Slutrapport – Final Report

SLF Projekt

Utveckling av hybridsystem för vete

Dnr SLF 147/00

Proj nr 0036001

Background

The application of Hybrid Breeding in wheat is so far hampered by the high costs of hybrid seed production which are due to the low cross fertilization and the low multiplication rate of wheat. Currently used systems for fertilization control in wheat have limited potential to cope with these biological limitations as they rely on strip cultivation. In general, systems that allow hybrid seed production in mixed plantings of male and female parental lines (interplanting systems) would be suited to improve the cross fertilization. Further, a low-input system for fertilization control increasing yield of hybrid seed per production area would render hybrid seed production in wheat economically viable. In conclusion, hybrid breeding in wheat faces two main challenges:

1. The lack of an economically viable system for fertilization control ('Hybrid System')
2. The low cross fertilization ability of wheat.

The project addressed these bottlenecks by working on three aspects to enable the establishment of a system that allows both, interplanting and hybrid seed production on both parental lines:

1. The investigation of a low-input system for fertilization control allowing hybrid seed production on both parent components in an interplanting field. This work on the development of an alternative hybrid system for wheat, was conducted in a research collaboration. To date, the results of this collaboration indicate the possibility to establish a novel approach to Hybrid Wheat with the perspective to develop a competitive hybrid system for wheat in a mid- to long-term perspective. These results shall be kept confidential until BASF Plant Science will file patents on the results obtained and will therefore not be reported here. It is expected that this will be elaborated within a timeline of two years from now on.
2. The investigation of traits related to cross fertilization ability (CFA). To date, the results indicate the possibility to establish a defined selection method for parental lines. These results shall be kept confidential until BASF Plant Science will file patents on the results obtained and will therefore not be reported here. It is expected that this will be elaborated within a timeline of one year from now on.
3. The investigation of GM and non-GM herbicide resistance traits for the use for selection of true hybrids from bulked seeding of pollinator and female lines (interplanting)

Investigation of GM and non-GM herbicide resistance traits

Any hybrid system based on genetically determined male sterility can be improved through combination with a herbicide resistance marker gene. For cost efficient hybrid seed production, a herbicide resistance trait needs to be combined with the female parent for the F1 hybrid. As a result, all F1 hybrids derived from a herbicide resistant female parent will be also resistant to the herbicide while plants derived from the non-resistant male parent will be succeptible. This allows to blend seeds of both F1 hybrid parental lines and planting in one production field (interplanting).

The seed harvested from an interplanting field will consist of herbicide resistant F1 hybrids and non-resistant selfings from the male parent. This allows to eliminate the unwanted self-seed and select F1 hybrids by the application of the herbicide in the farmer's production field (Fig. 1). This approach has two advantages: The seed producer can perform the hybrid seed production by blending both parental lines in one field (interplanting), thus skipping the expensive strip cultivation. The farmer combines the selection of the agronomically superior F1 hybrids with the weed control after emergence in his crop production field.

