocking construct), which is active and prevents seed germination by default. The block in seed germination can be reversed through the addition, in the same construct, of an inducible gene encoding an inhibitor of the lethal gene product. This strategy was demonstrated in tobacco using *barnase* under control of an embryo-specific promoter as the lethal gene, and the barnase inhibitor-encoding gene, *barstar,* under control of a heat-inducible promoter (Kuvshinov *et al.*, 2001). In order to further tighten control and prevent escape from the germination block through silencing and mutation, a further construct with two different promoter-barnase combinations in one construct was developed, and dubbed double RBF (Kuvshinov *et al.*, 2005).

11.3 Experimental evidence for efficacy of the technology for transgene containment

There are few publications describing the efficacy of the proposed strategies aimed at preventing transgene transmission through seed by seed lethality. Despite the intense debate on the use of 'terminator technology' realization of the original concept of a three-component system leading to inducible seed lethality has not been reported. Experience with inducible expression systems shows that reliance on induction for blocking transgene spread may not be prudent. Although 100% efficient blocking of transgene transmission may be of less importance for effective variety protection, penetration of the inducer and effective induction of the recombinase gene would have to approach 100% in order to be useful for biological containment. Although various chemical induction systems have been developed and to some extent demonstrated to work in the laboratory ((Moore *et al.*, 2006), their utility in the field is less well proven and many (such as those using vertebrate hormones or antibiotics) are not likely to be acceptable in view of environmental considerations. Experience with chemically-inducible transgene excision (see Chapter on Transgene Excision) suggests that acceptable efficiency with chemical inducers is hard to achieve.

The Recoverable Block of Function strategy reverses the need for induction of gene expression to block seed germination to the need for induction to recover the block of seed germination, i.e. the default state is block of germination and effective induction is required to achieve germination. Therefore the efficiency of cell lethal gene expression and of the promoter driving the cell-lethal gene is crucial for biological containment, rather than the efficiency of the chemical induction. The original publication describing RBF gives no details of the actual efficiency of the germination block in tobacco (Kuvshinov *et al.*, 2001). With double RBF in tobacco 0% germination was observed in the absence of induction of the recovery construct by heat, although the significance of this number could not be established because seed sample size was reportedly somewhere between 100 and 1200). In the best case recovery of germination upon heat treatment was up to 100% (Kuvshinov *et al.*, 2005).

12 Inhibiting seed dormancy

12.1 Conclusions

Dormancy of transgenic crop seeds after spilling or shattering may lead to volunteer emergence in subsequent years and to transgene flow through the seed. Thus, inhibition of seed dormancy may contribute to the decrease of transgene spread through seeds. Agronomic practices play an important role in the seed survival rate and may, when carefully chosen, contribute significantly to diminishing gene flow in this manner. Genetic variation for secondary dormancy in oilseed rape suggests that there is perspective for selection against secondary dormancy in breeding, although this has not received attention so far. A direct correlation between levels of dormancy and the extent of transgene flow through seeds has not been shown, although high levels of transgenic seeds in years following the cultivation of transgenic oilseed suggest that it is an important contributor.

12.2 Background

Shattering during harvest, predation in the field and losses during transport may all contribute to incorporation of seeds into the soil seed bank. If these seeds survive and germinate, they can give rise to volunteer populations and thus contribute to seed-mediated gene flow. The amount of seed coming into the seed soil bank, combined with seed survival in the soil determines the recurrence of volunteer populations. Primary dormancy, present in most plants, is generally considered essential for the normal agricultural practice of seed harvesting and storage. Secondary dormancy occurs in seeds that initially encounter conditions that are unfavorable to germination and growth, and is not as common. In the absence of secondary dormancy, seeds may germinate too early and be killed by unfavorable conditions such as freezing (fatal germination). The length of time that seeds remain in the soil bank depends on the crop's biology and agronomical practices such as tilling. Tilling induces fatal germination in wheat, which has no secondary dormancy and survival without tilling is higher (Nielson *et al.*, 2009). In oilseed rape, tilling induces secondary dormancy (reviewed in Warwick *et al.*, 2009). A long-term field experiment monitoring the occurrence of transgenic seeds in conventional oilseed harvests 5 to 8 years after growing the transgenic crop, showed that up to 18% of seeds were transgenic (Messéan *et al.*, 2007). Varieties differ considerably in their level of secondary dormancy, thus there is likely to be a genetic component determining dormancy, which in turn offers the possibility to breed for reduced dormancy (Gruber *et al.*, 2004; Gruber *et al.*, 2009). Lower dormancy of a crop plant in crop/wild relative-hybridization event may also negatively affect the introgression of transgenes into wild relative populations, if the trait is dominant (see chapter on Transgenic Mitigation).

