An exploration of the potential contribution of genetic modification and genome editing to the development of abiotic stress-tolerant crops as compared to conventional breeding

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An exploration of the potential contribution of genetic modification and genome editing to the development of abiotic stress-tolerant crops as compared to conventional breeding

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Preface

In plant breeding, much attention is paid to obtaining crops that are more tolerant to abiotic stress factors such as drought, heat or salinity. Due to climate change, temperatures in agricultural areas are increasing and desiccation is occurring. Salinization is also an increasing problem, not only in coastal areas but also in irrigated areas. In addition, the risk of local flooding due to short heavy rainfall increases. Crop yields are greatly adversely affected by these abiotic stress factors. Crops that are more tolerant to such growing conditions are therefore urgently needed in order to ensure food security in the future. The search for stress-tolerant crops takes place through conventional breeding, as well as through genetic modification and gene editing. According to some researchers, the latter two techniques are essential to obtain tolerances against abiotic stressors. The European Commission also believes that gene editing can contribute to achieving the objectives of the 'Green Deal' and the United Nations sustainable development goals for a more resilient and sustainable agri-food system.

The COGEM has commissioned a literature study on developments in stress-tolerant crops and the role that genetic modification and, in particular, gene editing play in this. The goal was (1) to obtain an overview of stress-tolerant GM crops that have been brought onto the market and their performance in the field, (2) on which other crop events are in the pipeline, and (3) on how realistic the expectations are for the new gene editing techniques in development of stress-tolerant crops compared to conventional breeding and selection. The overview is also important for the pending Biotechnology Trend Analysis 2022.

The report starts with an overview of molecular mechanisms that can enhance stress tolerance, and then discusses the limited number of GM crop events that have been commercialized and/or authorized. It continues with an overview of GM crop events for which there is information available in the literature about field performance. It finishes with a discussion of the limitations of strategies using single-gene approaches to improve stress tolerance without affecting yield of crops grown commercially in the field. At the very end of the report there is an informative list of information about field trials with GM crops in many countries around the globe.

The number of commercialized and/or authorized GM crops with improved stress tolerance is currently very limited. Whether many more GM crop events are in the commercial pipeline is difficult to say, since that information is not publicly available. The feeling that I got is that this number may also be very limited, due to the limitations discussed in the report. COGEM was also interested in whether new gene editing techniques based on CRISPR-Cas have already led or will lead in the near future to applications in improving stress tolerance of crops. This is also difficult to say, on the one hand because the information is not publicly available, and on the other hand because the techniques are still relatively new and the pipelines for commercialization take many years.

I wish you a pleasant reading,

Prof. dr. J. Memelink, Chairman Advisory Committee

Summary

This report explores trends in the use of genetic modification (GM), i.e., transgenesis and new genomic techniques (NGTs), notably targeted mutagenesis using genome editing, for developing abiotic stress tolerant crops. We focussed on transgenic events that are authorized for cultivation and on events that appeared to be promising for a pipeline towards commercialization. For the latter, we paid particular attention to the performance in field trials that are as representative as possible for the performance in commercial cultivation. We compared the results with developments in conventional breeding for abiotic stress tolerance. We used a combination of literature research, including reviews/overviews on transgenesis and genome editing in crop breeding, databases of field trial applications, other databases, such as ISAAA GM Approval Database and EUginius, country reports of OECD, and USDA FAS.

Worldwide, three types of transgenic events targeting abiotic stress tolerance have been authorized for cultivation, all increasing drought tolerance. DroughtGard® maize (MON87460) has been cultivated commercially in the USA since 2013 and is based on a bacterial cold shock protein gene (*cspB*) acting as an RNA chaperone; it is also being tested in field trials in Sub-Saharan Africa and has recently been authorized in several countries in that region (South Africa, Nigeria). It is allowed for import as food and feed in the EU. Drought-tolerant HB4 soybean has recently been authorized for cultivation in Argentina, Brazil and the USA, and HB4 wheat recently in Argentina. They are based on a transcription factor from sunflower involved in an ethylene signalling pathway. The events NXI-1T, -4T and -6T in sugarcane have been authorized for cultivation in Indonesia and are based on a bacterial gene, *betA*, for the synthesis of glycine betaine, which acts as an osmo-protectant. There is limited information on the performance of these authorized events in cultivation practice.

We describe several genes that appeared to lead to significant improvements in stress tolerance of GM plants in field trials. Most events concerned drought tolerance, followed by salinity and heat. It proved to be difficult to assess the fate of promising events towards commercialization. Commercially relevant information on events tested that was kept confidential often included the underlying genes. For many events recent information or publications did not exist. This absence may be because of failure in further testing towards commercialization, but the absence of recent information does not preclude that commercialization may still take place in the future.

Breeding varieties that are tolerant for abiotic stress is complex for several reasons. There is a fundamental trade-off for the plant between investing in stress protection and in growth, meaning that improvement of stress tolerance may lead to lower yield under favourable conditions. Complex signalling pathways and feed-back mechanisms are part of the plant responses to stress, and these will vary with developmental stage of the plant. Crops may be particularly sensitive at specific developmental stages of the growth cycle, such as flowering or grain filling. It proved difficult to identify traits that can be efficiently tested under controlled conditions (in growth chambers and greenhouses), and that at the same time have predictive value for improvement of stress tolerance under field condition, where stress is variable in occurrence and severity. Multi-site/multi-year trials, including stress testing in managed environments, are essential to successfully produce stress-tolerant varieties.

GM techniques, including genome editing, are helpful in assessing gene functionality in stress responses and in dissecting response mechanisms. The effect of inserting a single or a few genes using GM to increase protection against stress is not always straightforward. Changing gene expression patterns may influence signalling pathways for other necessary traits, such as responses to other abiotic and biotic (pathogens, pest) stressors, or growth. Nevertheless, next to the three commercialized events and the genes that confer significant improvements in drought tolerance in the field, also single locus introgressions and induced mutations in single genes have led to conventionally bred varieties with improved stress tolerance. Negative effects of constitutive changes in gene expression of a GM event may be addressed by employing subtle changes in gene expression, preferably in response to stresses. Genome editing enables introducing small changes in promoters, creating variation in gene expression. Moreover, there are fast developments in genome editing technology, including targeting several genes at a time (multiplexing). It is difficult to predict to what extent this will lead to targeting several of the promising genes together to adapt signalling pathways/stress responses in a balanced way.

Conventional breeding for abiotic stress tolerance has also made progress in crops such as maize and wheat, using multi-site, multi-year field trials. Genetic gain in drought tolerance in conventional breeding has increased with the development and implementation of, notably, DNA marker-assisted selection, genomic prediction, high-throughput phenotyping, and crop growth models. The latter two will also be of use to improve GM approaches by more efficient testing of new gene constructs and identifying useful traits, respectively. For instance, conventional maize breeding has resulted in the introduction of varieties with improved drought tolerance since 2011 in drought-prone areas of the USA corn belt. They have also led to drought-tolerant maize variety development by public sector breeding for tropical areas, i.e., Sub-Saharan Africa, South Asia and Latin America.

Concluding, GM approaches as well as conventional breeding must deal with the complexity of plant responses in the development of abiotic stress tolerant crops. Both approaches have produced drought stress-tolerant crop variants. Increasingly research on abiotic stress-tolerant plant lines, developed with genome editing, is published but results of more extensive field testing are not available yet. The potential of genome editing is therefore difficult to assess at present, also given continuous developments in technology and plant improvement concepts. Recently, large-scale analyses combining field trials, HTP genotyping and genomic prediction appear to have shown a way to improvements in assessing the genetic basis of traits that are adaptive to variable drought conditions in the field. In such approaches, also traits conferred by GM may be involved, as well as alleles generated using genome editing.

Samenvatting

Dit rapport verkent trends in het gebruik van genetische modificatie (GM) en nieuwe genomische technieken (NGT), met name het gericht aanbrengen van mutaties via het zogenaamde “genoom editen”, voor het ontwikkelen van gewassen die beter bestand zijn tegen abiotische stress. Onze focus lag op transgene varianten (“events”) die toegelaten zijn voor teelt, en op varianten (transgene en met genoom edits) die veelbelovend lijken voor een pijplijn richting commercialisering. Voor de tweede groep baseerden we ons op de resultaten van veldproeven omdat die het dichtst bij de omstandigheden in commerciële teelt komen. We vergelijken deze resultaten met ontwikkelingen in de conventionele veredeling voor abiotische stress tolerante rassen. We voerden daartoe een literatuuronderzoek uit en doorzochten databases van veldproefaanvragen en andere databases, zoals de ISAAA GM Approval Database en EUginius, en landenrapporten van OECD en USDA FAS.

Wereldwijd zijn drie typen van transgene varianten voor abiotische stress tolerantie toegelaten voor teelt, alle gericht op droogtetolerantie. DroughtGard® maïs (MON87640) wordt sinds 2013 commercieel geteeld in de VS en is gebaseerd op een bacterieel gen (*cspB*) dat codeert voor een koudeschok eiwit ter bescherming van RNA; deze variant wordt ook getest in Sub-Sahara-Afrika en is recent toegelaten in verschillende landen in deze regio (Zuid-Afrika, Nigeria). MON87460 maïs is toegelaten als voedsel en veevoer in de EU. Droogtetolerante HB4 soja is recent toegelaten voor teelt in Argentinië, Brazilië en de VS, en HB4 tarwe heel recent in Argentinië. Ze zijn gebaseerd op een transcriptiefactor uit zonnebloem die betrokken is bij een ethyleen signaalroute. Events NXI-1T, -4T en -6T in suikerriet zijn toegelaten voor teelt in Indonesië en zijn gebaseerd op een bacterieel gen, *betA*, dat betrokken is bij de productie van glycinebetaïne, dat functioneert als een beschermer tegen osmotische stress. Er is beperkte informatie over de prestaties van deze toegelaten events in commerciële teelt.

We beschrijven verscheidene andere genen die leiden tot significante verbetering van de stresstolerantie in veldproeven. De meeste genen dragen bij aan droogtetolerantie, gevolgd door zouttolerantie of verminderde hittestress. Het bleek moeilijk inzicht te krijgen in de voortgang van het traject van veelbelovende genen in de pijplijn richting commercialisering. Voor commerciële toepassingen van belang zijnde informatie, waaronder de onderliggende genen, is veelal vertrouwelijk. Voor veel events ontbrak recente informatie. Dit kan komen omdat recente testen richting commercialisering niet succesvol waren, maar afwezigheid van informatie sluit een aankomende commercialisering niet uit.

Veredelen van plantenrassen die tolerant zijn voor abiotische stress, is complex om verschillende redenen. Elke plant moet een balans vinden tussen investeren in bescherming tegen stress en in groei (opbrengst), waardoor een betere tolerantie tegen stress kan leiden tot minder opbrengst onder optimale omstandigheden. Complexe signaalroutes en terugkoppelingsmechanismen liggen ten grondslag aan de reactie van de plant op stress en deze kunnen verschillen in opeenvolgende ontwikkelingsstadia. Ook kunnen gewassen in bepaalde stadia, zoals bloei of graanvulling, bijzonder gevoelig zijn. Het blijkt ook moeilijk om eigenschappen te vinden die efficiënt getest kunnen worden onder gecontroleerde omstandigheden (in klimaatkamers en kassen) en die tegelijk een goede voorspellende waarde hebben voor verbeterde stresstolerantie onder veldomstandigheden, waar stress varieert in vóórkomen en ernst. Veldproeven op meerdere plaatsen en over meerdere jaren, inclusief stress-testen op proefvelden waar stress kan worden gestuurd, zijn noodzakelijk om succesvol stress-tolerante rassen te ontwikkelen.

GM technieken, waaronder genoom editen, zijn nuttig voor het testen van functies van genen en het ontrafelen van mechanismen van stressreacties. Het invoegen van een enkel gen of enkele genen via GM, kan signalen voor andere eigenschappen beïnvloeden, waaronder weerstand tegen andere abiotische en biotische (pathogenen) stressoren, of de groei. Negatieve effecten van veranderingen in genexpressie kunnen worden tegengegaan door subtiele veranderingen in genexpressie aan te brengen, bij voorkeur in reactie op het optreden van stress. Genoom editing maakt het mogelijk gericht kleine veranderingen in promotoren aan te brengen, wat leidt tot het creëren van variatie in genexpressie. Op dit moment zijn er snelle ontwikkelingen in genoom editing technologie, inclusief het tegelijkertijd editen van meerdere genen (zogenaamde “multiplexing”). Het is moeilijk te voorspellen in welke mate hiermee meerdere genen op een samenhangende wijze aangepakt kunnen worden voor het aanpassen van signaalroutes/stressreacties.

Conventionele veredeling voor abiotische stress tolerantie heeft voortgang geboekt in gewassen zoals maïs en tarwe, met veldproeven op meerdere plaatsen en over meerdere jaren. De genetische winst die in conventionele veredeling voor droogtetolerantie kan worden geboekt, is toegenomen dankzij ontwikkelingen in DNA merker-gestuurde selectie genomische selectie (“genomic prediction”), high-throughput fenotyperen en gewasgroeimodellen. De laatste twee zullen ook bruikbaar zijn voor het verbeteren van GM benaderingen, respectievelijk via het efficiënter testen van GM varianten en het identificeren van bruikbare eigenschappen. Bijvoorbeeld voor conventionele maïsveredeling heeft dit geleid tot de introductie van rassen met verbeterde droogtetolerantie in delen van de maïsteeltgebieden (“corn belt”) van de VS die gevoelig zijn voor droogte. Het heeft ook geleid tot ontwikkeling van droogtetolerante maïsvarianten door publieke sector veredeling voor tropische gebieden, te weten Sub-Sahara-Afrika, Zuid-Azië en Latijns-Amerika.

Concluderend hebben bij de ontwikkeling van abiotische stress tolerante gewassen zowel GM benaderingen als conventionele veredeling te maken met de complexiteit in de reacties van de plant. Beide benaderingen hebben rassen opgeleverd met verbeterde droogte-tolerantie. Over ontwikkeling van abiotische stress-tolerante lijnen met behulp van genoom editing wordt in toenemende mate gepubliceerd, maar resultaten van veldproeven zijn nog beperkt gepubliceerd. De potentie van genoom editing is daarom nog moeilijk in te schatten, mede gezien de continue ontwikkeling van de technologie en veredelingsconcepten. Grootschalige combinaties van veldproeven, high-throughput fenotyperen en genomic prediction kan leiden tot verbeteringen in het voorspellen van de genetische basis van eigenschappen die reageren op representatieve droogteomstandigheden in het veld, wat ook zou kunnen worden gecombineerd met het ontwikkelen van GM eigenschappen of genoom editen van de onderliggende genen.

Abbreviations

ABA	abscisic acid (plant hormone)
AI	artificial intelligence
ASI	anthesis silking interval (time between pollen shed and silk emergence in maize)
BR	Brassinosteroid
CaMV	cauliflower mosaic virus (origin of 35S promoter)
CBI	Confidential Business Information
CIMMYT	International Maize and Wheat Improvement Center (Mexico: Centro Internacional de Mejoramiento de Maíz y Trigo)
CK	cytokinin (plant hormone)
DSB	Double Strand Break (of DNA in genome editing)
DT	drought tolerance
ETH	ethylene (plant hormone)
GM	genetic modification
GxE	Genotype – environment interaction
HDR	homology-directed repair (repair of DNA double strand break (DSB) in genome editing
HI	harvesting index
HT	herbicide tolerance
IR	insect resistance
IRRI	International Rice Research Institute (Philippines)
MAS	(DNA) Marker-Assisted Selection
NGT	New Genomic technique
NHEJ	Non-Homologous End Joining (repair of DNA double strand break (DSB) in genome editing
NPBT	New Plant Breeding technique
RNAi	RNA interference (post-transcriptional gene silencing)
ROS	Reactive Oxygen Species
SDN	site-directed nuclease (general term for genome editors, such as ZFN, TALEN and CRISPR/Cas9)
SSA	Sub-Saharan Africa
TF	Transcription Factor
TPE	Target Population of Environments
WUE	water use efficiency

Contents

1. Introduction

Genetically modified (GM) crops were successfully introduced into cultivation in the mid-1990s, with two types of agronomic traits, herbicide tolerance (HT) and insect resistance based on Cry proteins from the bacterium *Bacillus thuringiensis* (Bt). Since then, these traits have remained the greater part of GM crop cultivation, although additional traits, such as improved product quality, have been introduced as well (ISAAA 2019). The big challenge for so-called second-generation GM crops is the successful modification of genetically complex traits, such as yield and tolerance for abiotic stresses, notably drought tolerance, but also salinity, heat, cold and flooding tolerance.

Since their introduction, there has been a debate about GM crops, mainly focussed on biosafety, sustainability, and socio-economic issues. With evident global climate change and the need to mitigate its consequences, stress-tolerant crops have gained more attention, and with that discussions about the pros and cons of GM technology also came to address the strategic value of GM technology for developing such crops. Abiotic stress tolerance is a complex trait, with many genes and signalling pathways involved, and this will make an approach of adapting crops using a single gene, or a combination of a few genes in so-called stacks, more difficult than with herbicide tolerance (HT) and insect resistance using Bt genes. Next to the need for gaining insights in the functionality of individual genes for stress tolerance in research under controlled conditions, practical stress tolerance in a crop depends on how the crop reacts to the local environmental conditions at various developmental stages. Certain stages, such as flowering and grain filling, may be particularly sensitive to stresses such as drought and heat, that often co-occur, leading to serious yield losses. Water deficits may be moderate or severe, and they may develop slowly or quickly, e.g., by dry winds. Droughts may also last for shorter or longer periods of time. Whether a gene can confer effective stress tolerance may be different for various environmental conditions or developmental stages. Basically, a trade-off can be expected between stress protection and growth [in the plant’s use of energy, carbon and other nutrients], which means that genetically improved performance of the crop under stress can be accompanied by yield penalties under favourable growing conditions.

