

An establishment of *Agrobacterium*-mediated transformation to rapeseed (*Brassica napus* L. cvs. WH3 and WH10) and functional analysis of *Arabidopsis* transcription factor *AtGRFs* in growth and developmental alternations of transgenic rapeseed

Ekhchimeg Vanjildorj¹, Hyun Jin Song¹, Jeong Hoe Kim², Yong Pyo Lim^{1*}
¹Department of Horticulture, Chungnam National University, Gung-dong, Yuseong-gu, Daejeon 305-764, Korea
²Department of Biology, Kyongpook National University, 1370 Sankyuk-dong, Buk-gu, Daegu 702-701, Korea
 Corresponding author's e-mail: yplim@cnu.ac.kr

ABSTRACT

Genetic transformation of rapeseed is stimulated by its high economic value and potential to expand of the crop usefulness. *AtGRF* proteins play a role in the regulation of cell expansion in leaf and cotyledon tissues. In this study, we transformed *AtGRF1* and *AtGRF2* genes to rapeseed genome using *Agrobacterium*-mediated transformation for increasing crop productivity, furthermore vegetable oil yield which is required to produce biodiesel from rapeseed. Transgenic rapeseed lines were generated from both cotyledon and hypocotyl explants on 20 mg l⁻¹ hygromycin-containing medium. All of the transgenic plants were phenotypically normal and produced fertile flowers and viable seeds, thus transformation efficiencies were 5.4% for *AtGRF1* and 4.2% for *AtGRF2*. Among the used two explants of rapeseed, hypocotyl has more ability (1.5-3-fold) to uptake transgene rather than cotyledon. PCR analysis showed that transgene was stably integrated into the genome of each transgenic plant. Of the tested six transgenic lines four gave 3:1 segregation ratio in the T₁ progenies after self-pollination, suggesting single copy integration of the transgene. One line gave 1:1 segregation ratio, suggesting semidominant and one line gave 15:1 segregation ratio, suggesting two copy integration of the transgene, respectively. The rapeseed transformants expressing each transgene displayed faster growth of plant (69.2% of transgenic plants already developed the fourth as well as initiated the fifth leaves; but only 12.5% of wild-type plants developed the fourth leaves. The fifth leaf did not emerge completely at 1-month after sowing the seeds). Area of cotyledon of transgenic plants were about 2-fold larger than wild-type plants at only early growth stages (until 21 days). However at one-month old seedlings, area of cotyledon was not significant different between wild-type and transgenic plants. But the leaf area of transgenic plants were about 2-fold larger in the first and second leaves, respectively, and about 3-15-fold larger in the third leaf compared to wild-type plants.