12.3 Experimental evidence for efficacy of the technology for transgene containment

There are no reports of attempts to actively modify seed secondary dormancy in the plant, although studies do show that agronomical practices (tilling etc.) are important in determining the level of persistence in the seed bank for different crops. Furthermore, there is genetic variation in the level of secondary dormancy between varieties. This also indicates that breeding or genetic modification for lower secondary dormancy can contribute to this lower persistence and hence to decreasing volunteer emergence and transgene flow through seeds. However, no direct correlation studies between level of dormancy and transgene flow through seeds have been reported.

13 Transgenic mitigation

13.1 Conclusions

Transgenic mitigation as a strategy does not by itself prevent transgene flow from transgenic crops to non-transgenic crops or wild relatives, but 'mitigates' the effects of such gene flow, i.e. its goal is to prevent the establishment of the transgene in volunteer populations or in populations of wild relatives if hybridization can occur. Thus it mostly mitigates the effect of, not blocks, transgene escape both by pollen as well as by seeds. In this strategy, the 'trait' gene (such as herbicide resistance or other desired traits) is closely linked to a gene that confers competitive disadvantage to hybrids or volunteers, in natural stands of wild relatives or agricultural fields with non-transgenic crops, respectively. The strategy depends on the two transgenes being so closely linked that they will not segregate during meiosis. Many mitigating genes have been suggested, but only one has been tested in practice, in both tobacco and oilseed rape. Short term competition experiments suggest that transgenic mitigation can work, thus contradicting more pessimistic scenario's from modeling studies, but these experiments will need to be complemented (like most other strategies for transgene containment) with long-term competition experiments under more realistic conditions to determine if the strategy holds up in real life situations.

13.2 Background

The establishment of transgenes in a wild relative population by introgression may be inhibited by transgenic mitigation. However, transgenic mitigation does not block the first step of transgene flow by pollen or hybridization with wild relatives and thus does not constitute true 'containment' (Gressel, 1999). Rather, in this strategy the further spread of the transgene is prevented by linking it to a mitigator gene (which if tightly linked would rarely segregate from the transgene) thereby lowering the fitness of a hybrid below that of the wild-type population. A mitigation gene in the form of a transgenic trait might already be present in the crop genome, thus the second transgene needs to be inserted close to the original transgene in order not to segregate from it during sexual reproduction. Such mitigation traits should be neutral or even favorable for crops, but deleterious to non-crop progeny due to negative selection pressure. Such traits might be dwarfism, uniform seed ripening, non-shattering fruits, lack of secondary dormancy and inhibition or delay of flowering (non-bolting) (Gressel, 1999). It should be noted that such mitigation genes should be dominant in order to work in hybrids, which may be problematic for most examples mentioned here. The rate of a single linked mitigation gene segregating away from the primary trait gene or mutating to an inactive form was estimated at 10^{-5} to 10^{-7}, and for two genes flanking the trait gene even as low as 10^{-12} (Gressel, 1999). Many different strategies for various crops using various mitigation genes have been reviewed elsewhere (Gressel, 1999; Gressel and Al-Ahmad, 2005b; Gressel and Al-Ahmad, 2005a; Weissmann *et al.*, 2008; Gressel and Valverde, 2009). The review below concentrates on those examples that have been tested (to some extent) in practice.

