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Spatial and temporal patterns of green fluorescent protein (GFP) fluorescence during leaf canopy development in transgenic oilseed rape, *Brassica napus* L.

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Abstract The green fluorescent protein (GFP) holds promise as a field-level transgene marker. One obstacle to the use of GFP is fluorescence variability observed within leaf canopies. In growth chamber and field experiments, GFP fluorescence in transgenic oilseed rape (*Brassica napus*) was shown to be variable at each leaf position over time and among different leaves on the same plant. A leaf had its highest GFP fluorescence after emergence and, subsequently, its fluorescence intensity decreased. GFP fluorescence intensity was directly correlated with the concentration of soluble protein. The concentration of the genetically linked recombinant *Bacillus thuringiensis* (Bt) *cry1Ac* endotoxin protein also was examined, and GFP fluorescence was positively correlated with Bt throughout development. The results show that GFP can be used as an accurate transgene marker but that aspects of plant developmental should be taken into account when interpreting fluorescence measurements.

Keywords Green fluorescent protein · *Bacillus thuringiensis* · Soluble protein concentration · Transgene monitoring · Canola

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Introduction

Monitoring transgenic plants under field conditions will become increasingly important as genetically modified (GM) crops are grown in large-scale agricultural systems. This would appear to be especially true for transgenic oilseed rape (*Brassica napus*). A significant amount of seed can be lost during oilseed rape harvesting (Thomas et al. 1991). Oilseed rape volunteers are abundant in subsequent years (Légère et al. 2001) and can have a relatively long period of persistence (Simard et al. 2002). Individual oilseed rape plants resistant to multiple herbicides have been produced under field conditions, evidently due to intraspecific hybridization (Hall et al. 2000; Beckie et al. 2003). In addition, a herbicide tolerance transgene from oilseed rape has been transferred to a naturally occurring wild relative population of *B. rapa* through hybridization (Warwick et al. 2003). These factors can all contribute to problems with management and containment of transgenes during oilseed rape production.

For a monitoring system to be effective, the marker technology should be accurate with few false positives and negatives, detectable throughout the life cycle of the plant, and able to inform on the status of specific genetically linked transgenes. The green fluorescent protein (GFP) has been proposed as a whole-plant marker for field-level applications (Stewart 1996). The GFP gene was first cloned from jellyfish (*Aequorea victoria*) in 1992 and has since been modified for specific applications (Prasher et al. 1992; Chalfie et al. 1994; Chiu et al. 1996; Siemering et al. 1996). GFP monitoring has the potential to track transgenes on large spatial scales utilizing the detection of the characteristic green fluorescence. The *mgfp5-er* variant gene has been shown to be a feasible transgene monitor in tobacco (*Nicotiana tabacum*) plants under field conditions (Harper et al. 1999). Plants synthesized the protein and remained fluorescent throughout the growing season. GFP has also been shown to be a feasible qualitative marker for the presence of a genetically linked synthetic *Bacillus thuringiensis* (Bt)

cry1Ac endotoxin transgene in tobacco and oilseed rape (Harper et al. 1999; Halfhill et al. 2001).

With this positive evidence in hand, the next step in the development of a GFP monitoring system is to more critically evaluate the system in oilseed rape and identify weaknesses that could limit its utility. The utility of a GFP detection system under field conditions could be constrained by differential fluorescence during the plant life cycle. In previous experiments with transgenic oilseed rape, leaf GFP fluorescence was not stable during the plant life cycle (Halfhill et al. 2001). In those experiments, GFP fluorescence tended to decline and become more variable as the plants matured. The inconsistency of GFP could be associated with the cauliflower mosaic virus (CMV) 35S promoter. Differential expression of transgenes regulated by the 35S promoter has been observed in studies with different plant species (Odell et al. 1985; Benfey and Chua 1990; Blumenthal et al. 1999; Harper and Stewart 2000). Alternatively, the variability of GFP could be associated with developmental factors within the leaf canopy.

In the investigation reported here, environmental chamber and field-level experiments were conducted to investigate whether changes in GFP fluorescence in transgenic oilseed rape were related to leaf aging. The experiments used transgenic oilseed rape with a Bt transgene genetically linked to GFP. This allowed evaluation of the extent that leaf developmental effects might compromise the use of GFP as a marker for linked transgene expression.