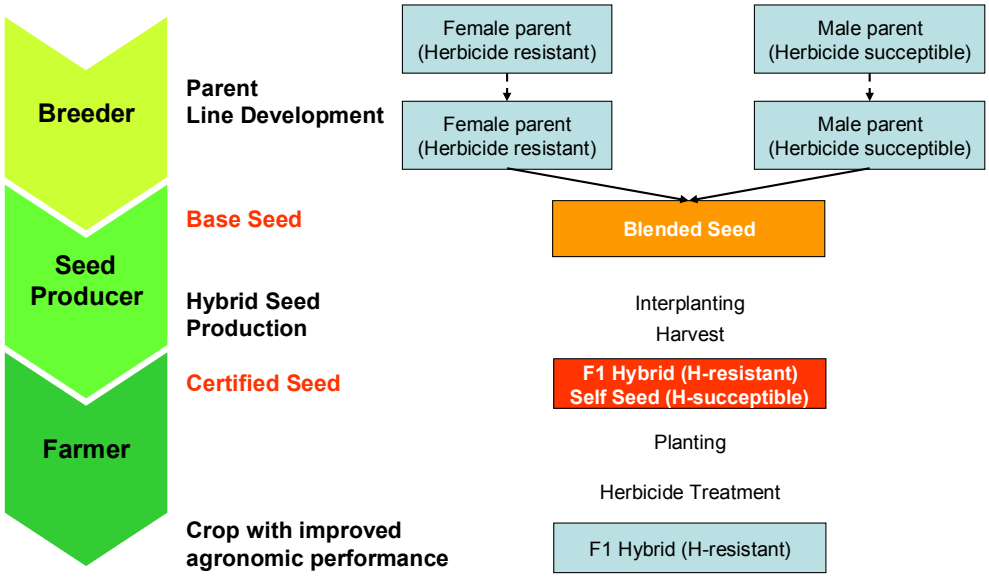

Figure 1: Use of herbicide resistance for efficient hybrid seed production

The approach to eliminate plants originating from selfings of the male component of hybrid seed productions using interplanting (bulked seeding of male and female lines) is currently investigated by several breeding companies (Peter Wilson, SunPrime Ltd., pers. comm.). In order to judge on possibilities to use herbicide resistance genes in hybrid wheat breeding, it is necessary to investigate whether such genes fullfil demands as i.e. stability over generations and environments as well as decent resistance levels. This is especially true for transgenic herbicide resistance traits. Therefore, herbicide resistant wheat plants of different origins (GM and non-GM) were investigated in the greenhouse. The aim was to obtain information on the efficiency of herbicide resistance transgenes in order to judge on the feasibility of these technologies for use in a hybrid system**.**

Materials and Methods

With regard to the combination of wheat hybrid systems with GM-herbicide resistance traits, transgenic herbicide resistant plants were generated from the elite spring wheat variety 'Canon' at Plant Science Sweden AB using Basta™ (bar) and an Imidazolinone resistance gene (Jianying *et al*., 2001) along with the reporter gene GUS (Jefferson et al., 1997). The transformation protocol utilizes Agrobacterium-mediated transformation of wheat.

The plant material produced was investigated by molecular genetic analysis employing Taqman technology (Ingham *et al*.,) and classical genetic methods (segregation analysis) in order to obtain information on the integration pattern and the stability of the transgenes. The phenotype of the plants was assessed by screening for the activity of the reporter gene GUS on the qualitative level and the expression of the *bar* gene using the Chlorphenolred test (Kramer *et al*., 1993).
Progenies of transgenic herbicide resistant plants were tested for herbicide tolerance in different generations in the greenhouse. As a reference, non-GM Imidazolinone resistant (CLEARFIELD™) plants were obtained through BASF Plant Science LLC, RTP, Raleigh, NC, USA. For this purpose, plants were sprayed in the 3-4 leaf stage in 2 concentrations (60 and 180 gai/ha) with the respective herbicides (Raptor™, Basta™) and scored on days 14 and 21 after treatment for 0 'resistant' to 9 'dead'

Results

From Agrobacterium-meditaed transformations with the Imidazolinone resistance gene a number of 32 T0 plants were produced in 2 independent experiments. Subsequent molecular and genetic analysis of T1 plants revealed 2 independent events were produced in each experiment. The T1 segregation of 4 T1 progenies are shown in Fig. 2. A χ^2 test proved event #1 with 1 intact transgene locus, event #2 with 2 intact transgene loci, event #3 with 2 partially intact (loss of 1 reporter gene copy) loci and event #4 with 2 intact loci (loss of both reporter gene copies). The latter was detected in T1 progeny of a T0 plant that is assumed chimaeric for events #3 and #4. Southern analysis partially confirmed the segregation analysis. However, not all progenies could be analysed by Southern.
The spraying of both transgenic and non-transgenic IMI tolerant T1 and T2 plants differentiated clearly between controls. The non-GM IMI-tolerant plants showed complete resistance towards both concentrations at both scoring dates (Fig. 3), indicating homozygosity for both loci. In contrast, the non-tolerant control 'Canon' proved to be completely succeptible.
The segregating T1 progeny from GM IMI-tolerant T0 plants showed segregation with a more clear result on day 21 after treatment (Fig. 4). However, the correlations between the two treatments were very low.