13.3 Experimental evidence for efficacy of the technology for transgene containment

The principle of transgenic mitigation has been tested with a tandem construct of an herbicide resistance gene ('the trait') and semi dominant dwarfing gene conferring gibberellic acid-insensitivity ('the mitigator') in both tobacco as well as in oilseed rape. In greenhouse experiments, hemizygous progeny of transgenic and wild type tobacco parents (TM plants) grown without herbicide use competed poorly in mixed stands with wild type competitor plants. With decreasing spacing between plants, increasing numbers of TM plants died and surviving plants did not flower, with exception of those at the periphery. At 1 cm plant distance, 100% of TM plants died, decreasing to 45% at 5 cm spacing and 1% at 10 cm spacing. Reproductive fitness (as measured by fruit number) was 0 at small distances and no more than 17% of wild type at 10 cm spacing (Al-Ahmad *et al.*, 2004). Curiously, the herbicide resistance conferred some fitness cost as well, contributing to decreased competition with wild type plants, but nonetheless

competitiveness of TM plants was also low as compared to transgenic plants carrying only the herbicide resistance gene. Models using the measured relative fitness of TM plants in a replacement series with increasing TM/wild type plant ratios, predicted that even with 90% TM plants in a mixed stand, transgenic plants would be extinct in three years using a 2.5 cm spacing and in 14 years using a 5 cm spacing (Al-Ahmad *et al.*, 2005). These observations contradict predictions by Haygood *et al.*, who suggested that even unfavorable crop genes may become fixed in a wild population and even lead to shrinkage of the wild population (Haygood *et al.*, 2003; Haygood *et al.*, 2004). However, the conditions used in the experiments by Al-Ahmad et al, particularly the very close spacing of the plants, are not likely to represent a realistic scenario.

The same construct was tested in transgenic oilseed rape (*B. napus*) plants, in greenhouses competition experiments with the non-transgenic oilseed rape, to assess the risk of establishment of a volunteer population in non-transgenic fields. Dwarfed herbicide resistant oilseed rape plants had a higher yield when grown alone, but they were increasingly unfit in competition experiments with wild type tall plants (relative reproductive fitness 0% at 2.5 cm and 4% at 5 cm planting distance). The higher yield in an unmixed stand for TM plants was confirmed under more realistic green-house conditions, where relative reproductive fitness was less then 11% (Al-Ahmad *et al.*, 2006). The fitness of crop/weed (*B. rapa*)-hybrids was also assessed with the same plants. While non-transgenic interspecific hybrids (F2; back-crossed once with *B. rapa*) had a fitness of 50-80% relative to *B. rapa*, that of the comparable transgenic interspecific hybrids was less than 2% of that of *B. rapa* in competition experiments, indicating that the dwarfing gene was the most important cause of the decrease in fitness of interspecific hybrids (Al-Ahmad and Gressel, 2006).

14 Inteins

14.1 Conclusions

Inteins are cis- or trans-splicing elements that allow the splicing together of two inactive halves of a protein into a complete and therefore, active protein within the plant cell. In theory, the expression of two inactive parts in different parents or in two different genomes (chloroplast and nuclear) will prevent and delay the transmission of active transgenes by pollen or seeds to related crops or wild relatives. It does not prevent the transmission of transgenes *per se*, since the components can still be transmitted through the maternal or both maternal and paternal lines. Although parts of the intein concept have been shown to work in the laboratory, its usefulness for biological containment has not yet been demonstrated.

