NOVEL ASPECTS OF THE ENVIRONMENTAL RISK ASSESSMENT OF DROUGHT-TOLERANT GENETICALLY MODIFIED MAIZE AND OMEGA-3 FATTY ACID GENETICALLY MODIFIED SOYBEAN

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SUMMARY

Background

To date, a novel generation of genetically modified (GM) plants is under development and at a pre-market stage. These GM plants are plants, which express pharmaceutical, nutraceutical or industrial compounds, have an increased tolerance to abiotic stress, like drought, or have new metabolic pathways, resulting for example in the production of non-native fatty acids. The nature of the genetic modifications of these novel GM plants clearly differs from that of current generations of GM plants with resistance(s) to a herbicide and/or insects, as they are genetically more complex and/or lead to significant metabolic shifts. As a result, the likelihood that these genetic modifications lead to unintended effects on the plant's genotype and/or phenotype could be higher for these novel generation GM plants than for current generations of GM plants. It can therefore be anticipated that their environmental risk assessment will need to address more uncertainties about their potential environmental effects.

Within a couple of years such novel GM plants will be submitted for EU market-approval. This will require an environmental risk assessment by the competent authorities of EU Member States, including the Netherlands. As the GMO Office, the executive office of the Dutch competent authority, foresees that the environmental risk assessment of such novel GM plants needs to take into account uncertainties of their genotype and phenotype, it selected two examples of novel GM plants that are currently in the mainstream of research and development efforts, in order to study whether their environmental risk assessment involves novel aspects compared to that of current generations of GM plants. These two novel GM plants are drought tolerant GM maize and omega-3 fatty acid GM soybean, both which are already being field-tested in the US.

Drought-tolerant GM maize

Since the 1930s conventional breeding of maize has led to a continuous improvement of grain yields. However, over the last few decades the variations in grain yield also increased markedly. Since weed control and nitrogen deficiency were no longer constraints to productivity during these last decades, the grain yield variability was mainly due to limited water supply and unfavourable temperatures. Yield stability rather than improvement of primary productivity became therefore an important additional target for maize breeders. As a result, conventionally breeding during the last twenty years resulted in maize cultivars with increased drought tolerance compared to older maize cultivars. Since the last decade, maize breeders' knowledge of the genetics and physiology underlying drought tolerance as a phenotypic trait expanded rapidly, to the extent that it has reached a level that enables to direct breeding of drought tolerance in maize, instead of only describing it.

Plants have several molecular mechanisms that enable them to respond to abiotic stress induced for instance by drought, and biotic stress caused by pathogens and herbivores. These molecular mechanisms are intricately associated with various plant physiological functions and morphological characteristics. Together, these molecular mechanisms constitute a multidimensional network system with many levels of gene expression and regulation. Biotic and abiotic stresses induce the expression of different but overlapping sets of genes in higher

plants. Understanding of this multidimensional network system is still a major challenge in plant biology, despite the research conducted over the last fifteen years.

Plant hormones such as abscisic acid (ABA), ethylene (ET), jasmonic acid (JA) and salicylic acid (SA) primarily regulate the protective responses to both biotic and abiotic stresses. While ABA is mainly involved in responses to abiotic stresses, it also governs a variety of growth and developmental processes, including seed development, dormancy, germination and stomatal movements. By contrast, ET, JA and SA play central roles in biotic stress responses. But there is also strong evidence for "crosstalk" between these two different stress response mechanisms.

The generation of reactive oxygen species (ROS) scavengers or other second messengers is another key process that is shared between abiotic and biotic stress response mechanisms. These second messengers often initiate a protein phosphorylation cascade that targets proteins directly involved in cellular protection against damage caused by stress or transcription factors, which control specific sets of stress-regulated genes. Synthesis of osmoprotectants is yet another mechanism that plants have evolved for responding to drought stress. Osmoprotectants are small molecules that stabilise proteins and cell membranes against denaturing effects of stress conditions on plant cellular functions. But many major crops lack the ability to synthesise special osmoprotectants, such as fructans, trehalose and glycine betaine, that are naturally accumulated by stress-tolerant organisms, such as certain plants, marine algae and bacteria.

In several cases genetic modification of a plant with a single trait involved in transcription control resulted in improved drought tolerance. Moreover, genetic modification of upstream signalling regulators also led to improved drought tolerance, although it often also activated a much wider network of genes, other than stress-specific ones, with deleterious effects on total plant performance. A third strategy for improving drought stress tolerance consists of genetic modification of (crop) plants with genes encoding the synthesis of special osmoprotectants. Over the last couple of years all these genetic modification strategies have been followed for improving drought tolerance in maize. Though, in several cases the genetic modification of maize was primarily aimed at further advancing scientific understanding various molecular and physiological mechanisms that underlie drought tolerance as a phenotypic trait.

In the US about 180 field trials with drought-tolerant GM maize have been conducted so far. More than 80 % thereof have been carried out by Monsanto and the rest by Pioneer Hi-Bred (DuPont), Syngenta, BASF, Stine Biotechnology and Biogemma. Monsanto and Pioneer Hi-Bred expect to commercially release their first types of drought-tolerant GM maize around 2011 – 2012. In the EU Biogemma has so far conducted 4 field trials with drought-tolerant GM maize, while Coop de Pau has carried out 2 field trials with drought-tolerant GM maize.

Information on laboratory and greenhouse experiments with drought-tolerant GM maize, the (drought stress) conditions in field trials in the US and the EU, the types of genetic modification and the resulting data on agronomic performance and potential environmental effects have as yet not been made publicly available. Only in two cases of field trials with drought-tolerant GM maize plants in the EU, information on the genetic modification has been made publicly accessible and some of the experimental results have been published in scientific literature.

Particularly for the environmental risk assessment (ERA) of an unconfined, large-scale or commercial release of a drought-tolerant GM maize plant in Europe, it should be reiterated that modern conventionally bred maize cultivars already exhibit a significantly enhanced tolerance to drought in comparison to maize cultivars that were in use a few decades ago. The baseline for comparing drought-tolerance of a GM maize plant to that of conventionally bred maize has thus been constantly evolving during the last decades and will continue to evolve further in the nearby future.

For an ERA of an unconfined, large-scale or commercial release of a drought-tolerant GM maize plant in Europe, there is a lack of data that allow a comparison of the performance of a drought-tolerant GM maize plant to that of the parental maize plant or a conventionally bred maize cultivar in commercial use under drought stress conditions. Relevant phenotypic parameters in this respect may include grain yield, barrenness (inability to produce viable ears), anthesis-silking interval (ear growth rate), leaf rolling, stomatal conductance (in relation to photosynthetic activity), water-use efficiency (biomass production per unit of transpiration), abscisic acid content in leaf and xylem, root characteristics, seed germination and dormancy, seedling survival and growth, pollen viability, activity of phospoenolpyruvate carboxylase (PEPC), ribulose-1,5-bipophosphate carboxylase oxygenase (Rubisco) and glycolic acid oxydase (GAO) and disease susceptibility.

Taking conventionally bred maize cultivars as the baseline for an ERA, it should be noted that in Europe that they already exhibit a significantly enhanced tolerance to drought in comparison to maize cultivars that were in use a few decades ago. This has not led to an persistence in agricultural habitats or invasiveness in natural habitats of modern maize cultivars in comparison to maize cultivars that were in commercial use a few decades ago.

Because the gene(s) inserted for drought-tolerance might also affect molecular response mechanisms to other forms of abiotic stress, like cold or salinity, it cannot be excluded that a drought-tolerant GM maize acquires a potential to survive at low temperatures in the winter and becomes more winter hardy. Consequently, this might lead to an increased potential of a drought-tolerant GM maize for persistency in agricultural habitats or invasiveness in natural habitats. In particular when the genetic modification targets changes in the abscisic acid (ABA) metabolism, which also regulates key processes in seeds, like dormancy and accumulation of storage lipids, it cannot be excluded that the seeds of a drought-tolerant GM maize plant might acquire a changed tolerance to cold. This in turn might result in an increased winter survivability, which could lead to an increased potential for persistency in agricultural habitats or invasiveness in natural habitats. Though, the overall likelihood of an increased potential for persistence in agricultural habitats or invasiveness in natural habitats of a drought-tolerant GM maize plant is extremely low, given the inherent characteristics of maize. In case cultivation of a drought-tolerant GM maize does lead to volunteers in the next crop, they can be controlled by usual volunteer control measures. And if they do spread and establish in natural habitats, (volunteer) control measures need to be extended to natural habitats.

There are no reasons to assume that a drought tolerance trait in a drought-tolerant GM maize will have a direct effect on maize predators, parasitoids, and pathogens and population levels of organisms that interact with them. First, because a drought-tolerance trait is not aimed to control maize pests at all. Second, because neither the drought-tolerance genes inserted and their products, like plant signalling factors or enzymes, nor the metabolites of these enzymes, like reactive oxygen species (ROS) scavengers or osmoprotectants, are known to have an effect on maize predators, parasitoids, and pathogens. On the other hand, a drought-tolerance trait in a drought-tolerant GM maize could have indirect impacts on maize predators, parasitoids, and pathogens and on population levels of organisms that interact with them. Because there is a potential crosstalk between molecular response mechanisms to abiotic and biotic stress in plants, it cannot be excluded that a drought-tolerant GM maize acquires a changed tolerance to biotic stress, which could result in changed interactions with maize predators, parasitoids, and pathogens. As a consequence, a drought-tolerant GM maize could have indirect impacts on population levels of organisms that interact with them. If a drought-tolerant GM maize plant acquires an increased susceptibility to maize predators, parasitoids, and pathogens, its cultivation may necessitate different phytosanitary measures, for instance an increased application of (chemical) crop protection means.

Moreover, because a drought tolerance trait in a drought-tolerant GM maize could potentially affect the metabolism of its pollen, it cannot be excluded that this might change the viability of its pollen, and consequently, the dispersal characteristics of its pollen. While there are no sexually compatible weedy or wild relatives of maize present in Europe, there is only a potential for gene transfer from drought-tolerant GM maize to non-GM maize. Hence, gene transfer through pollen flow from a drought-tolerant GM maize could confer a selective advantage to non-GM maize under abiotic stress conditions and/or either a selective advantage or disadvantage for the recipient non-GM maize plants under biotic stress conditions.

There are no reasons to assume that a drought-tolerant GM maize will have detrimental effects on human or animal health. Because neither the drought tolerance genes inserted and their products, like plant signalling factors or enzymes, nor the metabolites of these enzymes, like ROS scavengers or osmoprotectants, are known to have effects on human or animal health.

Finally, there are no reasons to assume that incorporation into the soil of root exudates, plant litter, seeds or pollen of a drought-tolerant GM maize will have effects on biogeochemical cycles. First, because horizontal transfer of the inserted genes from a drought-tolerant GM maize to soil microbes is extremely unlikely under natural conditions. Second, because expression of the inserted genes, which are under the control of eukaryotic promoters with very limited, if any, activity in prokaryotic organisms, is extremely unlikely in soil microbes. Third, because neither the drought tolerance genes inserted and their products, like plant signalling factors or enzymes, nor the metabolites of these enzymes, like ROS scavengers or osmoprotectants, are known to have effects on soil microbes.

Omega-3 fatty acid GM soybean

Over the last twenty years, there is an increasing interest in very long-chain polyunsaturated fatty acids (VLC-PUFAs), particularly the omega-3 group usually found in fish oils, because of their health-beneficial properties with respect to conditions, such as cardiovascular diseases and obesity. One major facet underplaying the dietary importance of VLC-PUFAs in human health is the very limited ability of mammals to synthesise these fatty acids themselves. As current sources of omega-3 VLC-PUFAs, predominantly oceanic fish oils, are in serious decline, there is an exhaustive search for an alternative (and sustainable) source of fish oils in human nutrition. To that end, the possibility of using GM crop plants engineered to synthesise

VLC-PUFAs in their storage seed oils has been thoroughly investigated over the past ten years.

Higher plant species are not capable to synthesise VLC-PUFAs. Hence, the conversion of plant fatty acids such as linoleic acid (LA) and α-linolenic acid (ALA) to VLC-PUFAs requires several non-plant native enzymes to generate omega-6 VLC-PUFAs such as arachidonic acid (ARA), and omega-3 VLC-PUFAs, like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Genes encoding such enzymes, i.e. desaturases and elongases, have so far been isolated from a number of marine microalgae and terrestrial fungi and transferred to several different plants, like flax, oilseed rape and soybean.

The production of significant levels of VLC-PUFAs in GM plants requires some of the most complex plant genetic engineering yet attempted. On the one hand, because multiple genes have to be inserted, on the other hand, because two generic bottlenecks, due to the presence of plant-specific fatty acid metabolic routes, need to be overcome. One of these bottlenecks has been described as ‘substrate-dichotomy’, that is a limited acyl exchange between fatty acids from the phosphatidylcholine (PC) pool and the coenzyme A (coA) pool. The other bottleneck is to ensure conversion of omega-6 fatty acids to their omega-3 counterparts, which is hampered by acyl channelling of potential substrates away from the PC and coA pool into the triacylglycerol (TAG) pool. Because of these bottlenecks, the first attempts to genetically modify plants for the production of omega-3 VLC-PUFAs led to very modest results.

However, continuous research has meanwhile resulted in GM plants, including soybean, with commercially worthwhile levels of omega-3 VLC-PUFAs. For instance, DuPont’s scientists have expanded the standard procedure of desaturating and elongating the shorter chain fatty acids by using co-expression of an omega-3 microsomal desaturase from the fungus *Sapgrolegnia diclinia* to convert the omega-6 VLC-PUFAs into omega-3 VLC-PUFAs. This led to a content of omega-3 VLC-PUFAs up to 40 % of the total fatty acid content in GM soybean seeds. In other experiments researchers from DuPont, the University of Nebraska, and the Plant Genetics Research Unit of the United States Department of Agriculture Agricultural Research Service transferred a gene encoding Δ15 fatty acid desaturase from *Borago officinalis* and the *FAD3* gene encoding Δ6 fatty acid desaturase from *Arabidopsis thaliana*, both under the control of soybean seed-specific β-conglycinin promoter, into soybean. Monsanto has also developed an omega-3 fatty acid GM soybean. This GM soybean produces stearidonic acid (SDA), which when consumed is converted to eicasopentanoic acid (EPA). Its commercial launch is projected to take place early in the next decade. It is conceivable but not certain that Monsanto used genes encoding Δ6 and Δ12 fatty acid desaturases isolated from the fungus *Mortiella alpina*, probably under control of a *napin* promoter isolated from oilseed rape – a strategy that previously resulted in an increase of SDA up to 23 % of the total fatty acid content in seeds of GM oilseed rape.

In the US, 5 fields trials with omega-3 fatty acid GM soybean plants have so far been notified by Pioneer Hi-Bred (DuPont), 5 fields trials by Monsanto and 4 field trials by the University of Nebraska. Furthermore, on 20 December 2006 Pioneer Hi-Bred International (DuPont) petitioned the US regulatory authorities to ‘deregulate’ an omega-3 fatty acid (high-oleic) producing GM soybean. This petition is currently pending. If the US regulatory authorities give their consent, it will be allowed to commercialise this high-oleic acid GM soybean in the US. Neither in this case, nor for the field trials with other omega-3 fatty acid GM soybean plants, information on laboratory and greenhouse experiments, the conditions in field trials in the US, the types of genetic modification and the resulting data on agronomic performance and potential environmental effects has thus far as yet not been made publicly available. In the EU field trials with omega-3 fatty acid producing GM soybean have thus far not been conducted, while applications for import, food and feed processing and/or cultivation of omega-3 fatty acid producing GM soybean have neither been submitted.

For an environmental risk assessment (ERA) of unconfined, large-scale or commercial release of omega-3 fatty acid GM soybean in Europe, it should be pointed out that some conventionally bred soybean cultivars have a high content of oleic acid (an omega-3 fatty acid), which can serve as baseline for assessing the environmental risks of high oleic acid GM soybean. However, with conventional breeding, it is not possible to develop soybean capable of producing VLC-PUFAs, such as stearidonic acid (SDA) or eicasopentanoic acid (EPA), because of the need to introduce genes encoding elongases and desaturases, which are not present in the gene pool of soybean.

For an ERA of an unconfined, large-scale or commercial release of a high oleic GM soybean or a VLC-PUFAs GM soybean in Europe, there is a lack of data that allow a comparison of the performance such omega-3 fatty acid GM soybeans to that of the parental varieties. It is likely that a GM omega-3 fatty acid producing trait will be put under the control of a seed-specific promoter. Nonetheless, relevant phenotypic parameters may include data not only on the duration of seed production, seed germination and dormancy, and seedling emergence, survival and growth. Other relevant phenotypic parameters may also include on re-growth from stubble, disease susceptibility, interaction with nitrogen-fixating symbionts, seed protein composition and levels of anti-nutritional factors.

Taking conventionally bred soybean plants as the baseline for an ERA, it should be noted that in Europe they are neither persistent in agricultural habitats, nor invasive in natural habitats. In the case of a high oleic acid GM soybean, there are no reasons to assume that it would become more persistent in agricultural habitats or more invasive in natural habits. First, because experience from the US indicates that conventionally bred high oleic acid soybean is not persistent in agricultural habitats. Second, because data from US field test with a high oleic acid GM soybean did not show differences in seedling emergence, re-growth from stubble and seed dormancy compared to the parental variety.

By contrast, in the case of a VLC-PUFAs GM soybean, there are reasons to assume that it could acquire an increased potential for persistency in agricultural habitats or invasiveness in natural habitats. First, because the plant (leaves) might have a changed proportion of saturated and unsaturated fatty acids, the stubble of a VLC-PUFAs GM soybean plant could have a better overwintering capability than that of its parental variety. This could potentially lead to VLC-PUFAs GM soybean volunteer plants in the next crop. Though, particularly if a seed-specific promoter controls the expression of the VLC-PUFAs trait, the likelihood might be negligible. Second, because seeds of a VLC-PUFAs GM soybean have lower proportions of saturated fatty acids in their oil than those of conventional soybeans, they might acquire changed dormancy and germination characteristics. This could potentially lead to volunteers in the next crop and/or invasions into natural habitats after transport by animals. In case cultivation of a VLC-PUFAs GM soybean leads to volunteers in the next crop, they can be controlled by usual volunteer control measures. And if they spread and establish in natural habitats, control measures can to be extended to natural habitats.

Furthermore, it is very unlikely that a high oleic acid producing trait or a VLC-PUFA producing trait confers any additional cross-fertilisation capacity to a GM soybean plant. Moreover, soybean has no sexually compatible weedy or wild relatives in Europe. In addition, the potential for gene transfer, through pollen flow, from a high oleic acid or a VLC-PUFA producing GM soybean plant to non-GM soybean plants is virtually zero, because soybean is a self-fertilising species with a cross-fertilisation capacity of less than one percent.

It is very unlikely that a high oleic acid GM soybean or a VLC-PUFAs GM soybean will result in direct effects on soybean predators, parasitoids and pathogens and population levels of organisms that interact with them. First, because neither a high oleic acid trait, nor a VLC-PUFAs trait is aimed to control soybean pests at all. Second, because oleic acid and VLC-PUFAs are not known to have detrimental effects on soybean predators, parasitoids and pathogens. On the other hand, when *FAD* genes are inserted, a high oleic GM soybean or a VLC-PUFAs GM soybean might have indirect effects on soybean predators, parasitoids and pathogens. Because insertion of *FAD* genes may affect ABA-responsive signalling, which plays a pivotal role in the crosstalk between biotic and abiotic stress response mechanisms in plants, a high oleic acid GM soybean or a VLC-PUFAs GM soybean might have changed interactions with soybean predators, parasitoids and pathogens. As a consequence, a high oleic acid GM soybean or a VLC-PUFAs GM soybean could have indirect impacts on population levels of organisms that interact with them. It should however be noted that data from US field trials with a high oleic acid GM soybean containing soybean-derived *FAD* genes did not show effects on beneficial organisms, such as bees and earthworms. If a high oleic acid GM soybean or a VLC-PUFAs GM soybean acquires an increased susceptibility to soybean predators, parasitoids, and pathogens, their cultivation might necessitate different phytosanitary measures, for instance an increased application of (chemical) crop protection means. And if a high oleic acid GM soybean or a VLC-PUFAs GM soybean acquires a changed interaction with nitrogen-fixating symbionts, this may necessitate a different usage of (chemical) nitrogen-fertilisers.

There are no reasons to assume that a high oleic acid GM soybean or a VLC-PUFAs GM soybean will have detrimental effects on human or animal health. First, because the genes inserted and their products, i.e. enzymes, like elongases and desaturases from microalgae, fungi, etc., are not likely to have detrimental effects on human or animal health. Second, because the metabolites of these enzymes, i.e. oleic acid or VLC-PUFAs, are considered beneficial to human or animal health upon consumption.

Finally, it is very unlikely that incorporation into the soil of root exudates, plant litter, seeds or pollen of a high oleic acid GM soybean or a VLC-PUFAs GM soybean will have effects on biogeochemical cycles. First, because horizontal transfer of the inserted genes from a high oleic acid GM soybean or a VLC-PUFAs GM soybean to soil microbes is extremely unlikely under natural conditions. Second, because expression of the inserted genes, which are under the control of eukaryotic promoters with very limited, if any, activity in prokaryotic organisms, is extremely unlikely in soil microbes. Third, because the gene products, i.e. enzymes, like elongases and desaturases from microalgae, fungi, etc., and the metabolites of these enzymes, i.e. oleic acid or VLC-PUFAs, are not known to have effects on soil microbes.

SAMENVATTING

Achtergrond

Op het moment wordt een nieuwe generatie van genetisch gemodificeerde (gg) planten ontwikkeld, die zich in de fase voorafgaande aan vermarkting bevinden. Deze gg-planten maken industriële, farma- of nutraceutische verbindingen, hebben een verhoogde tolerantie tegen abiotische stress, zoals droogte, of beschikken over nieuwe metabolische routes die bijvoorbeeld resulteren in niet-planteigen vetzuren. De aard van de genetische modificaties in deze gg-planten verschilt duidelijk van die van de huidige generaties van gg-planten met herbicide- en/of insectresistentie, omdat ze genetisch meer complex zijn en/of leiden tot wezenlijke metabolische verschuivingen. Het gevolg hiervan is dat de waarschijnlijkheid dat deze genetische modificaties leiden tot onbedoelde effecten op het geno- en/of fenotype van de plant hoger kan zijn dan voor de huidige generatie gg-planten. Daarom kan verwacht worden dat voor hun milieurisicobeoordeling met meer onzekerheden over mogelijke milieueffecten rekeningen moet worden gehouden.

Binnen een aantal jaren zullen dergelijke gg-planten worden ingediend voor een EU markttoelating. Dit zal een milieurisicobeoordeling vereisen door het bevoegde gezag van EU lidstaten, inclusief Nederland. Omdat Bureau GGO, het uitvoerend orgaan van het Nederlandse bevoegde gezag, verwacht dat de milieurisicobeoordeling van dergelijke nieuwe gg-planten onzekerheden over hun geno- en fenotypes in beschouwing moeten worden genomen, selecteerde het twee voorbeelden van gg-planten die zich momenteel in de hoofdstroom van het onderzoek bevinden, om na te gaan of met hun milieurisicobeoordelingen aspecten gepaard gaan, die nieuw zijn in vergelijking met die van de huidige generatie gg-planten. Deze twee nieuwe gg-planten zijn droogtetolerante gg-maïs en omega-3 vetzuur gg-soja, die alle twee in de VS al in het veld worden beproefd.

Droogtetolerante gg-maïs

Sinds de jaren dertig van de vorige eeuw heeft conventionele veredeling van maïs geleid tot een voortdurende verbetering van de graanopbrengst. De laatste tientallen jaren heeft dit echter ook geleid tot aanzienlijke variaties in graanopbrengst. Omdat onkruidbeheersing en stikstoftekorten voor de productiviteit niet langer meer beperkingen vormden, waren de variaties in graanopbrengst hoofdzakelijk het gevolg van watertoevoer en ongunstige temperaturen. Opbrengststabiliteit in plaats van verbetering van primaire productiviteit werd daardoor een belangrijk additioneel doel voor maïsveredelaars. Als gevolg hiervan resulteerde conventionele veredeling in maïscultivars met een verhoogde droogtetolerantie in vergelijking met de oudere maïscultivars. Sinds de laatste tien jaar is de kennis van veredelaars van de genetica en fysiologie van droogtetolerantie dusdanig snel toegenomen dat het een niveau bereikt heeft dat het mogelijk maakt om de veredeling te sturen in plaats van het alleen maar te beschrijven.

Planten hebben verschillende moleculaire mechanismen die hen in staat stellen om te reageren om abiotisch stress, zoals bijvoorbeeld droogte, en biotisch stress veroorzaakt door ziekteverwekkers en herbivoren. Deze moleculaire mechanismen zijn op een complexe manier geassocieerd met verschillende plantfysiologische functies en morfologische eigenschappen. Deze moleculaire mechanismen vormen samen een multidimensionaal netwerk met vele niveaus van genexpressie en –regulatie. Biotische en abiotische stress zorgen voor de expressie van verschillende maar elkaar overlappende sets van genen in hogere planten. Ondanks het onderzoek van de laatste vijftien jaar vormt het begrijpen van dit multidimensionale netwerk nog steeds een grote uitdaging voor de plantbiologie.

Planthormonen als abscisinezuur (abscisic acid, ABA) ethyleen (ET), jasmonzuur (jasmonic acid, JA) en salicylzuur (salicyclic acid, SA) zorgen voor de primaire regulatie van de reacties die planten tegen biotische en abiotische stress beschermen. Terwijl ABA voornamelijk betrokken is bij reacties tegen abiotische stress, regelt het ook verscheidenen groei- en ontwikkelingsprocessen, zoals onder meer zaadvorming, -dormantie en –kieming en het openen en sluiten van huidmondjes. In tegenstelling tot ET, JA en SA, die centrale rollen spelen in reacties tegen biotische stress. Maar er zijn ook sterke bewijzen voor "crosstalk" tussen deze twee verschillende responsmechanismen.

De vorming van vrije zuurstofradicalen (reactive oxygen species, ROS) en andere secundaire boodschappers is een ander sleutelproces dat wordt gedeeld door responsmechanismen tegen biotische en abiotische stress. Deze secundaire boodschappers starten vaak een cascade van reacties die eiwitten fosforyleren, die direct betrokken zijn in cellulaire bescherming tegen schade die wordt veroorzaakt door stress of transcriptiefactoren, die de expressie van specifieke sets van stressgereguleerde genen controleren. De aanmaak van 'osmoprotectants' is weer een ander mechanisme, waarmee planten zich tegen droogtestress kunnen beschermen. Osmoprotectants zijn kleine moleculen die eiwitten en celmembranen beschermen tegen afbraak van plantcellulaire functies als gevolg van stress. Maar vele belangrijke landbouwgewassen missen het vermogen tot aanmaak van speciale osmoprotectants, zoals fructanen, trehalose en glycinebetaïne, die van nature ophopen in stresstolerante organismen, zoals sommigen planten, zeealgen en bacteriën.

In verscheidene gevallen resulteerde de genetische modificatie van een plant met een enkele eigenschap die bij de transcriptiecontrole betrokken is, in een verbeterde droogtetolerantie. Hiernaast leidde genetische modificatie van upstream signaleringsfactoren ook tot een verbeterde droogtetolerantie, hoewel hierdoor ook vaak een wijder netwerk van genen geactiveerd werd met nadelige gevolgen voor het functioneren van de gehele plant. Een derde strategie voor het verbeteren van droogtetolerantie bestaat uit genetische modificatie van een gewas met genen die coderen voor de aanmaak van speciale osmoprotectants. De laatste paar jaren zijn voor het verbeteren van droogtetolerantie van maïs al deze genetische modificatie strategieën gevolgd. Maar in verschillende gevallen was de genetische modificatie van maïs primair gericht op het verder vergroten van het wetenschappelijk inzicht in de verschillende moleculaire en fysiologische mechanismen die bijdragen tot droogtetolerantie als een fenotypische eigenschap.

In de VS zijn tot dusver circa 180 veldproeven met droogtetolerante gg-maïs uitgevoerd. Meer dan 80 % hiervan zijn door Monsanto gedaan en de rest door Pioneer Hi-Bred (DuPont), Syngenta, BASF, Stine Biotechnology and Biogemma. Monsanto en Pioneer Hi-Bred verwachten dat de commercialisering van hun eerste types van droogtetolerante gg-maïs in 2011 – 2012 zal plaatsvinden. In de EU zijn tot nog toe 4 veldproeven met droogtetolerante GG maïs uitgevoerd door Biogemma en 2 door Coop de Pau.

Informatie over laboratorium- en kasproeven met droogtetolerante gg-maïs, de (droogtestress) condities in veldproeven in de VS en de EU, de types van genetische modificatie en de resulterende gegevens over landbouwkundig gedrag en mogelijke milieueffecten zijn vooralsnog niet publiek beschikbaar. Alleen in twee gevallen van veldproeven met droogtetolerante gg-maïs in de EU is informatie over de genetische modificatie publiek toegankelijk en sommige experimentele resultaten zijn in wetenschappelijke tijdschriften gepubliceerd.

Voor een milieurisicobeoordeling van een niet-ingeperkte, grootschalige of commerciële vrijzetting van een droogtetolerante gg-maïsplant in Europa, moet worden bedacht dat moderne, conventioneel veredelde maïscultivars al beschikken over een significant verhoogde droogtetolerantie in vergelijking met de maïscultivars die enkele tientallen jaren geleden in gebruik waren. De referentie (*baseline*) voor het vergelijken van de droogtetolerantie van een gg-maïsplant met die van conventioneel veredelde maïs is de laatste tientallen jaren dus voortdurend geëvolueerd en zal verder blijven evolueren in de nabije toekomst.

Voor een milieurisicobeoordeling van een niet-ingeperkte, grootschalige of commerciële vrijzetting van een droogtetolerante gg-maïsplant in Europa is er een gebrek aan gegevens, aan de hand waarvan het gedrag van een droogtetolerante gg-maïsplant en dat van de ouderplant of een commercieel in gebruik zijnde, conventioneel veredelde maïscultivar onder droogtestress vergeleken kunnen worden. Relevante fenotypische parameters hiervoor kunnen bestaan uit graanopbrengst, onvermogen tot kolfvorming (*barrenness*), kolfgroeisnelheid (*anthesis-silking interval*), bladkrulling, gedrag van huidmondjes (in relatie tot fotosynthese), watergebruikefficiëntie (biomassaproductie per eenheid van transpiratie), ABA concentratie in blad en xyleem, worteleigenschappen, zaadkieming en –dormantie, overleving en groei van zaailingen, pollenlevensvatbaarheid, ziektegevoeligheid en activiteit van enzymen als fosfoenolpyruvaatcarboxylase (PEPC), ribulose-1,5-difosfaatcarboxylaseoxygenase (Rubisco) en glycolzuuroxidase (GAO).