With the introduction of new genomic techniques (NGTs, also named new plant breeding techniques (NPBTs)), most prominently genome editing enabled by CRISPR-Cas systems, the issue of the possible contribution of genetic engineering for the development of stress-tolerant crops has become even more relevant. The COGEM has asked for an update on the subject, and in this report this is focussed on the following questions:

1. which crop and trait combinations ((transgenic) events) to increase abiotic stress tolerance have been authorized for cultivation and can thus be commercialized? What are the experiences with these events in cultivation practice?
2. which crop and trait combinations ((transgenic) events) may be likely to become relevant for a commercial pipeline of abiotic stress-tolerant varieties, that is, can we trace events that have shown success in protecting yield under (more or less unpredictable) stress conditions while also being productive under favourable conditions?
3. what can be said about the contribution of (transgenic or NGT) events in comparison to conventional breeding to successfully produce abiotic stress-tolerant crops?

GM crop development starts by identifying genes that could confer a desired trait to a crop. Often transformation into the crop is already part of assessing the functionality of specific genes in basic research. In the first instance, phenotyping for identifying and testing genes will comprise efficient tests that can be performed on large numbers of plants. In the case of abiotic stress tolerance this might for instance entail watering seedlings with solutions containing a specified level of NaCl in order to assess their salt tolerance. Subsequently, the best performing events will be tested further for analysing plant development, physiological markers for stress tolerance and biomass production under controlled conditions such as in greenhouses. In the end, the most promising events will be tested in field trials, preferably under conditions representative for commercial cultivation, in order to assess their value for cultivar development both under favourable and stress conditions.

There is a large body of literature describing genes that, when transformed into crop plants or when mutated, conferred stress tolerance under controlled conditions and in short-term experiments. However, relatively few of these genetic events have been tested in field trials, which form the final test of functional efficacy during cultivar development but are costly as multi-location/multi-year trials are necessary due to the inherent variability of cultivation conditions. This is a general feature of cultivar development, not only of GM cultivar development, but for stress tolerance it is particularly important as the level of stress varies across a growing season and thus field trials are an essential test for the efficacy of the event for crop yield. For that reason, we focus this literature study on publicly available data on GM abiotic stress-tolerant plants that have been researched and tested for field performance to identify variants that appear to be promising for a possible pipeline towards commercial cultivation. For this, we looked for published data in the scientific literature, international databases, country reports on developments in GM, and other sources. As expected, the majority is about drought stress, and that is therefore the stress we focussed on.

In our report, we first give a brief overview of mechanisms and types of researched genes involved in abiotic stress tolerance to provide background to the reader. Subsequently, the authorized/commercialized events are described, followed by an exploration of other events for which information on (successful) field trials were found. Finally, we discuss the effectiveness and limitations of biotechnological approaches in relation to conventional efforts to produce stress-tolerant varieties. A complicating factor in this discussion is that the baseline is shifting, since conventional breeding becomes more efficient in time as the breeders' toolbox is being enlarged by non-GM breeding tools, such as DNA markers.

2. General mechanisms of stress tolerance and types of genes targeted for enabling stress tolerance

2.1. General mechanisms of abiotic stress tolerance

Plants show a complex range of responses to abiotic stress to address damaging effects on for instance photosynthesis and growth. A basic feature is that plants will divert energy from growth to initiating protective mechanisms, the extent of which depending on the levels of abiotic stress that they are challenged with. This eventually results in decreased crop yield. Broadly speaking, for drought tolerance, two strategies may be distinguished: dehydration avoidance, i.e., maintaining water status by developing more extensive root systems, osmotic adjustment or reducing transpiration, and dehydration tolerance, aiming at maintaining plant function under low-water status by generating molecular protectants (Tuberosa, 2012). In the case of salt stress, there are two major components affecting growth that need to be addressed in breeding: the osmotic effect of salt on cells, as is also occurring with drought, and the toxic effects of the salt ions Na^+ (and Cl^-) when accumulating in the plant (van Zelm et al., 2020). The challenge for breeding lies in striking a balance between improved water use or protection, and growth that is underlying yield. Initially, biotechnological research was mostly focussed on improving protection mechanisms for plant survival, which came with negative effects on general plant performance. Later on, research moved its focus to improving protection while minimizing yield penalties or aiming at improving growth while delaying drought protection responses ("growth-centred") (Tenorio Berrío et al., 2022). Other ways of stress tolerance are improved recovery from severe stress, which is related to the improvement of protection mechanisms for plant survival and that may also come with large costs for growth, and escape mechanisms. Escape mechanisms are more in the realm of agronomy, for instance, changing timing of sowing and/or aiming at early harvesting, and some of these may generate new needs for traits to be adapted in breeding. This will be more extensively discussed in Chapter 5.

Plant responses to abiotic stress involve complex signalling pathways and thus many genes that have been tested in GM (transgenic) and NGT (new genomic techniques) approaches. One may make a distinction between genes coding for enzymes involved in the production of compounds that protect cells from damage and genes involved in modifying the development and physiology of the plant, which often are transcription factors that form part of signalling pathways. We describe them briefly below. More extensive overviews may be found in the scientific literature, e.g., in (Priya et al., 2019).

2.1.1 Cellular protection

Abiotic stresses can damage cells through osmotic stress from drought or salt, or through the formation of reactive oxygen species (ROS) by disturbance of photosynthesis and metabolism balance. ROS can damage cell components such as membranes, and can play a role in cross-linking cell wall components, but in a controlled fashion it has a function in signalling pathways (see below). Cold tolerance can entail damaging low temperatures, e.g., for (sub)tropical crops such as rice or tomato, or frost, which necessitates cell protection from drying and crystallization damage.

The plant can produce metabolites for osmo-protection and membrane stabilization, for ROS scavenging, and, in the case of salt stress, transporters for removal or sequestering of toxic levels of Na^+ and for optimizing K^+ levels (as K^+ is an important plant nutrient, the uptake of which is hampered by high Na^+ levels).

Important osmo-protectants, which protect against cell turgor loss due to external (salt) stresses, are proline, glycine betaine, trehalose and mannitol. Heat shock factors act as molecular chaperones, protecting protein or RNA structures (Janni et al., 2020). Late embryogenesis abundant (LEA) proteins, which accumulate during seed maturation, are also induced by stress in vegetative tissues, where they serve a protective role. They are highly hydrophilic proteins, such as dehydrins. Like proline, dehydrins also offer protection to oxidative stress (Halder et al., 2018). Part of the LEA genes are induced by cold and their products were therefore named as COR (cold-regulated proteins) (Kosová et al., 2021).

ROS scavenging counteracts ROS accumulation and is particularly effective under severe stress. It is effected by metabolites, such as glutathione and ascorbate, and enzymes, such as APX (ascorbate peroxidase), GPX (glutathione peroxidase), CAT (catalase), SOD (superoxide dismutase) and POX (peroxidases) (Choudhury et al., 2017).

Protection against cell toxicity of the salt component Na^+ can come from transporters which remove Na^+ from the sap stream or from the cell by transporting it into the apoplast or by sequestering it in vacuoles. For example, Na^+ can be removed from xylem vessels into neighbouring parenchyma cells through HKT1 transporters, and from the cytoplasm into vacuoles by NHX antiporters, in combination with H^+-ATPases and H^+-PPases for maintaining ionic balance (van Zelm et al., 2020). Furthermore, a K^+ transporter gene, *OsHAK21*, was shown to be involved in salt tolerance in rice at the germination and seedling establishment stage (Srivastava et al., 2020).

2.1.2. Plant responses at the tissue and plant level

Various protective mechanisms function against water loss at the level of plant organs. These include the composition of the cuticle and the presence of wax layers on the leaf surface, changes in stomatal density, and adaptations of root system architecture and anatomy/physiology, which can, amongst others, affect hydraulic conductance. Improved root system architecture is important for the uptake of water and nutrients but can consume photosynthates at the cost of plant growth and yield.

The plant's microbiome, particularly mycorrhiza, has gained increasingly attention for providing protection against abiotic stresses, but biotechnological approaches have only been applied to a limited extent. An example is the *NSP2* gene encoding a transcription factor that is involved in regulation of strigolactone production, a plant hormone that acts as a signalling molecule for attracting arbuscular mycorrhizal fungi in the rhizosphere (Müller and Harrison, 2019). Overexpression and gene-edited versions of *NSP2* in barley will be subjected to field trials at the Crops Science Centre in Cambridge (UK) in 2022 (https://www.cam.ac.uk/research/news/crop-science-centre-to-conduct-field-trials-of-genetically-modified-barley-that-could-reduce-need).

Thus, in these plant developmental and architectural traits (and also in interactions with beneficial microorganisms), transcription factors and signalling pathways play a role that can be targets of genetic improvement. These will be discussed in the next section.

2.1.3. Transcription factors and signalling components

Transcription factors (TFs) direct the transcription of genes through recognizing and binding to specific sequence elements in the promoter of the genes they are targeting. They are involved in practically all aspects of plant development, including responses to abiotic stresses. Several large TF families, such as NAC (name derived from the earlier identified NAM, ATAF1/2, and CUC2 genes) or WRKY (TFs containing the conserved amino acid sequence WRKYGQK combined with zinc-finger-like motifs), have multiple members demonstrated to be active in abiotic as well as biotic stress responses, where they can act as positive or negative regulators. Other important TF families are the bZIP (basic leucine zipper proteins) and HD-ZIP (homeodomain leucine zipper proteins), where also both positive and negative regulators can be found. In GM applications, transgenic overexpression or ectopic expression of a positive regulator may result in improved stress tolerance whereas gene silencing (by RNAi) or gene knockout (by genome editing) will decrease stress tolerance. This will be the other way round for negative regulators. Hence, negative regulators may be an attractive target for genome editing, as their knockout may lead to improved stress tolerance.

Prominent in abiotic stress responses are signalling pathways that involve plant hormones, notably abscisic acid (ABA) and ethylene (ETH). ABA is a pivotal hormone in abiotic stress response (Chen et al., 2020), most notably for osmotic stress responses, relaying stress signals from drought or salt and resulting in stomatal closure and other protective responses, including ROS scavenging, while inhibiting other hormonal pathways involved in growth. ETH can also inhibit growth in response to stress, but it is also involved in responses to biotic stresses (pathogens and pest organisms) and developmental pathways, such as etiolated growth and fruit ripening. Under flooding stress, ETH has a special role as it can quickly accumulate in submerged plant organs because of its poor solubility in water, in which case it promotes growth enabling leaves and inflorescences to reach above water levels (Sasidharan and Voesenek, 2015). AP2/ERF (gene family named after first identified APETALA2 and ETH-responsive element binding factors) TFs are intricately intertwined with ETH and ABA signalling. For instance, ERF-VII members that are stabilized by high ETH (Hartman et al., 2019), are involved in submergence tolerance, and may also be active in drought tolerance, e.g., SUB1A in rice, amongst others through enhanced ABA responsiveness (Tamang et al., 2021a). The DREB (Drought Responsive Element Binding) proteins (or CBF, C-repeat binding factor) that were among the first TFs identified as involved in plant responses to various stresses, such as drought, salinity or cold, also belong to the AP2/ERF superfamily (Feng et al., 2020). DREB TFs are functioning in abscisic acid (ABA)-independent pathways, whereas ABA-dependent pathways involve, e.g., AREB (ABA-Responsive Element Binding) proteins. They recognize specific promoter elements (CRT/DRE and ABRE, respectively) in the downstream genes (Zhang et al., 2022).

The hormonal role of brassinosteroids (BR) has been elucidated relatively recently. They generally promote growth. The BES1/BRF1 transcription factors are central mediators in BR signalling (Divi et al., 2016). Cytokinins (CKs) also promote growth and are involved in delaying drought-induced leaf senescence (the drought tolerance breeding trait “stay-green”). CKs are also involved in root system architecture, an important adaptive trait for water and nutrient uptake, but in an inhibitory role, which is counteracted by auxins. Auxin is also an important type of hormone promoting growth, but it does not appear to have played a prominent role in manipulating abiotic stress tolerance using GM technology, although auxin can play a role in the pathways impacted by events targeting stress responses (see below e.g., with the *HVA1* gene in root development in 4.2.1). Strigolactones play among others a role in root and shoot branching, and in plant-microbe interactions (see previous section) and in inducing germination of parasitic plants, such as *Striga* (Aliche et al., 2020).

Ca^{2+} plays an important role as secondary messenger relaying signals in the cell, including induction of stress responses. Ca^{2+}-fluxes inside cells, generated upon triggering of receptors by environmental cues, can also be passed on from cell to cell, thus moving as a wave through plants leading to systemic stress responses. An example is the SOS3-SOS2-SOS1 chain in salt stress (SOS: Salt Overly Sensitive). When signalled by a Ca^{2+} spike, the calcium sensor AtCBL4 (AtSOS3), a calcineurin B-like protein, recruits the CBL-interacting protein kinase AtCIPK24 (AtSOS2) to the plasma membrane, where it activates (via phosphorylation) the Na^+/H^+ antiporter AtSOS1 to remove Na^+ from the cell (Manishankar et al., 2018). The systemic response to abiotic stress can be initiated by a local Ca^{2+} influx into the cell from the apoplast and vacuole, activating cytoplasm CPK/CBL-CIPKs that in turn induce plasma membrane RBOH (NADPH oxidase/respiratory burst oxidase homologue) to produce ROS in the apoplast, leading to Ca^{2+} influx into neighbouring cells, resulting in a wave throughout the plant at up to 8.4 cm/min (Choudhury et al., 2017). In these signalling events, specific *MAPK* (mitogen-activated protein kinase) genes are involved as well. These kinases transmit signals in a chain of kinases phosphorylating other kinases, with MAPKKK phosphorylating MAPKK in turn phosphorylating MAPK, thus providing flexibility in regulating responses to external stimuli; MAPK can (in)activate target proteins, such as TFs (Majeed et al., 2022).

2.2. Ways to modify genes involved in stress tolerance pathways

In the classical transgenic approach, genes that can originate from a wide range of organisms are introduced into crop plants that confer a novel (dominant) trait. An alternative approach developed at the end of the 1990s is silencing the expression of a particular endogenous gene using an RNAi hairpin construct based on inverted sequences from the particular gene. Before that, an approach called antisense (expressing a complimentary copy that would hybridize to the mRNA targeted for silencing) was used to obtain similar effects (without knowledge of the exact silencing mechanisms present in the plant cell). The integrated RNAi construct genetically behaves as a dominant trait. RNAi can have a quantitative effect (“knockdown”) as well as the on/off effect of a knockout.

In most of the recent genome editing applications, endogenous genes are knocked out by introducing a small insertion or deletion; this leads to results that are similar to gene silencing with RNAi, but the edited gene behaves as a recessive trait (as usually all alleles and often also all functionally redundant family members of the gene need to be mutated for achieving the desired trait). This type of genome editing effecting small mutations is called SDN-1 (site-directed nuclease 1), which is based on making DNA double strand breaks (DSBs) and repair by the cell’s error-prone NHEJ (non-homologous end joining) DNA repair system. Other types are SDN-2 that is based on DSB repair using a small external template containing the desired change, and SDN-3 based on homology-directed repair using a larger external template that could for instance be a complete gene (Lusser et al., 2011; Schaart et al., 2016). SDN-2 and 3 have been far less used than SDN-1 in plants up to now, as is the case with more recent developments of base editing (Zhang et al., 2021) and prime editing (Zong et al., 2022) that are also more precise in introducing mutations than SDN-1.

An interesting variant of targeted mutations is introducing a change in the promoter region of a gene to affect its expression pattern. Depending on the type of change this may behave as a recessive trait (when the expression is reduced) or as a dominant trait (when the gene is more highly or ectopically expressed).

3. Searches for field trials in literature, databases and other sources

3.1. Search strategies

For this review, the basic approach was a literature study combined with searches in databases of field trials at competent authorities of countries or jurisdictions active in GM crop development, searches in other databases involving GM crops, such as EUginius and NGO/business association databases (e.g., Croplife), and in reports, such as USDA GAIN worldwide country reports and OECD reports, and information on company websites. For some countries, including Colombia, South Africa, Italy, and Switzerland, experts were consulted.

A particular limitation of this literature study was that breeding companies' pipelines are confidential, and also the data they must provide to competent authorities, are largely subject to confidentiality. For instance, records of public information on field trials often include no details on genes involved, as this type of information was removed as CBI (confidential business information) or CCI (commercially confidential information). The level and detail of information provided varied between countries.

As a proxy, we searched for (scientific) publications containing information on field trials, in particular those assessing yield under realistic conditions, which is the actual proof for cultivation success of novel stress tolerance traits. Yield mostly concerned grain or tuber yield as most GM work is performed on large crops, i.e., arable crops, such as maize, rice, wheat and potato. In practice, it proved difficult to select relevant publications using keywords such as 'field testing' or 'trial'; therefore, review papers were searched for information on field testing as well.

