# Positive effect of transgenes on Powdery Mildew resistance in *Symphyotrichum novi-belgii* under greenhouse conditions

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## Abstract

In order to reduce the susceptibility towards the powdery mildew *Golovinomyces cichoracearum*, two different genes related to pathogen resistance were introduced into two genotypes of *Symphyotrichum novi-belgii*, ‘Victoria Fanny’ and X1.105. The genes were *Ace-AMP1*, a non-specific lipid transfer protein from *Allium cepa* and *VvWRKY1*,a transcription factor from *Vitis vinifera*. Both known to be involved in resistance signalling and known from previous studies to increase resistance against a broad range of pathogens. In the current study, transgenes were expressed under the control of the CaMV 35S promoter and were introduced into *S. novi-belgii* via *Agrobacterium thumefaciens* mediated transformation. The natural level of susceptibility was higher in wild type ‘Victoria Fanny’ than in wild type X1.105. A significant reduction in the percentage of infected leaves was observed in three out of eight X1.105 lines containing *Ace-AMP1* but the infection level was similar for infected leaves. In ‘Victoria Fanny’ one line out of eight containing *VvWRKY1* had a reduced infection level.

## Keywords

*Ace-AMP1*; *VvWRKY1*; *Aster novi-belgii;* *Golovinomyces cichoracearum*; *Agrobacterium tumefaciens*;

## Running title

*Symphyotrichum novi-belgii*

## Introduction

*Symphyotrichum novi-belgii* (L.) G.L.Nesom (syn. *Aster novi-belgii* L.) is a popular autumn flowering perennial used as potted plant in Denmark and has been among the 10 most sold plants since 2004. *S. novi-belgii* is highly susceptible towards the powdery mildew *Golovinomyces cichoracearum* (Mørk et al. 2011) which is usually controlled by evaporation of sulphur in the horticultural industry. Through breeding some resistance has been achieved, but breeding programs and research have been restricted to intraspecific hybridisation (Kristiansen et al. 1997) and mutagenesis (Kristiansen and Petersen 2009). By introducing transgenes, the limitations of a narrow gene pool will be removed and open a large number of possibilities.

To improve the defence system in plants several different transgenic strategies can be applied (Collinge et al. 2008). The most commonly used approach has been to constitutively express one antimicrobial protein such as chitinase and thereby enhancing the resistance in a specific manner. Another way is to influence a large array of plant genes by over-expression of a transcription factor involved in defence regulation. Thereby, the plant’s own defence system is always active, even before the pathogen starts the intrusion. A variation of this is to constitutively over-express signals in the signalling pathway, which will also lead to a constitutively activated defence system, or to over-expression of receptor proteins.

In the present study, two candidates were approached for improving powdery mildew resistance in *S. novi-belgii*. The two candidate genes were: (1) *VvWRKY1*,coding for a transcription factor involved in resistance gene regulation, and (2) *Ace-AMP1*,a non-specific Lipid Transfer Protein (ns-LTP).

*VvWRKY1* was isolated as a full-length cDNA sequence from a *Vitis vinifera* L. cv. Cabernet Sauvignon grape berry library and encodes a polypeptide of 151 amino acids length with only one WRKY domain at the N-terminal end followed by a Cys2/His2-type zink finger motif (Marchive et al. 2007). Together with other structural motifs, VvWRKY1 is placed in the subgroup IIc of WRKY transcription factors (Eulgem et al. 2000). Marchive et al (2007) found that ectopic over-expression of VvWRKY1 in tobacco reduced the susceptibility against tobacco powdery mildew (*Erysiphe cichoracearum*) and *Pythium*, but the level of transcripts encoding Pathogenesis Related (PR) proteins was not changed. The gene is therefore assumed not to have a direct effect on PR gene expression. Similar results have been found for two close sequence homologs of *VvWRKY1* ; *FaWRKY1* from *Fragaria ×ananassa* and *AtWRKY75* from *Arabidopsis thaliana* (Encinas-Villarejo et al. 2009).