Uitgaande van conventioneel veredelde maïscultivars als referentie voor een milieurisicobeoordeling, moet worden opgemerkt dat deze in Europa al een significant versterkte droogtetolerantie vertonen in vergelijking met de maïscultivars die enige tientallen jaren geleden in gebruik waren. Dit heeft niet geleid tot persistentie in landbouwkundige habitats of invasiviteit in natuurlijke habitats.

Omdat de genen die voor droogtetolerantie worden ingebracht, ook van invloed kunnen zijn op de moleculaire responsmechanismen tegen andere vormen van abiotische stress, zoals kou of zilt, kan niet worden uitgesloten dat een droogtetolerant gg-maïs het potentieel verkrijgt om lage wintertemperaturen te overleven. Dit kan leiden tot een verhoogd potentieel van een droogtetolerante gg-maïs voor persistentie in landbouwkundige habitats of invasiviteit in natuurlijke habitats. In het bijzonder wanneer de genetisch modificatie ingrijpt op het ABA-metabolisme dat ook sleutelprocessen in zaden reguleert, zoals dormantie en ophoping van opslaglipiden, kan het niet worden uitgesloten dat de zaden van een droogtetolerante gg-maïs een veranderde vorsttolerantie verkrijgen. Dit kan op zijn beurt resulteren in een verhoogde overleving gedurende de winter, hetgeen kan leiden tot een verhoogd potentieel van een droogtetolerante gg-maïs voor persistentie in landbouwkundige habitats of invasiviteit in natuurlijke habitats. Toch is de overall waarschijnlijkheid van een verhoogd potentieel van een droogtetolerante gg-maïs voor persistentie in landbouwkundige habitats of invasiviteit in natuurlijke habitats zeer klein in het licht van de inherente eigenschappen van maïs. Mocht de teelt van droogtetolerante gg-maïs leiden tot opslag in het volgende gewas, dan kunnen de opslagplanten door gebruikelijke opslagbestrijding beheerst worden. En mochten ze zich verspreiden en vestigen in natuurlijke habitats, dan kan opslagbestrijding naar natuurlijke habitats worden uitgebreid.

Er zijn geen redenen om te veronderstellen dat droogtetolerantie als eigenschap in een droogtetolerante gg-maïsplant een direct effect heeft op maïspredatoren, -parasitoïden en –ziekteverwekkers en op populatieniveaus van organismen die hiermee in wisselwerking zijn. Ten eerste, omdat droogtetolerantie als eigenschap niet gericht is op bestrijding van maïsplagen. Ten tweede, omdat niet bekend is dat de ingebrachte droogtetolerantie-genen, hun producten, zoals plantsignaleringsfactoren of enzymen, en de metabolieten van deze enzymen, zoals opruimers van vrije zuurstofradicalen of osmobeschermers, een effect hebben op maïspredatoren, -parasitoïden en –ziekteverwekkers. Aan de andere kant kan droogtetolerantie in een droogtetolerante gg-maïsplant een indirect effect hebben op maïspredatoren, -parasitoïden en –ziekteverwekkers en de populatieniveaus van organismen die hiermee een wisselwerking hebben. Omdat er tussen moleculaire responsmechanismen tegen abiotische en biotische stress mogelijk ‘crosstalk’ plaatsvindt, kan het niet worden uitgesloten dat een droogtetolerante gg-maïs een veranderde tolerantie verkrijgt tegen biotische stress. Dat zou kunnen resulteren in veranderde wisselwerkingen met maïspredatoren, -parasitoïden en –ziekteverwekkers. Als gevolg hiervan kan een droogtetolerante gg-maïs een indirect effect hebben op de populatieniveaus van organismen die hiermee een wisselwerking hebben. Als een droogtetolerante gg-maïsplant een verhoogde gevoeligheid voor maïspredatoren, -parasitoïden en –ziekteverwekkers verkrijgt, dan zijn voor de teelt wellicht andere fytosanitaire maatregelen nodig, zoals bijvoorbeeld aan verhoogde toepassing van (chemische) gewasbeschermingsmiddelen.

Er zijn geen redenen om te veronderstellen dat een droogtetolerante gg-maïs nadelige effecten op de menselijke of dierlijke gezondheid zal hebben. Dit omdat niet bekend is dat de ingebrachte droogtetolerantie-genen, hun producten, zoals plantsignaleringsfactoren of enzymen, en de metabolieten van deze enzymen, zoals opruimers van vrije zuurstofradicalen of osmobeschermers, een effect hebben op de menselijke of dierlijke gezondheid.

Tenslotte zijn er geen redenen om te veronderstellen dat opname in de bodem van wortelsappen, plantafval, zaden of stuifmeel van een droogtetolerante gg-maïs effecten op biogeochemische kringlopen zal hebben. Ten eerste, omdat horizontale overdracht van de ingebrachte genen vanuit een droogtetolerante gg-maïs naar bodemmicroben zeer onwaarschijnlijk is onder natuurlijke omstandigheden. Ten tweede, omdat expressie van de ingebrachte genen, die onder controle van eukaryote promotoren staan met een zeer beperkte tot geen activiteit in prokaryote organismen, zeer onwaarschijnlijk is in bodemmicroben. Ten derde, omdat niet bekend is dat de ingebrachte droogtetolerantie-genen en hun producten, zoals plantsignaleringsfactoren of enzymen, en de metabolieten van deze enzymen, zoals opruimers van vrije zuurstofradicalen of osmobeschermers, een effect hebben op bodemmicroben.

Omega-3 vetzuur gg-soja

De laatste twintig jaar groeit de belangstelling voor zeer lange keten meervoudig onverzadigde vetzuren (very long-chain polyunsaturated fatty acids, VLC-PUFAs), in het bijzonder voor de omega-3 groep die gewoonlijk in visolie wordt aangetroffen, vanwege de gezondheidsbevorderende eigenschappen met het oog op hart- en vaatziekten en vetzucht. Een belangrijk aspect van het voedingskundig belang van VLC-PUFAs voor de menselijke gzondheid is dat zoogdieren nauwelijks het vermogen hebben om deze vetzuren zelf aan te maken. Omdat de huidige bronnen van omega-3 VLC-PUFAs, hoofdzakelijk zeevisoliën, ernstig in gevaar zijn, is een intensive zoektocht naar alternatieve (en duurzame) bronnen op gang gekomen. Voor dat doel is de laatste tien jaar de mogelijkheid om gg-planten te ontwikkelen, die VLC-PUFAs in de opslagoliën van hun zaden aanmaken, uitgebreid onderzocht.

Hogere planten zijn echter niet in staat om VLC-PUFAs aan te maken. De omzetting van vetzuren in planten, als linolzuur en linoleenzuur, naar VLC-PUFAs vergt daarom verschillende niet-planteigen enzymen voor de vorming van omega-6 VLC-PUFAs als arachidonzuur en omega-3 VLC-PUFAs EPA (eicosopentaenoic acid) en DHA (docosahexaenoic acid). Genen die voor dergelijke enzymen coderen – desaturasen en elongasen, zijn tot dusver uit verschillende mariene microalgen en landschimmels geïsoleerd en in verschillende planten, zoals vlas, koolzaad en soja, ingebracht.

De productie van significante hoeveelheden VLC-PUFAs in gg-planten vergt één van de meest complexe genetische modificaties die tot dusver in planten zijn geprobeerd. Aan de ene kant, omdat meerdere genen moeten worden ingebracht, en van de andere kant, vanwege twee generieke bottlenecks in planteigen routes voor vetzuurmetabolisme. Eén van deze bottlenecks is beschreven als ‘substraat-tweedeling’, hetgeen inhoudt dat er een beperkte uitwisseling plaatsvindt van acylgroepen van vetzuren uit de fosfatidylcholine-poel en die uit de coenzym A-poel. De andere bottleneck is om ervoor te zorgen dat omega-6 vetzuren in omega-3 vetzuren worden omgezet, hetgeen verhinderd wordt doordat acylgroepen hiervan vanuit de fosfatidylcholine-poel en de coenzym A-poel naar de triaglycerol-poel gekanaliseerd worden. Vanwege deze bottlenecks leidden de eerste pogingen om planten genetisch te modificeren voor de productie van VLC-PUFAs tot zeer bescheiden resultaten.

Ondertussen heeft voortdurend onderzoek echter geresulteerd in gg-planten, waaronder soja, met commercieel interessante niveaus van omega-3 vetzuren. Zo hebben bijvoorbeeld onderzoekers van DuPont de standaardprocedure voor het onverzadigen en verlengen van de kortere ketens vetzuren uitgebreid door coëxpressie van een omega-3 microsomale desaturase uit de schimmel *Sapgrolegnia diclinia* voor de omzetting van omega-6 vetzuren in omega-3 vetzuren. Dit heeft geleid tot een verhoging van het gehalte aan omega-3 VLC PUFAs tot 40 % van het totale vetzuurgehalte in de zaden van gg-soja. In andere experimenten hebben onderzoekers van DuPont, de Universiteit van Nebraska en de Plant Genetica Onderzoekseenheid van de Landbouwkundige Onderzoeksdienst van het Amerikaanse landbouwministerie een gen dat codeert voor Δ15 vetzuurdesaturase uit *Borago officinalis* and het *FAD3* gen dat codeert voor Δ6 desaturase uit *Arabidopsis thaliana*, beiden onder controle van een sojazaad-specifieke β-conglycinine promotor, in soja ingebracht. Monsanto heeft ook een omega-3 vetzuur gg-soja ontwikkeld. Deze gg-soja produceert stearinezuur (stearidonic acid, SDA) dat na consumptie wordt omgezet in EPA. De verwachting is dat deze gg-soja binnen een paar jaar gecommercialiseerd zal worden. Het is denkbaar maar niet zeker dat Monsato genen heeft gebruikt, die coderen voor Δ6 en Δ12 vetzuurdesaturases uit de schimmel Mortiella alpina, vermoedelijk onder controle van een *napin* promotor uit koolzaad – een strategie die eerder heeft geresulteerd in een stijging van het gehalte aan SDA tot 23 % van het totale vetzuurgehalte in zaden van gg-koolzaad.

In de VS zijn tot dusver 5 veldproeven met omega-3 vetzuur gg-sojaplanten door Pioneer Hi-Bred (DuPont) kennisgegeven, 5 veldproeven door Monsanto en 4 veldproeven door de Universiteit van Nebraska. Hiernaast heeft Pioneer Hi-Bred op 20 december 2006 de Amerikaanse regelgevende autoriteiten verzocht om een omega-3 vetzuur (hoog oliezuurgehalte) gg-soja te ‘dereguleren’. Dit verzoek is momenteel in behandeling. Als de Amerikaanse regelgevende autoriteiten hun instemming geven, dan is het toegestaan om deze gg-soja te commercialiseren. In dit geval, noch voor de veldproeven met andere omega-3 vetzuur gg-sojaplanten is informatie publiek beschikbaar gemaakt over de laboratorium- en kasexperimenten, de condities in de veldproeven in de VS, de types van genetische modificatie en de resulterende gegevens over het landbouwkundige gedrag en mogelijke milieueffecten. In de EU zijn tot dusver geen veldproeven met omega-3 vetzuur gg-sojaplanten uitgevoerd en ook zijn er nog aanvragen voor import, transport, verwerking en/of teelt van omega-3 vetzuur gg-soja ingediend.

Voor een milieurisicobeoordeling van een niet-ingeperkte, grootschalige of commerciële vrijzetting van omega-3 vetzuur gg-soja in Europa moet erop gewezen worden dat sommige conventioneel veredelde sojacultivars beschikken over een hoog gehalte aan oliezuur (een omega-3 vetzuur), die kunnen dienen als referentie (*baseline*) voor het inschatten van de milieurisico's van gg-soja met een hoog oliezuurgehalte. Het is echter niet mogelijk om met conventionele veredeling een sojaboon te ontwikkelen, die in staat is om VLC-PUFAs, zoals SDA of EPA, te produceren vanwege de noodzaak om genen in te brengen, die niet in de genenpoel van soja aanwezig zijn.

Voor een milieurisicobeoordeling van een niet-ingeperkte, grootschalige of commerciële vrijzetting van omega-3 vetzuur gg-soja in Europa is er een gebrek aan gegevens, aan de hand waarvan het gedrag van een omega-3 vetzuur gg-soja kan worden vergeleken met die van de ouderplant. Het is waarschijnlijk dat een omega-3 vetzuur producerende eigenschap onder controle van een zaadspecifieke promotor wordt gebracht. Desondanks kunnen hiervoor relevante fenotypische parameters niet alleen bestaan uit de duur van zaadproductie, zaadkieming en –dormantie, opkomst, overleving en groei. Ook fenotypische parameters als hergroei vanuit stoppels, ziektegevoeligheid, wisselwerking met stikstofbindende symbionten, zaadeiwitsamenstelling en gehalten aan antinutritionele factoren zijn mogelijk relevant.

Uitgaande van conventioneel veredelde sojaplanten als de referentie voor een milieurisicobeoordeling moet worden opgemerkt dat deze in Europa niet persistent in landbouwkundige habitats en niet invasief in natuurlijke habitats zijn. In het geval van een hoog-oliezuur gg-soja zijn er geen redenen om te veronderstellen dat die meer persistent in landbouwkundige habitats of meer invasief in natuurlijke habitats zou zijn. Ten eerste, omdat ervaring in de VS aangeeft dat conventioneel veredelde hoog-oliezuur soja niet persistent is in landbouwkundige habitats. Ten tweede, omdat gegevens uit Amerikaanse veldproeven met hoog-oliezuur gg-soja geen verschillen lieten zien in opkomst van zaailingen, hergroei vanuit stoppels en zaaddormantie in vergelijking met de oudervariëteit.
Daarentegen zijn er in het geval van een VLC-PUFAs gg-soja wél redenen om te veronderstellen dat die een verhoogd potentieel zou kunnen verkrijgen voor persistentie in landbouwkundige habitats of invasiviteit in natuurlijke habitats. Ten eerste, omdat de plant (bladeren) mogelijk een veranderde verhouding van verzadigde en onverzadigde vetzuren bevat, waardoor de stoppels van een VLC-PUFAs gg-soja een betere overwinteringscapaciteit kunnen verkrijgen dan de oudervariëteit. Dit kan mogelijk leiden tot VLC-PUFAs gg-sojaopslagplanten in het daarop volgende gewas. Hoewel, in het bijzonder als de expressie van de eigenschap voor VLC-PUFAs onder controle van een zaadspecifieke promotor staat, de kans hierop verwaarloosbaar is. Ten tweede, omdat zaden van een VLC-PUFAs gg-soja in hun olie een kleiner aandeel verzadigde vetzuren bevatten dan die van conventionele soja, kunnen die zaden veranderde dormantie- en kiemingseigenschappen verkrijgen. Dit kan mogelijk leiden tot opslagplanten in het daarop volgende gewas en/of invasies in natuurlijke habitats na transport door dieren. Mocht de teelt van een VLC-PUFAs gg-soja tot opslagplanten in het volgende gewas leiden, kunnen ze door gebruikelijke opslagbestrijding

beheerst worden. Mochten ze zich verspreiden en vestigen in natuurlijke habitats, dan kunnen deze beheersmaatregelen naar natuurlijke habitats worden uitgebreid.

Hiernaast is het zeer onwaarschijnlijk dat een eigenschap als een hoog oliezuurgehalte of productie van VLC-PUFAs voor extra kruisbevruchtingscapaciteit van een gg-sojaplant zorgt. Bovendien heeft soja in Europa geen seksueel compatibele onkruidachtige of wilde verwanten. Er bestaat dus alleen de mogelijkheid van genoverdracht van een omega-3 vetzuur gg-sojaplant naar niet-gg-sojaplanten door middel van stuifmeel. Het potentieel hiervoor is vrijwel geheel afwezig, omdat soja een zelfbevruchter is met een kruisbevruchtingscapaciteit van minder dan één procent.

Verder is het zeer onwaarschijnlijk dat een hoog-oliezuur gg-soja of een VLC-PUFAs gg-soja directe effecten zal sorteren op soja-predatoren, -parasitoïden en –ziekteverwekkers en op de populatieniveaus van organismen die hiermee in wisselwerking staan. Ten eerste, omdat noch de eigenschap hoog-oliezuurgehalte, noch de eigenschap VLC-PUFAs productie gericht is op de bestrijding van sojaplagen. Ten tweede, omdat bekend is dat oliezuur en VLC-PUFAs geen nadelige effecten hebben op soja-predatoren, -parasitoïden en –ziekteverwekkers. Aan de andere kant, wanneer *FAD* genen zijn ingebracht, kan een hoog-oliezuur gg-soja of een VLC-PUFAs gg-soja een indirect effect hebben op soja-predatoren, -parasitoïden en –ziekteverwekkers. Omdat insertie van *FAD* genen van invloed kan zijn op ABA-responssignalering, dat een sleutelrol speelt in de ‘crosstalk’ tussen responsmechanismen tegen biotische en abiotische stress in planten, kan een hoog-oliezuur gg-soja of een VLC-PUFAs gg-soja een veranderde wisselwerking aangaan met soja-predatoren, -parasitoïden en –ziekteverwekkers. Als gevolg hiervan kan een hoog-oliezuur gg-soja of een VLC-PUFAs gg-soja een indirecte invloed uitoefenen op populatieniveaus van organismen die hiermee in wisselwerking staan. Er moet echter worden opgemerkt dat gegevens uit Amerikaanse veldproeven met een hoog-oliezuur gg-soja met *FAD* genen uit soja geen effecten lieten zien op goedaardige organismen als bijen en aardwormen. Mocht een hoog-oliezuur gg-soja of een VLC-PUFAs gg-soja een verhoogde gevoeligheid verkrijgen voor soja-predatoren, -parasitoïden en –ziekteverwekkers, dan zijn mogelijk andere fytosanitaire maatregelen nodig, zoals bijvoorbeeld een verhoogd gebruik van (chemische) gewasbeschermingsmiddelen. En mocht een hoog-oliezuur gg-soja of een VLC-PUFAs gg-soja een veranderde wisselwerking verkrijgen met stikstofbindende symbionten, dan vereist dit wellicht een andere (chemische) stikstofbemesting.

Hiernaast zijn er geen redenen om te veronderstellen dat een hoog-oliezuur gg-soja of een VLC-PUFAs gg-soja nadelige effecten zal hebben op de menselijke of dierlijke gezondheid. Ten eerste, omdat het niet waarschijnlijk is dat de ingebrachte genen en hun producten – enzymen als elongasen en desaturasen uit microalgen en schimmels –effecten zullen hebben op de menselijke of dierlijke gezondheid. Ten tweede, omdat de metabolieten van deze enzymen – oliezuur en VLC-PUFAs – geacht worden na consumptie een gezondheidsbevorderend effect te hebben.

Tenslotte zijn er geen redenen om te veronderstellen dat opname in de bodem van wortelsappen, plantafval, zaden of stuifmeel van een hoog-oliezuur gg-soja of een VLC-PUFAs gg-soja effecten op biogeochemische kringlopen zal hebben. Ten eerste, omdat horizontale overdracht van de ingebrachte genen vanuit een hoog-oliezuur gg-soja of een VLC-PUFAs gg-soja naar bodemmicroben zeer onwaarschijnlijk is onder natuurlijke omstandigheden. Ten tweede, omdat expressie van de ingebrachte genen, die onder controle van eukaryote promotoren staan met een zeer beperkte tot geen activiteit in prokaryote organismen, zeer onwaarschijnlijk is in bodemmicroben. Ten derde, omdat niet bekend is dat de ingebrachte genen en hun producten – enzymen als elongasen en desaturasen uit microalgen en schimmels, een effect hebben op bodemmicroben.

1. SCOPE AND OBJECTIVES OF THE STUDY

1.1 Background

In the Netherlands the Ministry of Housing, Spatial Planning and the Environment, together with other relevant ministries, is the competent authority for the implementation of EU Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms (GMOs). The GMO Office of the National Institute for Public Health and the Environment is the executive office responsible for the handling of authorisation procedures for activities with GMOs, whose potential risks to human health and the environment must be assessed. In this context the GMO Office conducts activities directly related for settling permit applications, as well as activities that support regulatory policy making and optimise methodologies for environmental risk assessment. In order to support the development of methodologies for environment risk assessment, the GMO Office commissioned to Schenkelaars Biotechnology Consultancy a study with the title “Novel aspects in the environmental risk assessment of drought-tolerant genetically modified maize and omega-3 fatty acid producing genetically modified soybean”.

Genetically modified (GM) plants that have so far obtained an approval to be introduced on the EU market, consist of plants, which, due to genetic modification, are more tolerant to a plague insect, such as the European corn borer, or a non-selective herbicide, like glyphosate or gluphosinate. Moreover, a next generation of GM plants is now at different stages of the EU market approval procedure. These GM plants are GM plants, which are improved versions of 1st generation GM plants and GM plants, into which more than one of the aforementioned traits have been combined (‘stacked’).

To date, a novel generation of GM plants is under development and at a pre-market stage. These GM plants are plants, which, due to genetic modification, express pharmaceutical, nutraceutical or industrial compounds, have an increased tolerance to abiotic stress, like drought or salinity, or have new metabolic pathways, resulting for example in the production of non-native fatty acids. While risk assessors and regulators worldwide have thus far gained much knowledge and experience with the environmental risk assessment of current generations of GM plants with herbicide-tolerance and/or insect-resistance, this is not the case for the novel generation of GM plants. The nature of the genetic modifications of these novel GM plants clearly differs from that of current generations of GM plants, as they are genetically more complex and/or lead to significant metabolic shifts. As a result, the likelihood that these genetic modifications lead to unintended epistatic and/or pleiotropic effects on the plant’s genotype and/or phenotype could be higher for these novel generation GM plants than for current generations of GM plants. Put differently, because the relationship between genotype and phenotype of novel generation GM plants is less straightforward in comparison to that of current generations of GM plants, it can be anticipated that their environmental risk assessment will need to address more uncertainties about their potential environmental effects.

Within a couple of years such novel GM plants will be submitted for EU market-approval, which requires an environmental risk assessment by the competent authorities of EU Member States, including the Netherlands. As the GMO Office foresees that the environmental risk assessment of such novel GM plants needs to take into account uncertainties of their genotype and phenotype, it selected two examples of novel GM plants that are currently in the mainstream of research and development efforts, in order to study whether their environmental risk assessment involves novel aspects compared to that of current generations of GM plants. These two novel GM plants are drought-tolerant GM maize and omega-3 fatty acids producing GM soybean, both which are already being field-tested in the US.

1.2 The study objectives

For this study two examples of novel generation GM plants were selected by the GMO Office:

1. Drought-tolerant GM maize, whose drought tolerance is caused by genetic modification with one of more genes on one gene cassette, or by interference in one or more metabolic pathways through RNAi-techniques, and;
2. GM soybean with an increased content of omega-3-fatty acids, due to genetic modification with one gene derived, for instance, of a fish or algae.

Against this background the main questions are:

1. Whether the environmental risk assessment (ERA) of these novel generation GM plants, as required by EU Directive 2001/18/EC, involves novel aspects, which cannot be addressed by current ERA methodologies, and;
2. If so, how to consider these novel aspects adequately in the ERA.

With a view to these questions, an inventory should therefore be made of the information needed for the ERA of these novel generation GM plants, the possible bottlenecks in their ERA, and in which way targeted research might be conducted to address the possible bottlenecks.

1.3 Approach to the study

For both selected novel generation GM plants, a survey of peer-reviewed scientific literature has been conducted by searching PubMed, while additional data and information have been collected by accessing patent databases and field trials databases of US and EU competent authorities.

In addition, the study was overseen by an advisory committee, whose members included:

- Marco Gielkens, GMO Office;
- Hans Bergmans, GMO Office;
- Petra Hogervorst, Ministry of Housing, Spatial Planning and the Environment;
- Wilke van Delden, University of Groningen, and;
- Willem Brandenburg, Plant Research International, Wageningen University and Research Centre.

2. DROUGHT-TOLERANT GM MAIZE

2.1 Introduction

This section starts with a discussion of the concept of drought-tolerance, as this concept appears to be understood differently by different disciplines, varying from molecular genetics, plant physiology to plant breeding. Subsequently, advances made in increasing drought tolerance in modern maize varieties though conventional breeding techniques are presented, because these advances indicate that the baseline for comparing drought tolerance of GM maize to that of conventionally bred maize has been constantly evolving during the last decades and will continue to evolve further in the nearby future. Next, current scientific understanding of molecular and biochemical mechanisms of drought stress response in plants is evaluated. This subsection is followed by several examples of GM maize varieties with genetic modification conferring drought-tolerance, as well as an overview with available information about field trials with GM maize in the US and EU. Then, the views of several scientific and regulatory experts on the potential environmental risks of drought-tolerant GM crops, including maize, are discussed. The section is closed by points to consider for an environmental risk assessment of drought-tolerant GM maize in accordance with the principles for the environmental risk assessment (ERA) of GM higher plants as laid down in Annex II of EU Directive 2001/18/EC.

2.2 The concept of drought-tolerance

2.2.1 Need for clarifying the notion of drought-tolerance

In the face of a global scarcity of water resources and the increased salinisation of soil and water, abiotic stresses, like drought and salinity, are already a major limiting factor in crop production and will soon become even more severe as desertification covers more and more of the world's terrestrial area. The significance of abiotic stress has not gone unnoticed by plant breeders in private and public sectors over the last decade. As a consequence, there is now considerable work going ahead with the aim to develop crops with improved tolerance to abiotic stresses, including drought (CGIAR, 2003). In popular terms, both public and private sector breeders have initiated an intensive search for 'more crop per drop'.

Yet, as plant breeding, including the application of tools from molecular biology, is making headways into the development of crop cultivars tolerant to drought stress, the conceptual framework of what actually constitutes a viable target for selection in this respect is not always clear. According to an extensive review by Blum (2005), in scientific workshops dealing with breeding for drought-prone environments, there is a constant debate on putative drought-resistance mechanisms, water-use efficiency, and their interrelationship and associations with yield potential. Water-use efficiency (WUE) is often equated with drought resistance and the improvement of crop yield under drought conditions, which is not necessarily the case. Drought resistance is sometimes considered as a penalty towards yield potential, which is neither necessarily the case. Molecular biologists entering this discipline often report the effect of an exotic gene towards drought tolerance and advertise its expected value in breeding, which is rarely the case. Blum (2005) therefore suggests to define yield potential as the maximum yield realised under non-stress conditions, while drought-resistance in its physiological context should be defined by ‘dehydration avoidance’ or ‘dehydration tolerance’.

2.2.2 Yield potential

A classical plant breeding axiom is that a high-yielding crop variety will perform well in most environments. Yet, the problems lies in the definition of ‘most environments’ and the delineation of those environments where a high yield potential will not suffice for sustained performance. A delineation of such environments can be established by the so-called crossover interaction, where under a particular environmental stress a cultivar with a high yield potential produces less certain than another cultivar that has a lower yield potential but seems to be better adapted to stress. For many cereals grown under water-limited conditions the crossover occurs at a yield of around 2 – 3 tons per hectare, which is approximately one third of the yield potential. The main reasons for a crossover under conditions of variable water supply is an inherent difference among tested cultivars in drought resistances beyond difference in their yield potential. Such crossover interactions are often a source of frustration to breeders in their attempt to select in one environment and achieve good performance also in other environments. This has led breeders in drought-prone regions to develop dedicated programmes towards the development of drought-tolerant cultivars, using programmed stressed environments and other selection tools. When effective and successful selection for yield under stress is exercised, it most likely involves a genetic shift towards a dehydration-avoidant phenotype. Such a dehydration-avoidant phenotype is characterised by the maintenance of high plant water status under stress and it can present any of the following features: early flowering, smaller plant, small leaf area, or limited tillering (in cereals), all of which are in contrast to a high yield potential phenotype. A crossover interaction for yield might however be avoided, if a breeder succeeds to recombine drought-resistance with a high yield potential through selection of a high yield genotype with dehydration-avoidance factors that are not associated with lower yield potential. This is however far from easy.

2.2.3 Drought-resistance

When a genotype yields better than another one under drought stress, it can be considered relatively more drought-resistant. As already indicated, plants can resist drought by either dehydration avoidance and/or by dehydration tolerance. Notably, drought-resistance in terms of the physiology involved interacts with the magnitude and the timing of stress with respect to the stage of plant development when the stress occurs. For instance, drought-resistance in seedlings grown in a pot is something different than drought resistance during grain filling in the field.

Dehydration avoidance can be defined as the plant capacity to sustain high plant water status or cellular hydration under the effect of drought. By this mechanism the plant avoids stress because plant functions are relatively unexposed to tissue hydration. To avoid dehydration a plant can do the following:

- Enhance capture of soil moisture;
- Limit plant water loss and/or;
- Retain cellular hydration despite the reduction in plant water status, i.e. osmotic adjustment.

With a view to enhanced capture of soil moisture, the essence of the matter is where deep soil moisture is available, a long root to reach this moisture is simply as effective as a long rope in a deep well.

Genetic variation exists in potential root length (maximum root length measured under non-stress and non-restrictive soil conditions) However, when plants are exposed to a drying soil, root morphology and growth can change to the extent that the potential root length, whether it is short or long, becomes irrelevant. In cereals for instance, a drying, hard topsoil resists the penetration and establishment of adventitious (crown) roots, while existing roots receive all transient assimilates and grow deeper. Shoot/total dry matter ration increases under drought stress, not because of an increase in root mass but due to a relatively greater decrease in shoot mass. Root mass rarely increases under drought stress. However, root length and depth may increase in a drying soil even at reduced total root mass. Hence, total root dry matter or its ration to shoot dry matter is not helpful information towards selection.

According to Blum (2005), it is not clear whether the capacity for developing longer roots under drought stress is compatible with a high yield potential. Under favourable soil moisture conditions, plants do not need a large root, as this is a waste of dry matter. By contrast, under conditions of unsecured soil moisture, a potentially large root is required to ensure capture of moisture. This form of insurance may pose a load on yield potential, if a large root is expressed in large root mass, particularly where extensive tillering is an important component of high yield potential, like in the case of most cereals. However, the inherent developmental plasticity of (cereal) plants often allow a high tillering phenotype to penetrate deep soil when the top soil is drying for a sufficient length of time during the tillering phase.