Improving stress tolerance may overlap with general yield-improving traits, such as photosynthesis engineering. For example, engineering C4 photosynthesis is primarily aimed at increasing yield but will also be useful under stress for improving water use efficiency (Hibberd and Furbank, 2016; Wang et al., 2017b). Another example is overexpressing Photosystem II Subunit S (PsbS) in tobacco that decreased stomatal aperture, resulting in reduced water loss per unit CO_2 assimilated in the field (Głowacka et al., 2018). An alternative approach to improving stress tolerance is agronomic measures that can also be effective in handling stress to crops, for instance, by enabling stress avoidance. Using winter wheat to avoid terminal drought at the reproductive stage, may have a trade-off with the need for cold tolerance during winter (Passioura, 2012) or spring frosts with spring wheat grown over winter in Australia (Langridge and Reynolds, 2021). An interesting example from the subtropics is found in wheat in India, where earlier sowing in autumn, immediately after rice, is beneficial as the plants may use the remaining water from the monsoon season, thus saving irrigation for the dry season that follows, and avoiding terminal heat in the next spring. However, for early sowing to be feasible, varieties need to become heat tolerant at early growing stages as well, which was not required before (Kumar et al., 2021). Thus, the use of agronomic measures may lead to different requirements for breeding adapted cultivars, that may also involve GM or NGT approaches. Because the focus of our study is on abiotic stress tolerance in itself, this report does not give an extensive overview of what traits and events could be relevant when addressing abiotic stress with agronomic measures, or for yield in general. For this review, we focussed on traits specifically conferring abiotic stress tolerance, particularly for drought, heat and salt. We also briefly explored cold and flooding stress tolerance studies, and trees and bio-based (e.g., biofuel) crops.

The searches produced several overviews from scientific literature containing information on crop/trait combinations being authorized and pipelines, information on field trial permits and GM developments per country from country reports, and information from other databases, such as EUginius, A detailed overview of results is provided in Annex 1. We used a combination of these findings with those from the literature to describe events commercialized and to indicate those that could be relevant for pipelines in the next chapter. With regard to data quality, peer-reviewed scientific publications were considered more reliable than info without underlying data from overviews or websites etc., and information from scientific publications was also much more detailed. For this reason, we indicate the source of information as much as possible.

4. Examples of crop events with information on their field performance

In this chapter, examples from the sources described in the previous chapter are described in detail. As to pipelines, information from several sources was combined as much as possible to obtain indications of promising events. We refer the reader to Annex 1 for details on the information that we have gathered per country.

First, the authorized crop events will be discussed, subsequently other possibly interesting examples. An overview of the events discussed can be found in Table 1 in Annex 2.

4.1. Commercialized/authorized stress-tolerant GM crops

Four stress-tolerant crops (encompassing three types of transgenes) have been authorized: DroughtGard™ maize MON87460, HaHB4 in soybean and wheat, and sugarcane NX1, all providing drought tolerance.

4.1.1. Maize MON87460 "Genuity® Droughtgard™" based on an RNA chaperone protein

Maize MON 87460 developed by Monsanto (presently Bayer) is transgenic for *CspB* from the bacterium *Bacillus subtilis* encoding a cold shock protein thought to act as an RNA chaperone. The gene is driven by the rice *actin1* promoter (Castiglioni et al., 2008). There is yet limited insight into the underlying mechanism of the MON87460 maize drought tolerance. It is associated with reduced leaf growth during anthesis, which is accompanied by water saving through a reduced sap stream during the drought-sensitive silking stage, reflected in a trend of more water remaining in the soil (at 0.5 m depth). Normally, a shorter anthesis silking interval (ASI, an indicator of stress tolerance in maize, see next chapter) and an increased ear growth is associated with higher leaf expansion, but this correlation is apparently broken in MON87460 maize. Thus, the construct provides a higher water use efficiency (WUE), as supported by a higher kernel set and resulting harvesting index (HI, ratio of grain to total shoot dry matter) under drought (Nemali et al., 2015). It is not clear what the role of the CSP is in this, except that *CspB* expression was highest in the basal meristematic zone of the developing leaves, which may lower general transcription/translation, resulting in reduced leaf growth. There was little difference in other important physiological markers, such as stomatal conductance and ABA levels (Nemali et al., 2015).

In field plots, drought tolerance was assessed by withholding irrigation from mid-vegetative to mid-reproductive stages (with rescues when needed to prevent crop failure), while controls were irrigated to 100% compensation of evapo-transpiration (which is not the same as well-watered) in the years 2009 – 2011. Under those conditions, grain yield in the drought-tolerant maize was on average 6% higher than controls, i.e., the same F1 hybrids lacking the drought tolerant (DT) MON 87460 event. The annual results varied from no difference between DT and control in a mild year in which the controls lost 14% yield compared to well-watered plants, to 11.7% difference in a bad year in which the control lost 44% yield. There were no significant differences under well-watered conditions (Nemali et al., 2015).

After authorization for cultivation in Canada in 2010 and the USA in 2011, commercialization of MON87460 started with demonstration trials in 2012 in the USA, after which there was cultivation on 50 kha in 2013, followed by 275 kha in 2014, ~1.2 Mha in 2016, and 1.4 Mha in 2017 (ISAAA, 2017). According to (Brookes and Barfoot, 2020), US acreage was 1.4 Mha in 2018 (1.34 Mha in 2016), showing a net yield gain of ~2.5% and a small income gain of ~$20/ha. In the EU, MON87460 is allowed for import as food or feed since 2015.

MON 87460 maize was used in the WEMA (Water Efficient Maize for Africa) project that used both conventional (developed through MAS, Marker-Assisted Selection) and transgenic materials for improving maize yields in Sub-Saharan Africa. WEMA encompassed six years of field testing (Ricroch and Hénard-Damave, 2016). It was followed up in 2018 by TELA (name derived from Latin “tutela” meaning “protection”), which is focussing on transgenic traits, in a public-private partnership led by AATF (African Agricultural Technology Foundation) with CIMMYT (International Maize and Wheat Improvement Center) providing high yielding/DT conventional lines and expertise, and Bayer promoting regulatory approval and dissemination of IR (insect resistance via Bt) and IR/DT stacks royalty free. Participating countries are Ethiopia, Kenya, Mozambique, Nigeria, South Africa, Tanzania, and Uganda (TELA maize project CIMMYT, AATF news). The third trial of TELA on stacked IR/DT in 2021 showed yields of (8-)9 tons/ha compared to (2.5-)3 tons/ha for the best local varieties (AATF news: https://www.aatf-africa.org/nigeria-transgenic-maize-trial-produces-outstanding-results/). The results have not been described in a scientific publication yet. Based on the website data it is not possible to disentangle Bt from DT effects. Nigeria allowed environmental release and cultivation in 2021, South Africa already in 2015 (USDA FAS GAIN and OECD, not in ISAAA GM database).

The MON87460 is the only stress-tolerant event for which there is a multi-year experience in commercial cultivation in the USA. There will be more discussion about this in Chapter 5.

4.1.2. Sunflower *HB4* transcription factor in soybean and wheat

4.1.2.1. Soybean HB4

GM drought-tolerant soybean, Verdeca HB4, was developed by Bioceres (Rosario, Argentina) and Arcadia Biosciences (Davis, California) in their joint venture Verdeca, and is based on the gene *HaHB4* from sunflower, which encodes a homeodomain-leucine zipper (HD-Zip) transcription factor. HaHB4 is different from HD-Zip I members in other crop species as it has a relatively short carboxy-terminal region. Overexpression using the Cauliflower Mosaic Virus (CaMV) 35S promoter in Arabidopsis led to growth retardation, but under its own drought and salt stress-inducible promoter *HaHB4* conferred drought tolerance without loss in plant performance. HB4 was originally identified through better survival after harsh treatments of withholding water for 10-20 days. Even though this is not necessarily a good predictor of performance under moderate stress under cultivation (see next chapter), HB4 proved effective under a wider range of conditions, leading to its introduction into crops (González et al., 2020). HB4 was first introduced into soybean cv Williams 82 and tested in the field across several years, under various conditions at the critical period before anthesis and during grain filling, encompassing combinations of dry or wet and warm or cool conditions, totalling 27 trials on 14 sites. Across all conditions yield increase was on average 4%, under drought stress there was 8.6% increase. Yield increase was 5% at mild temperatures, and 11% when drought stress was combined with higher temperature. Therefore, HB4 also showed a yield increase in the particularly unfavourable combination of drought and heat. The gene is thought to be involved in an ETH signalling pathway. Plants show lower sensitivity to ETH, leading to delayed senescence during drought, so that photosynthesis and growth during drought are sustained longer (improved “stay-green”). Indeed, higher transpirational water use was shown in the field, even more so under well-watered conditions, which was associated with a larger xylem area. By contrast, under greenhouse conditions, the water use was similar to wild type plants, but this was measured in pots, which limit root growth. Nevertheless, higher WUE (yield per unit rainfall) was accompanied with lower water use under drought, indicating that there was some involvement of stomatal closure but to an extent that there still was relatively higher CO_2 uptake. The higher yield was based on higher grain numbers that were only partly offset by lower grain weight. Overall, there was no improvement of harvest index (HI) but a higher total biomass production during the sensitive period of pod development and grain filling (González et al., 2020). HB4 soybean has been approved for cultivation in Argentina, Brazil, and the USA, and for import for food in Canada, Australia and New Zealand (http://www.isaaa.org/gmapprovaldatabase/event/default.asp?EventID=403), and China, being an important export market for Argentina, thus in principle clearing the way towards commercial cultivation (Bioceres 220429 press release: https://investors.biocerescrops.com/news/news-details/2022/Bioceres-Crop-Solutions-Announces-Regulatory-Approval-of-HB4-Soy-in-China/default.aspx).

4.1.2.2. Wheat HB4

A wheat transgenic HB4 version using the constitutive maize UBI-1 promoter was developed by Bioceres (Rosario, Argentina) and Florimond Desprez (France) in a joint venture, Trigall Genetics. *HB4* wheat was tested in 37 field trials on 13 sites divided across four groups. The first group of trials was to select the best line, the second to extensively compare the HB4 line to the non-transgenic INDEAR (from Bioceres's R&D company) test set of germplasm, and the last two for assessing more detailed phenotypic traits in ecophysiology and floral/crop phenology, respectively, in the field under various conditions during the critical periods before anthesis and during grain filling, encompassing combinations of dry or wet and warm or cool conditions. The best line showed on average 16% higher yield under stress, whilst this amounted to 3% under normal conditions (González et al., 2019). Across all conditions, the yield increase was 6%, 16% under drought, and 20% when combined with higher temperatures, but 12% when combined with moderate temperature conditions. There was neither an effect on crop phenology or grain weight, nor an improvement in HI but there was higher biomass development during the sensitive period before anthesis and grain set. This was related to higher WUE (yield per unit rainfall), the more so under drought (González et al., 2020). *HB4* wheat IND-00412-7 was authorized as food and for cultivation in 2020 in Argentina, but awaited the last step of assessing its potential impact on markets (national and international). At the end of 2021, approval for import into Brazil, Argentina's main export market for wheat, was granted, in principle clearing the way towards commercial production. Seeds were grown on 55000 ha in 2021. HB4 wheat was authorized for import as food also in Australia and New Zealand in 2022. In addition, there were field trial applications for HB4 wheat in the respective databases of the US and the EU.

There were plans to develop HB4 in maize, alfalfa and sugarcane in joint ventures (Feeney, 2014) (Ricroch and Hénard-Damave, 2016), but no outcomes of that could be found in literature nor in a 2020 white paper by Bioceres.

The HB4 events in soybean and wheat have shown promising results for drought (and heat) tolerance in extensive field testing. They have only been recently approved for cultivation; therefore, little can be said yet about their impact in commercial cultivation.

4.1.3. Sugarcane NXI-Ts based on an osmo-protectant

Drought-tolerant sugarcane was developed in Indonesia by PT Perkebunan Nusantara XI, in cooperation with CDAST, Jember University. Drought is one of the main limitations to sugarcane cultivation. Three events have been authorized, all based on the *betA* gene producing choline dehydrogenase, which is involved in the biosynthesis of glycine betaine, an osmo-protectant: NXI-1T from the bacterium *Escherichia coli*, and NXI-4T and NXI-6T from the bacterium *Rhizobium meliloti*, according to ISAAA GM Approval database: EUginius mentions uncertainty of origin from either *E. coli* or *R. meliloti* for all three events and further lists the use of the constitutive CaMV 35S promoter. Cane yields were reported to be 10-30% higher under drought conditions than for the parental lines (Babu et al., 2021) and 20-30% higher sugar production under drought (Waltz, 2014). No further scientific publications could be found providing more specific details on transformation details and the drought testing. The GM sugarcane was authorized for cultivation in 2013 and grown on about 81000 ha in Indonesia in 2018; seeds are not freely available (USDA FAS GAIN report ID2021-0040).

4.2. Examples of GM crop events tested for their field performance

In this section we describe examples of transgenic plants that are potentially interesting for pipelines towards cultivation, based on a combination of sources providing indications of promising performance of the plants under both normal and stress conditions. Prominent among these sources of data are field trial results, mostly on drought. However, in contrast to the previous section, no firm indications for moving towards commercialization were found for any of these examples.

4.2.1. LEA proteins and molecular protection

Wheat transgenic for barley *HVA1*, encoding a LEA group 3 protein that acts as a molecular protectant, under the constitutive CaMV 35S and maize ubi1 promoters, was tested in the field during 6 years, the first two years at Bozeman MT by Montana State University in the USA under dryland conditions, both rainfed (~200 mm during growing season) and irrigated, then in Egypt during four winter seasons, also comparing rainfed, i.e., three seasons with 71-93 mm precipitation complemented with one irrigation at Giza, plus one season (the third in Egypt) at several locations with precipitation ranging between 150-260 mm, with irrigation. The field trials had limited success in the first seasons, but the line selected and tested in the fifth season (the third in Egypt) showed a higher grain yield per plot in two out of four locations, and two later lines performed even better in the sixth season (Bahieldin et al., 2005). In the commercialization overview of (Waltz, 2014), continued *HVA1* wheat field testing at AGERI in Egypt for building the dossier for approval was discussed, but there was not much optimism about the likelihood of commercialization at the time, and since then nothing could be found about authorization. (Ricroch and Hénard-Damave, 2016) also included these trials in their overview, and in addition they listed greenhouse tests on *HVA1* under rice *Act1* promoter in maize for salt and drought tolerance (Nguyen and Sticklen, 2013); no field tests on maize transgenic for *HVA1* were found in literature nor in the USA database.

In the literature, the barley *HVA1* gene expressed in rice also gave higher grain yield per plant in four out of five lines in a small field trial under non-irrigated conditions, i.e., with soil kept moist at 20-25% water content (v/v), and with no yield penalty under two normal irrigation trials. The *HVA1* gene was expressed under the composite, more specifically stress (ABA)-inducible promoter *ABRC321* that combines the ABRE element from *HVA22* with the CE element from *HVA1,* leading to accumulation in primary and lateral root primordia resulting in increased root system development. This was likely effected through auxin signalling (Chen et al., 2015).

Thus, the cell protectant LEA *HVA1* was shown to confer drought tolerance in wheat and rice in field trials. There were also indications for its effectiveness in salt tolerance, but no results from field trials were found on this.

4.2.2. Source-sink relationships

Source-sink relationships (for carbon partitioning) are an important theme in stress tolerance. (Nuccio et al., 2015) showed significantly increased maize grain yields by overexpressing rice *TPP* (trehalose-6-phosphate phosphatase) in developing kernels under a flower-specific promoter from the rice *OsMADS6* gene, thus decreasing T6P (trehalose 6-phosphate) levels both under well-watered conditions and under drought conditions in managed field trials (drought managed by irrigation). They tested several independent events from the OsMADS6::TPP construct over several locations and years. T6P is thought to be involved in sugar signalling. In the transgenic plants, sucrose levels were raised in young ears, which was associated with improved kernel set and harvest index (HI).

T6P synthase (TPS) is involved in balancing levels of T6P in interaction with sucrose levels (Wingler, 2018). We also encountered the *TPP* and *TPS* genes among the most recent US release notifications for maize mentioning heat tolerance and gene editing components Cas9 and TPS and TPP sgRNAs by the University of Florida, but could not find publications on this.

A special form of maize Sh2 (shrunken2) encoding an AGPase (ADP glucose pyrophosphorylase), which is involved in the starch biosynthesis pathway, combined a point mutation from a heat stable allele, HS33 with a 6-bp insertion from an allele showing reduced negative feedback from inorganic phosphate (Pi), Rev6, into one coding sequence (Smidansky et al., 2002). This sequence was transgenically expressed under the *Sh2* promoter in maize, which led to increased grain yield (up to 64%) from plots in field trials well-watered by rainfall or irrigation, but only under high temperatures (>33°C, archived weather data) during early seed development (Hannah et al., 2012). Increased yields were shown to be due to a higher number of ovules that developed into seed than in wild type. Transgenic versions in rice and wheat also showed increased grain yields under controlled conditions, but in wheat it showed only some yield advantage in field trials under favourable conditions (temperature was not addressed as factor) (Meyer et al., 2007). The point mutations and indel in this transgene would seem to be amenable to genome editing of the native alleles in maize as well as rice and wheat.

Another interesting example with regard to source-sink relations is *AtAVP1* from *Arabidopsis thaliana*. *AtAVP1* encodes a H^+-PPase (pyrophosphatase). In field tests with transgenic barley expressing *AtAVP1* under the constitutive CaMV 35S promoter, grain yields per plant were 23-34% higher than wild type at low salt and 79-87% higher at high salt levels. Low salt and high salt areas were localized in the trial field by measurements of soil electrical conductivity: around 161 and 1231 µS/cm, respectively. The high salt was so severe that for wild type plants grain yield could only be measured on a few representative survivors per plot, thus providing an overestimation (Schilling et al., 2014). No changes were found for transgenic barley in Na^+ levels of leaves, which was not in line with expectations of a role of H^+-PPase in sequestering Na^+ in vacuoles (Gaxiola et al., 2016).