*Ace-AMP1* encodes a protein similar to non-specific lipid transfer proteins (ns-LTP) (Tassin et al. 1998) and was isolated from immature *Allium cepa* seeds (Cammue et al. 1995). Even though the protein has an amino acid sequence similar to ns-LTP, Ace-AMP1 was unable to transfer phospholipids from liposomes to mitochondria (Tassin et al. 1998). Instead it was suggested that *Ace-AMP1* either functions by binding to pathogen elicitors leading them to the plant receptors ( Patkar and Chattoo 2006; Roy-Barman et al. 2006) or that Ace-AMP1is toxic to pathogens (Patkar and Chattoo 2006; Wu et al. 2011). *Ace-AMP1* has been inserted successfully into *Triticum aestivum*, *Oryza sativa*, *Rosa hybrida* and *Pelargonium sp.*, thereby reducing their susceptibility to different diseases (Bi et al. 1999; Li et al. 2003; Patkar and Chattoo 2006; Roy-Barman et al. 2006).

The aim of the current study was to test, if introduction of *Ace-AMP1* and *VvWRKY1* could reduce the susceptibility of two *S. novi-belgii* genotypes towards powdery mildew.

## Materials and Methods

### Plant materials

Two *Symphyotrichum novi-belgii* genotypes were selected for this experiment. One commercial seed producing cultivar (‘Victoria Fanny’) known to easily produce transgenic shoots and one pollen and seed producing breeding line (X1.105) to allow stacking and transfer of transgenes to other genotypes. Shoots from the two lines were established *in vitro* by nodal cuttings taken from greenhouse plants and grown according to Mørk et al (2012).

### Constructs

Two resistance genes, both driven by the 35s promoter were used for the current experiment. 1) *VvWRKY1* which was isolated from *V. vinifera* and coding for a transcription factor involved in disease resistance and kindly provided by Dr Virginie Lauvergeat, Institut des Sciences de la Vigne et du Vin, France (Marchive et al. 2007). 2) *Ace-AMP1* , isolated from *A. cepa* and coding for a non-specific lipid transfer protein and kindly provided by Dr Bruno Cammue, Centre of Microbial and Plant Genetics, Belgium (Cammue et al. 1995). The two genes were inserted into a pCAMBIA230035Su vector (Nour-Eldin et al. 2006) using the USER cloning protocol (Nour-Eldin et al. 2006).

### Transformation of *S. novi-belgii*

The binary vectors were introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation. Colonies were selected on YEP plates containing 100 mg l-1 kanamycin. The constructs were verified by plasmid purification, restriction enzyme digestion and sequencing. Transformation of the two genotypes of *S. novi-belgii* was performed as described by Mørk et al. (2012).

### Evaluation of transgenic lines

Plantlets that grew vigorously after successive subcultures on medium containing kanamycin (100 µg ml-1) were tested by PCR for the presence of *nptII* and gene of interest. The primers were as follows: Ace-AMP-F (5’-CAT GGT TCG CGT TGT ATC TTT ACT T-3’), Ace-AMP-R (5’- GCT TAT ATA CTT GGA CGC TGA GG-3’) giving an expected product of 397 bp, VvWRKY1-F (5’-TCA GTC TCT CTA ATG GAA GGC CAC C-3), VvWRKY1-R (5’-TGG AAA GAG TGG TGG ACA CCC ATA TCA TCT CT-3’) giving an expected product of 546 bp, NptII-F (5’ TCC GGC CGC TTG GGT GGA GAG-3’) and NptII-R ( 5’- CTG GCG CGA GCC CCT GAT GCT-3’) giving an expected product of 471 bp. The primers were transgene specific and did not give any amplification products from non-transformed *Symphyotrichum* DNA. The programs were: 94 °C for 1 min followed by 35 cycles of 94 °C for 1 min (denaturation), gene specific annealing temperature for 1 min (annealing), 72 °C for 1 min (elongation), 72 °C for 10 min (elongation). The annealing temperatures were: *Ace-AMP1,* 59 °C, *NptII*, 60 °C and *VvWRKY1*, 62 °C.