With a view to reduce water use, most plants are developmentally and physiologically designed by evolution to remain functioning under drought stress. Since crop production is a function of water use (WU), the issue for breeders is how to reduce water use under drought stress, while minimising the associated reduction of production. Apparently, water-use efficiency (WUE) for biomass production is not a fixed crop entity, which allows some room for manipulation by breeders. Reduced plant height, leaf area and leaf area index (LAI) are the major mechanisms for moderating water use and reducing crop injury under drought stress.

With a view to osmotic adjustments, there is growing body of evidence on the association between a high rate of osmotic adjustment (OA) and sustained yield or biomass production under water-limited conditions across different cultivars of crop plants, like barley, chickpea, cotton, millet, pea, sorghum, sunflower and wheat. Since osmotic adjustment helps to maintain higher leaf relative water content (RWC) at low leaf water potential (LWP), it is evident that osmotic adjustment helps to sustain growth, as the plant meets its transpirational demand by reducing its leaf water potential.

Beyond the effect on cellular hydration, other putative roles of osmotic adjustment have been recently discussed under the term of ‘osmeoprotection’, which points at possible roles for cell-compatible osmolytes in protecting enzymes against heat inactivation or securing cellular membrane stability. So far, there seems to be no solid evidence of a cost in yield potential for osmotic adjustment capacity, except possibly in rice.

Finally, stomata closure in response to leaf desiccation and a transported hormone signal produced in the root in response to root desiccation are yet other processes to support a high relative water content in the plant.

Dehydration (desiccation) tolerance can be defined as the plant capacity to sustain or conserve plant function in a dehydrated state. This is sometimes viewed as the second defence line after dehydration avoidance. As an effective drought resistance mechanism in crop plants dehydration tolerance is rare. However, it exists in the seed embryo, but once germinated the plant loses this mechanism. Extreme desiccation tolerance is known in so-called resurrection plants and some attempts are made in laboratories to use it for improving crop plants (Ingram *et al*., 1996). Thus far, limited studies of dehydration tolerance in crops have shown that genotypic variation in plant recovery from dehydration is positively correlated with plant water status retained during desiccation rather than with the capacity to retain function at a dehydrated state. Moreover, differences in dehydration tolerance among species are linked to different capacities for water acquisition rather than to differences in metabolism at a given water status.

In summary, both natural selection and selection by breeders have given preference to dehydration avoidance over dehydration tolerance as the major strategy of crop plants for coping with drought stress, with the exception of resurrection plants. Even exotic genes that are evaluated for function in experimental transgenic *Arabidopsis thaliana* or tobacco are often more expressed in dehydration avoidance and its components rather than in desiccation tolerance.[1] The only major exception that constitutes a form of an effective dehydration tolerance mechanism in crop plants is stem reserve utilisation for grain filling under drought stress. This is a whole-plant process that allows effective grain filling when whole-plant photosynthesis is inhibited by drought stress during grain filling. In dehydrated or over-heated cereal plants this mechanism can account for up to 90 % of total grain weight under stress. In addition, non-senescence (delayed senescence or stay-green) is also considered an important mechanism for sustaining yield under drought stress during grain filling, like for example in the case of maize and sorghum, where non-senescence contributes to sustaining a positive plant nitrogen balance.

2.2.4 Water-use efficiency

In essence, water-use efficiency (WUE), measured as the biomass production per unit of transpiration, describes the relationship between water use and crop production. Moreover, the basic physiological definition of water-use efficiency equates to the ratio of photosynthesis to transpiration, also referred to as transpiration efficiency. Although genetic variation for water-use efficiency has been observed in crop plants, its molecular dissection has only recently been initiated in *Arabidopsis thaliana*, where the *ERECTA* gene was found to be critical in altering transpiration efficiency by mechanisms including leaf epidermal and mesophyll differentiation (Karaba *et al*., 2007).

Yet, the notion of water-use efficiency (WUE) is often equated in a simplistic manner with drought-resistance without considering that it is a ratio between two physiological (transpiration and photosynthesis) or agronomic (yield and crop water use) entities. Blum (2005) therefore explains the intrinsic paradox in assuming that a high water-use efficiency would mean better yield under drought stress by pointing at an experiment comparing a high-yielding semi-dwarf cultivar of durum wheat with a landrace, both grown under drought stress and control conditions. In the control water use was the same, but under drought stress water

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[1] See for example http://www.plantstress.com/Files/Abiotic-stress_gene.htm.

use in the landrace was relatively higher than in the high-yielding semi-dwarf cultivar. On the other hand, biomass under drought stress was relatively higher in the landrace than in the high-yielding semi-dwarf cultivar. Moreover, water-use efficiency was the same in both in the control and it increased in both under drought stress. But water-use efficiency under drought stress was higher in the high-yielding semi-dwarf cultivar than in the landrace, because of the relative differences in their water use and biomass. Greater biomass production under drought stress was associated with relatively greater water use and lower water-use efficiency as seen in the landrace.

Based on this and other studies, the conclusion can be drawn that high yield potential and high yield under water-limited conditions are generally associated with reduced water-use efficiency, mainly because of high water use. Features linked to low yield potential, such as smaller plants, smaller leaf areas or shorter growth duration, result in a high water-use efficiency because they reduce water use.

By contrast, dehydration avoidance through enhanced capture of soil moisture by roots has been found to be associated with a low water-use efficiency in rice and pine. On the other hand, reduced transpiration in rice and sorghum is associated with a higher water-use efficiency.

Against this background, Blum (2005) warns that breeding for high water-use efficiency (based on the assumption that it equates with improved drought-resistance or improved yield under drought stress conditions) might bring serious negative consequences. High water-use efficiency is largely a function of reduced water use rather than a net improvement in biomass production. If low water use is the breeder’s target, it is better to select directly for this trait, instead of water-use efficiency.

2.2.5 Testing of drought-resistance

The effect of a single ‘drought-tolerant’ gene on crop performance in water-limited environments can thus be assessed only when the whole system is considered in terms of yield potential (YP), drought-resistance (DR) and water-use efficiency (WUE). In contrast to the standard tests that are available for assaying plant disease resistance or other selected traits in plant breeding, there is however not yet a standard system of testing stress-tolerance in general, and drought-tolerance in particular.

For the development of standard systems to assess drought-tolerance, the common test criterion is yield under drought stress conditions. This may be affected by the genetic makeup of yield potential and by specific genes affecting drought resistance. In order to elucidate the phenotypic effect of a specific genetic modification towards drought-resistance, field tests must separate between the effect of this genetic modification from the impact of the yield potential of the given genotype on yield under drought stress. In the view of Blum (1999), two major test systems could be further developed for this purpose: 1) the line source irrigation system, which allows to test different genotypes over a gradient of drought stress conditions, and; 2) the orthogonal comparison of genotypes between non-stress and stress conditions, by using irrigation to control stress at one site, or by testing genotypes at different locations that differ in their water regime.

2.3 Conventional breeding for drought-tolerance in temperate maize

2.3.1 General characteristics and uses of maize

Maize is a member of the *Maydeae* tribe of the grass family *Poaceae*. It is an allogamous plant that propagates through seed produced predominantly by cross-pollination and depends mainly on wind borne cross-fertilisation. The maize plant has pistillate inflorescences enclosed in numerous large foliaceous bracts (ears) from 7 to 40 cm long, with spikelets in 8 to 16 rows on a thickened axis (cob) in the leaf axils and staminate spikelets in long spike-like racemes that form large spreading terminal panicles (tassels). Generally, maize plants have a great plasticity adapting to extreme and different condition of humidity, sunlight, altitude and temperature (OECD, 2003).

Maize is an annual plant and the duration of the life cycle depends on the variety and on the environment in which the variety is grown. Maize cannot survive temperatures below 0 °C for more than 6 to 8 hours after the growing point is above ground (5 to 7 leaf stage). Damage from freezing temperatures depends on the extent of temperatures below 0 °C, soil condition, residue, length of freezing temperatures, wind movement, relative humidity, and stage of plant development. Although maize was domesticated and diversified mostly in the Meso-American region, at present it is typically cultivated mainly in (warm) temperate regions due to moisture level and number of frost-free days required to reach maturity.

Because maize is not tolerant to cold, it must be planted must be planted during spring in temperate zones. Its root system is generally shallow, so the maize plant is rather dependent on soil moisture. As a C4-plant, maize is a considerably more efficient water-efficient crop than C3-plants, like alfalfa and soybean. Maize is most sensitive to drought at the time of silk emergence, when the flowers are ready for pollination. Maize used for silage is harvested while the plant is still green and the fruit immature, whereas sweet maize is harvested in the 'milk stage', after pollination but before starch has formed, between late summer and early to mid-autumn. Field maize is left in the field very late in the autumn, in order to thoroughly dry the grain, and may sometimes not be harvested until winter or even early spring.

Maize has lost the ability to survive in the wild due to its long process of domestications, and it needs human intervention to disseminate its seed. Although maize from the previous crop year can over-winter and germinate the following year, it cannot persist as a weed. In the US the presence of maize in soybean fields following the maize crop from the previous year is a common occurrence. Measures are commonly taken to either eliminate these volunteer maize plants with the hoe or herbicides, but the volunteer maize plants that remain and produce seed usually do not persist the following years. Since maize is incapable of sustained reproduction outside domestic cultivation, it is non-invasive in natural habitats. In contrast to most weedy plants, maize has a pistillate inflorescence (ear) with a cob enclosed with husks. Consequently, seed dispersal of individual kernels does not occur naturally. Individual kernels, however, are distributed in fields and main avenues of travel from the field are harvesting operations and transports to storage facilities.

In industrialised countries maize is mainly used to feed animals, directly in the form of grain and forage or sold to the feed industry, and as raw material for industrial products. Though, over the last few decades human consumption of sweet maize has increased in industrialised countries. Maize breeders in the US and EU mainly focus on agronomic traits for its use in the animal feed industry and on a number of industrial traits, like high fructose corn syrup, fuel ethanol, starch, glucose and dextrose.

2.3.2 Conventional maize breeding

From the start of the large-scale adoption of hybrids by maize growers in the 1930s until the first decade of the 21st century maize grain yields have been steadily rising in the US and EU. The maize grain yield has increased by about 100 kg per hectare per year, or 2 % per year (Tollenaar *et al*, 1999). Yet, over the last few decades, the variations in harvestable yield have also markedly increased (Bruce *et al*., 2002). Much of the increase in yield variability can be attributed to varying environment stress conditions, improved nitrogen inputs and better weed control, and continuing sensitivity of different maize lines to the variation in input supply, especially rainfall. As drought stress alone can account for a significant percentage of average yield losses and water resources for agronomic use become limiting, the development of drought-tolerant maize lines becomes increasingly important.

Understanding the nature of higher grain potential and enhanced yield stability especially in (drought) stress-prone environments provides opportunities to improve the breeding process. From a historical review Duvick (2001) concluded that nearly half of the maize yield enhancement over the last century was due to widespread mechanisation, better farm management and inputs, and increasing planting densities, while genetic improvements through maize hybrid breeding contributed to the other half. After an examination of the performance of 36 maize hybrids commercially released at intervals between 1934 and 1991, Duvick (1997) showed that the newer hybrids produced higher yields than the older lines in four different environments that included both hot, dry conditions and wet, cool conditions. Hence, the improvements in yield over the years were partly due to improved tolerance to abiotic stress. He also demonstrated that the change in hybrid yield potential on a per plant basis from the older to the newer lines was not significant. The increases were due to better performance under higher planting densities. While imposing higher plant densities generates symptoms of stress response in maize and leads to a reduction of yield on a per plant basis, this reduction is typically compensated by the increase in the plant numbers per unit area that increases net yield.

According to Duvick (2001), improvements in heterosis or hybrid vigour *per se* have not contributed to greater yields. Heterosis as measured by the difference between the yields of the hybrids and the mid-point of the two parents has not increased since the 1950s. Instead the parental inbred lines have improved more in yield due to breeding selection as the hybrids themselves. Hence, selection for improved yield in parental lines and for improved yield stability in their hybrid progenies appears to be the key to past success. The apparent increase in stress tolerance in modern temperate maize germplasm (Duvick, 1997; Tollenaar *et al*., 1999) has been attributed to:

1. The occurrence of heat and drought in nurseries with no available irrigation;
2. The use of high plant densities during hybrid line development;
3. Large-scale broad-area testing that includes the use of winter nurseries, and;
4. The use of stable high-yielding progenitors to form the next crop of parental inbred lines.

Notably, the variance for maize yield averages in the US were small until about the 1970s. The low variation during the early years was most likely due to reduced nitrogen inputs and poor weed controls, while reflecting that hybrids were genetically more diverse. However, between the 1960s to 2000, the variance in grain yield increased significantly, nearly tripling from the previous decades, suggesting a greater volatility in average maize production. This fluctuation in grain yield may primarily be due to limited water supply and unfavourable temperatures, since weed control and nitrogen deficiency have been effectively removed as production constraints. However, the relative contributions of genetic gain and of gains due to agronomic and environmental influences are difficult to separate, as genotype x environment interactions is a prominent feature of yield improvement in maize. Nonetheless, it is now generally accepted that modern hybrids show an increased level of stress tolerance that counters the potential water limitations with significantly improved levels of crop productivity (Duvick, 1997; Tollenaar *et al.*, 1999). Improvement in the ability of maize plants to overcome both large and small stress bottlenecks, rather than improvement in primary productivity, has become the primary driving force of higher yielding ability of newer hybrids (Duvick, 2005). The current expectation is that the potential for future yield improvement through increased stress tolerance of maize is large, as yield potential is approximately three times greater than present commercial maize yields (Tollenaar *et al.*, 2002). The mechanism, by which maize breeders have thus far improved stress tolerance, is not known in full detail, but it is speculated that increased stress tolerance may have resulted from the selection for yield stability. Stability analyses on a number of high-yielding maize hybrids showed that they can differ in yield stability, but the results did not support the argument that yield stability and high grain yield are mutually exclusive.

2.3.3 Understanding of drought-tolerance in maize

Over the last decade numerous private and public researchers and breeders have devoted considerable efforts to gain a better understanding of possible drought stress tolerance mechanisms in maize with a view to exploit this knowledge for breeding purposes (Bruce *et al.*, 2002; Campos *et al.*, 2004). This does not imply that agronomic measures that aim to maximise water availability at key growth stages are not critically important, since genetic solutions are unlikely to close more than 30 % of the gap between potential and realised yield under water stress. While improved genetics can be packaged in a seed, which, at least from a seed industry point of view, has shown to be an effective means of delivering conventional and transgenic traits that contribute to improved yield and yield stability, improved agronomic practices depend more heavily on input availability, infrastructure and skills in crop and soil management.

As indicated, conventional maize breeding has resulted in improved drought stress tolerance, However, physiology has thus far only described generally what has been accomplished by breeding rather than directing the process. Yet, physiology, in combination with genomics, offers promise of improving the rate of gain for key traits, as especially those such as drought-tolerance that are difficult to phenotype. But while there has been an exponential increase in the number of genotyping initiatives in plant species over the past decade, and a concomitant decrease in the cost per data point generated, the ability and capacity to measure plant phenotypes for important traits have not kept pace, and this lag hampers the ability to describe gene-to-phenotype relationships for drought-tolerance in maize.

Nonetheless, some knowledge has already been gained about Quantitative Trait Loci (QTL) associated with a specific maize phenotype under drought stress. Over the last decade a series of studies has targeted a range of phenotypic parameters, among which grain yield and its components, anthesis-silking interval (ASI; ear growth rate), root traits, and measures of plant water use and status, such as stomatal conductance, and leaf and xylem abscisic acid (ABA) content.

What is a quantitative trait locus (QTL)?

Inheritance of quantitative traits refers to the inheritance of a phenotypic characteristic that varies in degree across a population of an organism and can be attributed to the interactions between two or more genes and their environment. While the single genes that contribute to polygenic traits follow patterns of Mendelian inheritance, and the associated phenotypes typically vary along a continuous gradient, often depicted by a bell curve. Human height is for example one such trait; humans clearly inherit a general tendency to be short or tall from their parents, but many genes contribute to this, as well as nutrition. Though not necessarily genes themselves, quantitative trait loci (QTLs) are regions of DNA that are closely linked to the genes that underlie the trait in question. These QTLs are often located on different chromosomes. The number of QTLs that explains variation in the phenotypic trait provides information about the genetic architecture of a trait. It may for example tell that plant height is controlled by many genes of small effect, or by a few genes of large effect.

QTLs are important in agricultural breeding programmes. Traits such as plant productivity, protein content, growth rate, or fat content in animals are all under the control of QTLs, and so breeding a plant or animal with the right collection of alleles at their QTLs is a major aim of crop and animal breeders. Mapping QTLs is therefore a major issue. A critical aspect to be discovered is the variance caused by the QTL. How much of the variability of the phenotypic trait is owing to genes as a whole, and to that QTL in particular? And is the variance additive with other genes? Statistical analysis is required to demonstrate the interaction of different genes with one another and to determine whether they produce a significant effect on the phenotype.

QTLs can also be used to identify candidate genes underlying a trait. Once a region of DNA is identified as contributing to a phenotype, it can be sequenced. The DNA sequence of any genes in this region can then be compared to a database of DNA for genes whose function is already known.

Campos *et al*. (2004) summarises the findings of these studies as follows:

- Individual drought tolerance associated QTLs generally explain less than 10 % of phenotypic variance for grain yield, anthesis-silking interval (ASI or ear growth rate) or barrenness under drought stress.
- QTLs for drought-resistance are often cross-specific; these QTLs often ‘disappear’ in crosses from different genetic backgrounds, which might be due to differences in the locations of specific QTLs may reflect disparities in colinearity of genes in the two parents rather than the presence of different genes. Though, it is more likely that variation in the location of QTLs in the genome is a function of the inherent levels of uncertainty in the mapping process, which relies on the stochastic process of recombination, particularly when small mapping populations are used.
- A clustering of QTLs for drought related traits in specific chromosomal regions is apparent, such as chromosome 1, which also includes QTLs related to grain yield and some root traits, and chromosomes 2 and 10.
- Most QTLs for drought are only detected under stress or non-stress (control) conditions. However, some QTLs for grain yield and ASI have been identified at the

same loci under both conditions, suggesting a constitutive rather than a stress responsive pattern of gene expression.
- QTLs for drought-tolerance obtained from inbreds need to be fully validated before assuming they will functions in the same manner in hybrids.

Outcomes of QTL analysis methods have thus provided population-specific statistical estimates of the effects of alternative alleles for candidate genes or genomic regions that may contribute to complex plant responses, like drought-tolerance. This approach appears to have worked reasonably well for simple traits, such as earliness of flowering, but may be less effective for more complex traits, like grain yield in relation to drought tolerance. Complicating factors, such as genotype x environment interactions and gene x gene interactions (epistasis), have been identified as important components of the genetic architecture of quantitative traits. As a consequence, these factors can contribute to a reduction in the predictability of phenotypes from QTL genotypes.

Furthermore, gene expression profiling techniques have been applied to explore responses of gene networks to water deficits, while still maintaining the resolution to measure the transcriptional activity of individual genes in tissue sampled at a particular developmental stage in stressed versus non-stressed plants. In maize considerable difference was observed in gene expression profiles between plants under drought stress in a limited rooting volume in the greenhouse (buckets) versus those stressed in an unlimited rooting volume in the field. When bucket-grown maize plants were drought stressed around flowering, they reached a water-deficit status sufficient to completely inhibit photosynthesis within 4 days, whereas field-grown plants required 21 days to reach a similar level of water stress. Moreover, far fewer (less than 2 % versus 27 %) genes showed a differential expression in maize ear tissue under a gradually developing stress than under sudden stress.

In addition, transgenic analyses have also been used as a tool to study genes and their effects on the (maize) plant phenotype and hypotheses regarding the role of key processes imparting drought tolerance (Sanguineti *et al.*, 1999; Jeanneau *et al.*, 2002; Sung *et al*, 2003; Shou *et al.* 2004; Young *et al.* 2004). Results from numerous transgenic analyses suggest that many transgenes putatively associated with drought tolerance do not directly affect the trait of interest, but rather depend in a complex manner on a chain of metabolic responses. For instance, increased grain yield (the primary trait) may be linked to a gene modulating assimilate flux, which itself is affected by a plant hormone. The lack of consistent transgene performance however may also be due to the long history of intensive improvement in the species. Consequently, transgenic drought-tolerant phenotypes that are relatively easy to generate in a non-selected plant like *Arabidopsis thaliana*, or in older maize cultivars, are less evident in today's elite maize hybrids (Bruce *et al.* 2002).

Notably, grain yield under stressed conditions is usually the primary trait for selection. In addition, experience in CIMMYT and at a Pioneer Hi-Bred indicates that key secondary traits for selection under drought stress are reduced barrenness (inability to produce viable ears), ASI (ear growth rate), stay-green, and to a lesser extent, leaf rolling. Other secondary traits, like changes in the root system, will only be worth adding when they have been field tested, especially because studies so far suggest that it is likely that vigorous root growth may involve a cost to grain production despite the improved advantage of water acquisition in dry soils (Bruce *et al.*, 2002).

Against this background, the use of genetic and genomics for breeding more drought-tolerant maize lines is advocated within an integrated framework that relies heavily upon critical input from disciplines, including plant and crop physiology, crop modelling, and precise field phenotyping (Campos *et al*. 2004). An integration of quantitative knowledge from diverse but complementary disciplines will allow researchers and breeders to more fully understand genes associated with drought-tolerance in maize, and more accurately predicts the consequences of modelling expression levels of those genes. Within such an integrated framework the combination of QTL analysis methods, gene expression profiling and transgenic analysis is expected to reduce the tens of thousands of candidate (trans)genes to a few (Bruce *et al*., 2002).

2.4 Molecular mechanisms for abiotic stress response

2.4.1 Introductory remarks

This subsection presents an overview of current scientific understanding of molecular mechanisms underlying plant response to abiotic stress, including drought, based on recent findings from experiments with different plant species. In many of these experiments *Arabidopsis thaliana* (thale cress) has been used as a model plant, but various crop species, such as maize, rice, wheat and barley, have also been subject of research aimed at advancing knowledge of plant molecular mechanisms for abiotic stress response.

The focus of this subsection is on plant molecular response mechanisms to drought stress, but it appears that these mechanisms are part of larger genetic networks and biochemical pathways, which also underlie molecular response mechanisms to other forms of abiotic stress, like cold or salinity, as well as to biotic stress caused by plant pathogens and herbivores.

2.4.2 Crosstalk between abiotic and biotic stress response

Plants have a series of subtle mechanisms for responding to environmental changes, which have been established during their natural evolution and domestication by humans. These mechanisms are involved in many aspects of anatomy, physiology, biochemistry, genetics, and development, in which the adaptive machinery related to molecular biology may be the most important. These molecular mechanisms at least include environmental signal recognition, signal transduction, signal output, signal response and phenotype realisation, and constitute a multidimensional network system, consisting of many levels of gene expression and regulation (Ingram *et al*., 1996; Vinocur *et al*., 2005; Fujita *et al*., 2006).

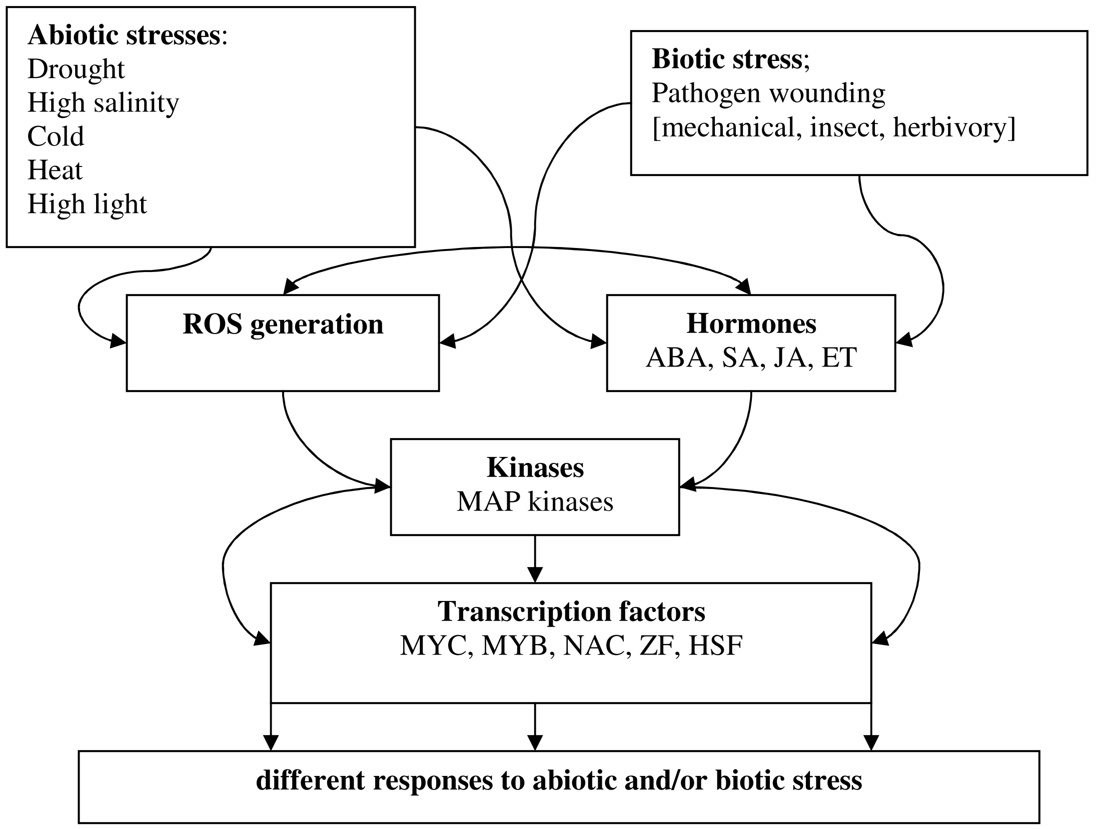
Plant hormones such as abscisic acid (ABA), ethylene (ET), jasmonic acid (JA) and salicylic acid (SA) primarily regulate the protective responses to both biotic and abiotic stresses via synergistic and antagonistic actions. While ABA is mainly involved in responses to abiotic stresses, like drought, cold and osmotic stress, it also governs a variety of growth and developmental processes, including seed development, dormancy, germination and stomatal movements. By contrast, ET, JA and SA play central roles in biotic stress signalling upon pathogen infection.

A study by Anderson *et al*. (2004) provides further details about the complex interplay between ABA and JA-ET signalling pathways. It is shown that exogenous ABA suppresses both basal and JA-ET-activated transcription from defense genes, whereas ABA deficiency as conditioned by mutation in the *ABA1* and *ABA2* genes encoding enzymes involved in ABA biosynthesis result in upregulation of basal and induced transcription from JA-ET responsive defense genes. It is further demonstrated that disruption of *AtMYC2* that encodes a basic helix-loop-helix Leu zipper transcription factor, which is a positive regulator of ABA signalling, leads to elevated levels of basal and activated transcription from JA-ET responsive defense genes. Moreover, the *jin1/myc2* and *aba2-1* mutants have increased resistance to the necrotrophic fungal pathogen *Fusarium oxysporum*. In addition, using ET and ABA signalling mutants, it is shown that interaction between ABA and ET signalling is mutually antagonistic in vegetative tissues.

Collectively, these results indicate that the antagonistic interactions between multiple components of ABA and JA-ET signalling pathways modulate defence and stress responsive gene expression in response to biotic and abiotic stresses. It is suggested that the antagonistic interaction between the ABA and JA-ET signalling pathways might be a strategy that plants employ to avoid simultaneous production of abiotic stress-related and biotic defense proteins. In addition, data show that defence gene suppression mediated by ABA cannot be reversed by exogenous application of methyljasmonate (MJ) and ET. Therefore, it is postulated that ABA action is a dominant process. One possible reason for this may be that drought stress affects plant survival in a more systemic and dramatic way than localised pathogen stress, and plants have evolved strategies to prioritise between these two stress responses. One would think that the antagonistic interaction between these two signalling pathways would compromise a plant's ability to tolerate both stresses should they occur simultaneously. However, simultaneous drought and necrotrophic pathogen attack may not happen very frequently in nature, because these pathogens require relatively humid conditions for successful infection and under such conditions water stress would not pose a significant threat.

Although there are differences in different higher plants and current knowledge is still far from complete, a basic model for abiotic and biotic stress responses in higher plants, including the "crosstalk" between these responses, has been conceived. Figure 1 provides a schematic overview of both these stress responses in higher plants.

Figure 1: Convergence points in abiotic and biotic stress signalling networks



Abbreviations: ABA = abscisic acid; ET = ethylene; JA = jasmonic acid; MAP = mitogen-activated protein; ROS = reactive oxygen species; SA = salicylic acid; MYC, MYB, NAC, ZF and HSF are acronyms given to different classes of transcription factors.
Source: Fujitia *et al.*, 2006

Nonetheless, ABA's involvement in plant diseases seems to be complex and also dependent on the type of the pathogen. For example, a study by Mauch-Mani *et al.* (2005) shows that ABA can also be implicated in increasing the resistance of plants towards pathogens via its positive effects on callose deposition. This finding provides further evidence that plant signalling pathways consist of elaborate networks with frequent "crosstalk", thereby allowing plants to regulate both abiotic stress tolerance and disease tolerance

The generation of ROS scavengers or other second messengers, like inositol phosphate, is considered another key process shared between biotic and abiotic stress responses. Second messengers can modulate intracellular Ca^{2+} levels, often initiating a protein phosphorylation cascade that targets proteins directly involved in cellular protection or transcription factors that control specific sets of stress-regulated genes. Environmental stress-inducible genes can generally be divided into two types:

1. Genes whose products directly confer the function of plant cells to resist environmental stress such as Late Embryogenesis Abundant (LEA) protein, anti-freezing proteins, osmotic regulatory proteins, enzymes for synthesising betaine, proline and other osmoregulators,
2. Genes whose products play an important role in regulating gene expression and signal transductions, like the transcriptional elements for sensing and transducing the protein kinases of mitogen-activated protein (MAP) and calcium-dependent protein (CDP), basic leucine transcription factor (bZIP), MYB, MYC, NAC, ZF and HSF.