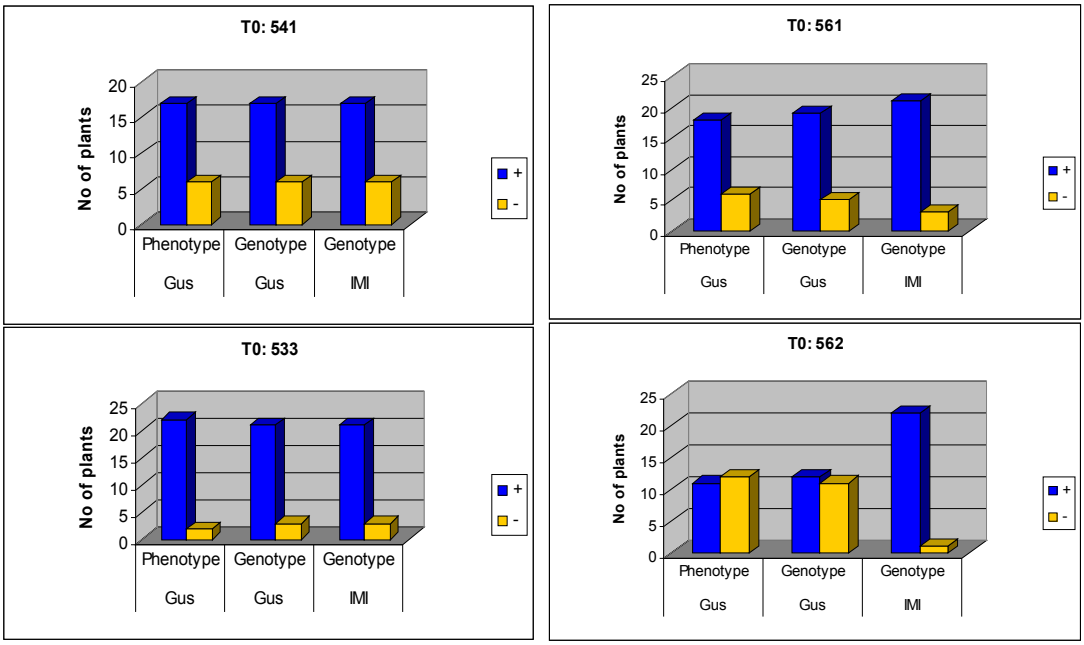

Figure 2: Segregation analysis of Imidazolinone-resistant T1 plants: T0 541 segregation for 1 locus, T0 533 segregating for 2 loci, T0 561 segregating for 2 partially intact loci, T0 562 chimaeric segregation pattern