### Expression of transgenes

Total RNA was extracted from plant tissue (leaves) using the RNeasy Plant Mini Kit (Qiagen, Copenhagen, Denmark). The manufacturer’s protocol was followed except that the lysis buffer was applied in minimum double amount and the transgenic line XA-4b was grinded in the presence of PVP. After control of RNA quality using 100 ng total RNA on a 1 % agarose gel, total RNA ≈5 ng was DNase treated with the Turbo DNase free kit (Ambion) according to the manufacturer’s protocol and subsequently used for cDNA synthesis using the iScript cDNA Synthesis Kit (Bio-Rad, Copenhagen, Denmark) according to the manufacturer’s protocol. Control of cDNA was done by PCR using primers amplifying the housekeeping gene coding for ubiquitin. PCR of the cDNA was conducted with the same primers as described above using a quick load PCR mix (New England Biolab). The PCR program for *VvWRKY1* was 95 °C for 30 sec 40 cycles of 95 °C for 30 sec (denaturation), 62 °C for 30 sec (annealing), 68 °C for 30 sec (elongation), 68 °C for 5 min (elongation), for *Ace-AMP1* the program was 95 °C for 30 sec 40 cycles of 95 °C for 30 sec (denaturation), 59 °C for 30 sec (annealing), 68 °C for 30 sec (elongation), 68 °C for 5 min (elongation), and the amount of template in each reaction corresponded to 25 ng total RNA. PCR products were visualised on 1 % agarose gels compared and to a 100 bp marker (New England Biolab).

### Acclimation and transfer to greenhouse

Shoots were multiplied and moved to MS basal medium without hormones for root formation. After two weeks, plantlets were transplanted to a peat based potting mixture (Pindstrup 2, Pindstrup Mosebrug, Pindstrup, 8550 Ryomgaard, Denmark) and covered with plastic for two weeks to acclimatise. After establishment, the plants were grown under greenhouse conditions at 20 °C and a 20 h photoperiod to keep them in the vegetative stage. Further experiments were conducted using these plants as stock plants for cuttings.

### Resistance analysis

On three subsequent days (replications) six cuttings of every transgenic and non-transformed line were planted in a peat based potting mixture (Pindstrup 2) and rooted under plastic. After four weeks, the plants were pinched and after additional two weeks the plants were inoculated with the powdery mildew *Golovinomyces cichoracearum* (Mørk et al. 2011) using an inoculation tower. Within each replication plants were divided into six blocks each containing one plant per line. The first 24 h after inoculation the plants were covered with plastic to ensure high humidity to facilitate spore germination. For colony formation plants were kept under greenhouse conditions for two weeks under long day conditions (20 h).

### Recordings and statistical analysis

One week after mildew inoculation individual leaves were photographed. Two fully developed leaves per shoot and two shoots per plant were used. The photos were analysed using WinRHIZO pro (Regent Instruments INC, version 2002c) to give an exact measure of the total leaf area as well as the area covered with powdery mildew. To be sure the image analyses were correct, a manual scoring of the infection level using a 6 level scale (0=0 %, 1=1-20 %, 2=21-40 %, 3=31-60 %, 4=61-80 %, 5=81-100 % infected leaf area) was performed as well. A linear correlation (R2= 0.7066; p<0.0001) was found between the two methods, and therefore the image analysis was regarded as a valid method for evaluating the area of infection. The infection level was calculated as the area of individual leaves covered with mildew divided by the total area of the leaf.

Means of all leaves per plant were made before variance homogeneity was tested by residual plots and data for infection level and percentage of infected leaves were arcsine-transformed prior to analysis of variance using a two-way analysis of variance and type III sum of squares (PROC GLM of SAS, SAS Institute, Cary, NC, USA). Dunnetts test was used to determine differences between the wild type and transgenic lines within each genotype and between wild types of the two genotypes. In figures and tables the back-transformed values are presented.

## Results and Discussion

### Transformation and Verification of Transgenes

## A total of 450 individual shoots were isolated after transformation of *S. novi-belgii* ‘Victoria Fanny’ and X1.105 with *Agrobacterium tumefaciens* LBA 4404 (Table 1). Each shoot represented an individual transformation event and was selected from calli, which were not attached to each other. The shoots were grown through several subcultures on kanamycin, and the most vigorous plants were selected for PCR. More shoots were regenerated from ‘Victoria Fanny’ (274) than from X1.105 (176) (Table 1) and in the present experiment around 44 % of the explants from ‘Victoria Fanny’ produced shoots. This number is consistent with previous studies on transformation of *S. novi-belgii* cultivars, in which a regeneration and transformation protocol was developed for ‘Victoria Fanny’ with GUS (β-glucoronidase) (Mørk et al. 2012).