2.4.3 Gene regulation and cell signalling

In *Arabidopsis thaliana* numerous genes that respond to dehydration stress have been identified and categorised as 'responsive to dehydration' (*rd*) genes and 'early response to dehydration' (*erd*) genes, which are involved in ABA-dependent and ABA-independent signalling pathways (Shinozaki *et al*., 1996).

A review by Valliyodan *et al.* (2006) discusses a series of studies in a series of different transgenic plants, mostly *Arabidopsis thaliana*. One of these studies indicates that a cis-acting element, the dehydration-responsive element/C-repeat (DRE/CRT), is involved in the ABA-independent regulatory systems. DRE/CRT also functions in cold- and high-salt-responsive gene expression. When the DRE/CRT-binding protein DREB1/CBF is overexpressed in transgenic *Arabidopsis thaliana*, changes in the expression of more than 40 stress-inducible genes were identified. Overall, these changes lead to increased tolerance to freezing, salt and drought.

Other important transcriptional regulators, such as the MYC and MYB proteins, are known to function in one of the ABA-dependent regulatory systems. The ABA-responsive element (ABRE) functions as a cis-acting element in the other ABA-dependent regulatory system, in which ABA-responsive element binding (AREB) basic leucine-zipper-type proteins (also known as auxin binding factors, ABFs) have been identified as transcriptional activators. The identification of these transcriptional factors of ABA signalling holds promise for genetic modification for enhanced drought tolerance.

Furthermore, it appears that a series of ABA synthesis genes, among which the zeaxanthin epoxidase gene (*ZEP*; also known as *LOS6/ABA1*), a 9-cis-epoxycarotenoid dioxygenase gene

(*NCED3*) and the aldehyde oxidase gene (*AAO3*) are upregulated by drought and salt stress in *Arabidopsis thaliana* but not obviously induced by cold.

In addition, two genes *AtMYB60* and *AtMYB61*, which encode transcription factors, have been found to be involved in the regulation of stomatal movement in *Arabidopsis thaliana*. Such findings might facilitate the engineering of stomatal activity to help crop plants survive water deficits.

Another study shows that transgenic rice plants constitutively expressing *CBF3/DREB1A* and *ABF3* genes from *Arabidopsis thaliana*, which have a function in ABA-independent and ABA-dependent pathways respectively, lead to activation of 12 and 7 target genes. These genes appear to confer stress tolerance. An additional 13 and 27 genes, respectively, are induced by drought stress in transgenic rice plants overexpressing *CBF3/DREB1A* and *ABF3*, while expression of *CBF3/DREB1A* improves tolerance to drought and high salinity and slightly improves tolerance to low temperatures.

In addition, the results of a field trial of transgenic maize plants, which express an *Arabidopsis thaliana* transcription factor (NF-YB class CGAAT-binding transcription factor) have been reported by Monsanto to show improved drought tolerance in the field.[2] The Monsanto data also suggest that the functions of selected transcription factors in drought tolerance are conserved across the dicot and monocot lineages, because they have similar impacts on specific phenotypes. Even though several genes that are associated with drought tolerance were identified and characterised, only a few drought tolerant transgenic maize plants were evaluated in the field trial.

Finally, genes from barley (*HVA1*) or wheat (*PMA80*), expressing late embryogenesis abundant (LEA) proteins, of which some, i.e. dehydrins, act as chaperones that stabilise vesicles, proteins and membrane structure in drought stressed plant. Both genes have also been used in developing transgenic rice or wheat plants with enhanced biomass productivity and water-use efficiency under water-deficit conditions.

Biotic and abiotic stresses thus induce the expression of different but overlapping sets of genes (Fedoroff, 2002; Fujita *et al.*, 2006). Up to now, hundreds of transcriptional elements of environmental stress-responsive genes in higher plants have been isolated, which regulate and control the stress reaction related to drought, salinity, cold, heat and pathogens (Sreenivasulu *et al.*, 2007) While in the genome of *Arabidopsis thaliana* and rice, about 1300 – 1500 genes encoding such transcriptional elements have been found, most of them have not yet been characterised in detail as to their specific function.

A review by Shinozaki *et al.* (2007) summarises findings from recent progress resulting from analysis of gene expression during drought-stress responses in plants. In this review the previous assumption that at least four signal transduction pathways exist in abiotic stress responses, of which two ABA-dependent and two ABA-independent (Shinozaki *et al.*, 1996) has been adjusted to the hypothesis that there are at least six signal transduction pathways, of which three depend on ABA whereas the other three function independent of ABA. Figure 2 presents an overview of these transcriptional regulatory networks of stress signals and gene expression.

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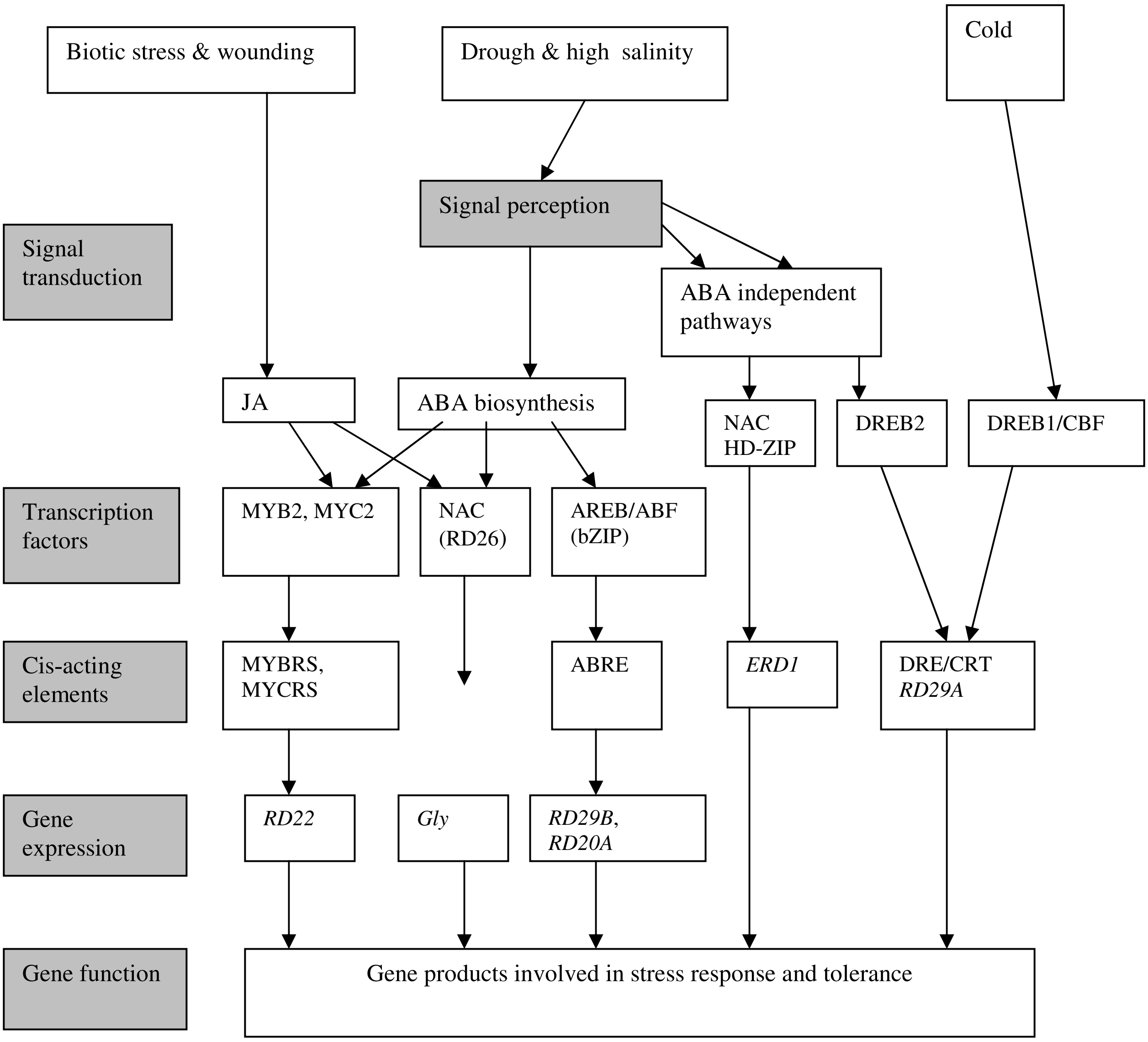
[2] By contrast to the other references in Valliyodan *et al.* (2006), the reference to “Heard, J. et al., Abstract L 8.02, Interdrought II, Rome, September 2005” could not be retrieved from PuBMed or Google Scholar.

Figure 2: Transcriptional regulatory networks of stress signals and gene expression

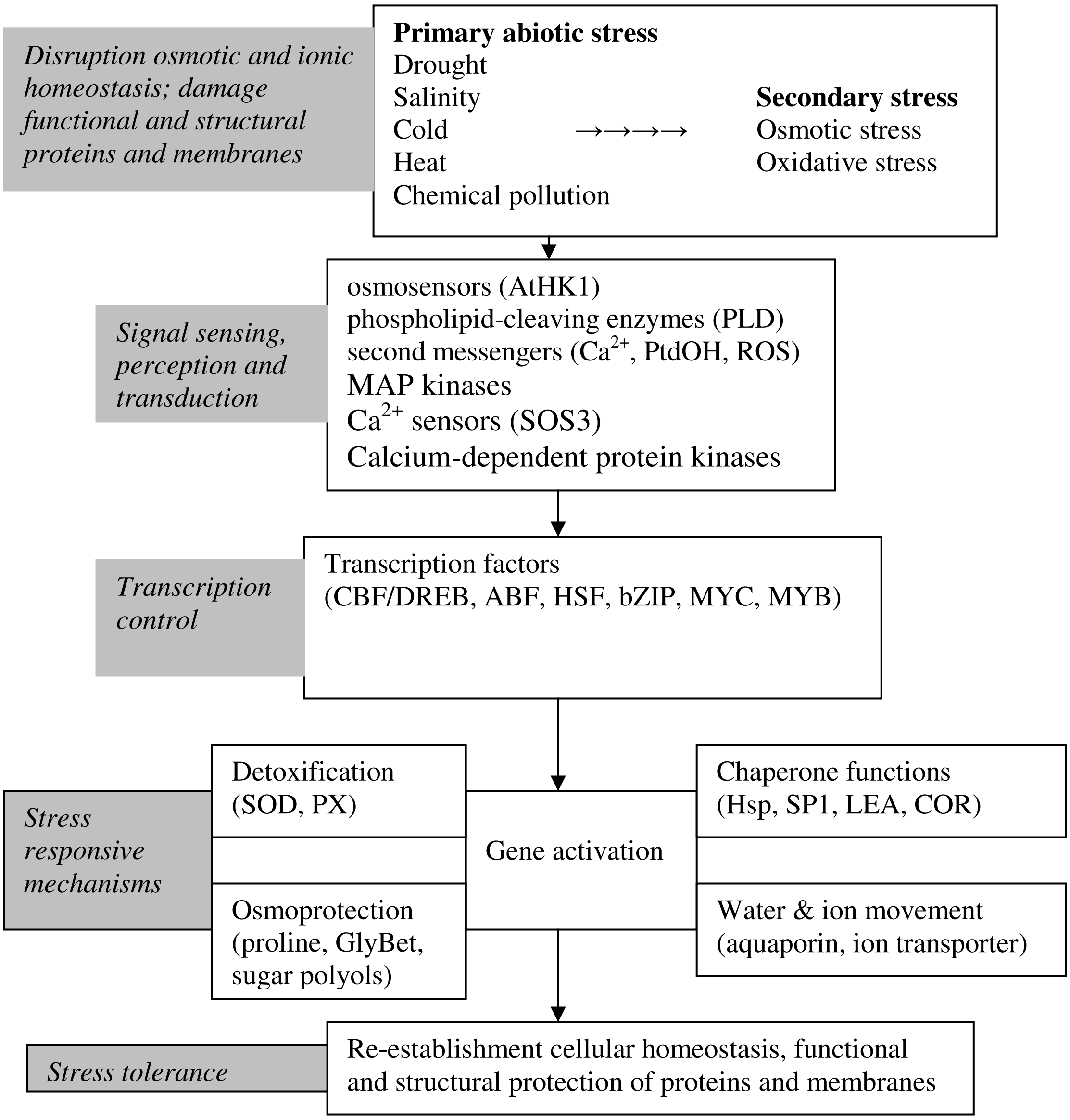


At least six signal transduction pathways exist in drought, high salinity and cold-stress responses: three are ABA dependent and three are ABA independent. In the ABA-dependent pathway, ABRE functions as a major ABA-responsive element. AREB/ABFs are AP2 transcription factors involved in this process. MYB2 and MYC2 function in ABA-inducible gene expression of the *RD22* gene. MYC also functions in JA-inducible gene expression. The RD26 NAC transcription factor is involved in ABA- and JA-responsive gene expression in stress responses. These MYC2 and NAC transcription factors may function in cross-talk during responses to abiotic stress and wound stress. In one of the ABA-independent pathways, DRE is mainly involved in the regulation of genes not only by drought and salt by also by cold stress. DREB1/CBFs are involved in cold-responsive gene expression. DREB2s are important transcription factors in dehydration and high salinity stress-responsive gene expression. Another ABA-independent pathway is controlled by drought and salt, but not by cold. The NAC and HD-ZIP transcription factors are involved in *ERD1* gene expression.
Abbreviations: ABF = ABRE binding factor; ABRE = ABA-responsive element; bZIP = basic leucine zipper; CBF = C-repeat-binding-factor; CRT = C-RepeaT; DRE = dehydration-responsive element ; DREB = dehydration-responsive element binding (protein); *ERD* = early responsive to dehydration gene; RD = responsive to dehydration gene.
Source: Shinozaki et al. 2007

2.4.4 Biochemical and molecular mechanisms of drought stress response

Understanding of the basic biochemical and molecular mechanisms for drought stress perception, transduction and tolerance is still a major challenge in plant biology. So far, plant modification for enhanced drought-tolerance is mostly based on the modification of either transcription and/or signalling factors or genes that directly protect plant cells against water deficit (Valliyodan *et al.*, 2006). Figure 3 provides a schematic overview of current knowledge of these basic biochemical and molecular mechanisms for abiotic stress perception, signal transduction and response mechanisms to abiotic stress.

Figure 3: Plant responses to abtiotic stress



Abbreviations: ABF = ABRED binding factor; AtHK1 = *Arabidopsis thaliana* histidine kinase; bZIP = basic leucine transcription factor; CBF/DREB = C-repeat-binding-factor/dehydration-responsive binding protein; CDPK = calcium-dependent protein kinase; COR = cold-responsive protein; Hsp = heat shock protein; LEA = late embryogenesis abundant; MAP = mitogen-activated protein; PLD = phospholipase D; PtdOH = phosphatidic acid; PX = peroxidise; ROS = reactive oxygen species; SOD = superoxide dismutase; SP1 = stable protein 1.
Source: Wang et al., 2003

Notably, molecular analyses of the signal transduction pathways have revealed a connection between changes in turgor pressures, synthesis of hormones, like ABA, and the induction of one set of genes involved in dehydration tolerance, while other genes are activated through a cellular dehydration signal transduction pathway that does not involve ABA.

Physiological and biochemical changes at the cellular level that are associated with drought stress include turgor loss, changes in membrane fluidity and composition, changes in solute concentrations and protein-protein and protein-lipid interactions. Plant tissues can maintain turgor during drought by avoiding dehydration, tolerating dehydration or both. These forms of stress tolerance are controlled by developmental and morphological traits, like root thickness, the ability of roots to penetrate in compacted soil layers, and root depth and mass. Constitutive phenotypic traits, e.g. root thickness, are present even in the absence of stress conditions.

By contrast, adaptive traits, such as osmotic adjustment and dehydration tolerance, arise in response to drought stress. Reduction of photosynthetic activity, accumulation of organic acids and osmolytes, and changes in carbohydrate metabolism are typical physiological and biochemical response to drought stress. The reduction of photosynthetic activity is due to several coordinated events, like stomatal closure and the reduced activity of photosynthetic enzymes.

Synthesis of osmoprotectants, osmolytes or compatible solutes is yet another mechanism that plants have evolved for responding to drought stress. Osmoprotectants are small neutral molecules that are non toxic to the plant cell at molar concentration and that stabilise proteins and cell membranes against denaturing effects of stress conditions on cellular functions. Many major crops lack the ability the synthesise special osmoprotectants, among which mannitol, fructans, trehalose, ononitol, proline and glycine betaine, that are naturally accumulated by stress-tolerant organisms, such as certain plants, marine algae and bacteria. It has been therefore hypothesised that engineering the introduction of osmoprotectant synthesis could be a potential strategy for improving the (drought) stress tolerance in crops (Rathinasabapathi, 2000; Quan *et al*., 2004; Almeida *et al*., 2007). However, the mechanism, by which these compounds provide protection, is not always completely understood. Usually, osmoprotectants are localised in the cytoplasm of plants and their active accumulation decreases the cell's osmotic potential and maintains cell turgor. However, genetic engineering for osmoprotectant accumulation does not always lead to osmotic adjustment in plants responding to stress.

Other responses, like the production of ROS scavengers, the induction of chaperone-like activities that protect protein structure and metabolic detoxification, have also been reported during drought stress. Field studies examining the association between osmolyte accumulation and crop yield have tended to show no consistent benefit. According to a review by Serraj *et al*. (2002), the few investigations with positive associations were obtained under severe water deficits with extremely low yields or conditions with special water-supply scenarios when much of the benefit is plant survival. The often-quoted benefit of turgor maintenance in plant cells is likely to result in crop behaviour that is exactly the opposite to what is beneficial to crop yield. One mechanism beneficial for crop yield responses to osmolyte accumulation is root development, in order to reach water that may be available in deeper soil layers.

2.4.5 Perspectives for drought-tolerant GM plants

Over the last decade transcriptomic, proteomics and metabolic analyses have identified and characterised numerous genes that are induced by drought stress and the associated signalling and regulatory pathways. Recent efforts on dissecting the crosstalk between drought stress and other major abiotic stress signalling pathways also provide potential candidate genes for multiple abiotic stress tolerance. Most of these studies were however conducted using model plants, like *Arabidopsis thaliana* and tobacco, and genetic engineering for drought-tolerance in crops is still in its early stages (Valliyodan *et al*., 2003).[3] As of 2003 the number of drought-tolerant GM crop plants that had undergone field trials and/or been tested under (natural) water-deficit conditions was considered rather modest (Sung *et al*., 2003). Tables 1, 2 and 3 provide examples of different types of drought-tolerant GM plants.

Table 1: Examples of drought-tolerant GM plant using signalling factors

Classification	**Gene name**	**Plant species**
Protein kinases		
CDPK	*OsCDPK7*	Rice
GSK3/shaggy	*AtGSK1*	*Arabidopsis thaliana*
MAPKKK	*NPK1*	Maize
SnRK2	*SRK2C*	*Arabidopsis thaliana*
Others		
Calcium sensor	*CBL1*	*Arabidopsis thaliana*
14-3-3 Protein	*GF1λ*	Cotton
CC-NBS-LRR	*ADR1*	*Arabidopsis thaliana*
Farnesyl-transferase	*ERA1*	*Arabidopsis thaliana*, oilseed rape

Source: Umezawa et al., 2006

Table 2: Examples of drought-tolerant GM plants using functional proteins

Mechanism	**Gene name(s)**	**Species**
Osmolyte metabolism		
Proline	*P5CS*	Tobacco, rice, petunia
	P5CR	Soybean
	ProDH	*Arabidopsis thaliana*
Polyamines	*ADC*, *SPDS*	*Arabidopsis thaliana*, rice
Myo-inositol	*IMT1*	Tobacco
Sorbitol	*stpd1*	Tobacco
Trehalose	*OtsA*, *Ots*, *B*, *TPSP*	Tobacco, rice, tomato
Galactinol	*AtGolS2*	*Arabidopsis thaliana*
Mannitol	*mtlD*	Wheat
Fructan	*SacB*	Tobacco, sugar beet
Glycine betaine	*COX*, *GSMT+DMT*	Tobacco, *Arabidopsis thaliana*
Antioxidants & detoxicants		
ROS-scavengers	*CuZn-SOD*	Tobacco

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[3] For an updated overview of genes for abiotic stress resistance in transgenic or mutant plants, please see http://www.plantstress.com/files/abiotic-stress_gene.htm

	Mn-SOD, *Fe-SOD*	Tobacco, lettuce, alfalfa
	GST, *GPX*	Tobacco
	MsALR	Tobacco
	PARP (RNAi)	Oilseed rape
	chyB	*Arabidopsis thaliana*
	AAR	Tobacco
Heat shock proteins & chaperones	*Hsp17.7*	Carrot
	Hsp21	*Arabidopsis thaliana*
	AtHSP17.6A	*Arabidopsis thaliana*
	DnaK1	Tobacco
	BiP	Tobacco
	SP1	Poplar
LEA-type proteins	*COR15a*	*Arabidopsis thaliana*
	HVA1	Wheat, rice, creeping bentgrass
	WCS19	*Arabidopsis thaliana*
	LEA	Chinese cabbage
Ion transport		
	AtNHX1	*Arabidopsis thaliana*, oilseed rape, tomato
	SOS1	*Arabidopsis thaliana*
	HAL1	Cucumber
	AVP1	*Arabidopsis thaliana*
Others		
ABA biosynthesis	*AtNCED3*, *CYP707A3*	*Arabidopsis thaliana*
Stomata	*Chl-NADP-ME*	Tobacco

Sources: Wang et al. 2003; De Ronde et al. 2004; Umezawa et al. 2006; Fu et al. 2007

Table 3: Examples of drought-tolerant GM plants using transcription factors

Classification	**Gene name**	**Plant species**
AP2/ERF familiy		
DREB1/CBF	*DREB1A/CBF3*	*Arabidopsis thaliana*, tobacco, rice
	DREB1B/CBF1	Tomato
	CBF4	*Arabidopsis thaliana*
	ZmDREB1A	*Arabidopsis thaliana*
	DREB1C/CBF2	*Arabidopsis thaliana*
AP2/ERF	*SHN1/WIN1*	*Arabidopsis thaliana*
	WXP1	Alfalfa
DREB2	*DREB2A*	*Arabidopsis thaliana*
Basic leucine-zipprer (bZIP) protein		
	AREB2/ABF4	*Arabidopsis thaliana*
	AREB1/ABF2	*Arabidopsis thaliana*
	ABF3	*Arabidopsis thaliana*, rice
MYB/MYC		
MYB, MYC	*AtMYC2*, *AtMYB2*	*Arabidopsis thaliana*
MYB	*CpMYB10*	*Arabidopsis thaliana*
R2R-MYB	*AtMYB60*	*Arabidopsis thaliana*
Zinc-finer proteins		
Cys2His2-type	*ZPT2-3*	Petunia
	CAZFP1	*Arabidopsis thaliana*

	STZ	*Arabidopsis thaliana*
Others		
NAC	*ANACO19/055/072*	*Arabidopsis thaliana*

Source: Umezawa et al., 2006

Advances in understanding of stress signal perception and transduction and the associated molecular regulatory networks, together with high-output transformation technology, are improving the possibility achieving the goal of developing drought-tolerant GM crops. More work on crop plants is still needed, in order to be able linking physiology, systems biology and field performance.

One the one hand, knowledge of traits in crop plants that are associated with root architecture and plasticity under water-deficit conditions, e.g. osmotic adjustments in roots, should be further increased. On the other hand, a major constraint to sustaining and improving crop productivity under drought stress is plant reproductive failure under stress. Most of the research on understanding drought-tolerance is focussed on plant developmental stages other than just before and after flowering, although the reproductive parts of many crop plants are of the most interesting with a view to harvestable yields.

While in several cases the modification of a single trait involved in transcription control resulted in significant improvements in drought stress tolerance, modification of upstream signalling regulators often activates a much wider network of genes, other than stress-specific ones, which may have deleterious effects on total plant performance, eventually becoming useless for agricultural practices. An ideal GM crop should possess a highly regulated stress-responsive capacity that does not affect crop performance when stress is absent (Wang *et al.*, 2003).

A review by Sung *et al.* (2003) identifies the following challenges to improve understanding of acquired tolerance to temperature extremes:

- Identify primary temperature sensor(s) or thermostat(s) and/or an array of sensing signals leading to an intertwined network.
- Better understand the role of signal transduction pathway crosstalk.
- Identify cis-elements and all transcription factors that regulate temperature stress responses.
- Better understand post-transcriptional and post-translational regulatory mechanisms in stress responses.
- Identify the genes and mechanisms of general stress processes as well as stress-specific responses.
- Identify the function of genes whose functions is presently unknown and determine role in tolerance.
- Understand nature of cross-protection.
- Identify all molecular and macromolecular targets of injury.
- Integrate disparate injury mechanisms and stress responses into a unifying perspective of tolerance.

2.4.6 Germination and dormancy of drought-tolerant GM maize seeds

Seed dispersal from crop plants is one of the routes that could potentially lead to (undesirable) volunteer plants in the next crop, i.e. persistency in agricultural habitats, and/or to invasion of (semi-)natural habitats. With a view to the environmental risk assessment of drought-tolerant GM maize plants in Europe and because seed spillage can occur during harvesting operations and transport to storage and processing facilities, the issue at stake is whether a GM drought-tolerance trait affects seed germination and dormancy in comparison to non-GM maize seed germination and dormancy.

Seed germination is one of the most important processes in the life history of plant, as it sets in motion the growth of the seedling. It is therefore not surprising that seed dormancy is a complex trait, influenced by numerous genetic and environmental factors that interact to maximise the long-term chances of survival of the seed. Dormancy is characterised by the inability of mature seeds to germinate under conditions relating to water, light and temperature that are favourable to germination. In dormant seeds, the failure of the embryo to resume growth following uptake of water (imbibition) can be caused by a variety of factors, arising from both embryonic tissues (embryo-based dormancy) and maternal tissues (coat-based dormancy). Dormancy is usually initiated during seed maturation (primary dormancy) and its maintenance in the mature seed depends on both environmental and genetic factors. Secondary dormancy can be initiated in mature non-dormant seeds by environmental conditions that do not favour germination, like high temperature. In the natural environment, the decay of dormancy for many seeds is promoted by several factors, including after-ripening (the exposure of dry seed to mild or hot conditions) and stratification (uptake of water at low temperature). Seed dormancy is an adaptive trait that promotes the survival of plants in hostile environments. In some plant species, dormancy is an adaptation to allow germination and seedling establishment to occur in more favourable seasons. The presence of dormant seed banks in soils provides an opportunity for germination to occur over several seasons, thereby maximising the chance of long-term survival (Koornneef *et al.* 2002). In important cereal crops, dormancy at harvest is a desired trait because it prevents the early germination of grains in the head following exposure to cool moist conditions, i.e. pre-harvest sprouting, which affects harvest yields.

Seed dormancy and germination are known to be mediated by hormones, with gibberellic acid (GA), ethylene (ET) and brassinosteroids all known to promote germination and ABA to promote dormancy (Koornneef *et al.* 2002) During seed development, ABA content increases and regulates many key processes, including imposition and maintenance of dormancy. In order for the seed to germinate, the high level of ABA must thus be reduced and studies have shown that this occurs when dormancy is broken by after-ripening and uptake of water at low temperature (Gubler *et al.* 2005). This presumably causes a switch to ABA catabolism, resulting in a decrease in ABA content in the embryo and a corresponding increase in inactive ABA metabolites such as phaseic acid (PA) and dihydrophaseic acid (DPA). A family of cytochrome P450s (CYP707A) has been identified that catalyse 8'-hydroxylation of ABA to PA during the early stages of water uptake in after-ripened seeds (Millar *et al.* 2006).

Furthermore, there is now strong evidence that changes in ABA and GA concentrations are causally linked during seed germination (Gubler *et al.* 2005). Studies in barley and sorghum suggest that ABA might block germination processes in dormant seeds by repressing GA biosynthesis. Some progress has also been made in identifying ABA-response genes that regulate dormancy, like for example *MARD1* (Mediator of ABA-Regulated Dormancy 1), *FsPP2C1*, encoding a phosphatase, and *LeSNF4*, encoding a kinase that might be involved in integrating hormonal response pathways.

It is clear that dormancy is a complex traits whose control involves many genes, with not only strong intergenic interactions, but also strong interactions between the genome and the environment. This makes studying these dormancy genes difficult. Against this background, quantitative trait locus (QTL) analysis is therefore another tool for dissection of dormancy, a trait that shows great variability among natural population of many plant species. In *Arabidopsis thaliana* 7 QTLs, termed DOG (Delay Of Germination) loci, have so far been found. Moreover, in rice 6 dormancy QTLs have been found that have strong intergenic interactions, whereas in barley up to 27 dormancy QTLs have been detected in different environments, but only 4 are major dormancy QTLs. Although advances have thus been made, current understanding of the molecular genetics of dormancy is still limited (Gubler *et al.* 2005).

Given the current level of scientific understanding of genetic and molecular mechanisms involved in seed germination and dormancy, it cannot be excluded that these mechanisms might be affected in seeds of maize plants that are genetically modified with drought-tolerance, in particular when changes in the ABA metabolism are involved. This might in result changed germination and dormancy characteristics of drought-tolerant GM maize seeds compared to those the parental maize seeds, which could eventually have an impact on the GM maize seeds' survivability.

2.4.7 Viability of drought-tolerant GM maize pollen

With a view to the environmental risk assessment of drought-tolerant GM maize, it should be noted that pollen dispersal is one route for transfer of genetic material from a plant to sexually compatible plants. In the case of a (GM) maize plant its pollen can be dispersed by wind to other maize plants. It should thereby be noted that in Europe maize has no wild or weedy relatives, although there is uncertainty about whether feral maize populations might be present in Southern Europe. Although during the dispersal process a certain percentage of the dispersing pollen will loose viability, i.e. died or lost the capability to germinate and achieve fertilisation, pollen viability is in general influenced by three main types of factors: 1) internal factors, such as pollen metabolism; 2) morphological factors, such as protected anthers or open flowers, and; 3) environmental factors, such as humidity, temperature and ultra-violet (UV) light (Smith-Kleefsman *et al.* 2005). Hence, the issue at stake here is whether, and if so, to what extent, a GM drought-tolerance trait in maize could potentially affect the viability of its pollen.