Materials and methods

Environmental chamber experiment

The environmental chamber experiment was performed in the Southeastern Plant Environmental Laboratory at North Carolina State University (Downs and Thomas 1991). Growth chambers were programmed for an 8/16-h (day/night) photoperiod utilizing high-pressure sodium lamps, and day/night temperatures were maintained at 22/18°C, respectively. Three homozygous T_4 GFP oilseed rape (*Brassica napus* cv. Westar) events were utilized in this study: GT 1 containing genetically linked GFP (*Aequorea victoria*, *mgfp5-er* variant; Haseloff et al. 1997) and Bt (*Bacillus thuringiensis*, synthetic Bt *crylAc*) transgenes; GFP 1 and GFP 2 containing only the GFP gene (Halfhill et al. 2001); non-transgenic oilseed rape (Westar). The GT 1 event was transformed with the pSAM 12 plasmid containing genetically linked GFP and Bt cassettes regulated by independent CaMV 35S promoters (plasmid described in Harper et al. 1999). A transgenic event was defined as the progeny of a single, independently transformed plant recovered from tissue culture. Four seeds from each transgenic event were sown in a standard greenhouse bark media mix in 0.6-l pots (11×11 cm) (three events grown concurrently, 48 pots in total). Five non-transgenic plants were maintained as controls. The pots were fertilized daily with a nutrient solution containing 7.6 m*M* N, 0.3 m*M* P, 2.8 m*M* K, 1.4 m*M* Ca, 0.5 m*M* Mg, and micronutrients in 1/4-strength Hoagland's solution. After germination, emerging seedlings were allowed to establish for 1 week and then culled to one plant per pot.

Four plants per transgenic event (three events grown concurrently, 12 plants total) were randomly harvested among all the plants in the environmental chambers at 8-day intervals beginning on the 18th day post-germination (four harvests in total). At each harvest, leaf area for each leaf position was recorded with a Li-Cor 3100 Leaf Area meter (Li-Cor Instruments, Lincoln, Neb.) for all measurable leaves (those greater than 5.0 cm^2). Wet mass for each leaf was recorded. GFP fluorescence and relative chlorophyll concentration were recorded with the GFP meter (Opti-Sciences, Tyngsboro, Mass.). The GFP meter is a self-contained, field-portable fluorescence detection and data-logging instrument (Millwood et al. 2003). For this study, a 465-nm filter with a bandwidth of 35 nm was used for GFP excitation. To quantify GFP emission, channel 1 (GFP channel) used a 530-nm filter with a bandwidth of 35 nm for emission and channel 2 (chlorophyll channel) used a 680-nm filter with a bandwidth of 35 nm for emission. For each leaf, two GFP measurements were recorded from the underside of the leaf, slightly off the midvein. On the last harvest date (42 days), GFP fluorescence was recorded for each leaf position for the five non-transgenic oilseed rape (Westar) plants. For the transgenic samples at each harvest date, two 1-cm-diameter leaf punches were collected from fresh leaves at the same position as the GFP measurements from each leaf with a 1.5-ml microcentrifuge tube. Each individual leaf was placed into a paper bag and dried in a convection oven at 60°C for 72 h. Dry weight of each leaf was recorded.

Soluble protein was extracted from the leaf punches, and Bradford analysis (Bio-Rad Laboratories, Hercules, Calif.) was used to quantify soluble protein concentration. The mass of each punch was recorded, and the sample was homogenized with 0.5-mm glass beads in a mechanical amalgamator (Silamat S5, Ivoclar Vivadent Clinical, Austria). After disruption of the leaf tissue, 400 μl of 0.1 *N* NaOH was added to each sample, and the samples were incubated on ice for 30 min. After incubation, 80 μl of 1 *M* Tris-HCl, pH 4.5, was added to neutralize each sample. The samples were centrifuged for 7 min at 7,000 rpm (Denville 260D Microcentrifuge, 24-sample rotor, Denville Scientific, Metuchen, N.J.), and the supernatant containing soluble protein was recovered. Bradford analysis was used to determine soluble protein concentrations in 96-well plates (EL 800 Universal Microplate Reader with the KC Junior software package, Bio-Tek Instruments, Winooski, Vt.). Bt protein concentration was determined by the use of a Bt ELISA (Cry1Ab/Cry1Ac Plate kit, catalog no. AP 003, Envirologix, Portland, Me.).