14.2 Background

Protein splicing elements, named inteins, which occur with proteins that can excise themselves from a larger precursor protein, are found in different domains of nature. The excised fragment is usually flanked by an N-terminal and a C-terminal splicing domain, which can undergo a splicing reaction, thereby deleting the intervening sequence. Although in nature this occurs in a single larger protein precursor (cis-splicing inteins), the N- and C-terminal splicing domains may also be present on two separate proteins (at the C-terminal and at the N-terminal end, respectively), so that splicing (trans-splicing) of the two proteins results in a fusion product. A complete, functional protein may result from the trans-splicing reaction when the two fused proteins are the N-terminal and C-terminal parts of a single protein (see Evans Jr *et al.*, 2005) for a review of the mechanisms and applications). The attraction of this system for transgene containment, which was recognized in an early stage, is that the two trans-splicing components can be expressed in different parents of a hybrid crop, so that neither parent can transmit an active protein encoding transgene, or in two different genomes from the same plant (nuclear and chloroplast). In the latter case, the nuclear encoded protein is transported to the chloroplast using a transfer peptide sequence, the two components meet in the chloroplast, and the active protein is produced in the chloroplast by splicing (Khan *et al.*, 2005). In such cases, transfer of the chloroplast-encoded part by pollen is unlikely in species where ptDNA transfer is mostly or strictly maternal.

14.3 Experimental evidence for efficacy of the technology for transgene containment

Trans-splicing of proteins has been demonstrated for several plant species, in nuclei and in chloroplasts, and for several proteins (reviewed in Evans Jr *et al.*, 2005; Khan *et al.*, 2005). All these experiments were performed in the laboratory and the general conclusion is that proper splicing needs to be optimized for the choice of splicing location that would yield an active protein and requires extensive optimization under environmental conditions. Large scale greenhouse or field trials have not been performed to assess the utility of the strategy for biological transgene containment. It should also be noted that the strategy aims at preventing the transmission of active transgenes and not of transgenes *per se*, thus the individual components can still be transmitted.

Concluding Remarks

From this report it may be obvious that there is an almost overwhelmingly large variety in methods proposed to limit undesired spread of transgenes beyond the crops and the fields for which they were intended. At the same time, it may become clear that very few of these have been thoroughly tested for their efficacy, stability, and for their usefulness in actual crops species, let alone in multiple crop species. Most strategies presented here were only tested in model plants such as *Arabidopsis* and tobacco, and far less in one or more crop plants.

Depending on the available information for each strategy, the reviewed strategies may be somewhat subjectively categorized as 'merely concept', 'proof of principle (in model plants)', and 'more extensively tested'. None of the strategies would comply for the category 'thoroughly tested in crop plants'. Auxotrophy, apomixis, parthenocarpy, reduced shattering, inhibiting seed dormancy, inteins and transgenic mitigation have hardly gone beyond the 'concept stage'. Blocking seed germination and transgene excision are in the 'proof of principle' stage. Inhibition of flowering, cleistogamy, chloroplast transformation and male sterility have been 'more extensively tested', also in some actual crops and in field experiments. Although the results obtained with model plants will have value for estimating the efficacy of certain strategies in real crops, the efficacy of a strategy may still differ between plant species.

None of the strategies reviewed in this report prevents or partially blocks all avenues for transgene spread. Whereas methods for decreasing gene flow through pollen, seed or both are relatively often studied, only one technology prevents transgene spread through vegetative reproduction (namely, auxotrophy), while transgenic mitigation may help to decrease competitiveness and invasiveness once a transgenic crop plant has escaped. Transgene escape through vegetative reproduction remains a possibility and could, hypothetically, in some cases be enhanced, when sexual reproduction has been fully blocked by blocking of flowering or by engineering complete male and female sterility. Although no indication of such effects were found in literature, such side-effects may have to be taken into account in a risk assessment. Other strategies prevent transgene spread through pollen or seed but not both. From this it follows that in the choice of strategy for prevention of transgene spread the ecology and agronomy of the crop need to be taken into consideration to determine where the effects of such strategies would be biggest. As the required level of containment gets higher, it will be more and more likely that two or more strategies will need to be combined to achieve the necessary level of containment, for example by blocking both transmission through pollen as well as through seeds.

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