Knowledge of pollen biology indicates that maize pollen is among the largest particles that are completely airborne. At anthesis (budding and unfolding blossom), water comprises about 60 % of the fresh weight of maize pollen. Maize pollen is generally considered dehydration intolerant (relative to pollen of other species), since it loses water rapidly and viability decrease sharply, because dehydration disrupts adenosinetriphosphate (ATP) formation. After anthesis, maize pollen dehydrates as it moves through the atmosphere until it lands on a receptive stigma. Upon landing on a receptive stigma, the maize pollen absorbs water from the stigma and proceeds to germinate. According to Luna *et al.* (2001), loss of maize pollen viability is faster at low relative humidity (RH) or high temperature. Experiments indicate that after release from anthers desiccation of maize pollen is a function of air temperature, relative humidity and time. Maize pollen's viability appears to decrease linearly with pollen moisture content (PMC) and is zero at a pollen moisture content about 30 %. Moreover, pollen death after release from the anthers is mainly due to dehydration, which is primarily controlled by the vapour pressure deficit of the air, and may be genotype-dependent (Fonseca *et al.* 2005).

As maize pollen viability depends both on environmental and genetic factors, it cannot be excluded that a GM drought-tolerance trait in a maize plant could potentially affect the metabolism in its pollen, which in turn might change its viability and therefore also its dispersal characteristics.

2.4.8 Conclusions

As a result of natural evolution and breeding, (crop) plants have acquired several molecular mechanisms that enable them to respond to abiotic stress, like drought, cold or salinity, and biotic stress caused by pathogens and herbivores. These molecular mechanisms include stress signal recognition, signal transduction, signal response and signal output, while they are intricately associated with various plant physiological functions and morphological characteristics. Together, these molecular mechanisms constitute a multidimensional network system with many levels of gene expression and regulation. Understanding of this multidimensional network system is still a major challenge in plant biology, despite the research conducted over the last ten to fifteen years.

Plant hormones such as abscisic acid (ABA), ethylene (ET), jasmonic acid (JA) and salicylic acid (SA) primarily regulate the protective responses to both biotic and abiotic stresses. While ABA is mainly involved in responses to abiotic stresses, it also governs a variety of growth and developmental processes, including seed development, dormancy, germination and stomatal movements. By contrast, ET, JA and SA play central roles in biotic stress responses. But there is also evidence that there is “crosstalk” between these two different stress response systems.

The generation of reactive oxygen species (ROS) scavengers or other second messengers, like inositol phosphate, is another key process that is shared between abiotic and biotic stress response mechanisms. These second messengers often initiate a protein phosphorylation cascade that targets proteins directly involved in cellular protection against damage cause by stress or transcription factors, which control specific sets of stress-regulated genes.

Synthesis of osmoprotectants is yet another mechanism that plants have evolved for responding to drought stress. Osmoprotectants are small molecules that stabilise proteins and cell membranes against denaturing effects of stress conditions on plant cellular functions. But many major crops lack the ability the synthesise special osmoprotectants, such as mannitol, fructans, trehalose, ononitol, proline and glycine betaine, that are naturally accumulated by stress-tolerant organisms, such as certain plants, marine algae and bacteria.

Biotic and abiotic stresses induce the expression of different but overlapping sets of genes in higher plants, which regulate stress responses to drought, salinity, cold or pathogens. So far, hundreds of transcriptional elements of stress-responsive genes have been identified, but most of them have not yet been characterised in detail as to their specific function.

Drought stress can induce several physiological and biochemical changes at the plant cellular level, like changes in turgor pressure, membrane fluidity and composition, concentration of organic acids and osmolytes, ABA synthesis, protein-protein and protein-lipid interactions, stomatal movements, photosynthetic activity, carbohydrate metabolism and the ability of roots to penetrate soil layers.

In several cases genetic modification of a (crop) plant with a single trait involved in transcription control resulted in improved drought-tolerance. Moreover, genetic modification of upstream signalling regulators in a (crop) plant also led to improved drought-tolerance, although it often also activated a much wider network of genes, other than stress-specific ones, with deleterious effects on total plant performance. A third strategy for improving drought stress tolerance consists of genetic modification of (crop) plants with genes encoding the synthesis of special osmoprotectants, such as mannitol, fructans, trehalose, ononitol, proline and glycine betaine.

Given the potential for crosstalk between abiotic and biotic stress response mechanisms, it is conceivable for all three types of genetic modification that a GM plant with a ‘drought-tolerance’ trait may also acquire a change in its susceptibility to other abiotic stresses, like salinity or cold, and/or biotic stresses caused by pathogens and herbivores. It is also conceivable, in particular when the genetic modification targets changes in the ABA metabolism, that the germination and dormancy characteristics of seeds of GM (maize) plants with a ‘drought-tolerance’ trait might be changed, which could eventually have an impact on the GM seeds’ survivability. In addition, a drought-tolerance trait in a GM maize plant could potentially also affect the metabolism in its pollen, which in turn might change its viability and, consequently, its dispersal characteristics.

2.5 Drought-tolerant GM maize: state of affairs

2.5.1 Examples of drought-tolerant GM maize in scientific journals

The previous section examined results of various studies aimed at improvement of current understanding molecular genetic and biochemical mechanisms for abiotic stress tolerance in plants in general. By contrast, this section focuses specifically on studies, which aim to advance drought-tolerance breeding, including genetic modification, in maize.

A paper by Laporte *et al.* (2002) presents the findings of experiments with transgenic tobacco expressing maize NADP-malic enzyme. Although tobacco was used as model plant in these experiments, they were conducted by a research group of Pioneer Hi-Bred International, a major player in the global seed business with a substantial interest in maize breeding. According to this paper, mechanisms that plants use to cope with drought can be grouped into two different strategies: drought-tolerance and drought-avoidance. This paper addresses the engineering of a drought-avoidance type phenotype, which allows for the conservation of water during plant growth. Since the majority of water loss from plant occurs through stomata, the experiment attempted engineering the opening-closure mechanism by inserting a single maize gene encoding NADP-malic enzyme (ME) into tobacco. As a result, the ME-transformed tobacco plants had decreased stomatal conductance and gained more fresh mass per unit water consumed than did the wild type, but they were similar to the wild type in their growth and rate of development. By contrast to other cited findings, which suggested that NADP-malic enzyme could be detrimental in the development of normal chloroplasts when expressed at high levels (20 – 70 fold increases) in a C3 plant, this experiment demonstrated that a 5 to 18 fold increased NADP-malic enzyme activity did not change the chlorophyll content in the transgenic tobacco plants under normal culture conditions. However, it could not be ruled out that higher levels of NADP-malic enzyme expression in tobacco may lead to alterations in chloroplast development, as, following exposure to drought, development of necrosis was more rapid in leaves with the highest NADP-malic enzyme expression than in leaves with moderate expression.

In addition, the extent to which stomatal conductance can be reduced without affecting plant growth or yield still has to be demonstrated in the field for crop species. Though, theoretical predictions and experimental results by others suggest that water-use efficiency can be improved with minimal sacrifice in productivity, particularly when the onset of drought through conservative water use during critical periods of plant development, including the days surrounding anthesis, can be delayed.

In another study by researchers from Pioneer Hi-Bred International, it is demonstrated that ABA, drought and heat induce expression of a cytokinin oxidase gene (called *Ckx* or *CKO*) (Brugière *et al.* 2003). The hypothesis is therefore that maize employs cytokinin degradation as a means to control growth and development under abiotic stress conditions. Cytokinin oxidase may have different regulating roles depending on the organ considered; one for cytokinins transiting in the xylem stream and another one for regulating cytokinin levels in primordia (root) or meristems during organ differentiation and environmental stresses. Although several genes might encode different cytokinin oxidases in maize, the expression of these genes under different abiotic stress conditions and their possible role in kernel sink-strength and cell division needs further study.

Since ABA plays an important role in a number of traits of agronomic importance, such as the (maize) plant's response to abiotic stress and the deposition of storage protein and starch during seed development, researchers from Pioneer Hi-Bred International also aim at advancing understanding of the mechanism of ABA-regulated gene expression. ABA-responsive element (ABRE) or G-box-like elements act as a cis-element for ABA-induced gene expression and basic domain Leucine Zipper (bZIP)-type transcription factors have a role in the regulation of the ABRE/G-box. Experiments were therefore conducted to identify the trans-factor interacting with other cis-elements, like Coupling Element1 (CE1), also called dehydration-responsive element2 (DRE2), which is involved in ABA-induced gene expression (Niu *et al.*, 2002). The experiments showed that the protein encoded by a seed-specific maize gene, *ABI4*, binds to CE1 in a number of ABA-related genes. Moreover, it also binds to the promoter of the sugar-responsive *ADH1* gene, demonstrating the ability of this protein to regulate both ABA- and sugar-regulated pathways.

In yet another study of 2004 by researchers from Pioneer Hi-Bred International results are presented from a study on the regulatory role of 1-aminocyclopropane-1-carboxylate (ACC) synthase in leaf performance and drought-tolerance in maize (Young *et al.* (2004). This enzyme has their interest, as it affects the first step in the biosynthesis of ethylene, which in turn regulates entry into several types of plant developmental cell death and senescence programmes besides mediating plant responses to biotic and abiotic stress. Since photosynthetic capacity increases during leaf expansion and declines with leaf age until a low level is reached prior to the onset of leaf senescence, the rate of initiation and execution of a senescence programme impacts the ultimate contribution that a leaves makes to a plant. For example, a delay in the onset of senescence in *Lolium termulentum* by just two days has increases the amount of carbon fixed by the plant by 11 %. This is of particular relevance to those crops where yield potential is reduced by adverse environmental conditions, such as drought, which can induce premature leaf senescence in older leaves.

Examples of delayed senescence can be grouped into two types of stay-green. The first stay-green type is functional in that the photosynthetically active lifespan of a leaf is extended. The second is cosmetic in that foliar chlorophyll is retained during the execution of an otherwise normal senescence programme. Several environmental and physiological conditions, like light deprivation and insufficient nutrients and water, can induce premature leaf senescence. Several hormones also play a regulatory role in the leaf senescence programme, like cytokinin and ethylene.

This study demonstrated that maize knock out mutants deficient in the gene encoding ACC synthase (ACS) are deficient in ethylene production and their leaves exhibit delayed senescence. Loss of *ZmACS6* expression reduces ethylene production by 85 – 90 %, whereas loss of *ZmACS2* expression reduces ethylene production by 45 %. Surprisingly, leaves with reduced *ZmACS* expression and ethylene production contain higher levels of chlorophyll and leaf protein as well as a higher rate of carbon dioxide assimilation, also in young and fully expanded leaves that are far from senescing. These findings suggest that *ZmACS* expression regulates the onset of senescence under normal growth conditions, and inhibits drought-induced senescence in older leaves. In addition, *ZmACS* expression serves to control aspects of leaf development that are independent of senescence and includes the regulation of leaf physiology and function.

In 2002 French researchers from the Institute of Plant Biotechnology at the University of Paris, the association of maize producers (AGPM) and the agro-biotechnology company Biogemma report about the use of transgenic maize plants, in order to understand better ABA synthesis in relation to drought tolerance (Jeanneau *et al.*, 2002). This study assesses the role of the maize-derived candidate gene encoding the Abscisic acid stress responsive protein (*Asr1*) as an explanation for genetically linked drought-tolerance QTLs, because Asr proteins may be involved in the protection of DNA during water loss or in gene regulation upon stress by changing the DNA topology. It is demonstrated that drought in the field results in leaf senescence increases in *Asr1*-overexpressing maize lines in comparison to non-transgenic controls. For some of the antisense lines the opposite is detected. No significance difference for leaf senescence is noticed between antisense and sense maize lines in irrigated fields. Based on this result, the hypothesis is that one of the functions of *Asr1* might be to participate in re-routing the metabolism from source to sink leading to the senescence of the source organs.

The researchers also modified carbon dioxide fixation rates in maize leaves through changes of C_4 phosphoenolpyruvate carboxylase (C_4-PEPC) activity using a gene from sorghum. This protein, which plays a pivotal role in the photosynthesis pathway, is one of the isoforms encoded by fives genes found in the maize genome. The C_4-cycle acts as a pump increasing carbon dioxide levels in the vicinity of the ribulose-1,5-biphosphatase (Rubisco), leading to a markedly reduced photorespiration. This explains why C_4-plants, like maize, when grown in their natural environment (hot climates with sporadic rainfalls) have a selective advantage over C_3-plants.

In maize subjected to drought stress, photosynthesis decreases, although the C_4-PEPC content slightly increases. Drought stress induces an increase in stomatal closure, thereby inhibiting photosynthesis. Since the C_4-PEPC enzyme is expected to contribute to the carbon flux control of the photosynthetic pathway, especially under carbon dioxide-limiting conditions, the researchers confirm the hypothesis that an increase in the C_4-PEPC content by genetic engineering may contribute to the improvement of drought-tolerance in maize.

In 2006 French researchers from the national institute for agricultural research INRA and the agro-biotechnology company Biogemma set out to assess the role of ABA and its possible interaction with ethylene in mediating leaf elongation response to soil water deficit (Voisin *et al*., 2006). The limitation of leaf elongation, together with reduction of stomatal conductance, is an important aspect of plant adaptation to drought stress, as it reduces transpiration, thereby saving water. In plant species, like maize, leaf water status is maintained under moderate water deficit as a result of efficient stomatal control combining hydraulic and chemical messages, in which ABA plays an important role through limiting ethylene production. The researchers used a set of maize genotypes with various levels of ABA either due to natural variability or to genetic transformation (three sense and three antisense) targeted on NCED (9-cis-epoxycarotenoid dioxygenase)/VP14, a key enzyme of ABA biosynthesis. Their results suggest that over a wide range, internal ABA levels have no clear effect on leaf elongation response to soil water deficit.

Except in the case of an antisense line with the strongest reduction in ABA accumulation, leaf elongation is slightly maintained during soil water deficit. Leaf ethylene production rate appears to be variable and not related to soil water deficit, except in the ABA-deficient transgenic lines where it increases by water deficit on average but not systematically. It is further shown that variability in ethylene production rate is not linked to variability in leaf elongation rate.

Overall, the researchers conclude that neither ABA nor ethylene seem to play a major role in the control of leaf elongation in response to soil water deficit.

A study by Shou *et al*. (2004) analyses the role of activation of oxidative stress signalling in improving drought-tolerance in major crops through genetically modifying maize with the tobacco *NPK1* gene encoding mitogen-activated protein kinase kinase kinase (MAPKKK). The study shows that *NPK1* expression enhances drought-tolerance in transgenic maize. Under drought conditions, transgenic maize plants maintain significantly higher photosynthesis rates than did the non-transgenic control, suggesting that *NPK1* induces a mechanism that protects the photosynthesis machinery from dehydration damage. In addition, drought-stressed transgenic maize plants produce kernels with weights similar to those under well-watered conditions, while kernel weights of drought-stressed non-transgenic maize plants are significantly reduced when compared with their non-stressed counterparts. It appears that in *NPK1* transgenic maize lines drought stress induces an elevated expression of a gene encoding a small chloroplast heat-shock protein (HSP), which protects thermo-labile photosystem II and whole-chain electron transport during heat stress.

It is further observed that *NPK1* maize lines have increased leaf number under drought stress, which may be attributed to the delayed maturation of these transgenic maize plants. Moreover, expression of *NPK1* also induces a set of other oxidative stress-related genes, including *GST*, which encodes glutathione S-transferase. This may explain why *NPK1* expressing transgenic maize plants also display enhanced freezing-tolerance, as it is demonstrated that two transgenic maize events survive longer under subzero temperatures than non-transgenic control maize plants and are able to withstand up to 2 °C colder temperature.

A study by Quan *et al.* (2004) describes the results of engineering the *E. coli betA* gene, which encodes choline dehydrogenase, into maize. This enzyme performs a key function in the biosynthesis of glycine betaine from choline. Glycine betaine acts as an osmoregulator, stabilises the structure and activities of enzymes and protein complexes, and maintains the integrity of membranes against damaging effects of excessive salt, cold, heat and freezing. Glycine betaine thus plays an important role in some plants, including maize, under conditions of abiotic stress, but different maize varieties vary in their capacity to accumulate glycine betaine. This experiment, which transforms an elite maize inbred line DH4866 with the *E. coli betA* gene, shows that the transgenic maize plants accumulate higher levels of glycine betaine. This leads to better drought-tolerance at germination and the young seedling stage compared to that of the non-transgenic maize plants.

Furthermore, the grain yield of these transgenic maize plants is significantly higher than that of the non-transgenic maize plants after drought treatment. This results from a combination of several factors, like greater root biomass, improved water absorption, larger leaf biomass, better carbon dioxide assimilation, and less inhibition of reproductive organs.

In addition, the results also indicate that the transgenic *E. coli betA* maize plants are more tolerant to salt stress than the non-transgenic maize plants.

A review by Sung *et al.* (2003) discusses about 10 transgenic attempts to enhance plant tolerance to heat stress, 20 to chilling stress and 19 to freezing stress. Two of these in total 49 transgenic attempts concerned maize.

In one of these cases it was shown that insertional knockouts of *Hsp101*, a gene encoding heat-shock protein 101 (HSP101) in maize, were found to be defective in basal and acquired thermotolerance (Nieto-Sotelo *et al.*, 2002). It was also demonstrated that HSP101 accumulation in mature embryos is independent of heat-stress induction and most likely dependent on developmental or dehydration stress-related signals that occur during embryo development and/or seed maturation. HSP101 is predominantly found in the nuclei and at low levels in the cytoplasm. In addition, the experimental data, together with similar findings in *Arabidopsis thaliana*, confirm the central role for HSP101 in the development of acquired thermotolerance in (maize) plants and suggest a negative regulatory role of HSP101 in the growth of primary roots at optimal and mild heat-shock temperatures.

In the other case it was shown that overproduction of *Arabidopsis thaliana* iron superoxid dismutase (FeSOD) in GM maize results in enhanced tolerance to oxidative stress, which is a major component of chilling stress. Though, oxidative stress was induced by application of methyl viologen in this experiment (Van Beusegem *et al*., 1999). Moreover, overexpression of *Arabidopsis thaliana* gene for FeSOD does not specifically enhance chilling stress tolerance, but provides the GM maize plants with generally improved growth characteristics, as measured by the ratio of the weight of the GM maize plants and the non-GM control maize plants grown at 15/17 ºC over that at 22/25 ºC.

2.5.2 Fields trials with drought-tolerant GM maize in the US and EU

According to the US data base with information about field trials of GM crops, 194 field trials have so far been conducted or are still being conducted with drought-tolerant GM maize in the US until June 2007. In about 20 cases the drought-tolerant GM maize plants also have (stacked) genes for tolerance to Lepidoptera insects or a herbicide. In all cases the (names of the) genes used for conferring drought-tolerance to the maize plants and their origin(s) are treated as confidential business information (CBI) by the Animal and Plant Health Inspection Service (APHIS) of the US Department of Agriculture (USDA). Moreover, in 184 cases an Environmental Assessment (EA) has not been required in the notification procedure for the field trials, whereas in the other 10 cases (Monsanto 5; BASF 3, and; Syngenta 2) an EA has been required, but has not been made publicly available. Table 4 gives and overview of the numbers of field trial with drought-tolerant GM maize in the US and EU until June 2007.

Table 4: Numbers of field trials drought-tolerant GM maize in the US and EU until June 2007

Company	**US**	**EU**
Pioneeer Hi-Bred International	5	-
Stine Seed Farm / Stine Biotechnology	3	-
Biogemma	9	4
Coop de Pau	-	2
Dekalb	1	-
Syngenta	14	-
BASF	4	-
Monsanto	158*	-

* 5 withdrawn & 1 denied

Sources: http://www.isb.vt.edu/cfdocs/fieldtests1.cfm/ and http://gmoinfo.jrc.it/

According to information on its website[4], Monsanto has two different types of drought-tolerant GM maize in its Research & Development (R&D) pipeline. One is in Phase 1 of the R&D pipeline, which is the phase of 'proof of concept' with an average duration of 12 to 24 months and an average probability of success of 25 %. The other type is in Phase 2, which is the phase of 'early product development' with an average duration of 12 to 24 months and an average probability of success of 50 %. None of these two types are thus in Phase 3 ('advanced development'), which includes regulatory data generation and has an average duration of 12 to 24 months with an average probability of success of 75 %, or in Phase 4 ('pre-launch), which includes a regulatory submission and has an average duration of 12 to 36 months with an average probability of success of 90 %. Though, it should be noted that these four phases can overlap, so that the total development time may vary from 4 to 8 years. Based

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[4] See http://www.monsanto.com/monsanto/content/sci_tech/prod_pipeline/productPipeline.pdf

on this information, it can be expected that Monsanto might commercially release its first type of drought-tolerant GM maize around 2011 – 2012.
Monsanto's website does however not provide any information about the genes used for conferring drought-tolerance to maize. Likewise, the websites of the other companies conducting field trials with drought-tolerant GM maize plants in the US do not reveal the (name of the) gene(s) used for conferring drought-tolerance. In a press release of 23 October 2006 posted on Crop Management, Pioneer Hi-bred indicates that it expects to commercialise a drought-tolerant GM maize after 2012.[5]

A non-exhaustive search on the website of the US Patent and Trademark Office (USPTO)[6] reveals numerous patents on the use of several types of genes conferring tolerance to abiotic stress. While many of these patents have been granted to public research institutions, including the Cornell University and Iowa State University, others have been granted to private companies, such as Pioneer Hi-Bred, Monsanto, Dekalb, Syngenta and BASF. Examples include: 1) 'disrupted' ACC synthase genes (*ACS*); 2) *CBF* genes; 3) *mt1D* gene encoding mannitol-1-phosphate dehydrogenase; 4) genes encoding tonoplast intrinsic proteins (aquaporins); 5) genes, such *ATK1* and *KAT*, encoding potassium channel proteins; 6) genes encoding enzymes involved in the biosynthesis of ABA, glycine betaine or trehalose, and; 7) (maize) plants with modified cytokinin expression or ethylene production. These findings do however not provide any clue about the genes inserted into drought-tolerant GM maize plants, which have been or are currently being field-tested in the US.

Compared to the US, the number of field trials with drought-tolerant GM maize has so far been very limited in the EU. According to the EU data base with information about field trials of GM crops, Coop de Pau notified 2 field trials with drought-tolerant GM maize with a gene for superoxidismutase in France in 1997, and Biogemma notified 4 field trials with drought-tolerant GM maize in the period from 2000 to 2005. As the EU data base only provided more detailed information about field trials with GM crops notified after 2002, only 2 of the 4 notifications of Biogemma are publicly accessible.

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[5] See http://www.plantmanagementnetwork.org/pub/cm/new/2006/DroughtTolerant/
[6] See http://www.uspto.gov/

2.6 Views on potential environmental risks of drought-tolerant GM maize

2.6.1 Views of the US National Research Council

In 2002 the US National Research Council issued a report on the environmental effects of transgenic plants, which was drafted by its Committee on Environmental Impacts Associated with Commercialization of Transgenic Plants (NRC, 2002). This committee examined the similarities and differences between crops developed by conventional and transgenic methods, the potential for commercialised transgenic crops to change both agricultural and non-agricultural landscapes and how well the US government had been regulating transgenic crops to avoid any negative effects.

One section of this report of 2002 focused specifically on the assessment of potential environmental risks posed by GM plants with tolerance to abiotic stress. Though, it was recognised that accurate assessments would not be possible until such GM plants would have been actually created, because the genetic mechanism of stress-tolerance greatly determines the scope of potential environmental risks. Though, it should be noted that about ten field trials with drought-tolerant GM maize had already been notified to the US regulatory authorities from 1998 to 2001.

Despite the uncertainty about the nature of stress-tolerant GM plants, they deserved attention because abiotic conditions, such as soil nutrient levels, water, cold, heat, salinity and metal toxicity, combined with their seasonal variations have strong determining effects on plant community structure, and the geographic distribution of many plant species is strongly influenced by these factors. Thus, when plants are transformed to better tolerate these abiotic conditions, it raises questions about the possible impacts on plant community structure and expansion of the geographic range of a plant species. While these issues are considered in the environmental risk assessment of herbicide- and/or insect-tolerant GM plants, these traits involved are not expected to alter their invasiveness or weediness compared to their non-GM parental plants.

While the committee expected that the environmental risks associated with abiotic stress tolerant GM plants to be complex and subtle, it focussed its further discussion on drought-tolerant crops that could be based on higher water-use efficiency, leading to greater biomass production per unit of water, or an increased ability to extract water from the soil. The committees expected that GM plants with improved water extraction will still require the same amount of water to grow. So, potential environmental effects may often be related to competition for sunlight or nutrients in the soil, due to the plant's metabolic needs associated with greater biomass. By contrast, a GM plant with a higher water-use efficiency would be a better competitor than a non-transformed plant. Such an improved competitive ability is the source of some concerns about the environmental risks of drought-tolerant GM plants, whether it is the crop or a wild relative that might receive the transgene(s) by horizontal gene flow.

But, according to the committee, competitive ability for water is not necessarily sufficient to cause a plant to expand its geographic range. A lupine plant in the oak savannah with better water-use efficiency may grow more luxuriantly than its conspecifics but might not expand into surrounding habitats because there is too much shade, water or soil nitrogen, which might neutralise its advantage in the savannah. A maize variety with higher water-use efficiency may grow better in the dryland production systems of parts of Nebraska and Kansas but may still not displace spring wheat in the neighbouring counties because it still needs water over a longer growing season than wheat. It is also possible that a farmer might clear droughty land and plant such a maize variety, leading to marginal increases in the area planted to maize and marginal decreases in the area in dry prairie remnants. It is unlikely that maize could be transformed into a plant that could grow in arid or semiarid environments without irrigation.

Thus, while the committee recognised that there is a clear potential for drought-tolerant GM plants to expand their geographic range, there is a limit to this potential due to the inherent characteristics of the plant, while there are also seasonal limitations of other abiotic and biotic factors that restrict it from expanding. Assessment of these risks therefore requires attention to the plant (species), trait and environment. Finally, the committee noted that certain salt-tolerant GM plants can also tolerate other abiotic stresses, such as chilling, freezing, heat and drought.

The report also discussed the environmental hazard of a GM 'whole-plant' due to a transgenic trait that may improve its fitness and ecological performance. In this context it is noted that many crop plants may pose little hazard, in so far as they are unable to survive without human assistance, also because crop plants frequently have characteristics, like lack of seed shattering and seed dormancy, which make them useful to humans but also reduce their ability to establish feral populations in either agro-ecosystems or non-agricultural habitats. Without major changes in its phenotype, maize is unlikely to survive for multiple generations outside agricultural fields, no matter what transgene is added to it.

2.6.2 Views of the Australian regulatory authorities

In the first half of 2007 the Australian regulatory authority issued two risk assessment and risk management plans (RARMPs) for limited and controlled releases of GM sugar cane with altered plant architecture, enhanced water or improved nitrogen use efficiency, and a drought tolerant GM wheat.[7] Besides an evaluation of the potential toxicity and allergenicity of the proteins encoded by the introduced genes for enhanced drought-tolerance or water-use efficiency (WUE), these RARMPs also examined the potential environmental risks of these GM sugarcane and GM wheat plants. For that purpose, both RARMPs also contained a concise introduction to plant molecular responses to drought stress.

The GM sugarcane lines contain one or more of the following genes encoding drought tolerance: 1) *MdS6PDH* from apple encoding D-sorbitol-6-phosphate-dehydrogenase; 2) *EcTPSP* from *E. coli* encoding trehalose-6-phosphate-synthase, and; 3) *AtMYB2* from *Arabidopsis thaliana* encoding for a myeloblastosis interacting protein.
With reference to previous RARMPs, the weed status of sugarcane is considered very low, as modern cultivars are not invasive in natural undisturbed environments. The ability of sugarcane to establish, spread and persist is unlikely due to factors, like competition with other plants, pest and diseases, soil type, moisture stress, sunlight requirements and low temperatures. It is therefore noted that in an environment, in which water availability is the main factor limiting the spread and persistence of sugarcane, expression of the genes for water-use efficiency could result in weediness of the GM sugarcane lines. However, under glasshouse conditions, necrosis is observed on the leaves of the GM sugarcane lines expressing *Md6PDH*, and these GM sugarcane lines are up to 30 % shorter compared to non-

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[7] RARMP for DIR 070/2006 – Limited and controlled release of GM sugar cane with altered plant architecture, enhanced water or improved nitrogen use efficiency, and RARMP for DIR 071/2006 – Limited and controlled release of GM drought tolerant wheat; see http://www.ogtr.gov.au/

GM parent lines. Therefore, GM sugar cane lines expressing *Md6PDH* are probably less fit plants that may be more vulnerable to attack by fungal and/or bacterial pathogens.

On the other hand, the *Md6PDH* gene is known to confer enhanced salt-tolerance to transgenic Japanese persimmon plants, while the *EcTPSP* gene confers tolerance to salt and low temperature in rice, as well as drought-tolerance, and overexpression of *AtMYB2* induces tolerance to both drought and salt stress in *Arabidopsis thaliana*. However, when a gene is expressed in different plant species, the same effect(s) on phenotype does not always eventuate, which may be the case in the GM sugarcane lines. As this is a 'proof of concept' field trial, the ability of the GM sugar cane lines to withstand abiotic stress throughout different stages of their lifecycle as compared to commercially available sugarcane cultivars is therefore unknown.

Moreover, the genes for enhanced water-use efficiency could potentially enhance resistance of the GM sugar cane lines to pests or pathogenic micro-organisms, leading to spread and persistence if pests or diseases were the main limiting factors. Though, the genes introduced for enhanced water-use efficiency are not known to confer enhanced tolerance to biotic stress. In addition, the high sugar content of sugarcane makes it an ideal host for a number of bacteria and fungi, leading to its degradation, and it is not expected that the introduced genes will alter this. But again, as this is a 'proof of concept' field trial, the ability of the GM sugar cane lines to withstand biotic stress throughout different stages of their lifecycle as compared to commercially available sugarcane cultivars is therefore unknown.

Furthermore, since the release would be of limited size and short duration and the applicant proposed a number of measures to limit spread and persistence, no risk in relation to weediness, spread or persistence is identified outside the trial site.

Finally, insertion of new genes and traits by conventional breeding or genetic modification can result in unintended and unexpected changes. Therefore, more data on the potential pleiotropic effects of the genetic modification on GM sugarcane lines selected for further development, and how these may affect potential weediness, toxicity and allergenicity, may be required to assess any future applications for a larger scale or commercial release.

The GM wheat lines contain one of six different genes for drought-tolerance. For each gene there are two constructs, one driven by a stress inducible promoter and one by a constitutive promoter, giving in total 12 different gene constructs. While the source organisms of the six genes are maize (1), *Arabidopsis thaliana* (1), *Saccharomyces cerevisiae* (2), *Physocomitrella patens* (2), the function of all six genes in the source organism is treated as confidential information.