At each harvest date, ANOVA was used to evaluate differences in GFP fluorescence, and Fisher's PLSD was used to determine whether significant differences occurred between leaf positions and collection dates (STATVIEW 5.0 for Windows, 1992–1998, SAS Institute, Cary, N.C.). Regression analysis was performed for GFP fluorescence by leaf area, leaf age, leaf wet and dry mass, wet mass per leaf area, chlorophyll concentration, Bt concentration per unit wet mass, and concentration of soluble protein per unit wet mass (STATVIEW 5.0 for Windows).

Field experiment

A field experiment was conducted at the Central Crops Research Station, Clayton, N.C. (35°39'N 78°27'W) in the spring of 2001. Ten events of GFP *B. napus* (GT 1–5, GT 8, 9, and GFP 1–3)(Halfhill et al. 2001) were hand-planted in ten 2×48-m plots, one event per plot (March 27, 2001). The planting rate was 100 seeds per square meter for a final stand density of 50 plants per square meter based on a 50% predicted germination frequency. Beginning 24 days post-germination, five plants per event were harvested from the field at 7-day intervals (50 plants per harvest, six harvests total). A Fluoromax-2 fluorescence spectrophotometer (Jobin Yvon & Glen Spectra, Edison, N.J.) with DATAMAX and GRAMS/386 software (Galactic Industries, Salem, N.H.) was used to quantify GFP fluorescence in the leaf tissue at each leaf position. Each leaf was excited at 385 nm, and emission spectra were recorded from 420–600 nm. A fiber optic cable was used to provide the excitation light to leaf tissue in vivo, and the intensity was measured at 508 nm (green light) in counts per second (cps). The 450-nm wavelength, outside the GFP fluorescence spectrum, was set as the

anchor for each sample. All samples were standardized to the average 450-nm value of non-transgenic oilseed rape to control for baseline variation (Millwood et al. 2003). Relative chlorophyll concentrations were estimated at the same position as GFP fluorescence using a Minolta SPAD-502 Chlorophyll meter (Spectrum Technologies, Plainfield, Ill.). Two 1-cm-diameter leaf punches were collected from fresh leaves at the same position as the GFP measurements from each leaf with a 1.5-ml microcentrifuge tube. Soluble protein was extracted, and soluble protein and Bt concentrations per unit mass were quantified as above. At each harvest date, ANOVA was utilized for evaluating differences in GFP fluorescence, and Fisher's PLSD was used to determine where significant differences occurred between leaf positions and collection dates (STATVIEW 5.0 for Windows). GFP fluorescence was correlated by regression to relative chlorophyll concentration, Bt concentration per unit wet mass, and concentration of soluble protein per unit wet mass (STATVIEW 5.0 for Windows).

Results

Patterns of GFP fluorescence

In the environmental chamber experiment, oilseed rape event GT 1 plants at the four-leaf stage (18 days) exhibited a similar GFP fluorescence among all leaf positions, with counts per second (cps) ranging from 975.9 to 1,067 (Fig. 1). As the plants developed, however, GFP fluorescence of individual leaves declined. After 26 days, fluorescence had decreased at leaf positions 1 and 2 by approximately 22%, and the youngest leaves at leaf positions 3–6 showed significantly higher GFP fluorescence (range: 1,026–1,174) than older leaves (Fig. 1). The developmental pattern continued as the experiment progressed. At the 34-day and 42-day harvests, an obvious ordering in fluorescence intensity was present, with younger leaves being higher (Fig. 1). The decline in GFP fluorescence at the older leaf positions continued until levels were similar to those of leaves on non-transgenic oilseed rape plants (approx. 551). A similar pattern of GFP fluorescence was present in plants growing in the field (data not shown).