INTRODUCTION

Rapeseed (*Brassica napus* L., AACB, n = 19), also known as rape, oilseed rape, rapa, rapeseed, is the typical bioenergy crop which is cultivated about 23,000,000 ha in the world. Rapeseed has received much attention worldwide and may soon be the most popular oilseed crop. Rapeseed is grown for the production of animal feed, vegetable oil for human consumption, and biodiesel. The establishment of a market of rapeseed oil was mainly due to the nutritional benefits associated with the low levels of saturated fatty acids such as aliphatic glucosinolates and erucic acid, making it appealing to health-conscious consumers. Because of the rapeseed has a high economic value, considerable and numerous transgenic researches have been conducted using various tissues as explants. These include cotyledons, hypocotyls and protoplasts. However, hypocotyl segments are the most desirable explants for tissue culture of most *Brassica* species because of their ability to regenerate. Several researches on transformation of genes into rapeseed with the aim to improve the herbicide and insect resistance, oil quality, in-crop biochemical factories, male-sterility, and abiotic tolerance have been reported. Even though rapeseed transformation protocols are well established, there is still a need for the development of efficient protocols for *B. napus* L. cvs. WH3 and WH10 (a Chinese rapeseed cultivars), because regeneration in rapeseed is highly variable and genotype-dependent. This genotype specificity is a limiting factor in *Brassica* regeneration, and in further transformation. Here we developed *AtGRF1* and *AtGRF2* transgenic rapeseed plants using *Agrobacterium*-mediated transformation. Previously *Arabidopsis* GROWTH-REGULATING FACTOR (*AtGRF*) gene family, which consists of nine members and encodes putative transcription activators involved in leaf and cotyledon growth, was identified and characterized. The increase in size of leaf blades was based on changes in cell size and not on changes in cell number. The leaves of both *AtGRF1* and *AtGRF2* overexpressors were larger and contained more palisade cells than those of wild-type plants. Palisade cells are grouped together to give the palisade layer of the leaf- this is the leaf tissue where most of the photosynthesis takes place. Moreover *AtGRF4* and *AtGRF5* mutants exhibit narrow leaf phenotypes due to decreases in cell number. These results indicating that increased leaf size in *AtGRF* overexpressors were based on differences in both cell size and cell number. Both *AtGRF1* and *AtGRF2* genes are involved in the increase of leaf photosynthetic area. However it seems that contribution of leaves to final seed yield in *Brassica* must be minor, because the leaves senescence during the period of rapid pod growth. In fact, both leaf and pod photosynthesis are complementary contribute towards seed yield, the positive correlations between leaf area indices and seed yield in oilseed *Brassicacae* have reported. Therefore our main objective of this study was to develop *AtGRF1* and *AtGRF2* transgenic rapeseed plants with increased oil as well as biodiesel yield via expanding leaf photosynthesis area.