AtAVP1 is located in the tonoplast as well as in the plasma membrane, and it may serve also other roles than in sequestering Na^+ in vacuoles. For example, wheat H^+-PPases (TaVP1) are found in plasma membranes of phloem sieve element-companion cell complexes in source leaves, where they are involved in apoplastic sugar loading for transport to sink tissues. It is supposed that they function as PPi synthase, providing PPi and so facilitating sucrose loading (Gaxiola et al., 2016). In line with such observations, wheat transgenic for *AtAVP1* showed increased carbon partitioning towards roots and grains, and this was associated with increased root biomass and 18-20% higher grain yields, also under normal field conditions (435 mm rainfall during growing season) (Regmi et al., 2020). In addition, post-germinative growth was increased, which may imply a more robust establishment of seedlings in the field. Stronger root development is associated with improved water and nutrient use efficiency, thus also explaining a role of AVP1 in drought tolerance (Gaxiola et al., 2012, 2016). *AtAVP1* was also subject of four field trial permit applications for wheat/barley by University of Adelaide in Australia from 2010 onwards, among which a recent one including testing under abiotic stress conditions under evaluation for 2022-2027. Also in the USA, field trial permits have been issued for *AtAVP1* transgenics in several crops, including creeping bentgrass (*Agrostis stolonifera*), tall fescue (*Festuca arundinacea*), and poplar (*Populus tremula* x *alba*). In the USA, (Pasapula et al., 2011) found an increase in cotton fibre yield of 20% during two years of dryland (430 mm annual precipitation) field trials without irrigation at Lubbock TX. (Qin et al., 2013) found higher peanut yields in two years of dryland field trials, also at Lubbock under low irrigation; under high irrigation the yields were comparable to wild type. In both crop species, the same construct under the constitutive CaMV 35S promoter was used.

Interestingly, by co-expressing *AtAVP1* (under the 35S promoter) with *OsSIZ1* (encoding a SUMO E3 ligase) (under a maize UBI promoter), increased drought, salt and heat tolerance was found in cotton, resulting in fibre yield increases of 133% in 2016 and 81% in 2017 compared to wild type in field trials under semi-arid agricultural conditions without irrigation. The smaller effect in 2017 may be related to higher rainfall during the growing season (Esmaeili et al., 2021). The combined effect of the two overexpressed genes was larger than that of the individual transgenic lines. Also for 2020, there was an acknowledged notification for release in the USA for testing *AVP1* and *OsSIZ1* by Texas Tech University.

Interestingly with regard to NGTs, the perennial ryegrass homologue of *AtAVP1* was used by (Templeton et al., 2008) in their “cisgenic” approach to confer drought tolerance, which actually represented an intragenic approach, i.e., a combination of a perennial ryegrass drought-inducible dehydrin promoter and the native *VP1* gene. This variant was listed by (Ricroch and Hénard-Damave, 2016) and in the Croplife Agricultural Innovation Database, but in the scientific literature only conference abstracts were found, e.g., (Templeton et al., 2008). Among the research partners in the Croplife Agricultural Innovation Database, Pastoral Genomics, Noble Foundation and University of Florida, the latter had acknowledged release notifications for drought tolerant perennial ryegrass in 2009 and 2010, but the genotypes were classified as CBI. No results on field performance were found in literature, nor indications for commercialization.

In summary, several genes show the relevance of improving source-sink relationships, i.e., sink strength of developing seeds, in drought and heat tolerance. These are as diverse as the *TPP* gene involved in sugar signalling (although the effect may be independent from the enzymatic effect on the sucrose signalling molecule T6P, (Claeys et al., 2019)) in maize and the *AVP1* H^+-pyrophosphatase in wheat, cotton and peanut. In addition, a special form of the *Sh2* AGPase contributed to heat tolerance.

AVP1 also showed its effectiveness for salt tolerance in field trials. The importance of sink strength will also be seen in the next section on cytokinin effects.

4.2.3. Cytokinin levels

With regard to hormonal pathways, a higher production of cytokinin through the introduction of a bacterial gene for isopentenyl transferase (*IPT*), a key enzyme in cytokinin biosynthesis, could, amongst others, lead to delayed senescence. Introduction of this gene has met with some success in field trials. Constitutive overexpression can affect plant development, for instance poor root growth, but this may be overcome by the choice of promoter.

IPT from *Agrobacterium tumefaciens* expressed under control of an *AtMYB32* promoter from Arabidopsis (that is most active in seedlings and gradually slows down during further plant development) led to delayed leaf senescence and higher biomass production in wheat. In two-year field trials in Australia, grain yields were higher under well-watered conditions using irrigation, though not consistently across lines and years, and yields were particularly higher (by 26-42%) under drought imposed during reproductive stages by using rainout shelters with rain sensors after early growth plant establishment (Joshi et al., 2019). Among Australian field trial permits, there also was *IPT* in canola (also under the *AtMYB32* promoter) but no literature on results was found.

In the overview by (Waltz, 2014), 12-17% higher grain yields in two years of USA field trials by Arcadia Biosciences under drought were reported for rice transgenic for *IPT* from *Agrobacterium tumefaciens* under the stress-inducible promoter of the senescence-associated receptor kinase SARK (Rivero et al., 2009). At the time, the hope was to bring it to the market from 2018-2020 onwards, but we could not find indications for commercial cultivation since then, nor could further results be found in peer-reviewed literature. The technology was also applied in canola and licensed by Arcadia Biosciences for use in soybean, wheat, rice, cotton, sugar beet, sugarcane and tree crops (Ricroch and Hénard-Damave, 2016), but no information could be found on results from these pipelines since then. The construct was also successful in improving yield under low irrigation in two years of dryland field trials (carried out as described above for *AtAVP1*) in peanut (Qin et al., 2011), but it had variable success in field trials for cotton. In Texas (Lubbock) field trials in 2010 and 2011, the cotton transgenic for *IPT* showed significantly higher yield than wild type and a null-segregant (i.e., progeny of a transgenic line lacking the transgene) under dryland conditions (average 44% and 27% more, respectively), whereas there were no differences under high irrigation. No difference was found in an Arizona (Maricopa) field trial in 2011 under water deficit applied during early flowering by lowering irrigation to half of the full irrigation to controls. This difference may have been due to higher root development during early or constant drought, an effect that is absent when drought comes in during the reproductive stage as based on additional greenhouse experiments (Zhu et al., 2018)).

In their review of a large company biotechnology programme for obtaining drought tolerant maize, (Simmons et al., 2021) gave an overview of what they called validated leads, that is, promising transgenic constructs based on the criterion of conferring at least 10% higher grain yields than wild type in field trials in the case of stress tolerance (see further discussion in next chapter). These included *IPT* leads, both bacterial and from maize itself. The bacterial version of *IPT* from *Agrobacterium tumefaciens* was combined with a maize promoter which is active during seed development, ZAG2-1 (Brugière, 2017). This was expected to increase sink strength during stress, based on results in an earlier publication (Brugière et al., 2008), where the expression pattern of the plant's own ZmIPT2 during kernel development suggested a role of *IPT* in grain sink strength, yet another example of the involvement of source-sink relationships in stress tolerance (see previous section). The *IPT* from maize itself was combined with a constitutive promoter, but in the referenced patent (Brugière, 2012) about abiotic stress tolerance apparently no report on the effect of the UBI1ZM promoter used in relation to any maize experiment was found (just expression of ZmIPT2 in maize tissues but apparently no field trial results).

Cytokinin has alternatively been targeted by affecting its catabolism. The chickpea *CKX6* gene under the chickpea *WRKY31* root-specific promoter led to lower cytokinin levels and stronger root development, which was associated with higher grain yield and improved drought tolerance in chickpea ((Khandal et al., 2020). A permit for a field trial in Czech Republic with barley containing *AtCKX1* from Arabidopsis under a rice root-specific promoter was issued in 2014, but no literature on results could be found, except for the proof of concept of increasing root to shoot ratio without affecting plant fertility by using a root-specific promoter (from the *PHT1-1* P transporter) (Mrízová et al., 2013). (Simmons et al., 2021) did find an advantage of such constructs for improving NUE (nitrogen use efficiency), but results on yields were inconsistent in their experiments.

In summary, changing cytokinin signalling by using the *IPT* gene involved in CK biosynthesis was shown to provide improved yields under drought stress in field trials for wheat, maize and rice. It appears to be critically dependent on using the right promoters to delay senescence or improve sink strength without negative pleiotropic effects. This also applies to a potentially interesting alternative way via the *CKX* gene involved in CK catabolism that led to stronger root development.

4.2.4. Ethylene signalling

A returning theme in manipulating hormonal signalling pathways to improve stress tolerance appears to be ETH signalling. The authorized HB4 events in soybean and wheat are related to ETH signalling (see section 4.1.2). Other examples are among the validated leads (see section 4.2.4) of (Simmons et al. 2021). Among these is silencing *ACS6*, encoding an enzyme performing a rate-limiting step in ETH biosynthesis. Multiple maize RNAi events selected for about 50% ETH reduction showed improved grain yield in managed drought stress and rain-fed field trials with respect to null comparators, showing a shorter anthesis-silking interval (ASI) and higher grain number per ear. Selected events also had increased yield under low nitrogen (Habben et al., 2014).

ARGOS8 was one of the striking validated leads of Pioneer (Simmons et al., 2021), which we describe in more detail in the next chapter. *ARGOS8* is an example of a gene involved in negatively regulating the ETH response in maize. Transgenic overexpression of *ARGOS8* using the maize *UBI* promoter gave more than 10% yield increase under drought without negative effects under optimal conditions in field trials in two years at several locations across the USA, including managed environments for drought and optimal conditions (Shi et al., 2015). The Pioneer researchers have explored alternative approaches, including exchanging the *ARGOS8* promoter for one from another maize gene, *GOS2*, using CRISPR-Cas9 and homology-directed repair (SDN-3), leading to moderately higher expression, also resulting in higher yields in field trials like for the transgenic overexpressor (Shi et al., 2017). In this way, the effect of *ARGOS8* could be optimized through adapting its expression levels. The resulting plants also showed higher grain yields under drought at anthesis without negative effects under well-watered conditions. Up to now, this is a rather rare example of applying SDN-3 in crop improvement, often cited in literature reviews.

In summary, several ways of adapting ETH signalling have led to increased yields under drought stress, including the HB4 authorized for cultivation in soybean and wheat. Other examples are *ARGOS8* and *ACS6*, the latter in a silenced version. *ARGOS8* is often cited as it also has a gene-edited version based on an exchange of promoters with another maize gene, which has been rarely applied yet.

4.2.5. ABA

Manipulating hormonal signalling pathways has met with variable success, likely because of the tight regulation of cellular hormone levels and the complex interaction with other hormonal pathways, including redundancies (Tenorio Berrío et al., 2022). ABA apparently shows a fine-tuned balance between synthesis and catabolism. Alternatively, there may be the possibility of adapting PYR/PYL (Pyrabactin Resistance/PYR-like) ABA receptors. However, ABA receptors have been targeted another way round. A specific rice triple PYL mutant (out of the total of 12 members of this gene family in rice) showed better growth and grain yield per plot under favourable paddy field conditions, which may possibly be seen as an example of a “growth-centred” improvement under conditions of no stress (Miao et al., 2018). One of the Pioneer validated leads in maize, the RING E3 ligase (involved in 26S proteasome degradation, a way to regulate cell responses by removing specific proteins) ZmXerico1, is likely working through regulating the ABA catabolic enzyme ABA 8’-hydroxylase (ABA8ox) (Brugière et al., 2017). On the other hand, Simmons et al. 2021 mention three maize genes for *ABA8ox* with confirmed biological effects in GM experiments, but no yield advantages in the field. Furthermore, an *OsABA8ox2* CRISPR knockout was promoting root development in a more vertical direction, associated with higher drought tolerance, whereas overexpression led to more drought sensitivity in rice. This has however not been tested yet in the field (Zhang et al., 2020).

In summary, ABA, an important hormone in plant abiotic stress responses, appeared to show few successful examples of direct manipulations for yield improvement under stress in the field. This may be related to the importance of fine-tuning to achieve a response not hampering growth. ABA will however play a role in signalling cascades addressed by other GM approaches, see for example ZmXerico1 discussed here and examples in the next section.

4.2.6. Other approaches

Transgenic plants with an *FTA* gene encoding the α-subunit of protein farnesyltransferase also showed the importance of using specific promoters to avoid negative pleiotropic effects. FT transfers a farnesyl group (a terpenoid) to a cysteine in proteins with a specific carboxy terminal CaaX-box motif. *FTA* silenced under a drought-inducible shoot-specific promoter, *AtHPR1*, in canola led to drought tolerance, most likely by increasing ABA responsiveness of stomatal guard cells, as originally identified through the ABA hypersensitive mutant *era1*, encoding the β-subunit of FT (the protein targets of FT are yet unknown). The effectiveness was confirmed by field trials at two sites in Alberta, Canada in 2006, with the best two transgenic lines showing significantly higher yields (11-20%) at both sites under limited irrigation, while there were no differences under well-watered conditions with a second irrigation two weeks after the first in the beginning of July (Wang et al., 2009). The event is licensed by Performance Plants as Yield Protection Technology (YPT), with the underlying idea that plants are protected from periods of drought by inducible higher stomatal sensitivity, while this induction is absent under well-watered conditions so it will not affect growth. The last field trials on stress tolerance in canola were mentioned in the Canadian field trials overview for 2009. A 26% higher yield was reported for canola in the pipeline overviews of (Gruskin, 2012; Waltz, 2014). YPT is presently one of the technologies targeting stress tolerance on the Performance Plants website. No information could be found about further development of the FTA-silenced canola after 2014. The Performance Plants website also mentions soybean showing 23% higher yield under heat and drought and optimal conditions (field trials on stress tolerance in soybean were mentioned in the Canadian field trials overview for 2020). In addition, licences for heat and drought tolerant and high-yield rice, drought and heat tolerant cotton, for heat and drought tolerant and high-yield wheat, with yield advantages of up to 32% were mentioned. No further information could be found on these yield advantages in the scientific literature, nor additional indications for commercialization.

Often it is not fully clear how a gene affects stress tolerance, and apparently a practical/empirical approach is followed to screen useful leads. An unexpected validated lead in (Simmons et al., 2021) was that a gene involved in rubber production in *Hevea brasiliensis*, *HbPT* encoding prenyltransferase, conferred drought tolerance on maize when constitutively expressed under the maize *UBI1* promoter. As IPP (and its isomer DMAPP) being transferred by HbPT are also the precursors of terpenoids that are in turn also precursors in the biosynthesis of several plant hormones (ABA, but also BR, CK, GA or strigolactones (Tarkowská and Strnad, 2018)), it was speculated that changing IPP/DMAPP fluxes in cell metabolism influences hormone biosynthesis routes (Hallahan and Keiper-Hrynko, 2009).

In summary, genes working with or through isoprenoid secondary metabolites were implicated in drought tolerance, but with limited knowledge on the molecular mechanism. In the case of *FTA*, the gene was identified through mutant analysis but for instance the specific protein(s) targeted for farnesylation appear to be unknown, yet also without details on the mechanism, useful genes can be established through testing, eventually in the field, like also the *HbPT*.

In the field of TFs, much work has been done on *CBF*/*DREB* genes, but with limited success in the field, see for a more detailed discussion the next chapter.

4.2.7. Other abiotic stresses and types of crops

Up to now we have mainly addressed large arable crops, for drought (the most commonly targeted stress), salt and heat stresses. Trees are more difficult for breeding as well as biotechnology because of their longer generation time and more complex field trials. Most work on trees has been done on various poplar species/hybrids (see (Thakur et al., 2021)). In China, a salt-tolerant poplar (*Populus* × *xiaozhannica* cv. 'Balizuang-yang') was developed using a bacterial mannitol dehydrogenase (*mtlD* involved in osmo-protectant production) that according to (Chang et al., 2018) would be permitted for large plantings; no further information on this could be found (e.g., in the ISAAA GM database). In the USA, transgenic research on poplar included many field trials on about 600 constructs, but just a few addressed abiotic stress tolerance as evidenced by release permits for *Populus tremula* x *alba* (see section 4.2.2., with *AVP1*) and for hybrid aspen. There may be hesitance with commercialization, as in an earlier case involving HT, due to the high demands of safety testing (Clifton-Brown et al., 2019). Gene flow likelihood is high in poplar as it is wind-pollinated. Much work has also been done on biological containment systems limiting pollen flow, e.g. (Klocko et al., 2016)).

A freeze-tolerant Eucalyptus was generated by transformation with *CBF2* under a stress-inducible promoter, *rd29A*, both from Arabidopsis (Hinchee et al., 2011). Field trials showed that the best lines could sustain temperatures down to -8.4^{0}C, which would mean that the Eucalyptus could be grown across a wider area in the US. For two events, authorization for cultivation was applied for in the USA in 2011, but this was not yet granted (Chang et al., 2018); as of 2022, the EIS (environmental impact statement) was still pending, the most recent document being from 2017 (USDA-APHIS petition 11-019-01p, https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/permits-notifications-petitions/petitions/petition-status, accessed 17-05-2022). Also see another DREB variant used in apple in section 5.1.3.

Trees such as poplar and willow have gained increasingly attention for bio-based applications, including biofuel production, but willow cannot be transformed yet. Other crops are *Miscanthus* and switchgrass (*Panicum virgatum*), but for both only limited transgenic work has been done, most for switchgrass (which also has potential problems with gene flow in its native area in the USA) (Clifton-Brown et al., 2019). Large crops such as maize and sugarcane, that are also used for biofuel, have been among the crops discussed in the trait sections above.