GFP fluorescence was correlated with soluble protein

Leaf tissues from plants growing in the environmental chamber and from the field were analyzed to identify factors that might be associated with the decreases in GFP fluorescence as leaves aged. There was no linear relationship between GFP fluorescence and leaf area, wet and dry mass, wet mass per unit area, and relative chlorophyll concentration at any of the harvest dates ($P>0.05$; data not shown). In contrast, GFP fluorescence was positively associated with soluble protein per unit wet mass ($\mu g/\mu l$ per gram tissue) in leaves of all ages (Fig. 2) and, in particular, the patterns of fluorescence decline in the two parameters were similar (Fig. 3). In environmental chambers, GT 1 exhibited a positive association over all plant harvest dates where GFP fluorescence varied over leaf positions (harvests 26 days to 42 days; Fig. 2). On the last harvest date (42 days), GFP fluorescence was directly proportional to the soluble protein per unit wet mass (GT 1, $R^2=0.967$; Fig. 2C). At the 42-day harvest, oilseed rape events GFP 1 and GFP 2 exhibited significant correlations between these indices ($P=0.0198$ and 0.0483, respectively), although the R^2 values for these regression lines were less robust ($R^2=0.563$ and 0.449, respectively; data not shown). The correlation of GFP fluorescence and soluble protein per unit wet mass was also evaluated under field conditions. On the last harvest (59 days), GFP fluorescence was positively associated with soluble protein per unit wet mass over eight combined oilseed rape events ($R^2=0.83$; Fig. 4A).

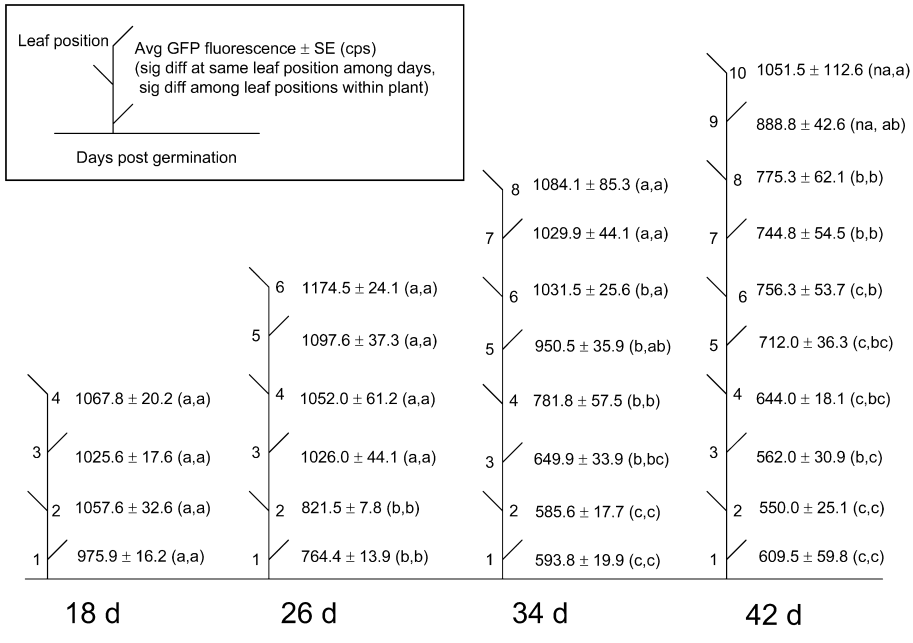

Fig. 1 GFP fluorescence intensity at each leaf position of transgenic oilseed rape. GT 1 oilseed rape was grown in environmental chambers over 42 days. GFP fluorescence was quantified with the GFP meter (units in counts per seconds, 530 nm). Non-transgenic oilseed rape leaves had an average fluorescence at 530 nm of 551.9±17.4 (± standard error) regardless of leaf position and age. *Different letters* represent significant differences (Fisher's PLSD, $P<0.05$), *na* not applicable