MATERIALS and METHODS

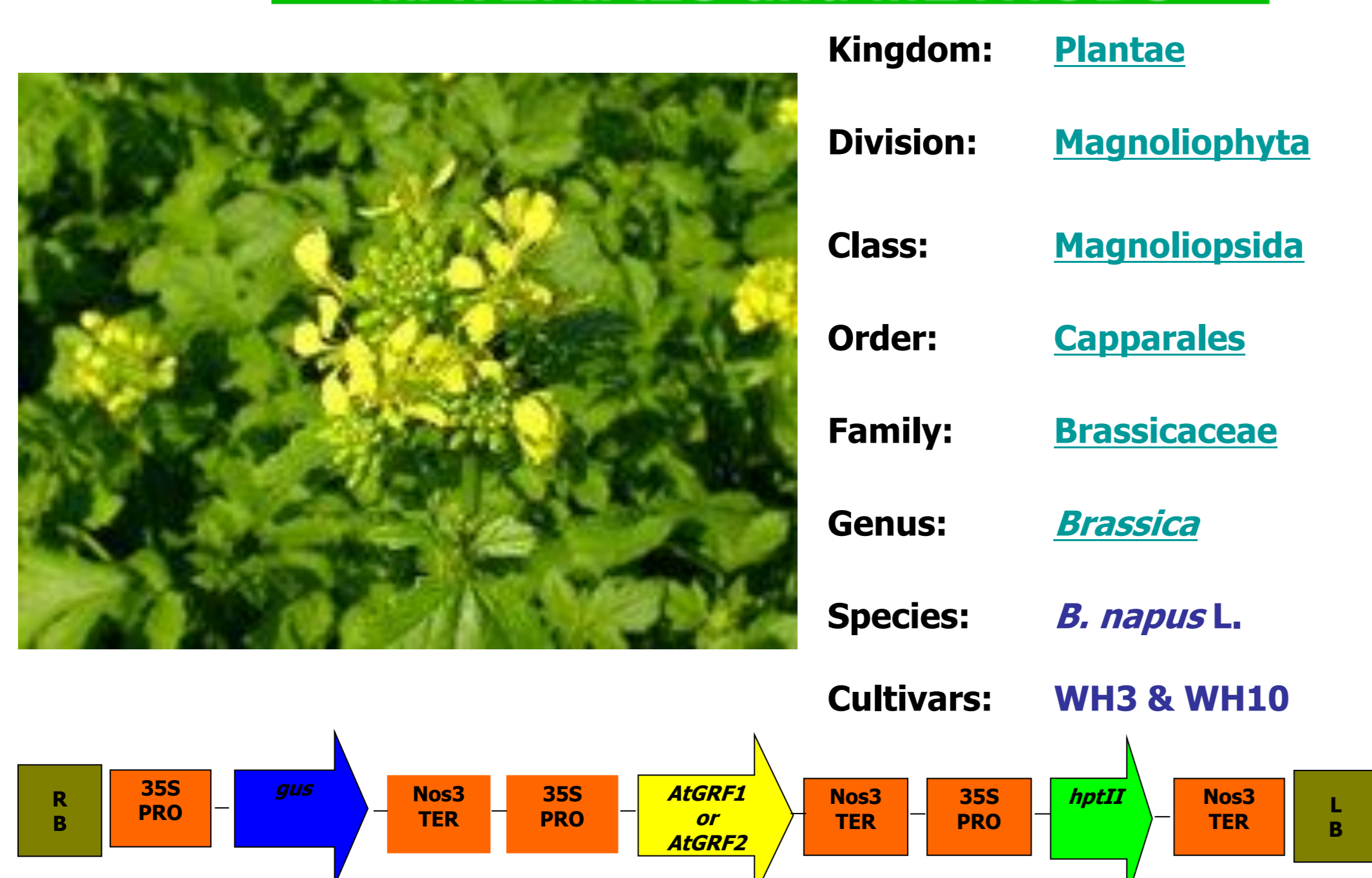


Figure 1. Schematic diagram of T-DNA region of binary vector pCambia 1300. *AtGRF1* or *AtGRF2*: *Arabidopsis* growth regulating factor1 or 2 genes, respectively, *gus*: gene for β -glucuronidase from *E. coli*, *hptII*: gene for hygromycin phosphotransferase from *E. coli*, 35S: promoters, NOS3: terminators.

Table 1. Transformation procedure of *Brassica napus* L.

Step	Duration	Media/Conditions
Cotyledon & hypocotyl explant	5-7 d	MS hormone free
Explant pre-cultivation	3 d	MS + 1 mg/L 2,4-D + 40 mg/L AS
<i>Agrobacterium</i> inoculation	10 min	MSRM (MS + 4 mg/L BA + 0.5 mg/L NAA + 3 mg/L AgNO ₃) + 50 mg/L AS
Co-cultivation	3 d	MSRM + 50 mg/L acetosyringone (AS)
Elimination	10 d	MSRM + 500 mg/L carbenicillin (CB)
Callus regeneration/selection	5-6 w	MSRM + 250 mg/L CB + 15 mg/L hygromycin (Hyg)
Shoot regeneration/selection	6 w	MS + 2 mg/L BA + 0.5 mg/L NAA + 5 mg/L AgNO ₃ + 250 mg/L CB + 15 mg/L hyg
Adventitious shoot regeneration	2 w	MS + 250mg/L CB + 15 mg/L hyg
Root regeneration	3 w	1/2 MS + 250mg/L CB + 15 mg/L hyg

RESULTS

Table 2. Transformation efficiency of *Brassica napus* L.

Trans-genes	Explants	(A)	(B)	(C)	(D)	T. E. (B/A*100%)
<i>AtGRF1</i>	Cotyledon	200	5	2	1	2.5
	Hypocotyl	392	27	4	1	6.9
	Total	592	32	6	2	Mean = 5.4
<i>AtGRF2</i>	Cotyledon	215	7	0	0	3.3
	Hypocotyl	428	21	4	1	4.9
	Total	643	27	4	1	Mean = 4.2

(A) No. of infected explants
 (B) Regenerated HygR calli
 (C) HygR plants with multiple shoots
 (D) HygR plants with adventitious shoots