With regard to other types of abiotic stress, flooding stress (waterlogging and/or submergence) has been addressed using gene technology only to a limited extent, mostly in rice where some landraces are adapted to incidentally high water levels of paddies but most widely cultivated varieties were not. An important locus conferring submergence tolerance, *SUB1A*, with no negative performance under normal conditions, has been identified and the candidate gene responsible (an ERF-like gene suppressing GA signalling and growth while preventing chlorophyll breakdown, thus saving energy for recovery after the flood) was corroborated by overexpression using the maize *Ubi1* promoter (Emerick and Ronald, 2019). However, the locus was essentially only used through marker-assisted introgression (Emerick and Ronald, 2019; Fukao et al., 2019). Research programmes listed by (Ricroch and Hénard-Damave, 2016), such as STRASA (Stress Tolerant Rice for Africa and South Asia, involving IRRI and CIMMYT), apparently also used *SUB1* introgression and further QTL (quantitative trait locus) identification ((Emerick and Ronald, 2019), https://strasa.irri.org/varietal-releases/submergence). The involvement of ETH signalling in submergence responses could perhaps be targeted by GM or genome editing, but we found no evidence that that has been done.

In summary, there are a few examples of trees modified for abiotic stress tolerance, though this is more difficult due to long generation times. An example of a cold tolerant Eucalyptus has been submitted for authorization in the USA, but there are apparently no new developments since the last EIS (environmental impact statement) in 2017. Trees will be more complicated in environmental releases because of the potential of gene flow into natural areas, such as with wind-pollinated poplars. There are examples of genes involved in flooding stress responses, but the successful example of *SUB1A* has been used in conventional introgression (by crossing) in rice.

4.3. Genome editing examples

Genome editing is a recent technological development, thus, the path towards commercialization has had less time compared to transgenic crops. Among abiotic stress tolerance traits in the USA “Am I regulated” database and (Ricroch et al., 2022) is a soybean edited for *Drb2a* and *Drb2b*. These two genes code for double-stranded RNA-binding protein 2. Proteomic profiling of an Arabidopsis mutant had shown many changes in production of proteins associated with drought stress or osmotic stress (and there were indications for better salt tolerance); nevertheless 21-day-old *Gmdrb2ab* soybean CRISPR/Cas9 knockout plants were more sensitive to drought than wild-type soybean plants with water withheld for a week in pots in a growth chamber. The genes could still be valuable targets for stress tolerance as many miRNAs dependent on Drb2 are involved in stress responses while being less important in basic plant development, and possibly also for other processes involving small RNAs according to (Curtin et al., 2018). No results of field testing were yet found in literature.

There are publications on CRISPR knockouts (SDN-1) conferring drought, salinity or heat tolerance, mostly in rice. Field testing is largely absent in publications, except testing to check for negative effects on plant performance under normal agronomical conditions, as for instance for rice with edited *OsRR22* (a type-B response regulator transcription factor involved in cytokinin signalling, (Worthen et al., 2019)) for salt tolerance (Zhang et al., 2019). Rice with edited *OsPQT3 E3* (an ubiquitin ligase) for salt tolerance, showed higher grain yield even under normal conditions (Alfatih et al., 2020).

CRISPR knockouts may build on knowledge of field testing using previous mutant or RNAi versions of the same genes. In the example of *OsRR22*, a salt-tolerant cultivar had been developed through classical mutagenesis of the *hst1* locus that encompasses *OsRR22* as the gene likely responsible for the stress tolerance (in the relatively short time of two years) (Takagi et al., 2015). Another example is *DRO1*/*qSOR1*, as *qSOR1* and *Dro1* were identified from a QTL. *Dro1* in rice promoted deeper rooting by increasing the root growth angle, as shown by crossing into a more superficially rooting variety where it improved drought tolerance (Uga et al., 2013). Four years of field trials on lines with a loss-of-function allele from cv Gemdjah Beton, resulting in shallower roots, had already shown significantly higher grain yields (kg/ha) in a saline paddy, while yields were similar under control conditions (Kitomi et al., 2020). CRISPR-Cas9 targeted mutagenesis of homologues *DRO1*, *qSOR1*/*DRL1*, and *DRL2* showed their involvement in developing a shallower root angle. This means that roots grow closer to the surface, which is helpful in avoiding salt in deeper soil layers, but which may be disadvantageous under drought, as indicated by the results on overexpressing *Dro1* above. Another example showing interesting trade-offs is the CRISPR knockout of *SlMAPK3* (encoding a signal transducing kinase) in tomato. This led to improved heat tolerance (Yu et al., 2019), but decreased drought tolerance (Wang et al., 2017a). In addition, resistance to *Botrytis* fungus was also reduced (Zhang et al., 2018).

Recently initiated field trials in Belgium featured CRISPR events in maize (https://vib.be/news/applications-submitted-new-field-trials-genome-edited-maize), apparently representing new mechanisms, based on DNA damage repair (among which a TF gene, *ZmNAC52*, about which no further information could be found in literature), and a linker histone protein affecting access to specific genes for transcription. Linker histone genes were reported to be induced by abiotic stress, a variant was reported to negatively regulate salt stress response in Arabidopsis through binding the promoter of *SOS1* and *SOS3* (Wu et al., 2022)).

An example in the Genetic Literacy GE tracker database (https://crispr-gene-editing-regs-tracker.geneticliteracyproject.org/) of a recent start-up company that is executing ambitious genome editing experiments on which apparently no scientific papers have been published yet, is Agrisea (presently Canada-based ALORA). It has started field (actually sea) experiments on the Bahamas and in Singapore with rice gene-edited for salt tolerance, adapting the expression of eight genes identified from salt-tolerant seagrass and mangrove species, with plans to extend their work to other crops. The idea is to grow this rice on sea using floating mini-farms, combining plant cultivation with fish nursery (which is, to some extent, similar to plans for seaweed production in Europe). There was also interest in salt-tolerant rice for the salinizing Mekong delta in Vietnam. From the information found in a recent review (Camerlengo et al., 2022), plus news items (Carey 210908 and Food Planet Prize 211029), it is difficult to assess how far the research has come towards practice.

In summary, the above gene-edited versions are based on gene knockouts and these will mainly work on negative regulators of abiotic stress tolerance. They have been subjected to field trials only to a limited extent as far as can be gathered from literature. Many positive regulators of stress tolerance have been tested using CRISPR/Cas9 as well, to study the functionality of the genes (i.e., knockout leading to poorer plant performance under stress) in research projects. For breeding stress-tolerant crops the approach would have to be modified. A notable example is the special approach for *ARGOS8* in maize described in section 4.2.5, which involved homology-directed repair (HDR, SDN-3) introducing a promoter sequence from another maize gene into the *ARGOS8* promoter region to moderately increase its expression level. This was already tested for its effectiveness in drought tolerance in field trials. It is a different approach from the other examples discussed here that introduced small mutations (gene knockouts, SDN-1) and is more reminiscent of an intragenesis approach where a new combination of a promoter and a gene from the same species is transformed into a crop. It has remained a rare example up to now, possibly since HDR (homology-directed repair) is still difficult to apply in plants.

4.4. Conclusions on events authorized and possibly in the pipeline

Several interesting examples exist of events that increase abiotic stress tolerance, but very few made it to authorization, MON87460 maize, HB4 soybean and wheat, and NXIs sugarcane. Even apparently promising ones in the field, e.g., company validated leads, have not been brought to commercialization yet, as far as could be gathered from literature, and approaches expected to be promising were not being commercialized in subsequent years, as far as we could establish from public information. It is hard to find clues for the reasons behind this. It may have to do with inconsistencies turning up in further field testing. Complex dossier building for authorization may also play a role, or a combination of field results and dossier building. Information needed for such inference is largely absent in literature (partly because the relevant data are considered as CBI and absent from any publication on the subject).

In an attempt to provide more background on possible reasons for lack of commercialization, we will discuss basic problems and limitations with GM and genome editing approaches for developing stress-tolerant crops in the next chapter.

5. Complexities with providing stress tolerance through GM in commercial cultivation

In the previous chapter, we have discussed the few examples of commercialized (or recently authorized) abiotic stress tolerant crop events and several examples that appeared promising based on field testing but did not (yet) appear as commercialized, mainly addressing drought, and a few concerning salt, heat (and cold or flooding). While it is normal to innovations that many exist in the early phases of development but only few make it to cultivation/commercial success, the question can be raised whether the selection is steeper for abiotic stress traits compared to other traits. There are some specific complexities with generating abiotic stress tolerance through biotechnology that will be discussed in this chapter. In addition to the basic notion that abiotic stress tolerance is based on complex multigenic plant responses, one specific point concerns the difficulty of efficiently selecting genes under controlled conditions that have predictive value for field conditions, where stress may follow a seasonal pattern but may also be unpredictable. The latter point is also a problem for physiological traits researched for conventional breeding approaches. This will be discussed in detail in the first section. Furthermore, there are practical problems related to technical limitations of genetic transformation and additional biosafety measures necessary during development and to authorization, which lead to stronger requirements for an event to be commercially valuable than with conventional breeding, as it also needs to compete with conventional approaches that incrementally also can achieve a certain stress tolerance during the same time path needed for GM development. This will be discussed in the second section.

5.1. Difficulties of testing genes for (drought) stress tolerance

5.1.1 Drought experiments under controlled conditions and in the field during a growing season

There are many papers testing the possible role of individual genes by ectopic (over) expression and silencing (by RNAi and more recently genome editing) (see Chapter 2 for an overview of the types of genes involved). Usually, they describe a limited number of markers for stress responses in seedlings or plants still at a vegetative stage under more or less controlled conditions, i.e., fresh and/or dry weight, chlorophyll content, stomatal conductance, ion leakage, malondialdehyde and ROS levels, and expression of marker genes. In addition, transcriptomics, proteomics, and metabolomics may be applied to obtain more insights on signalling pathways involved, by relating differences in gene expression with growth conditions applied and with levels of stress tolerance of plant lines tested. The difficulty lies in finding traits and or markers among such data that can be efficiently measured in the laboratory and greenhouse that at the same time have a good predictability for crop behaviour under conditions of commercial cultivation in the field. This also applies to physiological traits assessed under controlled conditions in conventional breeding approaches.

Already in 2012, Passioura discussed the difficulties associated with applying single gene transformations to achieve stress tolerance for commercial cultivation. Gene identification often involves subjecting plants (the model plant *Arabidopsis* as well as some major crops) to simply scorable experiments such as using osmotics like PEG (polyethylene glycol) or salt in hydroponics or applying severe drought stress on plants growing in pots in the greenhouse and looking for survival/recovery. This way of phenotyping a stress response may not be particularly relevant for agricultural practice (Passioura, 2012; Hall and Richards, 2013), where drought generally develops gradually and will often be moderate, as farmers will aim at avoiding complete plant failure by adapting their cropping systems (Tardieu et al., 2018). There are a few exceptions, such as sudden dry winds (or even sand storms), or when a sowing time optimal for other reasons must be chosen with the risk of early droughts, which would specifically require high survival capability of seedlings until rain falls (Passioura, 2012). In addition, the physiological basis of particular transgene effects are often not sufficiently clear to judge their relevance to the field (Hall and Richards, 2013).

With regard to greenhouse experiments, for instance testing plants in pots is quite distinct from field conditions, if only because the pots limit root growth. Water can be taken up more easily from the soft soils often used in pot experiments but it is also exhausted more quickly than under typical field conditions. Under those conditions faster stomata closure is advantageous, but that is not functional in the field where drought builds up gradually, except maybe with sudden dry winds (Passioura, 2012).

In recent years, high-throughput (HTP) phenotyping has evolved quickly and technology has been developed for both greenhouse and field trials (Tardieu et al., 2017; Yang et al., 2020). Modelling using data continuously collected throughout the growth season enables the detection of traits that contribute to yield under varying conditions in the greenhouse and the field, for instance, water conservation traits under greenhouse conditions that could be predictive for performance in the field, in particular grain yields. Such continuous measuring and modelling may partly bridge the gap between controlled experiments and field experiments. With regard to HTP phenotyping, low canopy temperature, in particular during grain filling, was found to be associated with higher yield (Saint Pierre et al., 2012; Langridge and Reynolds, 2021). However, high canopy temperature would not necessarily be disadvantageous, as it may also indicate lower stomatal conductance that is helpful in saving water use across the growing season (Hall and Richards, 2013).

In practice, relatively few events have been put to the test under field (cultivation) conditions, at least in published papers. and when they have, it may be limited to normal conditions for establishing any possible negative effects on plant growth and yield. This is also reflected by the observation that from all field trial permits, only for few genes the results could be retrieved in the scientific literature. Moreover, yield is a difficult trait to assess, with low heritability (i.e., a relatively small part of the variation found in the field can be explained by genetic factors (compared to environment and random errors)) in drought-prone environments, which demands multi-site/multi-year trials (Passioura, 2012).

Araus et al. (2019) criticized yield gains reported in small field trials on a per plant instead of the per area basis that would be more relevant for field harvesting practice. When the results claim a high % differences between transgenic and non-transgenic lines, it would be important to also compare the lines with a representative panel of conventional lines adapted to the targeted field conditions (Araus et al., 2019).

Assessing yield under field conditions representative for commercial cultivation is laborious and costly. In particular, applying drought is complex under field conditions, it may for instance necessitate the use of rainout shelters. Alternatively, sites with predictable low rainfall may be used, where drought levels can be adjusted by irrigation. Saline field trials may even be more difficult, as salt levels may be unevenly distributed within the field and change upon rainfall, draining salt from higher soil levels. Heat may also be difficult, but for instance CIMMYT (Mexico) uses an array of field sites across Africa, Latin America and South Asia where maize and wheat are being screened, including sites where heat stress can be tested for instance at anthesis by carefully choosing planting dates (Vikram et al., 2016; Prasanna et al., 2021). Sometimes, controlled experiments may shed light on difficulties with transgenic events encountered in field experiments, and so improvements could be made in an iterative way, in the course of time.

5.1.2. Drought tolerance strategy and breeding targets

In reviews, defining plant drought tolerance with relevance to field conditions is repeatedly discussed as a major difficulty due to the large variance in local field conditions (e.g., Nuccio et al. 2018). Definitions will depend on the target population of environments (TPE) for which breeding is performed. One could model plant growth for average conditions or aim for a specified percentage of years of stronger drought occurring (e.g., 10%, Passioura 2012), but often the occurrence of drought is quite irregular (Tuberosa, 2012). For instance, the drought-prone regions of the US Corn-belt show much variation between years in rainfall and its distribution across the season, leading to variable impacts on crop yield, with only some years (e.g., 2012) having a severe drought that substantially reduces yield (Cooper et al., 2014).

The way drought tolerance is defined therefore is closely linked with the breeding targets. In the field, water use efficiency (defined as plant dry weight produced per unit of water transpired) encompasses a broad array of characteristics, that is, being able to effectively take up water provided by rain or by irrigation, or present in the (deep) soil, and to use that water efficiently to produce biomass, in particular, grain filling. Crop simulation models (incl. genetic models (QTLs)) in combination with environmental records over an extended period could be of help (Hall and Richards, 2013).Timing of stresses like drought and heat is also important to breeding for tolerance. Water saving could mean slower growth at the beginning of the crop cycle to save water for terminal drought at the reproductive stage (grain filling) in semi-arid regions. Alternatively, plants continuing growth under stress (delaying leaf senescence, so-called "stay-green", see previous chapter for examples) may be more effective when enough water can still be extracted from subsoils or when water/irrigation can be expected later on (Passioura 2012). Plants growing faster (e.g., due to more open stomata) and flowering earlier would be more beneficial under Mediterranean conditions to avoid terminal droughts (Saint Pierre et al., 2012). Such a growth-centred approach was presented as a more modern research approach next to the approach of protecting yield under (periods of severe) stress while avoiding as much as possible growth penalties, indicated in a recent review by (Tenorio Berrío et al., 2022). The most sensitive stage for many crops is during flowering/grain filling. Both pollen and ovules can be affected, in which carbohydrate metabolism and transport are involved; in maize also mistiming of male and female flowering, the anthesis-silking interval (ASI), can ensue (Passioura, 2012).

(Tardieu et al., 2018) elaborated upon the problems around defining drought scenarios and the usefulness of experimentally identified physiological traits for improving yield in the field. For instance, larger root systems can be advantageous to obtain water from deep soil (this is part of the mechanism behind effects of for example *AVP1*), but a large root system in shallow soil or at early growth can result in water depletion, leaving too little for reproductive growth later in the season. Indeed, a strategy of saving water by lowering hydraulic conductance by selecting for smaller xylem vessel diameters proved effective in wheat cultivation in Australia. To address this context dependency of physiological traits, they propose a strategy where multi-year/multi-site yield trials with hundreds of genotypes are used to link drought scenarios under realistic environmental conditions to favourable alleles in a genome-wide association study. This is combined with HTP phenotyping and crop modelling to link effect of traits and allelic effects to environmental scenarios, using genomic prediction. This should lead to modelling patterns of environmental scenarios and combinations of alleles performing favourably in these scenarios, which may be used to evaluate the predictability of yield by these allele combinations and to assess their usefulness for variety improvement. (Millet et al., 2019) elaborated this approach for maize using European field trials along a climatic transect to establish the environmental factors affecting yield (mainly determined by grain number) and then relating these to genotypic responses based on whole genome marker scores, with plant development modelled from a HTP phenotyping platform. Genomic prediction normally is difficult if GxE interactions are high, but in this way they managed to obtain a good prediction of yields.