Insertion of the transgenes was verified by PCR with gene specific primers. Procedures for PCR amplification of the *Ace-AMP1*, *VvWRKY1* and *nptII* (kanamycin resistance)genes in transgenic plants were established and shoots were selected, if they were positive for both *nptII* and the gene of interest. A total of 20 transgenic lines were identified for further studies (Table 1) including three lines of ‘Victoria Fanny’/*Ace-AMP1* (FA1, FA2 and FA4); eight lines of X1.105/*Ace-AMP1* (XA2, XA3a, XA3b, XA3c, XA4, XA6, XA7a and XA7b); eight lines of ‘Victoria Fanny’/*VvWRKY1* (FV1, FV2, FV3, FV4, FV5, FV6, FV7 and FV8) and one line of X1.105/*VvWRKY* (XV1).

### Expression of transgenes

Expressions of the transgenes were examined using PCR on cDNA and examples are shown in (Fig. 1a). The quality of the total RNA used for cDNA synthesis was confirmed on agarose gels (Fig. 2b). A negative control was performed by PCR directly on RNA to confirm that the RNA was not contaminated by genomic DNA. A clear expression of *Ace-AMP1* was seen in FA1 and FA4 (Fig. 1a). Faint bands could be identified in FA2 and XA7b, whereas in the remaining lines no expression was seen. Varying expression levels between transgenic lines due to i.e. positioning effects and gene silencing is a generally observed phenomenon and also previously observed in *T. aestivum* and *Rosa hybrida* transformed with *Ace-AMP1* (Li et al., 2003; Roy-Barman et al. 2006). In *R. hybrida* it was demonstrated that lines with different expression levels could possess the same level of resistance towards the pathogen (Li et al., 2003). PCR on the cDNA from *VvWRKY1* transformed plants showed a clear expression in FV6 and FV7 and faint bands in FV1, FV3, FV5 and XV1 (Fig. 1a). Expression in FV4 and FV8 could not be confirmed. However, it is not uncommon that transcription factors have low expression levels even with a constitutive promoter. Insertion of *VvWRKY1* in tobacco also resulted in a low level of expression, even though the infection level with fungal pathogens was reduced (Marchive et al. 2007).

### Powdery Mildew Resistance Test

The 20 transgenic lines as well as non-transformed ‘Victoria Fanny’ and X1.105 were inoculated with powdery mildew maintained on *S. novi-belgii* kept under greenhouse conditions, and the average infection level and the percentage of infected leaves determined (Table 2). Large differences in the natural resistance level of the two non-transformed genotypes were found when determined both as the infection level (percentage of leaf area covered with mildew infection) and the percentage of infected leaves (Table 2). ‘Victoria Fanny’ had the highest susceptibility with an overall infection level of 26.2 % and 97.2 % infected leaves compared with 11.8 % and 80.9 %, respectively, in X1.105.

*AceAMP1*

In X1.105, the overall infection level (when non-infected leaves were included) was significantly reduced in four lines transformed with *Ace-AMP1* compared with the wild type (Table 2). However, when the infection level was calculated only for infected leaves none of the transgenic lines differed from the wild type (Table 2). The percentage of infected leaves was significantly reduced in three lines of X1.105 by transformation with *Ace-AMP1* and tended to be reduced in all X1.105 lines containing *Ace-Amp1*. This indicates that insertion of *Ace-AMP1* reduced the number of leaves that were infected with powdery mildew in X1-105. However if infection took place the colonies spread to the same area as in non-transformed leaves. In ‘Victoria Fanny’ neither the infection level nor the percentage of infected leaves were influenced by insertion and expression of *Ace-AMP1*.

Roy-Barman et al (2006) found up to 50 % reduction in the number of *Blumeria graminis* colonies in *Triticum aestivum* transformed with *Ace-AMP1* and in *Rosa hybrida* ‘Carefree Beauty’ transformed with *Ace-AMP1* a significant reduction in the leaf area infected with *Sphaerotheca pannosa* was also recorded due to a reduced spread of hyphae and conidial sporulation (Li et al. 2003). Several other studies using protein assays have shown that *Ace-AMP1* can reduce susceptibility towards at least 15 other plant pathogenic fungi (Cammue et al. 1995; Pillay et al. 2011; Wu et al. 2011). This is partly consistent with the findings in *S. novi-belgii* where *Ace-AMP1* reduced the number of infected leaves but not the area of infection in X1.105.