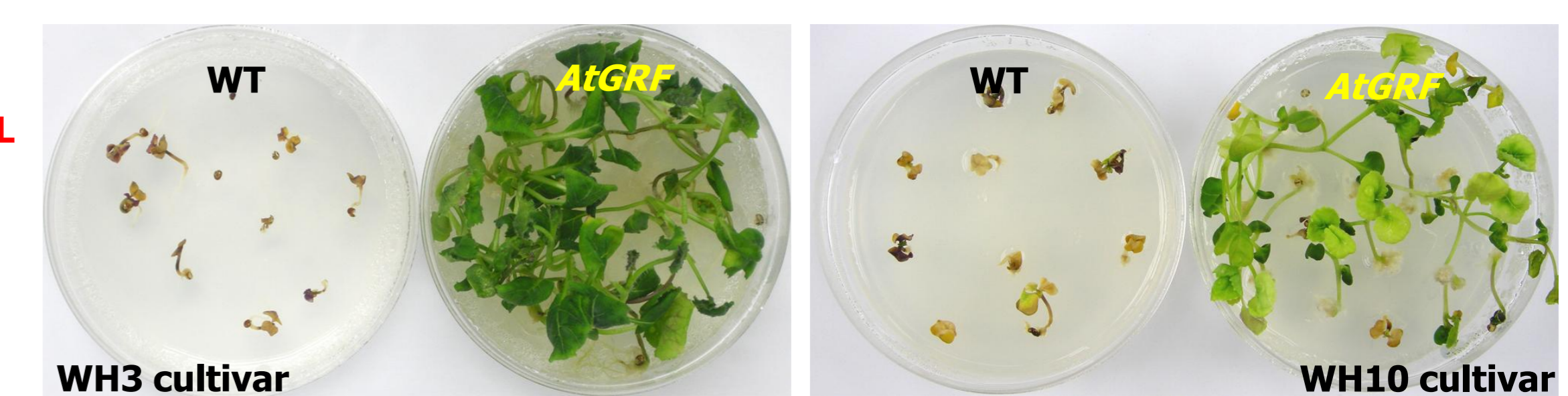


Figure 3. Transgene segregation to T₁ progenies. *AtGRF1* transgenic and wild-type (WT) seeds were germinated on MS+Hyg (25 mg l⁻¹) for 5 weeks.

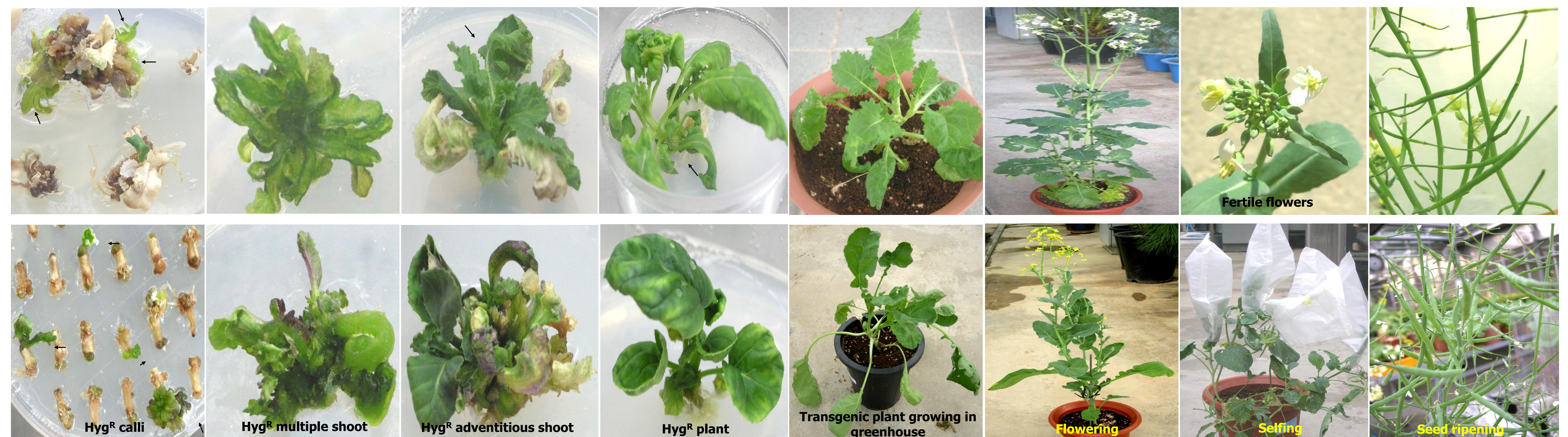


Figure 2. Regeneration of transgenic rapeseeds (*Brassica napus* L. cvs. WH3 (upper row) and WH10 (lower row)) from cotyledonary and hypocotyl explants, respectively.