The RARMP for this case is very similar to that for GM drought-tolerant sugarcane lines, also because it concerns a 'proof of concept' field trial and the survival of the GM wheat plants would still be limited by temperature, low intrinsic competitive ability, nutrient availability, pests and diseases and other environmental factors that normally limit spread and persistence of wheat plants in Australia.

The main difference is that a comparison is also made with several commercial non-GM wheat cultivars with drought-tolerance that are already available in Australia. For example, the variety "Gladius" released in February 2007 produces yields 20 – 30 % higher than the benchmark variety "Yipti" that may be grown as a non-GM variety during the proposed field trial. The GM wheat lines are derived from the wheat cultivar "Bobwhite 26", which is considered to be of lower quality than most commercial cultivars, and, therefore, the GM wheat lines are unlikely to be more competitive than existing elite varieties, even if an increase in drought-tolerance is achieved.

Finally, when large scale or commercial releases of one or more of these GM wheat lines will be applied in the future, additional information will be required, including: 1) a molecular characterisation; 2) additional data on the potential toxicity and allergenicity of the proteins encoded by the introduced gene and of plant materials, and; 3) physiological and agronomic characteristics indicative of weediness, including measurement of altered reproductive capacity, tolerance to drought and other environmental stresses, including salinity, and disease susceptibility.

2.6.3 Views of the French biosafety committee

Two notifications of field trials with drought-tolerant GM maize in France indicate that these field trials began on 1 April 2004 and might last to 30 November 2008. In one case (SNIF B/FR/05/02/02) the *Asr1* gene from maize is overexpressed in the GM maize plant for conferring drought tolerance. In the other case (SNIF B/FR/05/02/03) the PEPC gene from sorghum has been inserted into the GM maize plant, which improves photosynthesis under drought conditions (see for both cases also section 2.5.1).

Notably, in both cases the advices of the French biosafety commission CGB concerning B/FR/05/02/02 and B/FR/05/02/03 indicate that the novel feature, i.e. drought-tolerance, of these GM maize plants does not make them different from conventionally bred maize with regard to invasiveness and survivability, while possible volunteers can be controlled by common agricultural practice.[8, 9] These advices do however not elucidate the considerations that led the CGB to this conclusion.

2.6.4 Views of Kjellsson et al.

In 1994 and 1997 Kjellsson *et al.* published two extensive catalogues with test methods for the environmental risk assessment of transgenic plants (Kjellson *et al.* 1994; Kjellsson *et al.* 1997). In the case of GM plants with enhanced tolerance to drought and/or frost, they suggest one or more, depending on the type of genetic modification, of the following methods to study their potential environmental effects:

- Enzyme assays for determination of the activity of enzymes either in different life stages, different organs, or under different environmental pressures. This method can predict which changes in the metabolism the plant may have obtained, and hence the consequences for the plant's ability to survive and compete. In the case of maize assays of the activities of the enzymes phospoenolpyruvate carboxylase (PEPC), ribulose-1,5-bipophosphate carboxylase oxygenase (Rubisco) and glycolic acid oxydase (GAO) could yield relevant data (Luna *et al.* 1985).
- Germination tests to study long-term seed viability, germination phenology, and consequently the initial phase of plant establishment in a new habitat.
- Leaf area and leaf demography measurements to study seedling competition, seedling growth, plant competition, plant size, plant growth and density-based competition. Data from such measurements represent expression of plant production, plant vigour and the competitive ability of the plant.
- Microsite tests to study seed germination, seedling survival, seedling growth, invasion, disturbances and genotype-environment interactions.

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[8] http://www.ogm.gouv.fr/experimentations/evaluation_scientifique/cgb/avis_dossiers_2005/avisBFR050202.pdf

[9] http://www.ogm.gouv.fr/experimentations/evaluation_scientifique/cgb/avis_dossiers_2003/AV030304.pdf

- Moisture loss tests to study seedling growth, seedling survival and plant growth. In such a test, plants are grown under different, controlled moisture treatments, in order to correlate seedling and adult plant growth to the level of moisture stress.
- Seed burial treatment tests to study seed bank, seed dormancy, seed survival and seed germination, in order to obtain information on probabilities for long-term survival of seeds in different types of soil ecosystems.
- Soil water content measurements to study the effect of soil water content on the early phases of plant establishment (i.e. seed germination and seedling survival).

Given the potential for crosstalk between abiotic and biotic stress response mechanisms, it cannot be excluded that a GM drought-tolerance (trans)gene may negatively or positively impact a maize plant's resistance to pathogens or herbivores. To study the effects of a GM pathogen-resistance trait in a GM plant, it is suggested to conduct enzyme assays, leaf area and leaf demography measurements.

2.6.5 Views on potential weediness of GM maize

In the environmental risk assessments of limited and controlled field trials with GM sugarcane and GM wheat with enhanced drought-tolerance by the Australian authorities, it is pointed out that in the case of large-scale and commercial releases of both these GM crops they will require an evaluation of the physiological and agronomic characteristics indicative of their weediness, including measurement of altered reproductive capacity, tolerance to drought and other environmental stresses, including salinity, and disease susceptibility.

In this context it is useful to bear in mind that agronomists and ecologists use the terms 'weed' and 'weediness' in different ways, which is often a source of misunderstanding in relation to discussions surrounding the release of transgenic plants (Ammann *et al.* 2000). For agronomists, the problem of weediness is solved if the (aggressive) weed can be removed from the agro-ecosystem by means of chemical and/or mechanical measures. For ecologists, invasions of weeds into (semi-)natural plant communities are potentially risky, in particular as highly competitive invaders are able to disturb the species pattern and outcompete rare, i.e. endangered and/or protected, species. But it can be difficult to call a plant a weed, because one and the same plant species may be regarded in some parts of its area as a harmless component of natural vegetation, in others as a weed, and yet in others, even as a useful plant species.

Nonetheless, the ecological literature is replete with recommendations about 'weediness' traits that could or should be measured, although there is no clear understanding of which character or subset of characters can accurately predict the weediness potential of a GM crop. Some of the characters most commonly discussed include seed production, seed dormancy, germination ability, competitiveness and pollen dispersal (Purrington *et al.* 1995).

2.7 Environmental risk assessment of drought-tolerant GM maize

2.7.1 *Principles for environmental risk assessment of GM plants*

EU Directive 2001/18/EC puts in place a step-by-step approval process made on a case-by-case assessment of the risk to human health and the environment before any GMOs can be released into the environment, or placed on the market as, or in, products. While Part B of the Directive deals with the deliberate release of GMOs for any other purpose than for placing on the market, Part C deals with the placing on the market of GMOs. The Directive foresees that in both cases an application must include an environmental risk assessment (ERA). The principles for the ERA are laid down in Annex II.

In the case of GM higher plants, like maize, the ERA should reach conclusions on the following aspects: 1) likelihood of increased persistency in agricultural habitats and increased invasiveness in natural habitats; 2) selective (dis)advantage; 3) potential for gene transfer to related and non-related organisms; 4) potential impacts of interactions with target organisms; 5) potential impacts of interactions with non-target organisms; 6) potential effects on human health; 7) potential effects on animal health; 8) potential effects on biogeochemical cycles, and; 9) potential impacts of the specific cultivation, management and harvesting techniques.

It should further be stressed that the comparative approach constitutes an important element in the ERA strategy. The concept of familiarity therefore plays a pivotal role, based on the fact that most GM plants are developed from crop plants, which have gained a history of safe use, and of which the biology is well researched (OECD, 1993). These crops can serve as a baseline for the ERA of the GM plants.

2.7.2 Points to consider in an ERA of drought-tolerant GM maize in Europe

Particularly for the ERA of an unconfined, large-scale or commercial release of a drought-tolerant GM maize plant, it should be pointed out that modern conventionally bred maize cultivars already exhibit a significantly enhanced tolerance to drought in comparison to maize cultivars that were in use a few decades ago. Put differently, the baseline for comparing drought tolerance of a GM maize plant to that of conventionally bred maize has been constantly evolving during the last decades and will continue to evolve further in the nearby future. Hence, one of the essential issues at stake in the ERA is how much does a GM drought-tolerance trait in maize add on the already existing drought tolerance in modern maize cultivars. As far as known for Europe, this has not led to an persistence in agricultural habitats or invasiveness in natural habitats of modern maize cultivars in comparison to maize cultivars that were in commercial use a few decades ago.

It should further be noted that information on laboratory and greenhouse experiments with drought-tolerant GM maize, the drought stress and other conditions in field trials in the US and the EU, the types of genetic modification and the resulting data on agronomic and potential environmental effects has as yet not been made publicly available. Only in two cases of field trials with drought-tolerant GM maize plants in the EU, information on the genes that were inserted has been made publicly accessible and some of the experimental results have been published in scientific literature (see sections 2.5.1 and 2.5.2).

For an ERA of an unconfined, large-scale or commercial release of a drought-tolerant GM maize plant in Europe, there is a lack of data that allow a comparison of the performance of a drought-tolerant GM maize plant to that of the parental maize plant or a conventionally bred maize cultivar in commercial use under drought stress conditions. Relevant phenotypic parameters in this respect may include grain yield, barrenness (inability to produce viable ears), anthesis-silking interval (ear growth rate), leaf rolling, stomatal conductance (in relation to photosynthetic activity), water-use efficiency (biomass production per unit of transpiration), abscisic acid content in leaf and xylem, root characteristics, seed germination and dormancy, seedling survival and growth, pollen viability, activity of phospoenolpyruvate carboxylase (PEPC), ribulose-1,5-bipophosphate carboxylase oxygenase (Rubisco) and glycolic acid oxydase (GAO) and disease susceptibility.

1. Likelihood of a drought-tolerant GM maize plant becoming more persistent than the parental plant in agricultural habitats or more invasive in natural habitats

There are reasons to assume that a drought-tolerant GM maize plant might be more persistent in agricultural habitats or more invasive in natural habitats than its parental variety. Because the gene(s) inserted for drought tolerance might also affect molecular response mechanisms to other forms of abiotic stress, like cold or salinity, it cannot be excluded that a drought-tolerant GM maize plant’s acquires a potential to survive at low temperatures in the winter and becomes more winter hardy. Consequently, this might lead to an increased potential of a drought-tolerant GM maize plant for persistency in agricultural habitats and invasiveness in natural habitats.

In addition, when the genetic modification targets the ABA metabolism, which also regulates key processes in seeds, like dormancy and accumulation of storage lipids, it cannot be excluded that the seeds of a drought-tolerant GM maize might acquire a changed tolerance to cold. This in turn might result in an increased winter survivability of the seeds of a drought-tolerant GM maize, which could lead to an increased potential for persistency in agricultural habitats and invasiveness in natural habitats.

Though, given the inherent characteristics of maize, the overall likelihood of an increased potential for persistence in agricultural habitats or invasiveness in natural habitats of a drought-tolerant GM maize plant is extremely low.

2. Any selective (dis)advantage conferred to a drought-tolerant GM maize

A drought-tolerance trait confers a selective advantage to the GM maize plant under conditions of drought stress. First, because the drought tolerance trait in a drought-tolerant GM maize is intended to result in a selective advantage under drought stress conditions. Second, as the gene(s) inserted for drought-tolerance might also affect molecular response mechanisms to other forms of abiotic stress, like cold or salinity, it cannot be excluded that a drought-tolerant GM maize plant’s acquires a selective advantage under other abiotic stress conditions, like cold or salinity.

Moreover, there are reasons to assume that a drought tolerance trait confers a selective advantage or disadvantage to a drought-tolerant GM maize under conditions of biotic stress. As there is a potential crosstalk between molecular response mechanisms to abiotic and biotic stress in plants, it cannot be excluded that the genetic modification might affect the molecular response mechanisms of a drought-tolerant GM maize to biotic stress, which could results in either a selective advantage or disadvantage under biotic stress conditions.

3. Potential for gene transfer to the same or other sexually compatible plant species under conditions of planting a drought-tolerant GM maize plant and any selective advantage or disadvantage conferred to those plant species

There are reasons to assume that the potential for gene transfer from a drought-tolerant GM maize plant to sexually compatible plant species, through pollen flow, might be affected. As a drought-tolerance trait in a drought-tolerant GM maize could potentially affect the metabolism of its pollen, it cannot be excluded that this might change the viability of its pollen, and consequently, the dispersal characteristics of its pollen. While there are no sexually compatible weedy or wild relatives of maize present in Europe, there is only a potential for gene transfer from drought-tolerant GM maize to non-GM maize. This could confer a selective advantage to non-GM maize under abiotic stress conditions and/or either a selective advantage or disadvantage for the recipient non-GM maize plants under biotic stress conditions.

4. Potential immediate and/or delayed environmental impact resulting from direct and indirect interactions between a drought-tolerant GM maize plant and target organisms, such as predators, parasitoids, and pathogens

There are no reasons to assume that a drought tolerance trait in a drought-tolerant GM maize will have a direct effect on maize predators, parasitoids, and pathogens. First, because a drought-tolerance trait is not aimed to control maize pests at all, and therefore no target organisms can be defined. Second, as neither the drought-tolerance genes inserted and their products, like plant signalling factors or enzymes, nor the metabolites of these enzymes, like ROS scavengers or osmoprotectants, are known to have an effect on maize predators, parasitoids, and pathogens.

5. Possible immediate and/or delayed environmental impact resulting from direct and indirect interactions of a drought-tolerant GM maize plant with non-target organisms, including impact on population levels of competitors, herbivores, symbionts, parasites and pathogens

There are no reasons to assume that a drought-tolerance trait in a drought-tolerant GM maize plant will have a direct effect on population levels of competitors, herbivores, symbionts, parasites and pathogens. First, because a drought-tolerance trait is not aimed to control maize pests at all, and therefore no non-target organisms can be defined. Second, as neither the drought-tolerance genes inserted and their products, like plant signalling factors or enzymes, nor the metabolites of these enzymes, like ROS scavengers or osmoprotectants, are known to have detrimental effect on population levels of competitors, herbivores, symbionts, parasites and pathogens.

On the other hand, there are reasons to assume that a drought-tolerance trait in a drought-tolerant GM maize plant could have indirect effects on population levels of competitors, herbivores, symbionts, parasites and pathogens. As there is a potential crosstalk between abiotic and biotic stress mechanisms in plants, it cannot be excluded that a drought-tolerant GM maize acquires a changed tolerance to biotic stress, which could result in changed interactions with maize predators, parasitoids, and pathogens, and therefore also in indirect impacts on population levels of organisms that interact with them.

6. Possible immediate and/or delayed effects on human health resulting from potential direct and indirect interaction of a drought-tolerant GM maize plant and persons working with, coming into contact with or in the vicinity of the GM higher plant release(s)

There are no reasons to assume that a drought-tolerant GM maize will have detrimental effects on human health of persons working with, coming into contact with or in the vicinity of a release. Because neither the drought-tolerance genes inserted and their products, like plant signalling factors or enzymes, nor the metabolites of these enzymes, like ROS scavengers or osmoprotectants, are known to have detrimental effects on human health.

7. Possible immediate and/or delayed effects on animal health and consequences for the food/feed chain resulting from consumption of a drought-tolerant GM maize plant any products derived from it, if it is intended to be used as animal feed

There are no reasons to assume that a drought-tolerant GM maize as animal feed will have detrimental effects on animal health. Because neither the drought-tolerance genes inserted and their products, like plant signalling factors or enzymes, nor the metabolites of these enzymes, like ROS scavengers or osmoprotectants, are known to have detrimental effects on animal health.

8. Possible immediate and/or delayed effects on biogeochemical cycles resulting from potential direct or indirect interactions of a drought-tolerant GM maize plant and target and non-target organisms in the vicinity of a drought tolerant GM maize plant release

There are no reasons to assume that incorporation into the soil of root exudates, plant litter, seeds or pollen of a drought-tolerant GM maize will have effects on biogeochemical cycles. First, because horizontal transfer of the inserted genes from a drought-tolerant GM maize to soil microbes is extremely unlikely under natural conditions. Second, because expression of the inserted genes, which are under the control of eukaryotic promoters with very limited, if nay, activity in prokaryotic organisms, is extremely unlikely in soil microbes. Third, because neither the drought-tolerance genes inserted and their products, like plant signalling factors or enzymes, nor the metabolites of these enzymes, like ROS scavengers or osmoprotectants, are known to have effects on soil microbes.

9. Possible immediate and/or delayed, direct and indirect environmental impacts of the specific cultivation, management and harvesting techniques used for a drought-tolerant GM maize plant where these are different from those used for non-GM higher plants

There are no reasons to assume that cultivation of a drought-tolerant GM maize requires different volunteer control measures in agricultural habitats than for its parental variety. Although it cannot be excluded that a drought-tolerant GM maize acquires, due to an increased tolerance to cold, a higher potential for persistency in agricultural habitats than its parental variety, the resulting drought-tolerant GM maize volunteer plants inside agricultural habitats can be controlled by usual volunteer control measures. On the other hand, because it cannot be excluded that a drought-tolerant GM maize acquires a potential for invasiveness in natural habitats, this might require to extend (volunteer) control measures to natural habitats.

There are reasons to assume that cultivation of a drought-tolerant GM maize might require different (chemical) phytosanitary measures than for its parental variety. First, because it cannot be excluded that a drought-tolerant GM maize plant might acquire a changed tolerance to biotic stress caused by maize predators, parasitoids and pathogens. Second, because it cannot be excluded that a drought-tolerant GM maize might result in different population size levels of organisms that interact with maize predators, parasitoids and pathogens.

3. OMEGA-3-FATTY ACID GM SOYBEAN

3.1 Search for alternative sources of omega-3-fatty acids

Over the last twenty years, there is an increasing interest in very long-chain polyunsaturated fatty acids (VLC-PUFAs), particularly the omega-3 group usually found in fish oils, because of their health-beneficial properties with respect to conditions, such as cardiovascular disease, obesity and metabolic syndrome. One major facet underplaying the dietary importance of VLC-PUFAs in human health is the very limited ability of mammals to synthesise these fatty acids de novo themselves (Graham *et al.* 2007).

Whether VLC-PUFAs should be regarded as ‘essential fatty acids’ has become subject of a scientific debate. According to an extensive review by Cunnane (2003), the term ‘essential fatty acid’ is ambiguous and inappropriately inclusive or exclusive of many PUFAs. When applied most rigidly to linoleic acid (LA) and α-linolenic acid (ALA), this term excludes the now well accepted but conditional dietary need for two PUFAs, i.e. arachidonic acid (ARA) and docosahexaenoic acid (DHA), during infancy. In addition, because of the concomitant absence of dietary α-linolenic acid, essential fatty acid deficiency is a seriously flawed model that has probably led to significantly overestimating linoleic acid requirements. Linoleic acid and α-linolenic acid are more rapidly β-oxidised and less easily replaced in tissue lipids than the common ‘non-essential’ fatty acids (palmitic acid, stearic acid and oleic acid). Carbon from linoleic acid and α-linolenic acid is recycled into palmitic acid and cholesterol in amounts frequently exceeding that are used to make long chain polyunsaturated fatty acids. These observations represent several problems with the concept of ‘essential fatty acid’, a term that connotes a more protected and important fatty acid than those which can be made endogenously. The metabolism of essential and non-essential fatty acids is clearly much more interconnected than previously understood. Replacing the term ‘essential fatty acid’ by existing but less biased terminology, i.e. omega-3 and omega-6 PUFAs, or naming the individual fatty acid(s) in question would improve clarity and would potentially promote broader exploitation of the functional and health attributes of PUFAs. Table 5 shows the proposed new classification of the principal PUFAs.

Table 5: Proposed new classification of the principal PUFAs

Pregnancy, lactation, infancy, childhood		Adulthood (> 20 years old)	
Conditionally dispensable	Conditionally indispensable	Conditionally dispensable	Conditionally indispensable
Eicopentaenoate*	Linoleate	Linoleate	α-Linolenate
	α-Linolenate	Eicopentaenoate	
	Arachidonate	Arachidonate	
	Docosahexaenoate	Docosahexaenoate	

* Includes other intermediate PUFAs, i.e. γ-linolenate, dihomo-γ-linolenate, ω6-docosapentaenoate, and other PUFAs < 18 or > 22 carbons long
Source: Cunnane 2003

Whatever the eventual outcome of this scientific debate on the concept of ‘essential fatty acids’, there is now growing concern that current sources of omega-3 VLC PUFAs, predominantly oceanic fish oils, are in serious decline. Moreover environmental contamination of the marine environment has resulted in the presence of potentially toxic substances, such as heavy metals and dioxins, in fish oils. Also, aquaculture fisheries currently rely on wild fisheries as a source of VLC-PUFAs. As a consequence, there is an exhaustive search for an alternative (and sustainable) source of fish oils in human nutrition. To that end, the possibility of using transgenic crop plants engineered to synthesise and accumulate VLC-PUFAs in their storage seed oils has been thoroughly investigated over the past fifteen years (Napier 2007).

An example of such a research effort is the EU-funded Integrated Programme LIPGENE that runs from 2004 to 2009.[10] Its primary focus is to advance understanding of the interaction of nutrients and genotype in the metabolic syndrome, which is the term used to describe a clustering of several risk factors for cardiovascular disease, namely obesity, abnormal blood lipids, e.g. high blood cholesterol, and raised triglyceride levels, insulin resistance and high blood pressure (hypertension). It therefore includes research to understand the manner in which differences in the composition of dietary fats interacts with natural human genetic variation to influence the development of the metabolic syndrome. On the other hand, it aims at creating alternative transgenic plant sources, i.e. linseed, soybean, Indian mustard and oilseed rape, of VLC-PUFAs, by taking genes from marine algae to produce a seed-oil containing the VLC-PUFAs found in fish oil.

Other examples, as reported by Powel (2007), are the development of omega-3 soybean oil by Monsanto that added a transgene to soybean for production of stearidonic acid, which when consumed is converted to eicasopentanoic acid (EPA), and the development of omega-3 soybean oil by Solae (a joint venture between DuPont and Bunge) and Monsanto, which are following a number of approaches for producing fish-oil-like fatty acids.

In December 2006, Pioneer Hi-Bred International (DuPont) has a petitioned the US regulatory authorities to deregulate a omega-3 fatty acid ("high-oleic") producing GM soybean, so that will be allowed to commercialise. While this petition is currently pending, Monsanto's GM soybean producing stearidonic acid is at the 'late development' stage and its commercial launch is projected to take place early in the next decade. Other GM soybeans producing omega-3 oils are at the 'advanced research' stage.

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[10] See http://www.lipgene.tcd.ie/

3.2 Biosynthetic pathways of VLC-PUFAs in transgenic plants

Higher plant species are not capable to synthesise VLC-PUFAs. Hence, the conversion of plant fatty acids such as linoleic acid (LA, 18:2, n-6) and α-linolenic acid (ALA, 18:3, n-3) to VLC-PUFAs requires a minimum of three non-plant native enzymatic reactions to generate omega-6 VLC-PUFAs such as arachidonic acid (ARA, 20:4, n-6), and omega-3 VLC-PUFAs, like eicosapentaenoic acid (EPA, 20:5, n-3) and docosahexaenoic acid (DHA, 22:6, n-3). Table 6 provides an overview of the common, lipid en chemical nomenclature of omega-3-fatty acids.

Table 6: Common, lipid and chemical names of omega-3-fatty acids

Common name	Lipid name	Chemical name
α-Linolenic acid (ALA)	18:3 (n-3)	Octadeca-9,12,15-trienoic acid
Stearidonic acid (SDA)	18:4 (n-3)	Octadeca-6,9,12,15-tetraenoic acid
Eicosatetraenoic acid	20:4 (n-3)	Eicosa-8,11,14,17-tetraenoic acid
Eicosapentaenoic acid (EPA)	20:5 (n-3)	Eicosa-5,8,11,14,17-pentaenoic acid
Docosapentaenoic acid	22:5 (n-3)	Docosa-7,10,13,16,19- pentaenoic acid
Docosahexaenoic acid (DHA)	22:6 (n-3)	Docosa-4,7,10,13,16,19-pentaenoic acid

Source: http://en.wikipedia.org/wiki/List_of_omega-3_fatty_acids

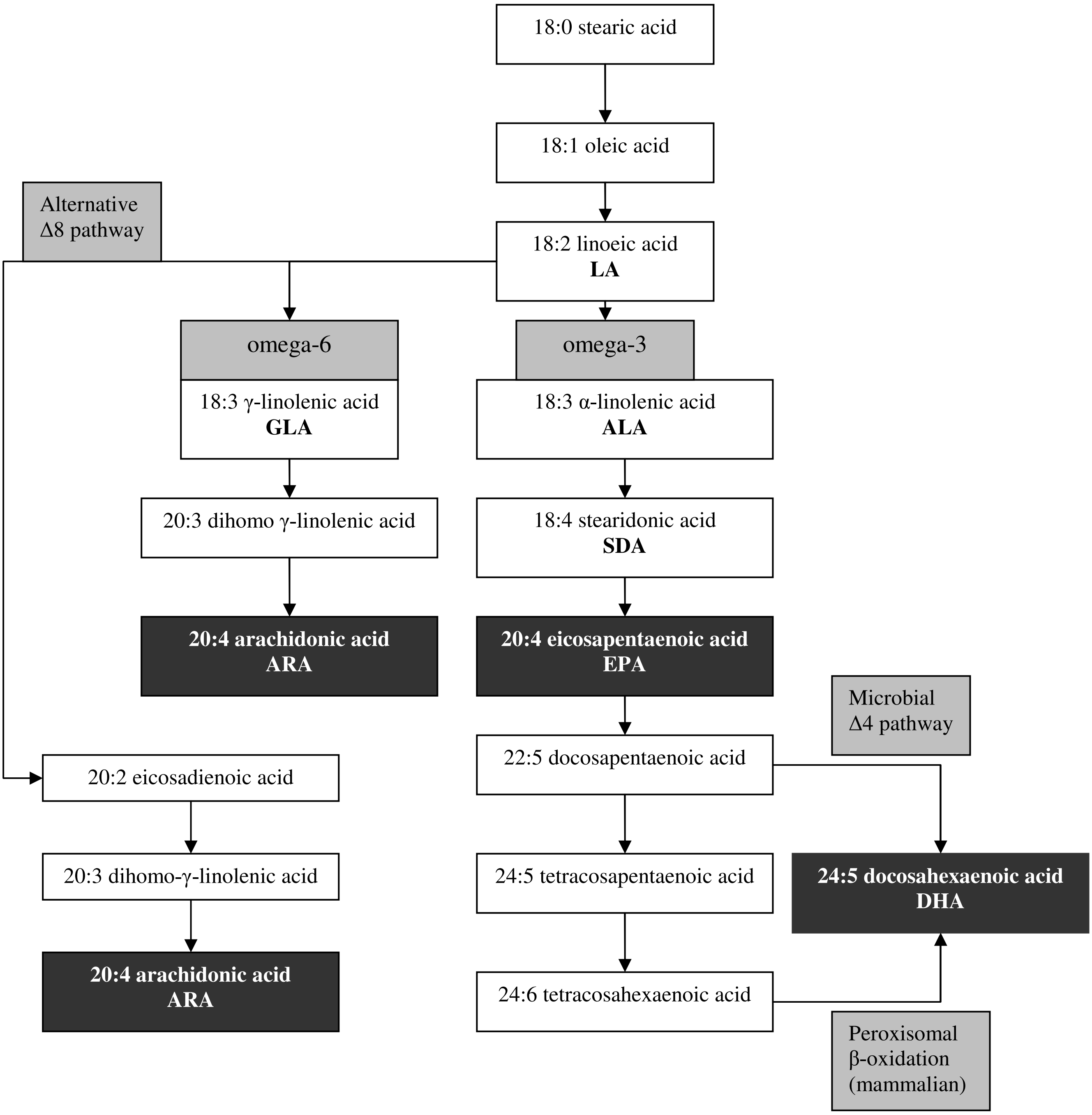
Notably, in the marine food web, microalgae and other organisms, such as thraustochytrids, which are consumed directly or indirectly by fish, are the primary source of omega-3 VLC-PUFAs. Several sets of genes encoding enzymesin the biosynthetic pathways of omega-3 VLC-PUFAs, i.e. desaturases and elongases, have so far been isolated from a number of marine microalgae, thraustochytrids and terrestrial fungi and transferred to several different plants (Robert 2006; Napier 2007).

The possibility of production of VLC-PUFAs in transgenic plants became first clear in 1996, when Reddy *et al.* (1996) transferred a gene encoding a Δ6-desaturase from a cyanobacterium into tobacco plants, resulting in the accumulation of low levels of γ-linolenic acid (GLA) and stearidonic acid (SDA). Much higher accumulation of GLA and SDA (combined Δ6-desaturated fatty acids level of about 20 % of total) was obtained by expression of a gene encoding Δ6-desaturase from *Borago officinalis*, one of the few plants species able to synthesise GLA (Sayanova *et al.* 1997).

Many higher plants synthesise 18:2(n-6) (linoleic acid; LA) and 18:3(n-3) (α-linolenic acid; ALA) in their seed oils but do not produce fatty acids further elongated or desaturated. To make 20:5(n-3) (eicosapentaenoic acid; EPA) or 22:6(n-3) (docosahexaenoic acid; DHA), addition of up to two elongation and three destauration activities are required. These enzymes can also act on omega-6 precursors and produce the long-chain omega-6 fatty acid 20:4(n-6) (arachidonic acid; ARA). To this end, Abbadi *et al.* (2004) transferred a Δ6 desaturase and a Δ5 desaturase from the diatom *Phaeodactylum tricornutum*, as well as a Δ6 elongase from the fungus *Physcomitrella patens* into flax in order to produce EPA. They were able to produce low but significant levels of AA (1.5 %) and EPA (1.0 %) in the seed, presumably by the action of the inserted transgenes on the precursor fatty acids LA and ALA, respectively. The reason for the low levels observed was probably that the algal desaturases use phosphatidylcholine (PC)-linked fatty acids as substrates, while elongation occurs on fatty acids esterified to coenzyme A (CoA). In flax, the seed triacylglycerols (TAGs) are rich in ALA and, to a lesser extent, in LA. The authors therefore suggest that LA and ALA are shunted efficiently into the acyl-CoA pool, by the action of a presumptive acyltransferase

(AT), prior to incorporation into TAG. This would make only low levels available in the PC pool for the first desaturation step by the introduced Δ6 desaturase and subsequently reduce the total yield of EPA and ARA. Moreover, the authors speculate that ATs that can efficiently transfer 18:3(n-6) and 18:4(n-3) from the PC pool to the CoA pool for a subsequent elongation and ATs that can transfer 20:3(n-6) and 20:4(n-3) from the CoA pool to the PC pool for a subsequent Δ5 desaturation do not exist in flax and the endogenous ATs may have a low affinity for these substrates. Figure 4 provides a schematic overview of various routes for VLC-PUFA biosynthesis in GM plants.