The precise role of *Ace-AMP1* is unknown, but several hypotheses have been published. Patkar and Chattoo (2006) and Wu et al (2011) suggested that *Ace-AMP1* acts as a toxin. Often, specific anti pathogenic metabolites are restricted to closely related plant species, and the pathogens adapted to a particular plant species can withstand these metabolites for example by detoxifying them. Therefore, by introducing toxic compounds from another plant species the pathogens are not adapted and capable of detoxifying them. The powdery mildew species that attacks *S. novi-belgii* (Mørk et al. 2011) is only found on members of the Asteraceae (Braun 1987) and is not adapted to toxins from *Allium*. However, if *Ace-AMP1* acts as a toxin, a similar effect would be expected in both *Symphyotrichum* genotypes, as a toxin acts independent of the natural resistance gene pool in the host plant. Therefore, it is more likely that *Ace-AMP1* in some way interact with the resistance gene pool of the host plant. This is in line with another theory stating that *Ace-AMP1* is involved in recognition of the pathogen (Patkar and Chattoo 2006; Roy-Barman et al. 2006). Because X1.105 has a natural lower susceptibility level, it can benefit significantly from an increased detection, while ‘Victoria Fanny’ with its natural high susceptibility level is still not capable of defeating the pathogen. The differences in susceptibility level between X1.105 and ‘Victoria Fanny’ containing *Ace-AMP1* can be due to differences in the regulation of expression of the transgene such as the integration point of the new transgene within the host chromosome (euchromatin or heterochromatin), regulatory sequences of the nearby host genes or the number of transgenes per integration site (Guo et al. 2010; Hassanein et al. 2005; Kishimoto et al. 2002b; Mercuri et al. 2000).

*VvWRKY1*

Introduction and over-expression of the transcription factor *VvWRKY1* in *S. novi-belgii* did not influencethe percentage of infected leaves in X1.105 whereas in one ‘Victoria Fanny’ line containing *VvWRKY1,* the area with mildew infection was reduced (Table 2). This may indicate that the primary effect of *VvWRKY1* was to prevent the powdery mildew fungus from getting established on the leaf. These results are similar to those found by Marchive et al. (2007) in tobacco, where ectopic over-expression reduced the susceptibility against the tobacco powdery mildew (*Erysiphe cichoracearum*) slightly, but significantly. Marchive et al (2007) argued that additional unknown pathogen-induced plant components would be needed to take full advantage of the *VvWRKY1* transcription factor. The same argument can be used in *S. novi-belgii.*

Due to the fact that the most susceptible genotype ‘Victoria Fanny’ did not show a significant decrease in the infection level and almost no leaves were uninfected after transformation with any of the transgenes, it can be concluded that ectopic expression of neither of the selected genes could decrease the level of susceptibility sufficiently to improve the natural resistance level in this cultivar. Because the two transgenes only improved mildew resistance in the *Symphyotrichum* genotype, with a low natural susceptibility, the transgenes cannot be used to improve powdery mildew resistance in all cultivars off *S. novi-belgii*. In general, the mechanisms that control disease resistance in plants involve many genes that are interconnected and contribute to the overall resistance level, and only a few results have been generated, where a single gene have a major effect like the barley *mlo* gene, which produce complete resistance against powdery mildew (Bent and Mackey 2007).

In all statistical tests, a significant effect of repetitions within block (the six inoculations per time) and of repeats in time was found. Even though, homogenous inoculation was attempted, differences in inoculation pressure between inoculations were found. This factor was included in the statistical analysis. By including this in the analysis, the effect of spore concentration was taken into consideration and did not mask the effect of the transgenes.

In the present study, the resistance experiment was set up to mimic a commercial greenhouse production whereas most tests of genetically transformed plants with disease resistance genes have been conducted in protected laboratory environments. These *in vitro* experiments or protein assays have shown clear effects of the transgenes (Cammue et al. 1995; Kishimoto et al. 2002a; Takahashi et al. 2005; Takatsu et al. 1999; Wu et al. 2011), while the present results did not provide the same level of significant differences when tested under greenhouse conditions. This may be due to more variable climate conditions in the greenhouse and unintended differences in inoculation level resulting in large variation between individual plants. However, to be of interest commercially, it is required that the effect of the transgene is strong enough to be used *in situ* in greenhouses or in the field.