Table 3. Segregation of hygromycin resistance in T₁ progeny of *AtGRF1* / 2 transgenic rapeseed (*Brassica napus* L.) cvs. WH3 and WH10

Cultivar	Transgenic line	Resistant (R) progeny	Susceptible (S) progeny	Ratio (R:S)	Expected ratio	χ^2 -value	p-value
WH3	GRF1-101	9.4 ± 0.7	0.5 ± 0.3	18.8 : 1	15 : 1	0.02	0.89
	GRF2-101	9.0 ± 1.5	4.1 ± 1.2	2.2 : 1	3 : 1	0.27	0.60
WH10	GRF1-101	9.2 ± 1.8	1.9 ± 0.9	4.8 : 1	3 : 1	0.29	0.59
	102	8.7 ± 1.0	3.3 ± 0.9	2.6 : 1	3 : 1	0.04	0.84
	GRF2-101	9.0 ± 2.8	6.0 ± 2.0	1.5 : 1	1 : 1	0.60	0.44
	102	9.7 ± 0.6	2.0 ± 0.8	4.9 : 1	3 : 1	0.42	0.52

Each value represents mean ± S.D. of four replicates. χ^2 indicates the significant fit to the expected ratio.

larger leaves and faster development of leaves. A: 1-month-old wild-type (WT) and WH10 T₁ plants of 35S::*AtGRF1* GRF1-1/-2 and 35S::*AtGRF2* GRF2-1/-2 transgenic lines are growing in the greenhouse. I-IV indicates first to fourth leaves. B: Mean of blade area of cotyledons and leaves (first to fourth leaves). Columns indicate the mean (n=10) and bars indicate ± SD.