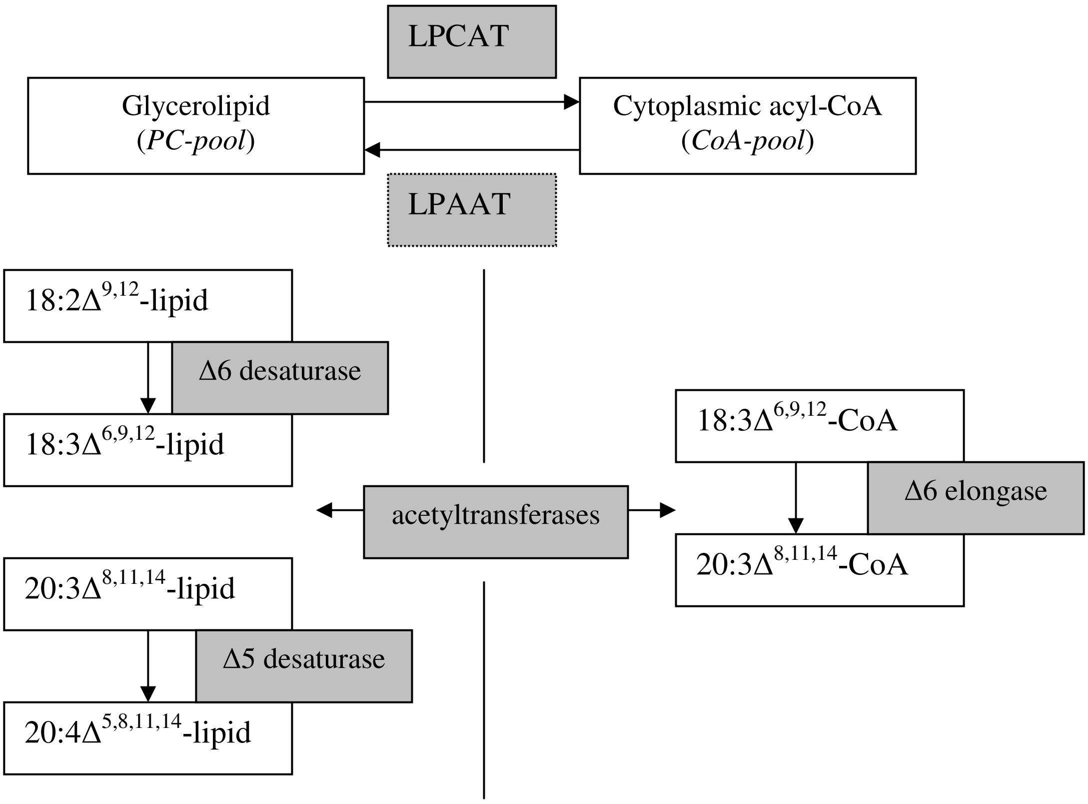
Figure 4: Schematic representation of VLC-PUFA biosynthesis in GM plants



Source: Napier 2007

According to Napier (2007), biochemical analyses of transgenic flax lines revealed several constraints that were preventing the synthesis of EPA. First, there appears to be limited acyl exchange from the PC pool of Δ6 desaturation into the CoA pool, as determined by an absence of Δ6 desaturated fatty acids in the CoA pool, but their abundance in the PC pool. Fatty acid elongation requires acyl-CoA substrates, and it is therefore essential to have efficient acyl exhange between the PC pool and the CoA pool to maintain a suitable flux of substrates for elongation. This represent a generic bottleneck in VLC-PUFA biosynthesis in transgenic plants, which has been described as 'substrate-dichotomy' because the two key enzyme activities require different acyl substrates. Figure 5 provides a schematic representation of this substrate-dichotomy.

Figure 5: Schematic representation of substrate-dichotomy



Non-animal fatty acid desaturation uses glycerolipid-linked substrates, whereas fatty acid elongation requires acyl-CoA substrates. The exchange of the fatty acids between phospholipids and the acyl-CoA pool is an enzyme mediated process (via acyltransferases). Non-native fatty acids, i.e. the products of transgenic VLC-PUFA activities, may not be efficiently exchanged bwteen these two metabolically active pools. (LPCAT= acyl-CoA:lyso-phosphatidylcholine acyltransferase; LPAAT= lipid-phosphatidylcholine acyltransferase).
Source: Napier 2007

In addition to this generic problem, detailed analysis of lipid species present in the transgenic flax lines also indicated the likelihood of species-specific factors that limit the potential to synthesise EPA in flax. On the one hand, the presence of a strong acyl-CoA-independent phospholipid:diacylglycerol acyltransferase (PDAT) activity channels fatty acids directly from their of desaturation on PC into triacylglycerol (TAG), compartmentalising them way from VLC-PUFA biosynthesis. On the other hand, flax probably lacks endogenous acyl-exchange activities that recognise non-native fatty acids as substrates. It is unlikely that this represents a generic problem, as other studies demonstrated strong influence of host plant species on the successful synthesis of ARA and EPA, presumable through some native activities that can overcome the substrate-dichotomy.

For example, Kinney *et al*. (2004) were successful in producing high levels of VLC-PUFA (19.6 % EPA, 2 – 3.3 % DHA) in soybean seed and embryos. This research group from DuPont transferred multiple genes, predominantly from fungi, to achieve EPA synthesis in soybean seed and DHA synthesis in regenerated soybean embryos. According to the patent description, the researchers used genes encoding Δ4 desaturase from *Schizochytrium aggregatum*, Δ5 desaturase and Δ6 desaturase from *Mortierella alpina*, Δ6 desaturase from *Saprolegnia diclina*, Δ15 desaturase from *Arabidopsis thaliana*, Δ17 from desaturase from *Saprolegnia diclina*, elongase from *Thraustochytrium aureum* and elongase *Mortierella alpina*. Noteworthy, the Δ17 desaturase from *Saprolegnia diclina* appears to be able to shunt the majority of biosynthesis in the omega-6 pathway over to EPA in the omega-3 pathway. In this way omega-3 fatty acids production was increased at the expense of ARA production and in spite of the lack of selectivity of other saturases and elongases used for omega-3 fatty acids as substrates.

In another attempt to circumvent the problems with acyl shuttling between PC and CoA pools, Robert *et al*. (2005) applied fatty acid desaturases that act on acyl moieties coupled to CoA. In addition, the aim was to go one biochemical step further and produce DHA in seeds. To this end, they transferred four genes into *Arabidopsis thaliana*, each under the control of the seed specific promoter from the *Napin* gene. These were a dual activity Δ5/Δ6 desaturase from the zebrafish *Danio rerio*, an elongase with Δ6 activity from the nematode *Caenorhabditis elegans*, and a Δ5 elongase and Δ4 desaturase from the prymnesiophyte *Pavlova salina*. Low levels of ARA (1.2 %), EPA (2.5 %) and DHA (0.5 %) were observed. This led to the speculation that the multiple use of the same promoter as well as the possible low level of 18:3(n-3) available for Δ6 desaturation contributes to low gene expression levels and LC-PUFA biosynthesis, respectively.

In yet another attempt to engineer plants for the synthesis of DHA, the results were also modest, with a level of 1.5 % in Indian mustard (Wu *et al*. 2005).

Given these modest results, Napier (2007) argues that this represents some of the most complex plant genetic engineering yet attempted, with five primary biosynthetic activities and several secondary enhancing ones, encoded by up to nine transgenes on two separate T-DNAs (Robert *et al*. 2005). It is also probable that the additional elongation and desaturation steps required to convert EPA to DHA represent an additional potential substrate-dichotomy bottleneck. Since both transgenic soy and Indian mustard efficiently accumulate EPA but not DHA, it seems likely that endogenous acyl-exchange activities are unable to mediate the second step, i.e. EPA to DHA. In that respect, it may be that even more additional transgenes encoding acyltransferases from VLC-PUFA accumulating organisms will be required to enhance accumulation of DHA in transgenic oilseed crops.

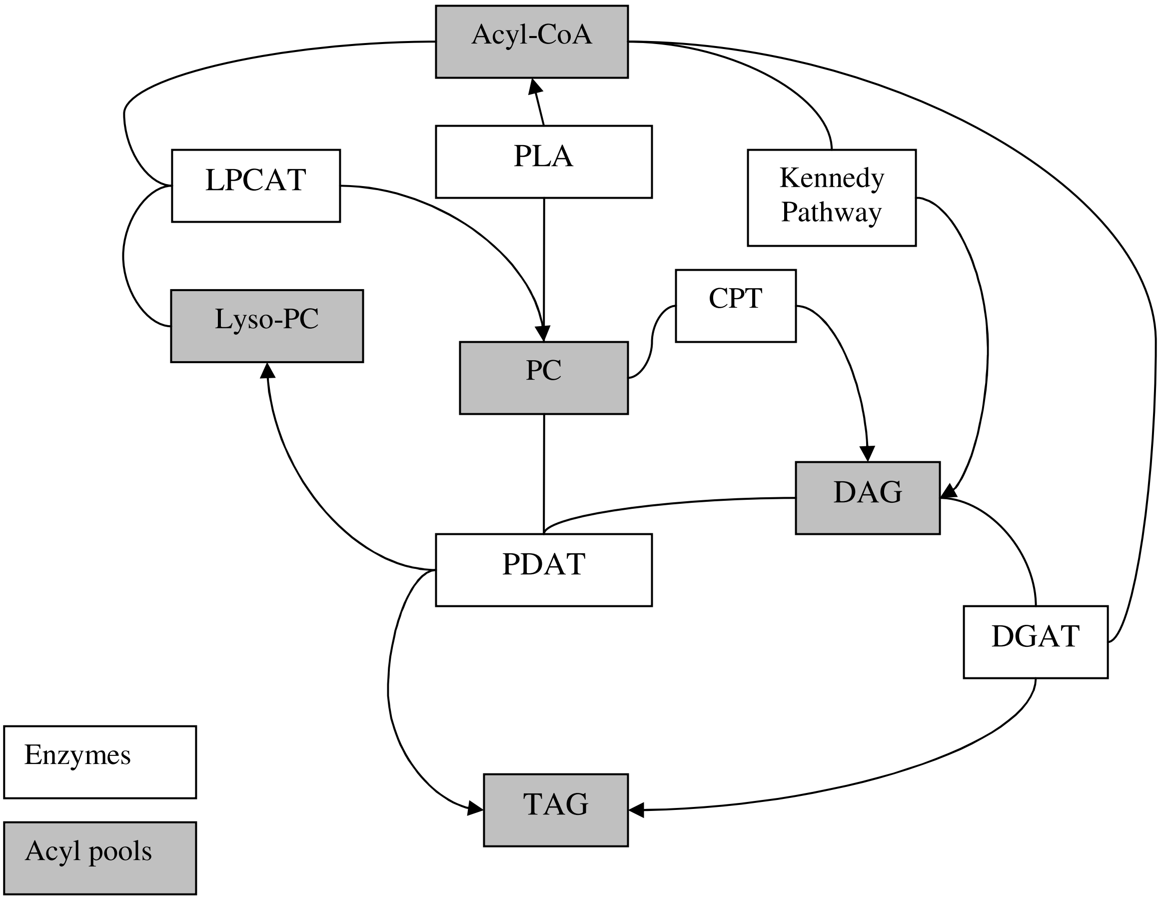
An interesting variation to the Δ6 pathway for ARA and EPA biosynthesis is the so-called Δ8 pathway, which has been reported in a taxonomically diverse range of organisms (see Figure 4). Qi *et al*. (2004) constitutively expressed an Δ9 elongase gene from the microalga *Isochrysis galbana* and two Δ8 desaturase genes – one from the microalga *Euglena gracilis* and one from the fungus *Morteirella alpine* – in *Arabidopsis thaliana*. This resulted in the production of ARA to a level of 6.6 % of total fatty acid content and EPA to a level of 3 %, albeit in the leaf tissue. Besides showing that the coupling of VLC-PUFA biosynthesis with the plant's endogenous fatty acid biosynthesis was indeed possible, this study also demonstrated for the first time that there exists an 'alternate' order, in which VLC-PUFA production by desaturation and elongation can occur.

The Δ8 pathway might potentially be more effective in the synthesis of ARA and EPA due to a reduced requirement for acyl exchange between the PC pool and CoA pool. Specifically, the first committed step in the Δ8 pathway is the C_2 elongation of endogenous LA and ALA in the CoA pool. In 2006 it was observed that these C_{20} elongation products (20:2Δ11,14 and 20:3Δ11,14,17) accumulate to very high levels in the CoA pool of transgenic *Arabidopsis thaliana* expressing the Δ8 pathway, indicating the inefficient transfer of the non-native fatty acids out of the CoA pool (Sayanova *et al.* 2006). According to Napier (2007), it will be important to determine whether this acyl exchange bottleneck is species-dependent, analogous to the situation in flax.

Although progress has been made in developing transgenic plants that produce VLC-PUFAs, considerable refinements are still required to generate a real substitute for fish oils, particularly because fish oils are not only rich in EPA and/or DHA, but they are also devoid of omega-6 fatty acids (Napier 2007). It is therefore desirable to ensure conversion of such omega-6 fatty acids, like GLA and ARA, to their omega-3 counterparts. Some success has been achieved through the use of omega-3 desaturases, for example, from the EPA-rich fungus *Saprolegnia diclina* (Pereira *et al.* 2004), the fungus *Phytophtera infestans* (Wu *et al.* 2005), or *Acanthamoeba castellanii* (Sayanova *et al.* 2006). However, the complete omega-3-desaturation of omega-6 fatty acids is hampered by the acyl channelling of potential substrates away from metabolically active pools and into TAG. Maintaining a continuous flux of substrates through the VLC-PUFA biosynthetic pathway(s) without significant loss to TAG constitutes a major challenge (Singh *et al.* 2005; Cahoon *et al.* 2007). Napier (2007) argues that this is obvious even in transgenic soy seeds that accumulate EPA to high levels, as these transgenic soy seeds also contain high levels of GLA and dihomo-GLA, presumably due to channelling of these fatty acids to TAG (Kinney *et al.* 2004). Similar loss of potential substrates was also observed by Wu *et al.* (2005) in transgenic Indian mustard, despite the utilisation of a transgene encoding an omega-3 desaturase.

It is not obvious how to solve this bottleneck, because it is likely that such channelling represents the sum of multiple different acyl exchange activities and because it is likely that each plant species has a different ratio of these activities. Figure 6 provides a schematic overview of routes involving various acyl exchange enzymes. One possibility may be through the use of desaturases with a preference for omega-3 substrates, such as have been identified from the *Primula* and *Echium* species (García-Maroto *et al.* 2006; Sayanova *et al.* 2006a). Another possibility may be to use transgenes encoding enzymes involved in acyl exchange and/or lipid channelling, such as phospholipids:diacylglyceroltransferase (PDAT) and diacylglycerol:acyltransferase (DGAT), which are involved in the biosynthesis of triaclyglycerol (TAG) and acyl-CoA:lyso-phosphatidylcholine acyltransferases (LPCATs) (Jako *et al.* 2001; Furukawa-Stoffer *et al.* 2003; Shockey *et al.*, 2006; Beermann *et al.* 2007; Cahoon *et al.* 2007; Graham *et al.* 2007). Yet, the results of these approaches have thus far been modest, as the amounts of VLC-PUFAs in transgenic oilseeds are not yet economically relevant. According to Cahoon *et al.* (2007), there is still a need to advance understanding of factors that mediate fatty acid flux between the PC, CoA and TAG pools.

Figure 6: Routes involving various acyl exhange enzymes



Abbreviations: LPCAT = acyl-CoA:lyso-phosphatidylcholine acyltransferase; PLA = phospholipase; DGAT = diacylglycerol:acyltransferase; PDAT = phospolipids:diacylglyceroltransferase: CPT = cytidine-diphosphate-choline:1,2-diacylglycerol cholinephosphotransferase; PC = phospatidylcholine; acyl-CoA = acyl-coenzyme A; DAG = diacylglycerol; TAG = triacylglycerol

Source: Singh et al. 2005

3.3 The biology of soybean

Soybean is commonly considered one of the oldest cultivated crops, native to North and Central China (OECD 2000). It is grown as a commercial crop in over 35 countries. The major producers of soybeans are the US, China, Democratic People's Republic of Korea and Republic of Korea, Argentina and Brazil. Soybean is grown primarily for the production of seed, has many uses in the food, feed and industrial sectors, and represents one of the major sources of edible vegetable oil and of proteins for livestock feed use.

Soybean is considered a self-fertilising species, propagated commercially by seed. Artificial hybridisation is used for cultivar breeding (OECD 2000), while genetic variability is very limited in soybean. Cross fertilisation is usually less than one percent. A soybean plant can produce as many as 400 pods, with 2 to 20 pods at a single node. Each pod contains 1 – 5 seeds. Neither the seedpod, nor the seed, has morphological characteristics that would encourage animal transportation.

None of the soybean varieties are frost tolerant, and they do not survive freezing winter conditions. Cultivated soybean seed rarely displays any dormancy characteristics and only under certain environmental conditions grows as a volunteer in the year following cultivation. If this should occur, volunteers do not compete well with the succeeding crop, and can be easily controlled mechanically or chemically. The soybean plant is not weedy in character. In North America, soybean is not found outside of cultivation. In managed ecosystems, soybean does not effectively compete with other cultivated plants or primary colonisers.
Moreover, soybean can only cross with other members of *Glycine* subgenus *Soja*. The potential for such gene flow is limited by geographic isolation. Wild soybean species are endemic in China, Korea, Japan, Taiwan and Russia. These wild soybean species are not naturalised in North America, and although they could occasionally be grown in research plots, there are no reports of their escape from such plots to unmanaged habitats.

According to Eckert *et al*. (2006), soybean is an ideal target for the production of novel fatty acids, including VLC-PUFAs, for a number of reasons. First, in soybean, unlike oilseed rape, a sufficient pool of linoleic acid (LA) is present in the oil. As a consequence, $\Delta 6$ desaturase activity alone is required for significant accumulation of γ-linolenic acid (GLA). Second, biological nitrogen fixation alleviates input costs associated with the production of the crop. Third, soybean meal, the protein content of the seed, is highly valued in feed supplements and does not contain the toxic glucosinolates found in trace amounts in other oil seeds, such as canola and rape seed, and typically soybean meal will command a higher price.

3.4 Examples of omega-3 fatty acid GM soybeans in scientific literature

A paper by Buhr *et al.* (2002) presents the results of experiments conducted by researchers from the University of Nebraska-Lincoln and DuPont with ribozyme termination of RNA transcripts to down-regulate seed saturated fatty acid genes in GM soybean. The aim was to investigate whether termination of transcripts with a self-cleaving ribozyme can serve as a tool to decrease specific plant gene expression. Here the soybean embryo-specific Δ-12 fatty acid desaturase *FAD2-1* gene was targeted, because its down-regulation elevates oleic acid of soybean seed storage lipids. Two ribozyme-terminated antisense constructs appeared to be capable of gene down-regulation, producing 57 % oleic acid compared with less than 18 % in the parental-type seed. In addition, ribozyme termination cassettes were also constructed to evaluate sense transcripts for single gene down-regulation and the simultaneous down-regulation of two soybean embryo-specific genes using a single promoter. Eight independent soybean transformants were screened that harboured a standard plus sense or ribozyme terminated *FAD2-1* cassette. Two of the eight ribozyme terminated transformants displayed oleic acids levels in the soybean seed storage lipids over 75 %, while none of the standard plus sense *FAD2-1* lines showed elevated oleic acid phenotypes. Also, the effects of dual constructs targeted at *FAD2-1* and the *FatB* gene encoding a palmitoyl-thioesterase were evaluated. Five GM soybean lines with the dual constructs had oleic acid levels of about 85% and saturated fatty acid levels of less than 6 %.

DuPont's focus has been on maximising both EPA and DHA in GM soybean (Daniells 2006). Its scientists have expanded the standard procedure of desaturating and elongating the shorter chain fatty acids by using co-expression of an omega-3 microsomal desaturase from the fungus *Sapgrolegnia diclinia*, to convert the omega-6 VLC-PUFAs into omega-3 VLC-PUFAs. They have achieved to raise the content of omega-3 VLC-PUFAs up to 40 % of the total fatty acid content in GM soybean seeds (Kinney *et al.* 2006). Furthermore, DuPont's scientist have also started to explore the use of bifunctional Δ12/ω3 fatty acid desaturases from *Fusarium moniliforme*, *Fusarium graminearum* and *Magnaporthe grisea* for improving the ratio of omega-3 to omega-6 fatty acids in both soybean and the oleaginous yeast *Yarrowia lipolytica* (Damude *et al.* 2006).

Another paper (Eckert *et al.* 2006) describes experiments by a consortium consisting of researchers from the University of Nebraska-Lincoln, USDA-ARS Plant Genetics Research Unit and DuPont, who transferred Δ15 fatty acid desaturase from *Borago officinalis* and a Δ6 fatty acid desaturase encoded by *FAD3* from *Arabidopsis thaliana*, both under the control of soybean seed-specific β-conglycinin promoter, into soybean; it is likely that field trials with the resulting GM soybean have been started in 2007. Notably, this paper also indicates that field studies on GM soybean event 420-5 with a borage Δ6 fatty acid desaturases were being conducted across multiple environments. This GM soybean produces GLA and SDA at levels from 24 – 30 % and 2 – 4 %, respectively, depending on the environment in which the seeds were harvested (Sato *et al.* 2004).

Powel (2007) reported about the development of an omega-3 fatty acid GM soybean by Monsanto. More specifically, this GM soybean produces stearidonic acid (SDA), which when consumed is converted to eicasopentanoic acid (EPA). Its commercial launch is projected to take place early in the next decade. It is conceivable but not certain that Monsanto uses transgenes encoding Δ6 and Δ12 fatty acid desaturases isolated from the fungus *Mortiella alpina*, probably under control of a *napin* promoter isolated from oilseed rape – a strategy that previously resulted in an increase of SDA up to 23 % of the total fatty acid content in seeds of transgenic oilseed rape (Knutzon *et al*. 2002; Ursin 2003).

3.5 Field trials with omega-3 fatty acids producing GM soybeans

According to the US field trial data base, 187 field trials with GM soybeans with an altered fatty acid profile have been notified between 1993 and 2007. In about 110 notifications the origin of the (trans)genes is treated as confidential business information (CBI), with a description of the phenotype of the GM soybean in terms, like ‘modified oil (quality)’, ’altered oil (profile)’, ‘lipid (profile) altered or ‘modified seed’, and in few cases combined with terms like ‘herbicide tolerance’, ‘increased yield’, ‘pathogen resistance’, ‘abiotic/drought/cold/salt (stress) tolerance’, ‘shade tolerance’, ‘nitrogen utilisation efficiency increased’. These notifications may or may not concern field trials with omega-3 fatty acid producing GM soybeans.

Table 7 gives an overview of the notifications with a description of the phenotype specifically with the term ‘omega-3 fatty acids produced’ or with a description of the transgenes/origin in terms, like ‘(fatty acid) desaturase/*Borago officinalis*’ and ‘(fatty acid) desaturase (*FAD3*)/*Arabidopsis thaliana*’. In these cases there is a high degree of certainty that the notifications concern omega-3 fatty acids producing GM soybeans.

Table 7: Field trials with omega-3 fatty acid GM soybean in the US

Notifier	**Omega-3 fatty acids**	**Denied or withdrawn**
Pioneer Hi-Bred	5	
Monsanto	5	2
University Nebraska	4	

Source: http://www.isb.vt.edu/cfdocs/fieldtests1.cfm/

Notably, 4 of the 5 notifications from Pioneer Hi-Bred (Dupont) concern GM soybean with ‘altered fatty acid profile’ combined with a series other GM traits, including ‘yield increased’, ‘herbicide tolerance’, ‘insect resistance’, ‘pathogen resistance’, ‘carbohydrate metabolism altered’, flavinoid level altered, ‘protein quality altered’ and/or ‘phytate reduced’. In these 4 cases an Environmental Assessment (EA) has been conducted, but these EAs have not been made publicly accessible. In all other cases listed in Table 7 an EA was not required. Information on laboratory and greenhouse experiments with omega-3 fatty acids GM soybean, the conditions in field trials in the US, the types of genetic modification and the resulting data on agronomic performance and potential environmental effects has thus as yet not been made publicly available.

Furthermore, on 20 December 2006 Pioneer Hi-Bred International (DuPont) petitioned the US regulatory authorities to ‘deregulate’ an omega-3 fatty acid (high-oleic) producing GM soybean.[11] This petition is currently pending. If the US regulatory authorities give their consent, it will be allowed to commercialise this high-oleic acid GM soybean in the US. Notably, the petition document with information on laboratory and greenhouse experiments with this omega-3 fatty acid GM soybean, the genetic modification, agronomic performance and potential environmental effects, is as yet not publicly accessible.

It should also be noted that in 1997 the US regulatory authorities have already granted a petition for determination of non-regulated status of a high-oleic acid GM soybean to DuPont

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[11] See http://www.aphis.usda.gov/biotechnology/status.shtml

(see paragraphs 3.6.1 and 3.6.2), although in this case, in contrast to the recent petition from Pioneer Hi-Bred International (a subsidiary of DuPont), the term ‘omega-3 fatty acid producing’ was not specifically used. However, this high-oleic acid GM soybean has never been commercialised.[12]

In the EU field trials with omega-3 fatty acid producing GM soybean have thus far not been conducted, while applications for import, food and feed processing and/or cultivation of omega-3 fatty acid producing GM soybean have neither been submitted. [13]

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[12] See http://www.biotradestatus.com/

[13] See http://gmoinfo.jrc.it/

3.6 Views on potential environmental risks of omega-3 fatty acids GM soybean

3.6.1 Views of the US regulatory authorities

In 1997 the US regulatory authorities received a petition for determination of non-regulated status of a high-oleic acid GM soybean from DuPont. Though, the term 'omega-3 fatty acid producing' was not used here. In this case the omega-6 fatty acid content of soybean was reduced and oleic acid was increased by transformation with the *GmFad2-1* gene from soybean, which encodes a Δ12 desaturase. The *GmFad2-1* gene was put under control of the soybean seed-specific β-conglycinin promoter. It caused a coordinate silencing (or sense suppression) of itself and the endogenous *GmFad2-1*, resulting in a GM soybean whose oil has an oleic acid content that exceeded 80 %, while conventionally bred soybean have an oleic acid content of 24 % . A second, constitutive *GmFad2-1* gene was also inserted, which was expressed in all tissues of the GM soybean plant, where it suppressed endogenous *GmFad2-1*, resulting in increased oleic acid content only in the seed.

Some indirect effects on seed fatty acid in the GM soybean were observed, like a very low content of linoleic acid and significantly lower linolenic acid and palmitic acid contents compared to the parental soybean line. Pleiotropic effects on seed storage proteins were also noted. In the GM soybean the concentrations of β-conglycinin α and α' were reduced and replaced with glycinin subunits as a result of silencing of the α and α' subunit genes mediated by the β-conglycinin promoter. But it was anticipated that increasing the content of glycinin subunits and decreasing the content of β-conglycinin subunits will improve the functionality of soy proteins in various foods.

The US regulatory authorities further noted that the parental plant, i.e. conventional soybean (*Glycine max*), does not show any weediness characteristics. Only the nearest wild relative of soybean, i.e. *Glycine soja*, is listed as a common weed in Japan, but it is there neither a harmful weed on cultivated land, nor on pastures and meadows. In addition, *Glycine gracilis*, known from Northeast China, is not listed as a weed.

Moreover, the high oleic acid production trait in the GM soybean was considered unlikely to cause or increase weediness, as there would have to be selection pressure. In addition, data submitted by DuPont showed no significant differences between the mean seedling emergence rates for the GM soybean lines and the parental variety, while the data also showed no volunteers from the GM soybean seed, re-growth from stubble, or increase in seed dormancy.

Since there are no relatives of cultivated soybean in the continental US, although some wild perennial species in US territories in the Pacific may be found, and soybean is almost exclusively a self-fertilising plant, the US regulatory authorise considered the formation of hybrids between the GM soybean lines and *Glycine* species in nature, is considered highly unlikely. Even if cross-pollination between the GM soybean lines with wild or cultivated soybeans would occur, there would still be no significant impacts, because any potential effect of the high oleic acid content trait would not alter the weediness potential of the resulting hybrids.

Finally, the US regulatory authorities had no reason to believe that deleterious effects on non-target organisms, including beneficial organisms such as bees and earthworms, would result from the cultivation of the GM soybean lines. The enzyme, the genes inserted and oleic acid in the GM soybean are normally present in soybean and are not known to have any toxic properties, while field observations of the GM soybean lines confirmed the absence of effects on non-target organisms. Also, the levels of anti-nutritional factors normally present in conventionally bred soybean, such as trypsin inhibitors, phytic acid and the oligosacchrides raffinose and stachyose, were similar in the GM soybean lines.

Based on these considerations, DuPont received a determination of non-regulated status of the high-oleic acid GM soybean from the US regulatory authorities. However, as noted above in section 3.5, this GM soybean has never been commercialised.

3.6.2 Views of the US National Research Council

In 2002 the US National Research Council issued a report on the environmental effects of transgenic plants, which was drafted by its Committee on Environmental Impacts Associated with Commercialization of Transgenic Plants (NRC, 2002). This committee examined the similarities and differences between crops developed by conventional and transgenic methods, the potential for commercialised transgenic crops to change both agricultural and non-agricultural landscapes and how well the US government had been regulating transgenic crops to avoid any negative effects.

In one section of the 2002 NRC report, the committee focused specifically on the environmental assessment of DuPont's high oleic acid GM soybean by the US regulatory authorities (see paragraph 3.5.1). Notably, the 2002 NRC report pointed out that there were little experimental data in the environmental assessment to allow an independent critique. It was also argued that some environmental questions had not been sufficiently addressed by the US regulatory authorities.