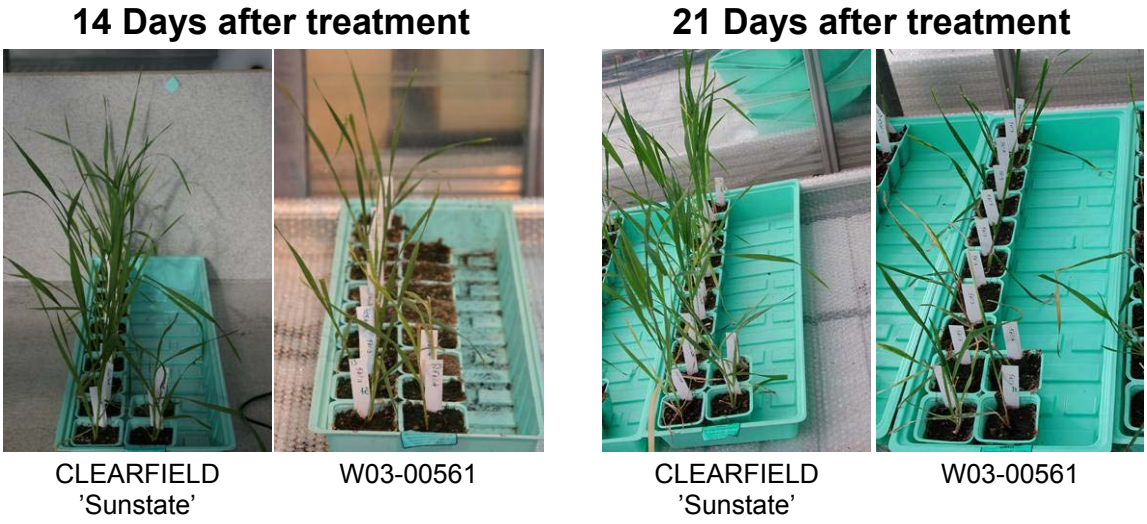

Figure 3: Effect of a spray with Raptor™ on the non-GM IMI tolerant variety 'Sunstate' and T1 progeny from transgenic IMI tolerant T0 plants

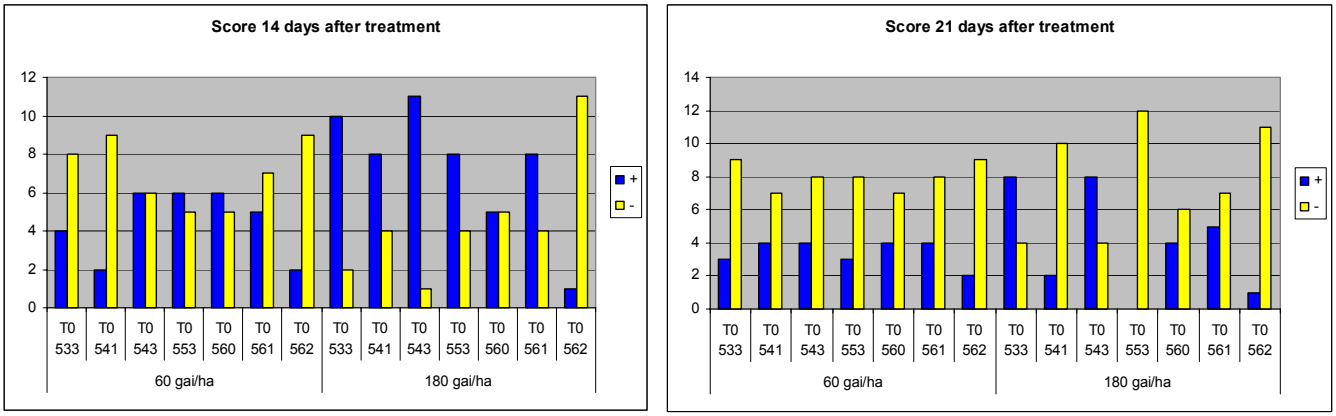

Figure 4: Segregation for IMI tolerance of T1 progenies from transgenic IMI tolerant T0 plants 14 and 21 days after treatment. + = resistant, - = succeptible

From Agrobacterium-meditaed transformations with the Basta™ resistance gene we focussed on progenies of T0 plants with either 1 or 2 transgene loci. The molecular genetic and segregation analysis of Basta™ resistant transgenic T2 progenies confirmed the molecular analysis only for T0 #10 plants with 1 copy/locus while progeny of T0 #12 segregated according to 2 loci (Tab. 1).