5.1.3. Results and issues from research examples: DREB and ion transporter genes

As an example how these problems worked out in GM research, a characteristic group of TFs is discussed here, namely the DREB proteins. The *DREB* or *CBF* genes are good early examples in GM development of genes that were identified by imposing severe stresses in controlled experiments. They encode transcription factors recognizing dehydration-responsive elements in promoters of genes involved in an ABA-independent response pathway (Zhang et al., 2022). They have been shown to offer effective protection under severe stress but with a negative effect on plant performance under control conditions, which could be mitigated by using stress-inducible promoters. For example, higher survival under severe stress by transgenic overexpression of *TaDREB2* and *TaDREB3* was shown to be partly due to wheat and barley plants growing slower, thus using less water but at the cost of productivity under normal conditions. This could be mitigated by using the drought-inducible promoter ZmRab17 (which may also provide higher frost tolerance) (Morran et al. 2011). The effectiveness of transgenic constructs may also vary between different genetic backgrounds. When crossed into different elite wheat lines, variation in TaDREB3 expression under the Rab17 promoter was encountered, and lines backcrossed from two of a total of three crosses with elite cultivars showed significantly higher grain yield of 18.9% and 21.5%, respectively, in large containers mimicking field conditions; for each of these two crosses this applied to just one of the two backcross progeny tested (Shavrukov et al., 2016). In these progenies, no induction of the transgene by cold was found and thus no improvement of frost tolerance (Shavrukov et al., 2016).

Even when yield penalties were mitigated by the use of stress-inducible promoters in controlled experiments, performance in field trials often appeared to be ineffective in terms of grain yield (Khan et al., 2016). In a recent compilation of 53 published transgenic *DREB* plant lines, 10 contained information from field studies. Six of these 10 field studies assessed grain yield (Sarkar et al., 2019). In a saline field test, higher grain yield per wheat plant transgenic for *GmDREB1* from soybean was found; this was due to a larger number of tillers per plant (Jiang et al., 2014). For peanut, higher seed weight and HI (harvest index) per plant was found for most of the *DREB1A* transgenic lines in a small field trial (Bhatnagar-Mathur et al., 2014). The yields from plots that are more representative for cultivation, were, however, not significantly improved in studies involving *AtDREB1A* in soybean (Rolla et al., 2014) and in wheat (Saint Pierre et al., 2012). In the wheat *AtDREB1A* case, a relationship was found between WUE under greenhouse conditions and more stable performance in field tests (Saint Pierre et al., 2012), in particular with respect to grain yields. Another example not discussed in Sarkar et al. (2019), concerning *CBF1* under a stress-inducible promoter in potato, also did not show improved tuber yield (kg/plot) in four years of field testing (Nichol et al., 2015).

Interestingly, in the USA “Am I regulated” database, a cisgenic version of *DREB1A* in rice is present (actually intragenic as it refers to a new combination of the *DREB1A* gene and an actin promoter). No further literature could be found for this event. Among the APHIS permits in the USA also a transgenic soybean with *AtDREB1D* was found; a study on its physiology (Guttikonda et al., 2014) (not in Sarkar et al. 2019) mentions ongoing field trials but no paper was found on that. Among the validated leads at Pioneer (Corteva) ((Simmons et al., 2021), see further below), *ZmSRTF18* turned out to be a truncated version of a maize *DREB2* gene. The truncated version lacked a functional N-terminal CBF domain/nuclear localization signal, and as such was shown to improve maize grain yield under drought without negative effects under normal conditions (Ayele et al., 2016).

DREB genes are also involved in cold tolerance, but very few field tests have been performed. A recent review listed examples of transgenic plants comprising several families of TFs (apart from CBF/DREB, also MYB, NAC, WRKY and bZIP), of which very few were tested in the field, including none for yield, testing was performed mostly using cold rooms (Mehrotra et al., 2020). A potentially interesting example was a small field trial that followed an apple tree line transgenic for peach *CBF1* under a constitutive promoter (CaMV 35S) for three years. The transgenic line showed reduced growth, early senescence (start of winter dormancy) and delayed bud break, which could be advantageous to avoid cold damage. Improved freezing tolerance was, however, only directly tested on leaves collected in June, showing an improvement compared to wild type of -2^{0}C (this was -4^{0}C with seedlings under greenhouse conditions) (Artlip et al., 2014).

Comparable results were found for several groups of genes involved in salt tolerance by (Kotula et al., 2020), who reviewed the effects of transgenic crops for Na^+ and K^+ transporters and H^+-ATPases and H^+-PPases (H^+-pyrophosphatases). The latter two create electrochemical gradients to facilitate Na^+ transport, e.g., into the vacuole by the NHX1 Na^+/H^+ antiporter or to the apoplast through the SOS1 Na^+/H^+ antiporter, to protect the cell. Out of 51 examples in which growth parameters were assessed quantitatively, 48 cases involving 26 crops showed improvement, but only six studies assessed yield and only two were tested in the field. The vacuolar Na^+/H^+ antiporter AtNHX1 in wheat led to grain yield (t/ha) that was 137% of wild type under salt stress, with leaves showing Na^+ and K^+ levels of 65% and 176%, respectively, of wild type (Xue et al., 2004)). *AtNHX1* expressed in cotton led to higher fibre yields than wild type under salt stress in the greenhouse and under irrigation in a dryland experimental farm (He et al., 2005).

Thus, several studies have addressed *DREB* genes for several types of abiotic stresses and ion transporter genes for salt tolerance, but few have assessed efficacy in the field. In most cases, these field trials did not show significant improvements in stress tolerance.

5.1.4. Example of a commercial programme targeting stress (drought) tolerance

The truncated *DREB* gene version *ZmSRTF18* was mentioned as a validated lead in a recent paper of Corteva (Pioneer) researchers. This paper contains an interesting description of the route from identifying genes involved in stress tolerance to field validation for commercial cultivation in maize in a breeding company (Simmons et al., 2021). From a programme from 2000 to 2018, they show 22 validated leads on yield enhancement and abiotic stress tolerance (and nitrogen use efficiency NUE), that resulted from field testing 1671 genes involving 3863 combinations of transgenic construct and year tests in grain yield harvesting plots. For yield, an increase of at least 2-3 bushel per acre (=0.13-0.20 ton/ha) advantage was set as threshold, for drought 10% yield increase under moderate-severe drought conditions. They followed a mixed strategy, including plot designs at statistical limits for budget reasons, and with an important role for managed stress sites. For instance, for drought they used sites with predictable low rainfall where stress could be applied at the pivotal reproductive stage and grain filling through manipulating irrigation. Of these 22 validated leads, 14 concerned stress tolerance. These leads were often hormone related, i.e., ABA, ETH and CK. The selection of genes started from basic tests in mostly *Arabidopsis* and also in rice, using activation tagging and overexpression, and this was extended with -omics, QTL studies, and bioinformatics. Literature leads became less important over time for the already mentioned limitations of (severe) stress-growth trade-offs and limited applicability to maize. In total, the pre-screens were estimated to add up to 35000 genes. In maize, greenhouse testing was important for enabling HTP phenotyping, but these greenhouse tests proved to be a poor prediction of field results, at most useful for picking out poorly growing plants. It was also hard to find helpful component traits (except for the water conservation mechanism, also see DREB discussion above), as most genes had a positive effect under severe stress but were performing poorly in other conditions. Drought-tolerant leads mostly were discarded because of negative performance under normal conditions. Most events were transgenic overexpression, but RNAi was also attractive as it generates a (semi)dominant trait, see e.g., ACS6 silencing affecting ETH signalling in 4.2.5. Genome editing had produced only a few events in 2018 but for its relative ease of use it can be expected to become attractive for possibilities of testing promising genes in larger sets of germplasm. There were also examples of interactions of transgenic events with elite germplasm it was introduced into, so that the same construct could act positively as well as negatively depending on the genetic background (e.g., strigolactone-related AtCXE20, which reduces free strigolactone concentration, a.o. leading to a smaller ASI, a marker for drought tolerance).

5.2. Practical problems with the GM approach in view of progress in conventional breeding

5.2.1 Progress compared to conventional breeding

A well-known problem is that for transformation often only one or a few well-transformable cultivars can be used. These are generally older cultivars that lag behind in yield performance compared to modern cultivars. Moreover, even if up-to-date cultivars could be used for the GM development process, conventional breeding would further improve during the period needed for assessing the GM cultivar, including dossier build up for authorization. Conventional breeding may add 0.5-1% yield improvement per year, which may offset gains of the GM variant during the total period of bringing the GM cultivar to market. Conventional breeding has been indicated to provide improvements of water-limited yields of ~0.5% (up to 0.87%) per year for wheat and maize (Hall and Richards, 2013). (Simmons et al., 2021) also discuss the problem of few genetic backgrounds being amenable to transformation, but during their programme improved protocols increased the range of usable germplasm available. Moreover, their crossing programme with elite germplasm worked in practice faster than transformation, the latter taking 3-4 years.

Langridge & Reynolds (2021) made a rough estimate that yield improvements of transgenic stress-tolerant crops should be well above 10% to warrant the extra regulatory costs in performing extensive field evaluations, particularly in wheat where no commercial GM crop has yet been grown (Langridge and Reynolds, 2021). This is in line with the pre-conditions set by (Simmons et al., 2021) for granting validated lead status to a drought tolerance event in their company breeding programme on maize, also partly in view of regulatory costs (for conventionally developed AQUAmax maize, the required yield advantage was set at 2% (https://www.pioneer.com/us/products/corn/traits-technologies/aquamax.html)). One way of handling this limitation was to try to obtain the best transgenic event before moving into the regulatory process, but nevertheless a large-scale optimization of constructs and transgene positions for a few genes (e.g., *ARGOS8*) had limited success.

Basically, a more optimal strategy in a breeding programme would be to introduce transgenes as new alleles into the ongoing breeding cycle of crossing and selecting, as their performance would be tested across different genetic backgrounds (for instance in heterotic groups of hybrid parental lines). With transgenes, it is, however, more practical to keep particular events/lines separate from the rest of the breeding programme so that confined testing is possible and safety dossiers can be built. Consequently, a transgene is introduced into elite plant material only after being proven successful in a thorough testing process. In the more optimal strategy, smaller-effect transgenes might be introduced and combined, as is done for large effect QTLs. Genome editing could offer an alternative option for this (Simmons et al., 2021). Genome editing could also be used for the development of safe harbour sites in the genome facilitating efficient transgene introductions into elite materials.

An alternative for a transgenic program is mining a crop's germplasm for alleles of the native homologues of genes after they have been proven to confer stress tolerance in a transgenic approach in the crop. An example is the *AtAVP1* gene, where the researchers tested it successfully in a GM context (see section 4.2.3) but also searched for allelic variation in native wheat H^+-PPases, *TaVP*s, as approval of GM variants in wheat on the short term was not likely forthcoming (Menadue et al., 2021). Such an alternative approach for transgenics in the form of allele mining in the crop's germplasm was not successful for *ARGOS8*, for which reason the researchers turned to genome editing (Shi et al., 2017) (see section 4.2.5). For *AVP1* and *TaVPs*, also genome editing was proposed as alternative where natural variation from allele mining would not deliver sufficient improvements (https://www.plantphenomics.org.au/over-expression-of-three-wheat-genes-linked-to-yield-gains/).

5.2.2. Breeding for drought tolerance and markets

More generally, drought tolerance is also a problematic trait from a commercial point of view (Simmons et al., 2021). As droughts do not occur every year, many farmers do not have a guaranteed advantage in every year of cultivation (see discussion in previous section). Most drought-prone fields will not be planted with maize when commodity prices expectations are low. In the USA maize practice, there are mostly problems at the grain filling stage, but the effects are usually small. At flowering, there are stronger effects, and to prevent that problem the conventional AQUAmax® cultivars were developed (Cooper et al., 2014). This could be done relatively efficiently because flowering stress is more easily scorable than grain filling in experimental plots. Furthermore, (Cooper et al., 2014) used managed environments across two hemispheres enabling two field experiments per year, with drought applied as 0.66 supply/demand during flowering and grain filling for which correlations with the TPEs (target population of environments) were established. Physiological experiments showed advantages for DT hybrids that more slowly deplete soil water accompanied by slower growth yet in the end producing higher grain yields by higher remaining water availability at the reproductive stage. Breeding was done with the help of genomic prediction of the well-established indicator of shorter ASI (anthesis-silking interval) using HTP genotyping of SNPs across the whole genome (Cooper et al., 2014). Another trait addressed was seedling emergence under stress. In this way, 5-9% higher yield (in 2011 7.3% and in 2012 9.7% at conditions leading to yields below 6 t/ha, in 2013 2.3% under conditions leading to yields above 6.5 ton/ha (drought overall specified as resulting in yields smaller than 9.5 ton/ha) (Gaffney et al., 2015). Thus, AQ yields 37 g/m^2 more under drought while not showing negative performance under favourable conditions. AQ also performs better with an unusually high planting density of 6.9 plants/m^2 so as to increase available water use, which leads to a 50 g/m^2 advantage above normal hybrids. The improved yield was likely not due to better water uptake but to maintaining HI under high plant density and water stress (Messina et al., 2020). Genetic gain under drought of 1-1.6%/year was higher than previous estimates of 0.7%/year (also see Hall & Richards 2013 above) (Messina et al., 2020). Crop growth models combined with whole genome prediction to tackle G x E x M (genotype x environment x management) interactions in breeding predictions was seen as key to success (Messina et al., 2020).

Public sector maize breeding for the tropics at CIMMYT and partner organizations directed at smallholders in Sub-Saharan Africa (SSA), South Asia and Latin America started already in the seventies with selection for drought tolerance at field sites and more recently increased genetic gain with improvements such as doubled haploids (DH), HTP phenotyping, and MAS. With regard to problems with QTLs being too many and inconsistent across sites, they also turned to genomic selection that would show a 43% improvement compared to phenotypic selection. Genetic gains of 2-2.57% per year were estimated under managed drought stress in SSA in 2013-2018. Multiple stress tolerant lines were released in SSA, South Asia and Latin America, for instance under the STMA programme, 218 lines to SSA between 2016 and 2019. Also data management is becoming increasingly important as is use of machine learning/decision support tools (Prasanna et al., 2021). In wheat, there is relatively more public breeding, with a large array of field sites across the world used by CIMMYT and IWIN (International Wheat Improvement Network). Facing similar problems with QTL, genomic selection, though relatively recent in wheat, also appears to be an improvement in wheat (Langridge and Reynolds, 2021).

Some of the problems in cultivation practice may also be seen in the only transgenic drought tolerant maize commercialized up to now, Bayer's MON87460 (DroughtGard). (Araus et al., 2019) noted that the income gain was marginal compared to total gains of transgenic maize in the USA based on figures from (ISAAA, 2017). In terms of acreage, MON87460 covered ~4% of total GM maize acreage in USA that was 33.84 Mha of herbicide tolerance and/or insect resistance traits (mostly stacked), representing 93.4% of total maize in the USA in 2017 (ISAAA, 2017). A report based on the 2016 Phase II ARMS (Agricultural Resource Management Survey) survey of maize (a survey performed every 6 years) by McFadden et al. (2019) put this in the perspective of other drought-tolerant maize in the USA. Drought-tolerant maize adoption was 40% in drought-prone regions, i.e., the Mid-West (western Corn Belt), which represented 22% of the total maize acreage. Of all drought-tolerant maize, 80% was conventionally bred, e.g., AQUAmax® from Pioneer and Artesian® from Syngenta, that are otherwise also mostly GM for herbicide tolerance and insect resistance (Bt). Early GM drought tolerance adoption up to 2016 was largely in line with the earlier years of adoption of HT and Bt, but from then on it has remained stable at 3-4% of the maize acreage or 20% of the drought tolerant maize area. This may be due to other favourable traits in the GM or conventional drought tolerant variants offered in certain areas. Differences in technology fees as part of the seed prices could also play a role. Drought tolerance is mostly combined with HT and Bt, and stacked traits are less costly than the traits separate in different lines added up, which may influence adoption. There is a price premium for drought tolerance (~$10/bag on total of ~$264/bag); this could not be estimated separately for GM, although it may be more costly than conventional drought tolerance. Another factor could be that the cultivars lagged behind in yield, as this also occurred in the early years of GM herbicide tolerance and Bt, but this was solved by crossing the construct into the latest varieties (also see discussion in previous section) (McFadden et al., 2019). Conventional drought tolerance was introduced in 2011, the GM drought tolerance in 2012 (commercially in 2013). Companies providing conventional drought tolerance are also researching GM approaches, e.g., Pioneer (Corteva), see Section 5.1.4.

After extensively discussing their biotechnology programme improving yield, drought tolerance and NUE, (Simmons et al., 2021) concluded that in the end focussing on yield may have been a better strategy, for the best yield-enhancing events are also performing well under stress. Apart from *ARGOS8*, e.g., also ROS-related genes such as *AtDTP6* improved yields under optimal growing conditions. In a review of the conventionally bred Pioneer drought-tolerant maize AQUAmax, (Cooper et al., 2014) discuss indications that already before the start of their programme, drought tolerance was improved indirectly by selecting in the TPE including drought-prone areas of the US Corn-belt, for example by improving root system architecture. However, extensive field studies showed that the rate of yield improvement under favourable conditions was higher than under drought (as gathered from the slope of curves from 1930-2010); also, in TPEs there can be large periods of no drought occurrence disturbing phenotyping efforts for drought tolerance. Drought tolerance will already have been implicitly improved in breeding for yield under particular conditions where there are drought periods; on the other hand, most breeding is performed under optimal inputs (Nuccio et al., 2018).

6. Discussion

This study’s exploration of genetic modification for the development of abiotic stress tolerant crops aimed at listing examples of genes that are successful in commercial cultivation or that are promising based on results in field experiments. Drought was the most prominently studied stress, with salinity and heat following behind, and we also briefly explored examples of cold and flooding tolerance. Three types of transgenic events have been authorized for cultivation, all providing drought tolerance. They are based on a bacterial cold shock factor introduced in maize, on a bacterial biosynthesis enzyme for an osmo-protectant introduced in sugarcane, and a plant transcription factor, HB4, introduced in soybean and in wheat, respectively. Many transgenes or gene edits have been researched, mostly under controlled conditions in small-scale experiments, but it proved difficult to obtain indications of commercialization of these events in the near future.