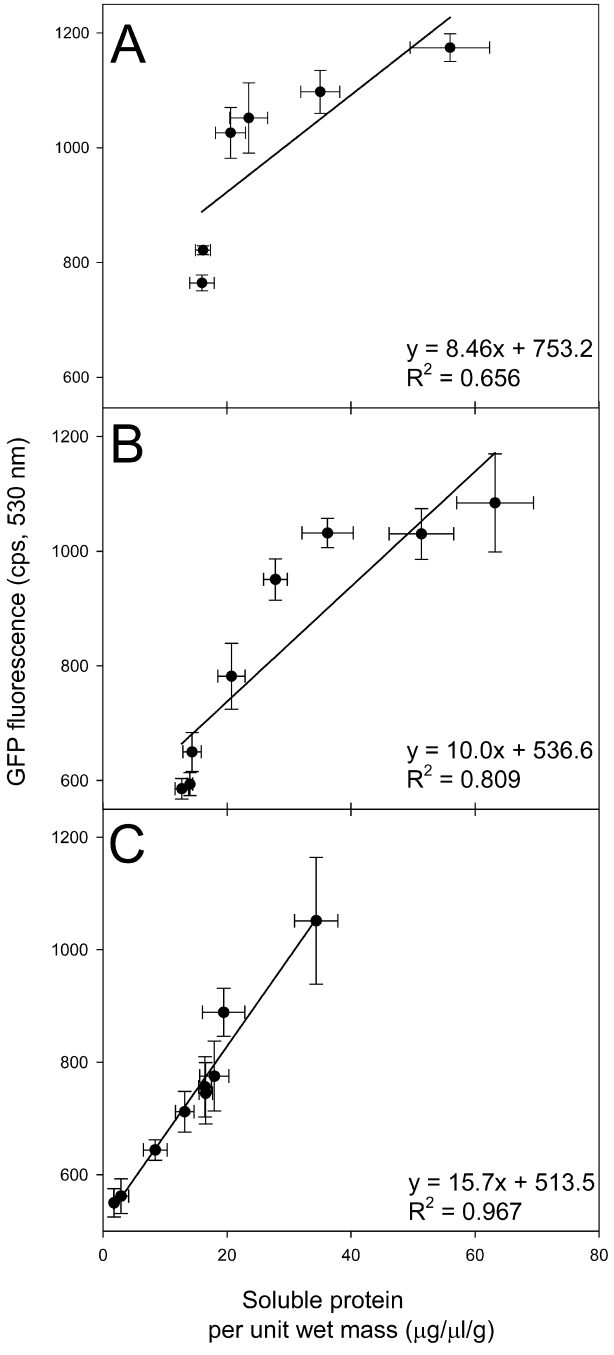

Fig. 2A–C Comparison of GFP fluorescence to the concentration of soluble protein per unit wet mass. Three harvest dates from oilseed rape event GT 1 grown in environmental chambers are represented (**panel A** 26 days, **panel B** 34 days, **panel C** 42 days). Data points represent the average GFP and soluble protein values for each leaf position. Regression analysis was performed for GFP fluorescence by soluble protein per unit wet mass over all leaf positions

Predictive value of GFP fluorescence for the concentration of a genetically linked protein

For all GFP/Bt events in both experiments, GFP fluorescence was positively associated with Bt concentration (Fig. 4). In the environmental chamber experiment, GFP fluorescence was associated with Bt concentration (μg Bt/g tissue) at the last harvest date (48 days, R^2=0.604; data not shown). Under field conditions when the eight GT events were combined, the GFP fluorescence intensity was strongly associated with Bt concentration at the last harvest date (59 days, R^2=0.853; Fig. 4B). In this latter case, the concentration of both genetically linked proteins exhibited the same pattern of variation with the highest concentrations in youngest leaves.

Discussion

Variable GFP fluorescence in the leaf canopy was noted during the life cycle of transgenic oilseed rape in previous research (Halfhill et al. 2001), but the physiological basis for the variability was unknown. The results of the experiments described here indicate that changes in leaf GFP fluorescence in the transgenic plants followed two specific patterns. First, the fluorescence intensity of individual leaves was highest when a leaf was relatively young, and intensity decreased over time as a leaf aged. Second, as a result of the changes in individual leaves, a fluorescence intensity profile existed for the leaf canopy at any particular time. The highest fluorescence was in young leaves near the apical meristem and the lowest in older leaves. The decline in fluorescence intensity with leaf aging was a steady progression to the low, background levels characteristic of control leaves in non-transgenic plants. The aging effect was similar, regardless of whether plants were growing in controlled environment chambers or the field.