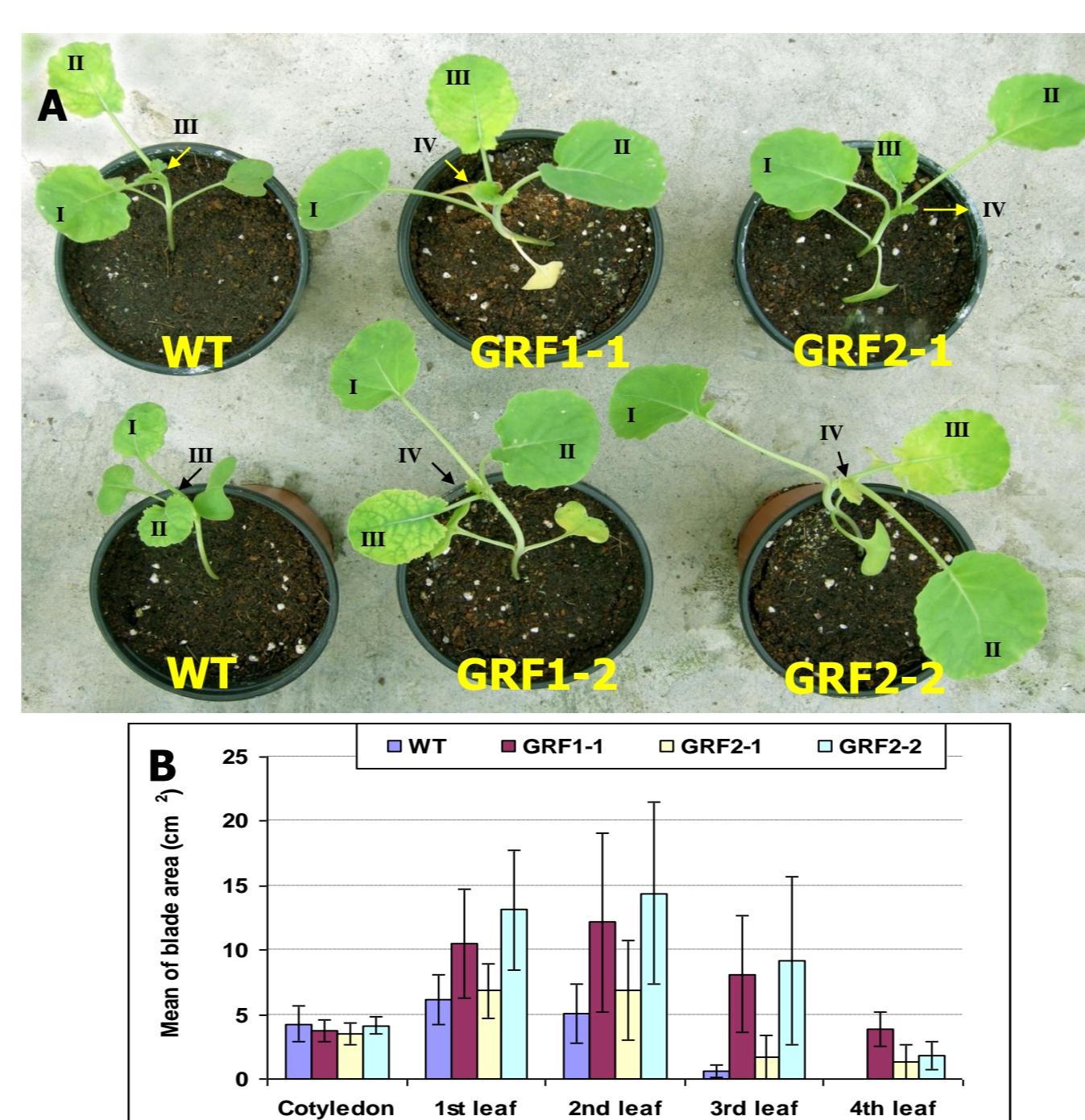


Figure 4. Overexpression of *AtGRFs* regulates larger leaves and faster development of leaves. A: 1-month-old wild-type (WT) and WH10 T₁ plants of 35S::*AtGRF1* GRF1-1/-2 and 35S::*AtGRF2* GRF2-1/-2 transgenic lines are growing in the greenhouse. I-IV indicates first to fourth leaves. B: Mean of blade area of cotyledons and leaves (first to fourth leaves). Columns indicate the mean (n=10) and bars indicate ± SD.

DISCUSSION

- The highest number of shoot per explant was regenerated on 4 mg/L BA, 0.5 mg/L NAA and 3 mg/L AgNO₃ containing medium.
- Application of 2,4-D and acetosyringone pretreatments prior to co-cultivation were induced severe callus growth during the subsequent experiments and increased transformation efficiency.
- Wild-type of *B. napus* L. has a higher hygromycin resistant than those of *B. rapa*. Therefore hygromycin selection pressure (20 mg/L) was used for selection of transgenic.
- From wild-type plant seed cultivation to development of *in vitro* transgenic plant has taken about 4 months. Transgenic lines did not show abnormality, all of them were fertile and transgene inherited to T₁ progenies as a Mendelian segregation pattern.
- Transformation efficiencies were 5.4% for *AtGRF1* and 4.2% for *AtGRF2*, respectively.
- Among the used two explants of rapeseed, hypocotyl has more ability (1.5-3-fold) to uptake transgene rather than cotyledon.
- Expression of *AtGRF1* / 2 genes involved in the regulation of meristemic activity of shoot apical meristem and regulate faster initiation of leaf from transgenic plants than did from wild-type.
- Expression of *AtGRF1* / 2 genes resulted larger leaves when compared to wild-type plants.
- In the further study will detect whether the larger leaf is caused by cell number or cell size alternations.