We have described a number of GM events that appeared to be promising for a combination of reasons (see Table 1 in Annex 2). The most important reason was that they confer improved yields in field trials under stress without negative effects under favourable conditions. Additional clues were sought in databases, overviews or news with plans of commercialization, or listings in field trial applications. However, without further clear indications, it remains hard to predict whether these events will achieve commercialization in the end. First, it turned out to be difficult to extract a pipeline of events that could become commercialized, as a large part of R&D is kept confidential as commercially relevant information. Thus, the identity of underlying genes researched will often not be released. A recent JRC report on market applications of NGTs identified 15 events addressing abiotic stress tolerance at the advanced R&D stage, meaning ongoing field trials and a likelihood to achieve commercialization in the medium term (by 2030). For reasons of confidentiality of the information, underlying traits/genes were not specified (Parisi and Rodriguez-Cerezo, 2021). Some companies published a retrospective of their experiences with developing transgenics for stress tolerance, for instance (Nuccio et al., 2018; Simmons et al., 2021), and these overviews gave some insight in the strategies and results obtained by companies in their stress GM R&D pipeline. Second, it also proved difficult to obtain an overview of those gene constructs that have been thoroughly tested in the field for yield improvement under abiotic stress from the available literature and data. For some genes, the apparent absence of reports from field testing after initial reports on gene identification and/or announcements of field testing/introduction into crops, may indicate that they were not successful. Still, publications may sometimes be released years later and successful development of varieties and building dossiers for authorization also takes time. For these reasons, our description of possibly promising genes for a commercial pipeline should be viewed as indicative and cannot be regarded as exhaustive (or predictive).

In discussions on the sustainability and regulation of new breeding technologies, an argument that is regularly put forward is the potential relevance for the development of resilient crops with regard to climate change adaptation. Resilience is a broad term that is often translated to abiotic stress-tolerant crops, but it also encompasses resistance to pathogens, pest and weeds, the patterns of occurrence of which may also change with global warming. In an analysis of many political documents about NGTs, and those from science organizations and scientific literature, resilience was by far the most frequently mentioned term, followed by terms partly being aspects of resilience, i.e., yield, pathogens and weeds, then abiotic stress tolerances to drought, heat, salinity, and plant nutrition (nutrient deficits), in a study by (Hüdig et al., 2022). These discussions take place in the wider context of an ongoing societal debate on the desirability of cultivating GM crops, with widely varying opinions ranging from that there is no use for GM crops in an environmentally safe and sustainable manner to that we cannot do without them at all to achieve a sustainable cultivation with sufficient productivity. Scientific reports have concluded that GM can contribute to sustainable agriculture, where sustainability depends on the trait, local conditions and institutional context, and provided that good agricultural practices are followed (Franke et al., 2011; National Academies of Sciences, Engineeering, and Medicine 2016). In this chapter, we will discuss, based on the results of this study, to what extent we may be able to draw conclusions on the possible contribution of genetic modification, that is, classical transgenesis and NGTs, particularly genome editing, for the development of abiotic stress tolerant crop varieties.

In the scientific literature, some authors question the value of GM approaches for improving abiotic stress tolerance that basically start from targeting single genes, even though in further research they can be combined with others (stacked). For instance, in their prognosis for genetic improvement of (water-limited) yield, (Hall and Richards, 2013) stated concisely: “Most traits we may wish to alter to increase yield or tolerance to abiotic stress are complex and it is unlikely that single genes will be important over a range of species and environmental conditions as we scale up from single plants to crop canopies and beyond where seasonal conditions are variable and unpredictable.” Apart from indications that drought signalling varies between species, searching for a gene with a universal effect across plant species may not be essential for breeding, as breeding and selection takes place within each crop. Within a crop, successful single gene approaches may still be rare when applied under the variable conditions in the field. This was illustrated for instance by the results of Simmons et al. (2021), who identified only 22 validated leads (successful transgenic traits in terms of yield in field testing, of which 14 under stress conditions) among 1671 genes tested for yield and stress tolerance. The difficulties with single-gene approaches may in principle not be surprising. Genes with a strong effect on abiotic stress tolerance operate in complex signalling and developmental pathways where they will often have pleiotropic effects that hamper the plant’s performance under other conditions.

Single gene approaches have been successful in resistance to biotic stressors, i.e., pests and pathogens, and these included GM examples in cultivation from early on in the introduction of gene technology, e.g., Bt against specific insect groups. Applications of NGTs as proof of concept include late blight resistant potato based on cisgenically introducing R genes (Haverkort et al., 2016) and bacterial blight resistant rice based on targeted knockout of pathogen-induced expression of S (susceptibility) genes (Eom et al., 2019; Oliva et al., 2019). In these cases, transgenics or genome editing was successfully applied, because (i) there were good options to use a single gene that would lead to resistance without strong negative pleiotropic effects on plant performance (a single R gene inserted in potato or a targeted promoter mutation in the case of the rice S genes), and (ii) their efficacy could be predicted from pre-testing within the crop concerned under controlled conditions and/or at early growth stages. This is not universally the case in developing resistance against biotic stressors, as also for diseases, the available resistance mechanisms can be complex and their selection or introduction likewise difficult.

As complex and interrelated signalling pathways are involved in plant responses to abiotic stress, changing the expression of a single gene may affect other genes involved in responses to other environmental or developmental cues and thus affect the plant’s performance. A second hurdle is the difficulty of predicting genes successful in yield improvement under field conditions on the basis of success in controlled experiments with plants in growth chambers and greenhouses. In this regard, the traits that are needed in the field were often not explicitly defined or researched. Defining traits for the field is hard because during a growing season stress often occurs irregularly and may considerably vary between years. Nevertheless, strategic choices in researching stress tolerance of gene constructs under controlled conditions also appear to have changed in time with regard to the weighing of the fundamental trade-off between increased plant protection (that takes away energy from other plant functions) and continued plant growth (necessary for yield) (Tenorio Berrío et al., 2022). It appears that during the early years of GM research in abiotic stress tolerance, gene identification was more targeted at traits for protection, which may come with a significant yield penalty under good conditions. More recently, the strategies have extended towards more growth-centred approaches, e.g., by adapting sink strengths. These approaches are projected to have larger potential to be effective across a growing season, but for the greater part this still needs to be proven by successes in field trials and in cultivation. Conventional approaches also have difficulty to identify/circumscribe physiological traits indicative for yield performance in the field under stress, such as canopy temperature or water use efficiency under controlled conditions. An example that was used successfully in several cases in maize is the silking interval (ASI, the time between male and female flowering).

(Tardieu et al., 2018) have proposed a way forward in linking physiological traits to yield performance in the field, by selecting combinations of alleles and/or traits adaptive to specific environmental drought scenarios in the target population of environments (TPE) of a breeding programme. For this, meta-analyses are performed to establish scenarios of environmental variation using sensor networks on tens of field trials. These field trials are used for assessing yield on hundreds of genotypes, while the genetic architecture of the traits is assessed by high-throughput (HTP) phenotyping under controlled and field conditions, combined with crop growth models and genomic prediction. Genomic prediction uses a combination of large-scale marker data across whole genomes and phenotypic data to predict breeding values of genotypes so that valuable combinations of alleles can be selected without extensive phenotyping. HTP phenotyping enables efficient assessment of traits during the whole crop growth cycle in both the greenhouse and the field. There have also been developments in traits that are particularly complicated to phenotype, such as root system architecture (see for example work on balancing improved uptake of water and nutrients with energy and nutrient investments in roots (Lynch, 2015; Schneider et al., 2020)). Such a combined approach of assessing environmental scenarios, phenomics and genomic prediction of yields could also involve traits obtained by GM (Tardieu, 2022).

Crop growth models combined with genomic prediction, based on several field-testing locations including managed environments, already has led to progress for drought tolerance in conventional breeding. It was reported to have led to increased genetic gain and has resulted in the introduction of varieties with improved drought tolerance in 2011 in drought prone areas of the USA corn belt, such as AQUAmax (Cooper et al., 2014). It has also led to further drought-tolerant maize variety development for tropical areas, i.e., Sub-Saharan Africa, South Asia and Latin America, in public breeding programmes by CIMMYT and IITA (Prasanna et al., 2021). Conventional breeding has also become faster and more efficient by other plant breeding innovations and tools, for instance, with the use of doubled haploids (DH) instead of inbreds produced through selfing, and employing speed breeding to shorten generations (Watson et al., 2018; Jähne et al., 2020).

GM and conventional breeding are normally combined at breeding companies to produce cultivars, by introducing transgenic traits such as HT or Bt into lines optimized in conventional breeding for their TPEs (target population of environments), but the programmes are not fully integrated. Transgenes will for example not be integrated like QTLs at an early stage of plant line development to test them in different genetic backgrounds in the context of an ongoing breeding programme. The practical limitation with transgenes is that they not only need standard field testing like conventional lines, but also need to be assessed for their safe use in field trials, for which it is practical to keep them apart from other germplasm during the greater part of their development. This makes developing a transgenic crop a more costly process, and thus the required yield advantage conferred by the construct needs to be higher to make it commercially competitive to continuing progress in conventional breeding lines during the transgenic version’s development time. A 10% increase was indicated as lower limit for wheat and maize (Langridge and Reynolds, 2021; Simmons et al., 2021). Genome editing-generated traits may be integrated and tested more efficiently in breeding programmes and evaluated along with all other variation in large field trials, whenever they are sufficiently similar to conventionally attained traits to warrant treating them as such in a regulatory context. This appears already to be the case in some jurisdictions, including the USA.

These concrete and supposed limitations do not mean that single gene approaches cannot work. Three types of transgenic events have been authorized for cultivation, one of which, the HB4, is effective in at least two crops, soybean and wheat. HB4 is a transcription factor of the homeodomain-leucine zipper (HD-Zip) type originally identified in a classical (severe) drought test under controlled conditions. As these two HB4 crops have only recently been authorized, there is yet little known of experiences in commercial cultivation. There were indications that HB4 would also be employed in other crops such as maize, but we did not yet find publications about results. MON87460 maize and NXI sugarcane events contain “protectants” from stress, an osmo-protectant and an RNA chaperone, respectively. We could find only little information about the performance of sugarcane NXI events; the MON87460 maize acreage has grown since its commercial introduction in 2013 (after a large demonstration trials year in 2012) to 20% of the drought-prone area in the USA, while the other 80% is occupied by various conventional drought-tolerant varieties introduced from 2011 on (McFadden et al., 2019). For transgenic drought-tolerant varieties, we did not find scientific publications performing meta-analyses on experiences with the effectiveness (yield performance) in practical cultivation, like has been done for e.g., Bt crops. Among the possible pipeline genes appearing to be helpful in protecting yield in field trials, *AVP1* showed improvements in several crops (see section 4.2.3). Moreover, there are also examples of single locus (non-transgenic) stress tolerance traits that were successfully introgressed using conventional breeding, e.g., for improving flooding tolerance (*SUB1*). In this regard, also classical mutations can be mentioned, e.g., salt tolerance based on *hpt1* (with *OsRR22* as gene-edited variant).

In recent years genome editing has increasingly contributed to research efforts for developing abiotic stress tolerance and this gave the field a new impetus. Although genome editing in the first instance starts from single genes, it may offer new, faster and/or more efficient possibilities in the field of abiotic stress tolerance. As a relatively recent development, their results have had less time to prove themselves under field conditions than transgenic approaches. One potentially interesting development, perhaps partially overcoming single gene limitations, is introducing a combination of several stress-related (trans)genes/alleles. A transgenic example is discussed in section 4.2.3, the combination of *AVP1* and *OsSIZ1*. Such combinations may become more feasible through the development of multiplex genome editing approaches. Multiplex genome editing entails producing targeted mutations in several genes in one round using specially developed delivery systems. Moreover, CRISPR genome editing may be used to make extensive mutant collections for large-scale screening of genes, although this is as yet little developed in plants (as opposed to mammalian/human cell systems) (Gaillochet et al., 2021). Genome editing also enables modifying promoters, which, by modifying or deleting specific promoter elements, allows subtle adaptations of gene expression during specific phases of plant development, in specific tissues and/or upon certain environmental cues. For transgenic research it was indicated that relatively little work has been done on promoters compared to testing candidate genes (Nuccio et al. 2018). By adapting promoters, negative side-effects of constitutively expressed abiotic stress-related genes on plant performance may be mitigated, as was done for instance with the SWEET S genes in rice against bacterial blight (Li et al., 2012). Modulation of gene expression to address quantitative genetic variation appears to have gained more attention through targeted mutation of promoters and other cis-regulating elements (enhancers) by genome editing, see for instance (Rodríguez-Leal et al., 2017). Moreover, genome editing has led to development of new concepts, such as faster domestication by introducing known domestication traits using genome editing into wild species or underdeveloped (“orphan”) crops that already have good abiotic stress tolerances (Lemmon et al., 2018; Li et al., 2018). In this report, we have not specifically discussed research on general yield improvement and on increasing the efficiency of photosynthesis (see e.g., (Eisenhut and Weber, 2019)), which may lead to successful traits under stress conditions, although this also remains to be seen in further field testing. In view of practical problems with the limited amenability of plant genetic resources/elite germplasm to transformation (including for genome editing, see chapter 5), it is not surprising that currently several institutes and companies also develop methods and (generic) strategies to improve both the transformation frequency and the amenability of a broad range of germplasm for regeneration (Atkins and Voytas, 2020).

In conclusion, all approaches to generate stress-resistant crop varieties have to deal with the complexity of stress responses under field conditions. Despite the inherent limitations of single-gene approaches in GM, there are examples of both conventional and transgenic crops with single-gene introgressions being cultivated. Recently, a combination of two transgenes was shown to be useful for improving stress tolerance in field trials as well. With regard to phenotyping in view of the irregular occurrence of stress during a crop season, conventional breeding has made progress through extensive field testing involving managed-stress environments, and this was further improved by new tools such as marker-assisted selection, more recently in combination with genomic prediction. This type of field testing will also apply for GM and genome-edited lines. There has been relatively little time to see published examples of genome editing fully tested in the field. For genome editing, there are also promising technical developments that still have to establish their value in practice, such as adapting promoters to change gene expression in a quantitative manner. Genome editing could address more genes at a time, but it will still be complex to optimize a balance in expression of combinations of genes, which will bring about the need for further developing knowledge. Therefore, it remains difficult to predict what progress may be expected from genome editing, as it is continuously evolving in methodology and plant improvement concepts. The field may steadily progress by increasing possibilities of large-scale data analysis, e.g., by AI (artificial intelligence)/machine learning. Recently, large-scale analyses combining field trials, HTP genotyping and genomic prediction appear to have shown a way to use physiological traits in improving stress tolerance. In such approaches, also traits conferred by GM may be involved, as well as alleles generated using genome editing.

7. References

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Annex 1: Results from searches for field trials from literature, databases and other sources

Field trial information in country websites of competent authorities or from country reports.

Databases and country reports were queried for information on field trials and projects involving crop abiotic stress tolerance. Results are given per country.

Argentina
The USDA FAS GAIN report BR2021-0047 mentions “The list of field trial applications currently being assessed is not public information”.

Australia
Among 141 crop Dealings involving Intentional Release (DIRs, most recently DIR 189, last accessed 220120 on the website of the OGTR Office of the Gene Technology Regulator), 20 applications were for field trials involved abiotic stress. Most were on wheat (13) and barley, crops that are grown under relatively harsh conditions in Australia. The applications encompass a range of genes, some of them classified as CBI; for just two of these genes (*AtAVP1* and *ipt)* peer-reviewed publications involving field testing results were found.

Bangladesh
The USDA FAS GAIN report BG2021-0038 mentions on-farm evaluation of 12 potential salt-tolerant wheat varieties with inserted Nax (=HKT1;4 & HKT1;5) genes by BWMRI jointly with Australian ACIAR, CSIRO. These have likely been obtained through introgression, see (Munns et al., 2012). The durum wheat varieties in which the genes from the wild relative *Triticum monococcum* were introgressed, showed improved yield in field trials (Deinlein et al., 2014; van Zelm et al., 2020). The University of Western Australia and the University of Dhaka are preparing regulatory dossiers for field trials at the National Institute of Biotechnology (NIB) for the screening of rice transgenic lines, including the genes PDH45 (helicase), G-protein, SNAC1, OsNHX1 and amiRNA-DST; these genes had been tested under controlled conditions (Touhidul Islam et al., 2009; Amin et al., 2012; Liu et al., 2014; Faisal et al., 2017); SNAC1 had been field-tested elsewhere (Hu et al., 2006).

Brazil
EMBRAPA (Brazilian Agricultural Research Corporation of Min Agr) and Corteva Agriscience have a partnership on the development of genome-edited crops, which includes, amongst others, drought-tolerant soybean (USDA FAS GAIN report BR2021-0047). EMBRAPA also has a partnership with Benson Hill Biosystems on the latter’s CRISPR 3.0 technology. Research on drought-tolerant soybean will involve knockout of genes involved in ABA metabolism (Embrapa formalizes agreement to increase genetic variability through gene editing - Portal Embrapa). A recent review report discusses work on drought tolerance in soybean using a.o. DREB and AREB genes ((Fuganti-Pagliarini et al., 2020)).The OECD mentions 31 field trials for 2020, including maize, soybean, cotton, sugar cane, eucalyptus, rice and citrus, and involving drought tolerance, but it is not clear for which crop(s).

Canada
No information is given on genes underlying the trait categories distinguished in the overview of field trials of plants with novel traits at the website of the CFIA (Canadian Food Inspection Agency). An interesting trend with time was seen here. Between 2007 and 2012, there were several hundreds of trials involving abiotic stress tolerance, mainly on canola (*Brassica napus*). The majority was performed by Monsanto, followed by Pioneer, Dow Agriscience, Bayer and a few public institutes and SMEs. After 2012, there have only been a few field trials involving abiotic stress tolerance, e.g. maize and wheat in 2014, potato in 2016, and soybean in 2020.