Based on the analysis of leaf tissues from growth chamber and field-grown plants, it is evident that the changes in GFP fluorescence were related to changes in the concentration of soluble protein. GFP fluorescence and soluble protein declined at similar times and to similar extents in individual leaves at different positions within the canopy. The close relationship between the two factors would suggest that the GFP decline was the result of general changes in leaf physiology and not a specific effect on the transgene or its expression when controlled by the 35S promoter.

The pattern of decline in GFP fluorescence and the concentration of soluble protein with leaf aging appears to be similar to that expected with "sequential senescence" of leaves, which was described many years ago (Woolhouse 1967). After leaf emergence, soluble protein levels typically increase during leaf elongation, reach an upper threshold, and then decrease with time until the leaf is eventually shed. Changes in the concentration of specific proteins that exist within the total soluble protein pool also have been previously tracked using other methodologies. In experiments with barley (*Hordeum vulgare*), for example, concentrations of ribulose bisphosphate carboxylase were high in primary leaves early in their development and then decreased over time in parallel with decreases in soluble protein (Friedrich and Huffaker

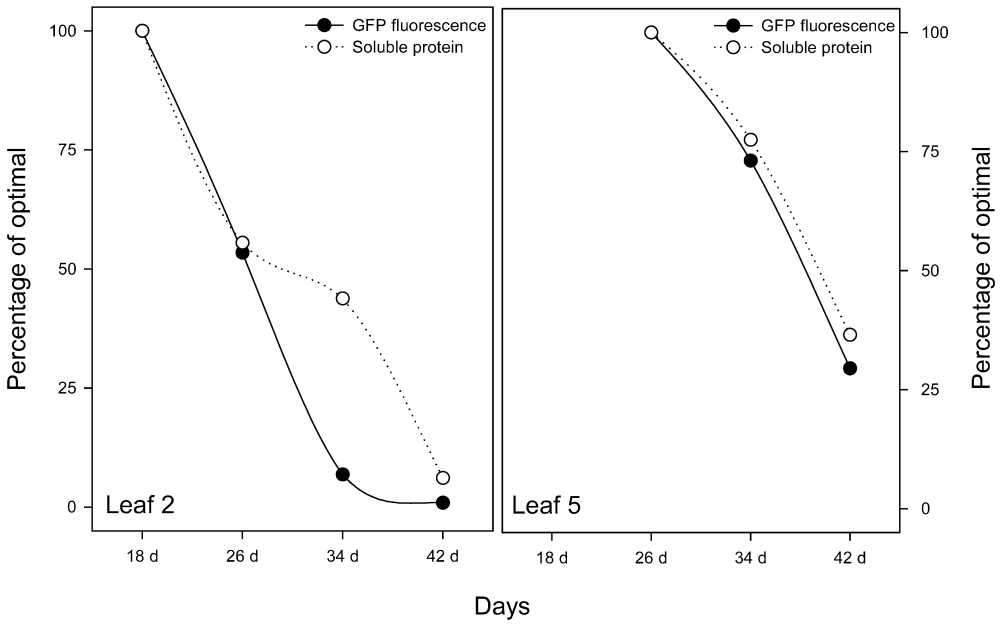

Fig. 3 Percentage of optimal soluble protein and GFP fluorescence at leaf positions 2 and 5. Optimal values were the highest average value for each measurement at each harvest date. The samples were from oilseed rape event GT 1 grown in environmental chambers. GFP fluorescence values were calculated by subtracting the wild-type oilseed rape (Westar) fluorescence at 530 nm. The decrease in the concentration of soluble protein and GFP fluorescence was consistent with the process of sequential senescence