For example, since temperate plant species, like soybean, tend to have a higher proportion of polyunsaturated fatty acids in the seed oil than do more tropical or subtropical crops, the NRC committee referred to studies that had investigated the effects of fatty acid profile changes on the degree of cold tolerance in plants. It cited papers by Kodama *et al.* (1994, 1995), which noted that transgenic tobacco plants with increased amounts of polyunsaturated fatty acids had an alleviation of cold-induced growth suppression. If polyunsaturated fatty acids help the seed survive cold winters, perhaps the GM soybean, with such a deficit of polyunsaturated fatty acids, would have reduced winter survival capacity. According to the NRC report, the data required to address this question might have been considered but were not included in the decision document. The US regulatory authorities reported that DuPont provided evidence on overwintering but without any elaboration. Possibly, the GM soybean suffered more winterkill than the non-GM soybean. But since this segment of the environmental assessment dealt with increased weediness potential, that fact might not have been considered important. Also, this question might have been considered irrelevant by the US regulatory authorities; that is, reduced overwintering potential of the GM soybean would represent a reduced ecological fitness and hence no increased threat. The NRC report considered it unlikely that the GM soybean had acquired a changed overwintering capacity because the seed oil was modified and not the plasma membrane lipids, which are more relevant for cold tolerance. The US regulatory authorities might have been aware of this fact and therefore did not think it necessary to mention. But by not mentioning the possibility, the US regulatory authorities left itself open to the charge that they had overlooked it, according to the NRC report.

The NRC report further pointed at the presence in the market of non-GM soybean varieties with similar high oleic acid attributes. In this context, it was argued that the environmental risk potential and differences between such conventional soybeans and the GM varieties are uncertain, not because of incomplete information and regulatory scrutiny of the GM lines, but because of almost complete lack of information on the conventional lines, which manifest an unstable increase in oleic acid in the seed oil. The genetic mechanisms by which this is achieved is not known. They could be a result of genetic changes in the soybean genome, due to mutagenesis that may have altered the amount of DNA, either by destroying portions of the genome or by causing a duplication (perfect or imperfect) of portions of the genome. According to the NRC report, it is therefore likely that several genes were altered, not just those regulating oleic acid content. Some of the altered genes may relate to environmental fitness, production of anti-nutritional factors, or other undesirable consequences. But the US regulatory authorities did not assess these new, conventionally bred cultivars, as the trigger for regulatory oversight is the use of recombinant-DNA breeding methods. Yet, the NRC report did not suggest that new crop varieties developed by solely conventional breeding should be regulated as stringently as GM varieties, because real damage to the environment of conventionally bred crops is rare. But it wished to make the point that knowledge of the genetic changes in the GM varieties allowed more confident and reliable predictions of environmental effects than conventional cultivars with high oleic acid content.

Overall, the NRC report concluded that there is no indication that the risks associated with the high oleic acid GM soybean lines differ in any material way from those of the same species with similar but non-GM attributes.

3.6.3 ISB Workshop 2002

In 2002 the Information Systems for Biotechnology (ISB) at the Virginia Polytechnic Institute and State University organised a two-day workshop for regulators and industry and academic scientists to discuss and evaluate current knowledge and research on secondary effects of transgenes for stress tolerance or novel metabolic pathways in crops (ISB 2002).

At this workshop, one group focussed on two cases of ‘oil modification’ that had already been deregulated, i.e. high oleic GM soybean and high laurate GM canola, and also discussed in general future oil modifications. Given that most modifications to plants will not increase total oil levels dramatically, this group emphasised that plants engineered with changes in oil metabolism were unlikely to have altered fitness characteristics. This group had no reason to believe that modified oils will be inherently more toxic to non-target organisms. In anticipating new risks, it was considered important to consider promoters. It was expected that new genes will always be under control of seed specific promoters. Nonetheless, as new plants engineered for modified oil content were progressing into large scale field testing toward commercialisation, changes in seed parameters that may have effects on fitness should be examined. These parameters include: 1) duration of seed production; 2) seed dormancy; 3) seed germination, and; 4) seed emergence under various conditions.

The group also identified several areas of research that might produce information useful in helping to asses the risks of plants intended for commercialisation:

- Effects of distances and other parameters on pollination frequencies for various crops – such data would be helpful in validating current isolation distances required for various crops engineered with various categories of transgenes.

- Baseline data for ecological studies – this type of data is necessary to interpret ecological changes that may be detected during field testing. Such data would help to define normal ranges in agricultural settings and would be useful in determining whether such changes are beneficial, neutral or deleterious.
- Transcript profiling – appears to be very useful as a tool for academic research, but the group did not see an immediate application as a screen for safety.

A recurring theme from this and the other breakout groups (altered flowering, cold tolerance, disease resistance, altered ripening and lignin modification) was that phenotypes and not specific genes are ultimately the relevant criteria for environmental risk assessment of field testing and commercialisation. Although changes in metabolism, signalling, or transcription may in turn bring about additional changes in gene expression or metabolic profiles, specific information about those changes is less important than the translation of those changes into relevant phenotypes, such as those influencing flowering, pollen biology, or persistence properties. The potential of these new genes for more broad-reaching effects should stimulate to look beyond the primary expected phenotype when establishing regulatory conditions for field trials and commercialisation.

3.6.4 Altered fatty acid profile and survivability

A paper by Linder *et al.* (1995) describes field and greenhouse experiments that were designed to assess the risk that seed-oil modification (high stearic acid content) transgenes will increase the persistence of feral oilseed rape (*Brassica napus*) and interspecific hybrids between *B. napus* and *B. rapa*, a weedy relative. The rationale behind these experiments was that seed-oil-modification (trans)genes are particularly likely to affect seedling performance, as seed oils in many angiosperms are critically important to dormant seeds and to establishing seedlings prior to initiation of photosynthesis because no other energy or carbon sources are available. Genetically modified oil content may therefore cause altered mobilisation and metabolisation of seed oils, changing the proportion of seeds surviving in the soil, the proportion of seeds emerging following germination, the timing of emergence and seedling vigour.

At two different sites, it was tested in the field whether buried seeds of transgenic high-stereate canola had increased survivability and dormancy compared to non-transgenic canola seeds. At one site, in California, no differences in initial proportions of dormant seeds and rates of exit could be detected, suggesting low probability that high-stearate canola will form larger or more persistent seeds banks than its non-persistent controls. At the other site, in Georgia, it was found that although high-stearate canola initially had as low or lower proportions of dormant seeds than its controls, high-stearate seed exhibited no detectable exit from the seed bank, whereas the controls had significant rates of exit. It was therefore concluded that escaped high-stearate seed may persist for a longer period than its controls at this site, while the differences between the sites highlighted the need to conduct risk assessment over the range where a transgenic crop will be commercialised. Furthermore, the greenhouse studies measured the relative ability of transgenic high-laurate canola and wild x crop hybrids to emerge from four depths in the soil and their subsequent seedling vigour. The results were that transgenic high-laurate canola's total emergence and timing of emergence could not be distinguished from its control, whereas high-laurate hybrids emerged more rapidly and had greater biomass at 2 weeks than their hybrid controls. It was therefore concluded that high-laurate hybrids emerged from shallow depths may experience performance advantages that will allow them to perform as well as their persistent, wild parents.

According to another paper by Linder (2000), structural, energetic, biochemical and ecological information suggests that germination temperature is an important selective agent causing seed oils of higher-latitude plants to have proportionately more unsaturated fatty acids than lower-latitude plants. Germination temperature selects relative proportions of saturated and unsaturated fatty acids in seeds oils that optimise the total energy stores in a seed and the rate of energy production during germination. Saturated fatty acids store more energy per carbon than unsaturated fatty acids. However, unsaturated fatty acids have much lower melting points than saturated fatty acids. Thus, seeds with lower proportions of saturated fatty acids in their oils are able to germinate earlier and grow more rapidly at low temperatures, even though they store less total energy than seeds with higher proportions of saturated fatty acids. Seeds that germinate earlier and grow more rapidly therefore have a competitive advantage. At higher germination temperatures, seeds with higher proportions of saturated fatty acids are selectively favoured because their oils provide more energy, without a penalty in the rate of energy acquisition. Macroevolutionary biogeographical evidence from a broad spectrum of seed plants supports this theory, as do microevolutionairy biogeographical and seed performance studies within species of *Helianthus*.

A series of papers published since 1992 and referenced by Khodaskovskaya *et al.* (2006) suggest that chilling resistant plants have a greater abundance of unsaturated fatty acids. During acclimation to cold temperature, the activity of desaturase enzymes increases and the proportion of unsaturated fatty acids rise. This modification allows membranes to remain fluid by lowering the temperature at which the membrane lipids experience a gradual phase change from fluid to semi-crystalline. Thus, desaturation of fatty acids provides protection against damage from chilling temperatures.

The papers by Kodama *et al.* (1994, 1995) presents results of experiments with transgenic tobacco with the *Arabidopsis thaliana FAD7* gene under control of the constitutive CaMV 35S promoter. This *FAD7* gene encodes a chloroplast omega-3 fatty acid desaturase that is responsible for the formation of trienoic fatty acids (TAs), like α-linolenic acid (ALA) (18:3), or a combination of 18:3, and hexadecatrieonic (16:3) acids, in leaf tissues. As a result, the transgenic tobacco plants had a decrease in the dienoic fatty acids (DAs) and an increase in the trienoic fatty acids (TAs), which are the major constituents in plant membrane lipids. Particularly, chloroplast membranes contain very high proportions of TAs. As the leaves of these transgenic tobacco plants had significantly less injury during chilling, it is concluded that the increase of TAs production during chilling acclimation is one of the prerequisites for normal leaf development at low, non-freezing temperatures. Notably, the evaluation of transgenic and non-transgenic plants showed differences in low-temperature tolerance in young seedlings but no discernible differences in the performance of mature plants.

Based on experiments with mutant *Arabidopsis thaliana* lines with negligible levels of TAs, Routaboul *et al.* (2000) find that at 22 °C no differences in photosynthetic activity compared to the wild-type. However, long-term growth at 4 °C leads to serious decrease in photosynthetic activity, chlorophyll content and thylakoid membrane content in the mutants relative to the wild-type. These detrimental effects appear to be strongly correlated with TAs content. About one-third of wild-type TAs content is sufficient to sustain normal photosynthetic activity at low temperature. These results therefore suggest that TAs are important to ensure correct biogenesis and maintenance of chloroplast function during growth of plants at low temperatures.

These experiments can be viewed as examples of a large number of studies conducted over the last decade, with the aim to advance understanding of the molecular mechanisms of chilling sensitivity in plants, because of agricultural demands for improvements in cold tolerance of crops. A review by Sung *et al.* (2003), for instance, lists three studies with *Arabidopsis thaliana FAD7* and *FAD8* engineered into tobacco, leading to increased heat tolerance, as well as four experiments with transgenic rice, tobacco and/or *Arabidopsis thaliana* with a transgene encoding glycerol-3-P-acyltransferase, resulting in altered fatty acid unsaturation and, as a consequence, altered chilling sensitivity, heat tolerance and/or changed photosynthesis rate. Moreover, based on experiments with transgenic tobacco, Khodakoskaya *et al.* (2006) confirms various studies that in *Arabidopsis thaliana*, three gene products, *FAD3*, *FAD7* and *FAD8* mediate the synthesis of TAs from 18:2 and 16:2 fatty acids.

Notably, one session at the 17th International Symposium on Plant Lipids was specifically devoted to "fatty acid modification, desaturases and stress adaptation" (ISPL 2007). For instance, one of the presentations discusses the effects of changing temperatures and humidity conditions on interrelated activities of *FAD2* and *FAD3* genes and the subsequent impacts on the levels of linoleic acid and linolenic acid in sunflower and soybean seeds (ISPL 2007; page 199 – 201). Another presentation proposes two different mechanisms by which temperature regulates *FAD2* activity in sunflower seeds: a direct effect and an indirect effect by which temperature determines the availability of oxygen, which, in turn, regulates *FAD2* activity (ISPL 2007; page 214). Yet, another presentation elaborates on experimental findings that greater saturation and longer fatty acid chains of triacylglycerol (TAG) in *Cuphea* seeds increases their susceptibility to deterioration during storage and water uptake (imbibition). This observation is counter-intuitive because ageing has traditionally been considered a result of peroxidative reactions, making seeds with polyunsaturated fatty acids most susceptible (ISPL 2007; page 237).

Although plants have evolved mechanisms to correctly target different fatty acids to plasma membrane lipids and (seed) storage lipids, it is not clear whether these targeting mechanisms function in a similar way when plants are engineered with transgenes for the production of non-native (VLC) PUFAs in their seeds (Millar *et al.* 2000). Moreover, change in the plasma membrane lipid composition as a consequence of cold acclimation represents one of the many ends of different signalling pathways in plants, including abscisic acid (ABA)-dependent and ABA-independent pathways (Bohn *et al.* 2007).

It is therefore conceivable that expression of *FAD* genes engineered into a plant for the production of polyunsaturated fatty acids may affect ABA-responsive signalling, also because ABA regulates many key processes in seeds, including imposition and maintenance of dormancy, although the role of ABA signalling in the biosynthesis and accumulation of storage lipid is not yet fully understood (Kim *et al.* 2006; see also chapter 2). Given the pivotal role of ABA in the crosstalk between biotic and abiotic stress response mechanisms in plants, it can not be excluded that transgenic plants engineered to produce polyunsaturated fatty acids might obtain a different disease susceptibility as a consequence thereof.

3.7 Environmental risk assessment of omega-3 fatty acid GM soybean

3.7.1 Principles for environmental risk assessment of GM plants

EU Directive 2001/18/EC puts in place a step-by-step approval process made on a case-by-case assessment of the risk to human health and the environment before any GMOs can be released into the environment, or placed on the market as, or in, products. While Part B of the Directive deals with the deliberate release of GMOs for any other purpose than for placing on the market, Part C deals with the placing on the market of GMOs. The Directive foresees that in both cases an application must include an environmental risk assessment (ERA). The principles for the ERA are laid down in Annex II.

In the case of GM higher plants, like soybean, the ERA should reach conclusions on the following aspects: 1) likelihood of increased persistency in agricultural habitats and increased invasiveness in natural habitats; 2) selective (dis)advantage; 3) potential for gene transfer to related and non-related organisms; 4) potential impacts of interactions with target organisms; 5) potential impacts of interactions with non-target organisms; 6) potential effects on human health; 7) potential effects on animal health; 8) potential effects on biogeochemical cycles, and; 9) potential impacts of the specific cultivation, management and harvesting techniques.

It should further be stressed that the comparative approach constitutes an important element in the ERA strategy. The concept of familiarity therefore plays a pivotal role, based on the fact that most GM plants are developed from crop plants, which have gained a history of safe use, and of which the biology is well researched (OECD, 1993). These crops can serve as a baseline for the ERA of the GM plants.

3.7.2 Points to consider in an ERA of omega-3 fatty acid GM soybean in Europe

For an ERA of unconfined, large-scale or commercial release of omega-3 fatty acid GM soybean in Europe, it should be pointed out that some conventionally bred soybean cultivars have a high content of oleic acid (an omega-3 fatty acid), which can serve as baseline for assessing the environmental risks of high oleic acid GM soybean. However, with conventional breeding, it is not possible to develop soybean capable of producing VLC-omega-3 PUFAs, such as SDA, EPA and DHA, because of the need to introduce genes encoding elongases and desaturases, which are not present in the gene pool of soybean. Hence, by contrast to GM soybean with high oleic acid content, there is no familiarity in the case of VLC-omega-3 PUFAs producing GM soybean.

It should further be noted that information on laboratory and greenhouse experiments with omega-3 fatty acids GM soybean, the conditions in field trials in the US, the types of genetic modification and the resulting data on agronomic and potential environmental effects has as yet not been made publicly available.

For an ERA of an unconfined, large-scale or commercial release of an omega-3 fatty acid GM soybean plant in Europe, there is lack of data that allow a comparison of the performance an omega-3 fatty acid producing GM soybean to that of the parental soybean. It is likely that a GM omega-3 fatty acid producing trait will be put under the control of a seed-specific promoter. Nonetheless, relevant phenotypic parameters may include data not only on the duration of seed production, seed germination and dormancy, and seedling emergence, survival and growth. Other relevant phenotypic parameters may also include on re-growth from stubble, disease susceptibility, interaction with nitrogen-fixating symbionts, seed protein composition and levels of anti-nutritional factors (trypsin inhibitors, phytic acid, raffinose and stachyose).

1. Likelihood of the omega-3 fatty acid GM soybean plant becoming more persistent than the parental plant in agricultural habitats or more invasive in natural habitats

In the case of a high oleic acid GM soybean plant, there are no reasons to assume that it might be more persistent in agricultural habitats or more invasive in natural habitats in comparison to the recipient or parental plant. First, because US experience indicates that conventionally bred high oleic acid soybean is not persistent in agricultural habitats or invasive in natural habitats. Second, because data from US field tests with a high oleic acid GM soybean do not show significant differences in seedling emergence, re-growth from stubble and seed dormancy between high oleic acid GM soybean and the parental variety.

In the case of a GM soybean plant with very long chain polyunsaturated fatty acids (VLC-PUFAs), there are reasons to assume that it could acquire an increased potential for persistency in agricultural habitats or invasiveness in natural habitats in comparison to the recipient or parental plant. First, because the proportion of saturated and unsaturated fatty acids in the plant (leaves) may be changed due to increased production of unsaturated fatty acids in a VLC-PUFAs GM soybean plant, it cannot not be excluded that this results in an increased cold tolerance of the stubble and thus a better overwintering capability compared to stubble of conventional soybean. This could potentially lead to VLC-PUFAs GM soybean volunteer plants in agricultural habitats. Though, particularly if a seed-specific promoter controls the expression of the VLC-PUFAs trait, the likelihood might be negligible. Second, because seeds of a VLC-PUFAs GM soybean plant have lower proportions of saturated fatty acids in their oil than those of conventional soybeans, it cannot be excluded that they might acquire changed dormancy characteristics and might potentially germinate earlier and grow more rapidly at low temperatures compared to conventional soybean seeds. As a consequence, seeds of a VLC-PUFAs GM soybean plant might potentially lead to volunteers in the crop in the year following its cultivation and/or invasion into natural habitats after transport by animals.

Furthermore, in particular when *FAD* genes are inserted, there are reasons to assume that this might result in changed tolerances of a VLC-PUFAs GM soybean plant to abiotic stress, like cold, and/or biotic stress. First, because insertion of *FAD* genes may affect ABA-responsive signalling, which plays a pivotal role in the crosstalk between biotic and abiotic stress response mechanisms in plants, it cannot be excluded that a VLC-PUFAs GM soybean plant acquires a changed tolerance to biotic stress. Second, because insertion of *FAD* genes may also affect ABA-regulated key processes in seeds, like dormancy and accumulation of storage lipid, which might result in changed seed dormancy and altered accumulation of seed storage lipid, it cannot be excluded that seeds of a VLC-PUFAs GM soybean acquire a changed tolerance to abiotic stress, like cold. In conclusion, the insertion of *FAD* genes could result in an increase or decrease of the potential of a VLC-PUFAs GM soybean for persistence in agricultural habitats or invasiveness in natural habitats in comparison to the parental variety.

2. Any selective (dis)advantage conferred to an omega-3 fatty acid GM soybean plant

There are no reasons to assume that a high oleic acid producing trait confers a selective advantage or disadvantage to a high oleic acid GM soybean plant. First, because there is no selection pressure for high oleic acid content. Second, because data from US field trials with a high oleic acid GM soybean show no significant difference between seedling emergence, re-growth from stubble and seed dormancy between a high oleic acid GM soybean and its parental variety.

In the case of a VLC-PUFAs producing trait, there are reasons to assume that this trait might confer a selective advantage to a VLC-PUFAs GM soybean plant under conditions of cold. First, because the proportion of saturated and unsaturated fatty acids in the plant (leaves) might be changed due to increased production of unsaturated fatty acids in a VLC-PUFAs GM soybean plant, which could lead to an increased tolerance to cold and thus a better survival capacity under conditions of (winter) cold. Though, particularly if a seed-specific promoter controls the expression of the VLC-PUFAs trait, the likelihood might be negligible. Second, because seeds of a VLC-PUFAs GM soybean plant have lower proportions of saturated fatty acids in their oil than those of conventional soybeans, which could lead to changed seed dormancy characteristics, as well as earlier seed germination and more rapid seedling growth at low temperatures compared to conventional soybean seeds.

Furthermore, in particular when *FAD* genes are inserted, there are reasons to assume that this might result in a selective advantage or disadvantage for a VLC-PUFAs GM soybean plant under biotic and abiotic stress conditions. First, because insertion of *FAD* genes may affect ABA-responsive signalling, which plays a pivotal role in the crosstalk between biotic and abiotic stress response mechanisms in plants, it cannot be excluded that a VLC-PUFAs GM soybean plant acquires a selective advantage or disadvantage under biotic stress conditions. Second, because insertion of *FAD* genes may also affect ABA-regulated key processes in seeds, like dormancy and accumulation of storage lipid, which might result in changed seed dormancy and altered accumulation of seed storage lipid, it cannot be excluded that seeds of a VLC-PUFAs GM soybean acquire a selective advantage or disadvantage under conditions of cold.

3. Potential for gene transfer to the same or other sexually compatible plant species under conditions of planting an omega-3 fatty acid GM soybean plant and any selective advantage or disadvantage conferred to those plant species

There are no reasons to assume that a high oleic acid producing trait or a VLC-PUFA producing trait confers any additional cross-fertilisation capacity to a GM soybean plant. Moreover, soybean has no sexually compatible weedy or wild relatives in Europe, while the potential for gene transfer, through pollen flow, from a high oleic acid or a VLC-PUFA producing GM soybean plant to non-GM soybean plants is virtually zero, because soybean is a self-fertilising species with a cross-fertilisation capacity of less than one percent.

4. Potential immediate and/or delayed environmental impact resulting from direct and indirect interactions between an omega-3 fatty acid GM soybean plant and target organisms, such as predators, parasitoids, and pathogens

There are no reasons to assume that a high oleic acid GM soybean or a VLC-PUFAs GM soybean will result in direct effects on soybean predators, parasitoids and pathogens. First, because neither a high oleic acid trait, nor a VLC-PUFAs trait is aimed to control soybean pests at all, and therefore no target organisms can be defined. Second, because oleic acid and VLC-PUFAs are not known to have detrimental effects on soybean predators, parasitoids and pathogens.

5. Possible immediate and/or delayed environmental impact resulting from direct and indirect interactions of an omega-3 fatty acid GM soybean plant with non-target organisms, including impact on population levels of competitors, herbivores, symbionts, parasites and pathogens

There are no reasons to assume that a high oleic acid GM soybean or a VLC-PUFAs GM soybean will result in direct effects on population levels of competitors, herbivores, symbionts, parasites and pathogens. First, because neither a high oleic acid trait, nor a VLC-PUFAs trait is aimed to control soybean pests at all, and therefore no non-target organisms can be defined. Second, because oleic acid and VLC-PUFAs are not known to have detrimental effects on population levels of competitors, herbivores, symbionts, parasites and pathogens.

When *FAD* genes are inserted, there are reasons to assume that a high oleic GM soybean or a VLC-PUFAs GM soybean might have indirect effects on population levels of competitors, herbivores, symbionts, parasites and pathogens. Because insertion of *FAD* genes may affect ABA-responsive signalling, which plays a pivotal role in the crosstalk between biotic and abiotic stress response mechanisms in plants, it cannot be excluded that a high oleic acid GM soybean or a VLC-PUFAs GM soybean acquires a changed tolerance to biotic stress, which could result in changed interactions with soybean predators, parasitoids and pathogens, and therefore also in indirect impacts on population levels of organisms that interact with them. It should however be noted that data from US field trials with a high oleic acid GM soybean containing soybean-derived *FAD* genes did not show effects on beneficial organisms, such as bees and earthworms.

6. Possible immediate and/or delayed effects on human health resulting from potential direct and indirect interaction of an omega-3 fatty acid GM soybean plant and persons working with, coming into contact with or in the vicinity of an omega-3 fatty acid GM soybean plant release

There are no reasons to assume that a high oleic acid GM soybean or a VLC-PUFAs GM soybean will have detrimental effects on human health of persons working with, coming into contact with or in the vicinity of a release. First, because the genes inserted and their products, i.e. enzymes, like elongases and desaturases from microalgae, fungi, etc., are not likely to have detrimental effects on human health. Second, because the metabolites of these enzymes, i.e. oleic acid or VLC-PUFAs, are considered beneficial to human health upon consumption.

7. Possible immediate and/or delayed effects on animal health and consequences for the food/feed chain resulting from consumption of an omega-3 fatty acid GM soybean plant any products derived from it, if it is intended to be used as animal feed

There are no reasons to assume that use of a high oleic acid GM soybean or a VLC-PUFAs GM soybean as animal feed will have detrimental effects on animal health. First, because the genes inserted and their products, i.e. enzymes, like elongases and desaturases from microalgae, fungi, etc., are not likely to have detrimental effects on animal health. Second, because the metabolites of these enzymes, i.e. oleic acid or VLC-PUFAs, are considered beneficial to animal health upon consumption.

8. Possible immediate and/or delayed effects on biogeochemical cycles resulting from potential direct or indirect interactions of an omega-3 fatty acid GM soybean plant and target and non-target organisms in the vicinity of an omega-3 fatty acid GM soybean plant release

There are no reasons to assume that incorporation into the soil of root exudates, plant litter, seeds or pollen of a high oleic acid GM soybean or a VLC-PUFAs GM soybean will have effects on biogeochemical cycles. First, because horizontal transfer of the inserted genes from a high oleic acid GM soybean or a VLC-PUFAs GM soybean to soil microbes is extremely unlikely under natural conditions. Second, because expression of the inserted genes, which are under the control of eukaryotic promoters with very limited, if any, activity in prokaryotic organisms, is extremely unlikely in soil microbes. Third, because the gene products, i.e. enzymes, like elongases and desaturases from microalgae, fungi, etc., and the metabolites of these enzymes, i.e. oleic acid or VLC-PUFAs, are not known to have effects on soil microbes.

9. Possible immediate and/or delayed, direct and indirect environmental impacts of the specific cultivation, management and harvesting techniques used for an omega-3 fatty acid GM soybean plant where these are different from those used for a non-GM soybean plant

In the case of a high oleic acid GM soybean there are no reasons to assume that its cultivation will require other volunteer control measures inside and outside agricultural habitats than for its parental variety. First, because it is unlikely that a high oleic acid GM soybean acquires an increased potential for persistency in agricultural habitats. Second, because it is unlikely that a high oleic acid GM soybean acquires an increased potential for invasiveness in natural habitats.

In the case of a VLC-PUFAs GM soybean there are no reasons to assume that its cultivation might require different volunteer control measures in agricultural habitats than for its parental variety. Although it cannot be excluded that a VLC-PUFAs GM soybean acquires, due to an increased tolerance to cold, a higher potential for persistency in agricultural habitats than its parental variety, the resulting VLC-PUFAs GM soybean volunteer plants in agricultural habitats can be controlled by usual volunteer control measures. On the other, because it cannot be excluded that a VLC-PUFAs GM soybean acquires, due to an increased tolerance to cold, a potential for invasiveness in natural habitats, this might require to extend (volunteer) control measures to natural habitats.

Furthermore, in the case of a high oleic acid GM soybean and a VLC-PUFAs GM soybean, in particular when *FAD* genes are inserted, there are reasons to assume that their cultivation might require different (chemical) phytosanitary measures than for their parental varieties. First, because it cannot be excluded that a high oleic acid GM soybean or a VLC-PUFAs GM soybean acquires a changed tolerance to biotic stress caused by soybean predators, parasitoids and pathogens. Second, because it cannot be excluded that a high oleic acid GM soybean or a VLC-PUFAs GM soybean might result in different population size levels of organisms that interact with soybean predators, parasitoids and pathogens. In addition, it cannot be excluded that a high oleic acid GM soybean or a VLC-PUFAs GM soybean acquires a changed interaction with nitrogen-fixating symbionts, which might necessitate a different usage of (chemical) nitrogen-fertilisers compared to their parental varieties.

LIST OF ABBREVIATIONS

ABA	abscisic acid
ABF	ABRE binding factor
ABFs	auxin binding factors
ALA	α-linolenic acid
ARA	arachidonic acid
AREB	ABA-responsive element binding
ASI	anthesis-silking interval
ASR	abscisic acid stress reponsive
AT	acyltransferase
AtHK1	*Arabidopsis thaliana* histidine kinase
bZIP	basic leucine transcription factor
CaMV	caulimosaic virus
CBF/DREB	C-repeat-binding-factor/dehydration-responsive binding protein
CoA	coenzyme A
COR	cold-responsive protein
CPD	calcium-dependent protein
CDPK	calcium-dependent protein kinase
CPT	cytidine-diphosphatecholine:1,2-diacylglycerol choleinephosphotransferase
DA	dienoic fatty acid
DAG	diacylglycerol
DGAT	diacylglycerol:acyltransferase
DHA	docosahexaenoic acid
DM	dry matter mass
DPA	dihydrophaseic acid
DREB	dehydration responsive transcription factor
DRE/CT	dehydration-responsive element/C-repeat
HSF	heat shock factor
Hsp	heat shock protein
EA	environmental assessment
EFA	essential fatty acid
EFSA	European Food Safety Authority
EPA	eicosapentaenoic acid
ERA	environmental risk assessment
ET	ethylene
FM	fresh mass
GA	gibberellic acid
GLA	γ-linolenic acid
GM	genetically modified
GMO	genetically modified organism
JA	jasmonic acid
LA	linoleic acid
LAI	leaf area index
LC	long chain
LEA	late embryogenesis abundant
LPAAT	lipd-phosphatidylcholine acyltransferase
LPCAT	acyl-CoA:lyso-phosphatidylcholine acyltransferase
LWP	leaf water potential

MAP	mitogen-activated protein
MAPKKK	mitogen-activated protein kinase kinase kinase
MJ	methyljasmonate
OA	osmotic adjustment
PA	phaseic acid
PC	phosphatidylcholine
PDAT	phospholipids:diacylglyceroltransferase
PEPC	phosphoenolpyruvate carboxylase
PLA	phospholipase
PLD	phospholipase D
PMC	pollen moisture content
PML	percentage moisture loss
PtdOH	phosphatidic acid
PUFA	polyunsaturated fatty acid
PX	peroxidase
QTL	quantitative trait loci
RARMP	risk assessment and risk management plan
RH	relative humidity
ROS	reactive oxygen species
RWC	relative water content
SDA	stearidonic acid
SOD	superoxide dismutase
SP1	stable protein 1
SA	salicylic acid
TA	trienoic fatty acid
TAG	triacylglycerol
YP	yield potential
VLC	very long chain
WM	wilted mass
WUE	water-use efficiency

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