Chile

US seed companies have operations in Chile, including work on drought resistance, particularly in maize. Only GM seed production can take place, no commercialisations are expected in the coming five years (USDA FAS GAIN report CI2020-0019). Chile is a large GM sowing seed producer, but cultivation for the domestic market is not allowed; on the other hand, early on it has enacted an assessment procedure for NPBT products as GM or not like Argentina (Turnbull et al., 2021). Among the plants assessed are two maize events conferring drought tolerance using RdDM (Sánchez, 2020) for which no further details could be found in literature.

China

No public information was available on field trials (USDA FAS GAIN report CH2021-0128). China recently changed its regulation policy for genome editing crops, which appears to now be less tight than for transgenic crops. This might lead to interesting developments in pipelines towards cultivation, in view of the large efforts in research as reflected in patents and scientific publications (see below).

Colombia

In 2018 and 2020, field trials on sugarcane with abiotic stress tolerance (among other traits) have been allowed (ICA Resolutions 26479 and 82361). There was no detailed information on the genes used in the three events targeting drought, salt and aluminium tolerance. In 2010, a field trial of rice tolerant to drought was approved (USDA FAS GAIN report CO2021-0027). No further information on the event could be found in ICA resolution 4041.

EU

The present EU database on field trial permits went back to 2002. In the first decade (2002-2012) there were more than 100 trials per year, but in the most recent decade (2012-2022) this has dropped to around 10 per year. We checked all 100 field trial notifications for the period 2012-2022 individually. In addition, queries were performed for (abiotic) stress, drought, salt or salinity, heat, and stoma(ta*). The resulting information comprised a limited number of trials involving events conferring abiotic stress tolerance, seven of them from the last ten years, including multiplication of drought-tolerant HB4 wheat. Apart from the recently authorized HB4, none of them left significant traces in the literature, only in vitro/greenhouse studies could be found. Also here, sometimes the underlying genes were CBI. Interestingly, the most recent trials in Belgium (by VIB) involve gene-edited maize lines.

India

Indian companies and public sector are developing GM crops, and also abiotic stress tolerance is mentioned, in a wide range of crops. Research into GM for application in India was suspended or outsourced, due to policy uncertainty, so the focus was on genomics and marker-assisted breeding in India. Apart from GEAC (Genetic Engineering Appraisal Committee), also State governments have to approve field trials through NOCs (No Objection Certificates); only a few states (Punjab, Haryana, Delhi, Rajasthan, Gujarat, Maharashtra, Karnataka, and Andhra Pradesh) have issued such permits. Illegal cultivation of unapproved GMOs occurs, however, no abiotic stress tolerant crops (USDA FAS GAIN report IN2021-0121).

Indonesia

The Indonesian government has issued a roadmap for GM seed research targets for 2020-2025 and 2026-2035, as a guide for government and stakeholders in development and production of crops. The research targets include the development of drought-tolerant sugarcane and salinity-tolerant sugar cane, and rice and soybean with abiotic stress tolerance, while production is envisioned for 2036 and later (USDA FAS GAIN report ID2021-0040).

Japan

A strategic innovation promotion programme stimulates genome editing in Japan. Recently, a tomato edited for increased GABA content has been allowed and released for consumption, which represents one of the first cases of marketing of genome editing. There was no mention of research on abiotic stress tolerance in USDA FAS GAIN report JA2021-0140.

Republic of Korea

Research is mainly focused on second and third generation traits, including drought resistance. An "environmental stress-tolerant rice" is under development; no further details were given on this research or the stage it is in (USDA FAS GAIN report KS2021-0025). An overview of the National

Program for GM crops (NCGC, started in 2011) mentions a drought-tolerant rice for which safety assessment had been completed (Park et al., 2018), but the only reference found to field studies for drought tolerant rice was a field study assessing physiological parameters under drought but not yield, apparently on lines transgenic for CaMrsB2 (Siddiqui et al., 2015).

Mexico
There have been no new authorizations for cultivation since 2018 (and actually only GM cotton is grown). There was no information on field trials in USDA FAS GAIN report MX2021-0087.

Paraguay
OECD mentions two field trials on maize, but these were not about drought tolerance.

The Philippines
Various GE crops have been approved for import; drought tolerant maize is still under application since 2017. The Philippines is characterized as the "regional biotechnology leader". A new Crop Biotechnology Center was inaugurated at the Philippine Rice Research Institute (USDA FAS GAIN report (RP2021-0063). The DA Biotechnology website containing the Status of application for field trials only listed a Golden Rice line at IRRI.

Russia
Field trials are generally not granted (USDA FAS GAIN report RS2020-0069). The OECD mentions that there are plans to use genome editing technologies to develop economically useful plants.

South Africa
Field trials with drought-tolerant maize MON87460 in 2019 and 2021 have been approved. At SASRI, research is ongoing on drought-tolerant GM sugarcane (USDA FAS GAIN report SF2021-0060). The OECD mentions that new GM approvals include stacks with MON87460 and that there are research projects using CRISPR for improving drought tolerance, including grapevine (in a cooperation with Italy). The grapevine was still at an early stage, there have not yet been field trials involving plants from NPBTs.

Switzerland
No field trials targeting abiotic stress tolerance were present among the experimental releases licensed by FOEN.

Turkey
There are no events allowed for cultivation, nor field trials (USDA FAS GAIN report TU2021-0043).

USA
Of a total of about 22000 entries for permits or notifications for release or interstate/release, 1040 issued/acknowledged referred to drought tolerance, of which only 58 were not CBI; for salt there were 114 (57 not CBI), and heat 55 (15 not CBI) (cold 338, 10 not CBI: CBF1 and ROS (AOX1, APX1, CAT, superoxide dismutase), choline oxidase). The entries with no CBI were from universities. Most company entries were classified as CBI, the great part concerning maize, with soybean as second. The USA was the first to have a gene-edited crop to be commercially cultivated (high oleic soybean oil).

Other databases

The USDA APHIS “AM I regulated” database was found to contain nine events (in seven queries) relating to abiotic stress tolerance (described in more detail below). The “Am I regulated” database was updated until the summer of 2020, after that the SECURE rule was applied, with a regularly updated Table of exempt trait - Mechanisms of action and confirmation letters, among which no abiotic stress tolerance events have been identified up to March 2022.

The EUginius database is a database maintained by the Federal Office of Consumer Protection and Food Safety (Berlin, DE) and Wageningen Food Safety Research, with known GMOs with information on modification, detection and authorisation in the EU. GE crops that are commercialised somewhere in the world or that have market-relevant traits are included in the database. Apart from the few authorized events conferring abiotic stress tolerance featured in the ISAAA GM Approval Database and literature, it contains information on MON87460, HB4 and NXI sugarcane, the well-known *ARGOS8* maize is mentioned, plus one rice (gene-edited for *OsRR22*) and one potato (gene-edited for the coilin gene).

Scientific literature providing overviews of (possible) events in the pipeline and field trials

Ricroch et al. 2022 recently provided an extensive overview of traits developed by GM or GE between 2015-2020. The marketed and pipeline events that they presented, were based on the ISAAA GM Approval Database and the USDA “Am I regulated” database: abiotic stress tolerance represented 5.5% of all products, including the well-known classical GM MON 87460 (maize) and HB4 (soybean and wheat) and sugarcane NXI, plus two miscanthus lines under CBI, a “cisgenic” (actually intragenic) rice (DREB1A), and a gene-edited maize (under CBI) and soybean (edited in the DRB genes Drb2a & b). In addition, an extensive list of 1736 patents for the 45 most important crops was retrieved, the greatest part being from China. Of the patents, 75% were on classical GM (transgenes), 11% on RNAi (gene silencing), and only 14% on genome editing, though with a large increase in genome editing from 2020 onwards. 30% of patents referred to abiotic stress tolerance, the majority of which focussed on drought (38% of abiotic stress) and salt (32%). Nevertheless, little commercialization was found for these traits in China, even in rice for which China holds 91% of the patents.

In an earlier paper on new traits up to 2016, Ricroch and Hénard-Damave presented a number of examples of abiotic stress tolerances based on field trials and company or institute websites (Ricroch and Hénard-Damave, 2016). The examples partly overlap with a brief overview by (Waltz, 2014). They encompass the three already named authorized examples, plus a number that appear to have not (yet) made it into cultivation, some of which have silencing constructs (FTA (farnesyltransferase) and ACS), and HVA1, ipt, DREB1A, and VP1 (pyrophosphatase like AVP1).

(Waltz, 2014) published a brief overview in a News Feature on drought tolerance towards commercialization, also containing information from interviewing researchers involved. Like with (Ricroch and Hénard-Damave, 2016), the follow up of these cases in later years may be interesting for understanding how the pipeline develops. In the chapter on events in commercialization and field trials, information on this follow up is given, based on what could be found in literature, databases and websites.

A recent report on market applications of NPBTs by JRC identified 23 abiotic stress tolerance traits at the early R&D stage and 15 at the advanced R&D stage, out of 292 traits in total in early R&D stage and 117 at the advanced stage, respectively. They were not specified as to underlying genes, as the underlying information was partly based on confidential information (Parisi and Rodriguez-Cerezo, 2021). We queried databases (Genetic Literacy GE tracker database, https://crispr-gene-editing-regs-tracker.geneticliteracyproject.org/) used in this study that were not in the above list in an attempt to find additional data on abiotic stress-tolerant variants.

In 2016, JRC published a paper on the global pipeline of GM (transgenic) crops (Parisi et al., 2016). It contained the well-known commercialized GM MON 87460 (maize), sugarcane NXI (at that time still pre-commercial) and HB4 (wheat at advanced R&D stage, soybean apparently still CBI). In addition, it had six abiotic stress tolerant events at advanced R&D stage, and 12 at the early R&D stage, all CBI. Interestingly, three events were at the regulatory stage, but since then no evidence of actual authorization could be found.

Annex 2: Overview of events involving abiotic stress tolerance

Table 1: Events involving abiotic stress tolerance authorized for cultivation or potentially interesting due to yield improvement in field trials, discussed in chapter 4.

Gene	**Promoter**	**Origin**	**OE, S or editing**	**Crop**	**Stress trait**	**gene product**	**notes**	**reference**
CspB	actin1 from rice (constitutive)	Bacillus subtilis	OE	maize	Drought	cold shock protein, RNA chaperone	MON 87460 event (Genuity® DroughtGard™) authorized for cultivation in Canada, USA, South Africa, Nigeria; cultivated in USA since 2013	Castiglione et al. 2008; Nemali et al. 2015
HaHB4	own stress-inducible promoter	Sunflower	OE	soybean	Drought	HD-Zip I TF, involved in ETH signalling pathway	IND-00410-5 event (Verdeca HB4) authorized for cultivation in Argentina, Brazil and USA	González et al. 2020
HaHB4	UBI-1 from maize (constitutive)	Sunflower	OE	wheat	Drought	HD-Zip I TF, involved in ETH signalling pathway	IND-00412-7 event authorized for cultivation in Argentina	González et al. 2020
EcBetA	CaMV 35S (constitutive)	E. coli	OE	sugarcane	Drought	choline dehydrogenase, involved in glycine betaine (osmo-protectant) biosynthesis	NXI-1T event authorized for cultivation in Indonesia	Marshall 2014; Waltz 2014; Babu et al. 2021

RmbetA	CaMV 35S (constitutive)	R. meliloti	OE	sugarcane	Drought	choline dehydrogenase, involved in glycine betaine (osmo-protectant) biosynthesis	NXI-4T authorized for cultivation in Indonesia	Marshall 2014; Waltz 2014; Babu et al. 2021
RmbetA	CaMV 35S (constitutive)	R. meliloti	OE	sugarcane	Drought	choline dehydrogenase, involved in glycine betaine (osmo-protectant) biosynthesis	NXI-6T authorized for cultivation in Indonesia	Marshall 2014; Waltz 2014; Babu et al. 2021
HVA1 (Aleurone 1)	Act1 from rice (constitutive)	Barley	OE	maize	Salinity	LEA group 3 protein (confers osmotolerance)		Nguyen & Sticklen (2013)
HVA1 (Aleurone 1)	ABRC321 (composite specifically (ABA) stress-inducible)	Barley	OE	rice	Drought	LEA group 3 protein (confers osmotolerance)		Chen et al. 2015
HVA1 (Aleurone 1)	CaMV 35S & ubi1 (constitutive)	Barley	OE	Wheat	Drought	LEA group 3 protein (confers osmotolerance)		Bahieldin et al. 2005; Waltz 2014)
TPP	OsMADS6 (flower-specific promoter from rice)	Rice	OE	maize	Drought	trehalose-6-phosphate phosphatase, involved in sugar signalling (source-sink)		Nuccio et al. 2015
Zm Sh2 - HS33/Rev6	Sh2	Maize	OE	maize	Heat	AGPase (ADP glucose pyrophosphorylase), catalyses rate-limiting step in starch synthesis, involved in determining seed sink strength		Smidansky et al. 2002; Hannah et al. 2012; Meyer et al. 2007;

AtAVP1	UBI from maize (constitutive)	Arabidopsis	OE	wheat	Drought/ salinity	H+-PPase (pyrophosphatase) located in plasma membrane (likely functioning as H+-PPi-synthase promoting source-sink sugar flow by raising phloem loading and transport)		Schilling et al. 2014; Gaxiola et al. 2016; Regmi et al. 2020; Menadue et al. 2021
AtAVP1	CaMV 35S (constitutive)	Arabidopsis	OE	cotton, peanut	Drought	H+-PPase	further improvement by combining with OsSIZ1 (encoding a SUMO E3 ligase)	Pasapula et al. 2011; Qin et al. 2013; Esmaeili et al. 2021
IPT	AtMYB32 (promoter most active in seedlings, gradually lower during development)	Agrobacterium tumefaciens	OE	wheat	Drought	isopentenyl transferase catalyses rate-limiting step in cytokinin biosynthesis, involved in delaying leaf senescence		Qiu et al. 2012; Joshi et al. 2019
IPT	SARK promoter from bean (stress-inducible)	Agrobacterium	OE	cotton, rice	Drought	isopentenyl transferase catalyses rate-limiting step in cytokinin biosynthesis, involved in delaying leaf senescence		Zhu et al. 2018; Waltz 2014; Ricroch & Hénard-Damave 2016
IPT	ZAG2-1 PRO (active in developing seed at lag phase, insensitive to stress)	Agrobacterium	OE	maize	Drought	isopentenyl transferase catalyses rate-limiting step in cytokinin biosynthesis, likely involved in seed sink strength		Simmons et al. 2021 (US9663796B2; US7531723B2)
CKX6	WRKY31 (root-specific)	Chickpea	OE	chickpea	Drought	cytokinin oxidase/dehydrogenase degrading cytokinin, leading to stronger root development		Khandal et al. 2020

ACS6	UBI from maize (constitutive	Maize	S (RNAi)	maize	Drought	ACC synthase 6, catalyses rate-limiting step in ETH biosynthesis		Habben et al. 2014, Simmons et al. 2021
ARGOS8	UBI from maize (constitutive)	Maize	OE (& SDN-3)	maize	Drought (yield)	Auxin Regulated Growth of Organ Size 8, negatively regulating ETH response, increasing biomass by increased cell expansion and proliferation	also expression moderately increased through promoter exchange (SDN-3)	Shi et al. 2015, 2017
ZmXerico1	ACTIN from rice (constitutive)	Maize	OE	maize	Drought	RING Domain E3 ligase (involved in 26S proteasome degradation of specific proteins, likely increasing ABA through regulating ABA8ox)		Brugière et al. 2017; Simmons et al. 2021
FTA	AtHPR1 drought-inducible shoot-specific	Arabidopsis	S (RNAi)	canola	Drought	farnesyltransferase alfa subunit, transfers farnesyl (terpenoid) group to specific proteins, effect likely through changing ABA signalling pathway		Wang et al. 2009; Waltz 2014
HbPT	UBI from maize (constitutive)	Hevea brasiliensis	OE	maize	Drought	prenyltransferase, mechanism not clear, perhaps related to changing metabolite fluxes to plant hormone synthesis		Simmons et al. 2021 (US20090288226A1)
CBF2	rd29A from Arabidopsis (stress-inducible)	Arabidopsis	OE	Eucalyptus	Cold (freeze)	CBF/DREB TF (C-repeat Binding factor/Drought Responsive Element Binding, involved in stress responses through ABA-independent pathway)		Chang et al. 2018 (USDA-APHIS petition 11-019-01p)

Drb2a and Drb2b			CRISPR-Cas9 SDN-1	soybean	Drought & salinity	Glycine max DOUBLESTRANDED RNA-binding protein2 (Drb2a and Drb2b), involved in stress responses		Curtin et al. 2018
OsRR22			CRISPR-Cas9 SDN-1	rice	Salinity	B type response TF involved in cytokinin signal transduction & metabolism	previously, salinity-tolerant EMS mutant identified, hst1, likely being based on OsRR22	Zhang et al. 2019
OsPQT3			CRISPR-Cas9 SDN-1	rice	Salinity	E3 ubiquitin ligase, higher oxidative stress tolerance associated with higher expression of ROS scavenging enzymes		Alfatih et al. 2020
DRO1			CRISPR-Cas9 SDN-1	rice	Salinity	DEEPER ROOTING 1 involved in gravitropic responses through negatively regulating auxin signalling affecting root system architecture		Kitomi et al. 2020
SlMAPK3			CRISPR-Cas9 SDN-1	tomato	Heat	MAPK serine-threonine protein kinase, negatively regulating heat stress tolerance (positively regulating drought stress tolerance)		Yu et al. 2019 (Wang et al. 2017)

OE = transgenic over-expression, S = silencing by RNAi