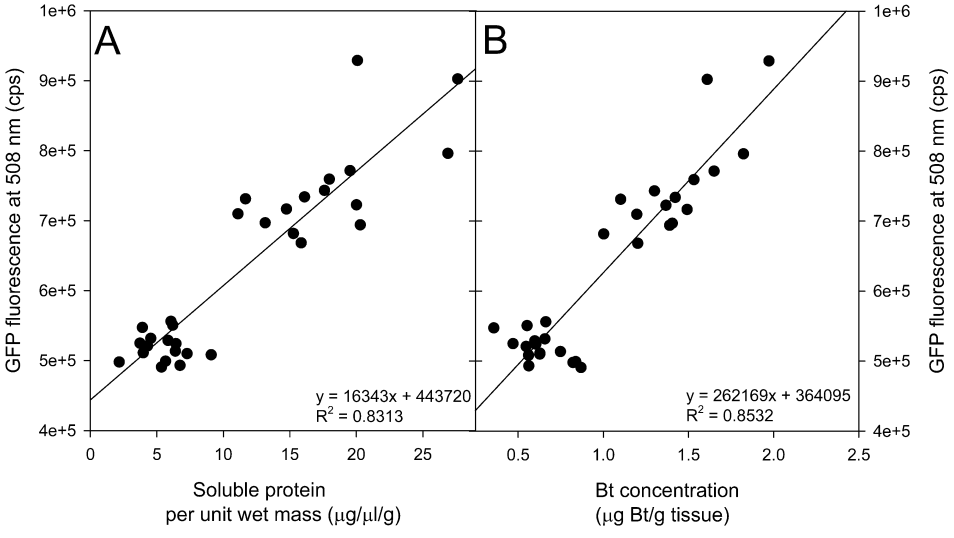

Fig. 4A, B Comparison of GFP fluorescence to the concentrations of soluble protein per unit wet mass and *Bacillus thuringiensis* (Bt). *Data points* represent leaf positions 3, 5, 9, and 11 from eight combined transgenic GFP/Bt oilseed rape events (GT 1–6, 8, and 9) harvested 59 days post-germination under field conditions. Regression analysis was performed for GFP fluorescence by soluble protein per unit leaf wet mass (**panel A**) and Bt concentration (**panel B**) over all data points

1980). When the process of sequential senescence is considered, it seems reasonable to conclude that the reduction in GFP fluorescence is simply the result of leaf aging— i.e., the programmed decrease in soluble protein, accompanied by a proportionate reduction in GFP.

It has been speculated that changes in the activity of the 35S promoter could have been responsible for variable GFP fluorescence. While one cannot dismiss the possibility that temporal reductions in 35S activity contributed to the decrease in GFP fluorescence over time, the effect would be confounded with the decreasing soluble protein concentration during sequential senescence. It has been observed that the 35S promoter produces a consistent percentage of recombinant protein per unit total soluble protein (Blumenthal et al. 1999; Harper and Stewart 2000). It is important to note that the correlations between GFP fluorescence and changes in physiology should be expected to be promoter-specific. Senescence-associated promoters, such as the *SAG12* promoter (Gan and Amasino 1995), when fused to GFP would be expected to exhibit different patterns in older tissues with potential increases in fluorescence with age.

Halfhill et al. (2001) suggested that increasing chlorophyll concentration in maturing leaves could cause variable GFP fluorescence by interfering with GFP fluorescence detection. Chlorophyll generally tends to remain closer to maximal concentrations longer than soluble protein as leaves age. This would perhaps explain why the chlorophyll concentrations were not correlated with, and thus probably not a cause of, the decline in GFP fluorescence under growth chamber or field conditions in these experiments.

One of the key aspects of using GFP fluorescence in a monitoring system for transgene expression is that it

accurately quantifies the concentration of genetically linked proteins of interest. The results of the present study show that GFP fluorescence accurately predicted the concentration of Bt endotoxin, even as both parameters changed over time. The results also imply, however, that false negatives could be generated if one solely assays older leaves, which characteristically have low concentrations of soluble proteins. That problem can be minimized if GFP fluorescence measurements are made on younger leaves near the apical meristem. Additionally, it has been known for some time that sequential senescence and the decline in soluble protein proceeds at a faster pace under water stress or nitrogen deficiency (Woolhouse 1967). Therefore, the observation that changes in GFP fluorescence are correlated with those in soluble protein concentration may prove to be a significant tool for monitoring particular plant stresses.

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