In conclusion this study showed a significant reduction in the percentage of infected leaves in three out of eight X1.105 lines transformed with *Ace-AMP1* whereas the area with infection was similar on infected leaves from all lines including the wild type. In ‘Victoria Fanny’, a highly mildew susceptible cultivar, none of the transgenes influenced the percentage of infected leaves but one transgenic line, FV1, showed a reduced area with mildew infection compared with the wild type.

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Fig. 1 Expression of RNA from selected transgenic lines and related controls (÷ = cDNA from XV1 for *Ace-AMP1* and cDNA from FA1 for *VvWRKY1*, W = H2O, + = genomic DNA from FA1 397 bp for *Ace-AMP1*/FV8 546 bp for *VvWRKY1*), where a) shows PCR of cDNA, b) quality control of total RNA (100 ng loaded) and c) negative control to confirm that no genomic DNA was present in the RNA.

**Table 1** Number of transformed *S. novi-belgii* shoots regenerated after transformation with *A. tumefaciens*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Genotype | Transgenes | No. of regenerated shoots | No of lines tested with PCR | Positive for *nptII* | Positive for gene of interest | No of lines for further studies |
|  | *Ace-AMP1* | 134 | 17 | 9 | 13 | 3 |
| ‘Victoria Fanny’ | *VvWRKY1* | 140 | 25 | 20 | 8 | 8 |
|  | Wild type | 8 | - |  |  |  |
|  | *Ace-AMP1* | 115 | 18 | 8 | 14 | 8 |
| X1.105 | *VvWRKY1* | 61 | 27 | 18 | 1 | 1 |
|  | Wild type | 11 | - |  |  |  |

Table 2 Effect of transformation with different resistance genes on the percentage of uninfected leaves and the area of infection based on all leaves or only infected leaves.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| S. novi- belgii genotype | Transgene | Line | | Infected leaves (%) | Infection level  ( all leaves1) | Infection level  ( infected leaves) |
| **‘Victoria Fanny’** | **Wild type** | **V. Fanny** | | **97.2a2** | **26.2a** | **27.0a** |
|  | |
| ***VvWRKY1***  ***Ace-AMP1*** | FV1 | | 100 | 15.3\* | 15.3\* |
| FV2 | | 95.4 | 20.6 | 21.6 |
| FV3 | | 94.2 | 19.5 | 20.7 |
| FV4 | | 95.8 | 19.4 | 20.2 |
| FV5 | | 93.1 | 25.1 | 27.0 |
| FV6 | | 92.8 | 28.4 | 30.6 |
| FV7 | | 91.4 | 21.2 | 23.2 |
| FV8 | | 92.6 | 20.6 | 22.5 |
|  | |  |  |  |
| FA1 | | 98.6 | 23.2 | 23.5 |
| FA2 | | 100 | 25.3 | 25.3 |
| FA4 | | 95.4 | 25.6 | 26.8 |
| **X1.105** | **Wild type** | **X1.105** | | **80.9b** | **11.8b** | **14.5b** |
| ***VvWRKY1*** | XV1 | | 62.5 | 8.8 | 14.1 |
| ***Ace-AMP1*** | XA2 | | 60.3 | 5.6\* | 9.4 |
| XA3a | | 58.6 | 6.5\* | 11.2 |
| XA3b | | 55.7\*3 | 6.7\* | 12.1 |
| XA3c | | 61.1 | 9.7 | 15.9 |
| XA4b | | 66.7 | 7.8 | 15.6 |
| XA6 | | 50.0\* | 8.5 | 13.9 |
| XA7a | | 65.3 | 8.9 | 13.6 |
| XA7b | | 50.8\* | 6.6\* | 13.0 |

**1 Means are ratings from image analysis of disease development on 4 leaves per plant, 6 plants per repetition and 3 repetitions.**

**2 Different letters indicate significant differences between wild types (P < 0.05) according to Dunnetts test.**

**3 Asterisks indicate significant differences between wild type and transgenic lines within genotype and transgene (P < 0.05) according to Dunnetts test**