Table 1: Analysis of T2 segregation from transgenic Basta™ resistant T0 plants

T1	T0		T2 Observed			Chi Square			
Line/Ref.no.	Genotype	CN	Total	+	-	no seg	15:1	3:1	Conclusion
W03-00566	10	1	8	8	0	1,000	0,465	0,102	homozyg
W03-00568			9	6	3	0,000	0,001	0,564	3:1
W03-00578			9	4	5	0,000	0,000	0,034	3:1
W03-00579			10	6	4	0,000	0,000	0,273	3:1
W03-00580			9	5	4	0,000	0,000	0,178	3:1
W03-00581			10	7	3	0,000	0,002	0,715	3:1
W03-00584			10	5	5	0,000	0,000	0,068	3:1
W03-00585			10	8	2	0,000	0,072	0,715	3:1
W03-00586			10	10	0	1,000	0,414	0,068	homozyg
W03-00587			9	9	0	1,000	0,439	0,083	homozyg
W03-00625	12	1	8	8	0	1,000	0,465	0,102	homozyg
W03-00626			10	10	0	1,000	0,414	0,068	homozyg
W03-00627			9	9	0	1,000	0,439	0,083	homozyg
W03-00628			10	9	1	0,000	0,624	0,273	15:1
W03-00638			10	10	0	1,000	0,414	0,068	homozyg
W03-00639			10	10	0	1,000	0,414	0,068	homozyg
W03-00640			9	8	1	0,000	0,547	0,336	15:1
W03-00641			10	10	0	1,000	0,414	0,068	homozyg
W03-00644			10	6	4	0,000	0,000	0,273	3:1
W03-00645			10	10	0	1,000	0,414	0,068	homozyg

Figure 5: Basta™ test spray: Control (left) and segregating T3 plantlets (right)

The spraying of transgenic Basta™ tolerant T3 plants differentiated clearly succeptible and resistant plants (Fig. 5). The effect was clearly visible already 14 days after treatment. The non-tolerant control genotype 'Canon' proved to be completely succeptible at both concentrations used. The phenotype correlated perfectly with the genotype that was assessed by q-PCR before spraying.

Discussion

Progenies from primary transformants (T0) from transformation with both, the Imidazolinone Resistance Gene (Jianying *et al*., 2001) and the bar/ bialaphos (BASTA™) resistance gene showed simple integration patterns (low copy number) and mendelian inheritance of the transgene. This result shows that the protocol for *Agrobacterium*-mediated transformation of wheat developed at Plant Science Sweden AB is very well suited to produce transgenic wheat plants that meet regulatory demands. The technology used for *Agrobacterium*-mediated transformation of wheat at Plant Science Sweden AB is thus suited to produce events with commercial qualtiy and can be used for product development.

In general, some T0 plants originating from experiments with the IMI tolerance gene appeared to have considerable rearrangements within the inserted T-DNA cassettes. The expression of reporter genes in segregating progenies corresponded to the molecular results obtained. It is thus important to further optimize transformation constructs for *Agrobacterium*-mediated wheat transformation and to carefully select events that meet commercial standards. However, additional results obtained within our wheat transformation pipeline indicate, that 80% of the produced events fulfill these criterion.

Regarding the expression of the herbicide resistance genes, the IMI tolerance seems less suitable for use as a trait in wheat plants as compared to Basta™ resistance as the test sprays differentiated less clearly in transgenic IMI tolerant progenies. Also, the resistant treated plants required a recovery phase after the spray, that caused a delay in development as compared to the non-treated control. In contrast, non-GM IMI tolerant wheat proved to be feasible for use as a trait as the

treated plants showed no difference in development as compared to the non-treated control. Therefore, both non-GM IMI tolerant wheat as well as GM Basta resistant wheat may be considered for use as trait genes in wheat.
In conclusion, the combination of hybrid systems with both, transgenic and non-GM herbicide resistance genes in wheat appears to be feasible. The next step is now to investigate whether the described herbicide resistance traits are suited to select non-resistant wheat plants from production fields in which both herbicide resistant F1 hybrids and non-resistant self plants from the male component occur.

References

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Ingham, D., Beer, S., Money, S. and Hansen, G. (2001) Quantitative real-time PCR assay for determining transgene copy number in transformed plants. – Biotechniques 31(1):132-134, 136-140.