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Frequencies, Timing, and Spatial Patterns of Co-Suppression of Nitrate Reductase and Nitrite Reductase in Transgenic Tobacco Plants[1]

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Frequencies, timing, and spatial patterns of co-suppression of the nitrate (*Nia*) and nitrite (*Nii*) genes were analyzed in transgenic tobacco (*Nicotiana tabacum*) plants carrying either *Nia* or *Nii* cDNAs under the control of the 35S promoter, or a *Nii* gene with its own regulatory signals (promoter, introns, and terminator) cloned downstream of two copies of the enhancer of the 35S promoter. We show that (a) the frequencies of transgenic lines affected by co-suppression are similar for the three constructs, ranging from 19 to 25%; (b) *Nia* and *Nii* co-suppression are triggered stochastically during a phenocritical period of 2 weeks between germination and flowering; (c) the timing of co-suppression (i.e. the percentage of isogenic plants affected by co-suppression reported as a function of the number of days of culture) differs from one transgenic line to another; (d) the percentage of isogenic plants affected by co-suppression is increased by growing the plants in vitro prior to their transfer to the greenhouse and to the field; and (e) at the end of the culture period, plants are either unaffected, completely co-suppressed, or variegated. Suppressed and nonsuppressed parts of these variegated plants are separated by a vertical plane through the stem in *Nia* co-suppression, and separated by a horizontal plane in *Nii* co-suppression.

During the past 10 years, gene silencing has become a common theme in plant molecular biology. Although many transformants that did not have correct expression of their transgene were put aside, the assumption being that position effect was probably responsible for poor expression, some scientists have come to "treasure their exceptions" and have developed a primary classification of transgene silencing events (Matzke and Matzke, 1995; Phillips et al., 1995).

Gene silencing can be due either to a transcriptional block (Meyer et al., 1993) or to a posttranscriptional process leading to an increase in mRNA turnover (Van Blockland et al., 1994). In both cases, silencing has been observed in *cis*, affecting single or multiple copies inserted at one locus (Linn et al., 1990; Meyer et al., 1993; Dehio and Schell, 1994; Ingelbrecht et al., 1994; Elmayan and Vaucheret, 1996) or in *trans*, affecting allelic or ectopic copies (Matzke et al., 1989; Meyer et al., 1993; Vaucheret, 1993; Park et al., 1996). Co-suppression belongs to the latter case and has been defined as the posttranscriptional silencing of transgene(s) and host gene(s) expressing the same coding sequence (Jorgensen, 1990; Van Blockland et al., 1994). This phenomenon has been observed in several plant species with different transgenes (for review, see Flavell, 1994; Dougherty and Parks, 1995; Matzke and Matzke, 1995). In each case, silencing of the transgene(s) and host gene(s) is characterized by a decrease in mRNA steady-state level, but the transcription rate is not affected.

In our laboratory, transgenes derived from the tobacco (*Nicotiana tabacum*) *Nia* and *Nii* genes were built to overexpress NR and NiR, respectively (Vincentz and Caboche, 1991; Vaucheret et al., 1995; P. Crété, M. Caboche, C. Meyer, unpublished data). Introduction of these transgenes into transgenic tobacco plants led either to co-expression or co-suppression of host genes and transgenes (Dorlhac de Borne et al., 1994; Palauqui and Vaucheret, 1995; Vaucheret et al., 1995). Plants that overexpress either NR or NiR activity exhibit a normal green phenotype, whereas NR or NiR deficiency leads to a cell-autonomous chlorotic phenotype visible at any stage of development (Gabard et al., 1987; Saux et al., 1987; Vaucheret et al., 1992, 1995; Dorlhac de Borne et al., 1994). This allows the analysis of the setting and resetting of co-suppression.

Several independent transgenic tobacco lines homozygous for the construct consisting of *Nia2* cDNA under the control of the 35S promoter (*35S-Nia2* cDNA) reproducibly showed co-suppression during successive field trials. After sowing, seedlings grew normally for a period of up to several weeks, expressing both the host genes and the transgenes. Then, co-suppression of *Nia* host genes and transgenes progressively affected an increasing number of plants before reaching a plateau at flowering. The percentage of plants affected by co-suppression remained the same at each generation in the same culture conditions, showing that the ability to trigger co-suppression was inherited

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Abbreviations: CaMV, cauliflower mosaic virus; *Nia*, nitrate reductase gene; *Nii*, nitrite reductase gene; NiR, nitrite reductase; NR, nitrate reductase; SAM, *S*-adenosyl-L-Met.

rather than being the inactivated state (Dorlhac de Borne et al., 1994), a result also found in the case of chitinase co-suppression (Hart et al., 1992). The percentage of plants affected by co-suppression was increased when plants were grown in vitro prior to their transfer to the greenhouse and to the field (Palauqui and Vaucheret, 1995), suggesting an effect of environmental factors, a result also found in the case of chitinase, SAM synthetase, and glucanase co-suppression (Hart et al., 1992; Boerjan et al., 1994; de Carvalho-Niebel et al., 1995).

Similarly, co-suppression of *Nii* host genes and transgenes was observed cyclically at each generation in a transgenic tobacco line homozygous for a construct consisting of the native *Nii1* gene cloned downstream of two copies of the enhancer of the 35S promoter (*70enh-Nii1* gene). After sowing, seedlings grew normally and co-expressed host genes and transgenes. Subsequently, co-suppression affected all of the plants almost synchronously between the 2nd and 4th week of growth (Vaucheret et al., 1995).

Here we show a comparative analysis of the frequencies, timing, and spatial patterns of co-suppression in transgenic tobacco plants carrying either the *35S-Nia2* cDNA or the *70enh-Nii1* gene constructs, and in plants carrying a new construct consisting of the tobacco *Nii1* cDNA under the control of the 35S promoter (*35S-Nii1* cDNA). We show that frequencies and timing are comparable for *Nia* and *Nii* co-suppression, whereas spatial patterns differ completely between the two genes, irrespective of the regulation of the transgene (constitutive *35S*-cDNA versus regulated *70enh*-gene).

MATERIALS AND METHODS

Transgenic plants were obtained by *Agrobacterium*-mediated leaf disc transformation of tobacco (*Nicotiana tabacum* cv Paraguay PBD6). Constructs 27, 30, and 34 carry complete or partial sequences of the tobacco *Nia2* cDNA inserted between the 35S promoter and terminator sequences (*35S-Nia2*) (Dorlhac de Borne et al., 1994; Palauqui and Vaucheret, 1995). Construct 475 carries the complete sequence of the tobacco *Nii1* cDNA inserted downstream of the 35S promoter (*35S-Nii1*) (P. Crété, M. Caboche, C. Meyer, unpublished data). Construct 461 carries the complete sequence of the tobacco *Nii1* gene (including introns, promoter, and terminator sequences) cloned downstream of the double enhancer of the 35S promoter (*70enh-Nii1*) (Vaucheret et al., 1995). Constructs 27, 30, 34, and 461 are carried on the binary vector pBin 19 (Bevan, 1984). Construct 475 is carried on the binary vector pBiB-Hyg (Gritz and Davies, 1983).

Genetic Analysis

The generation of transgenic lines homozygous for a single *35S-Nia2* transgene locus was described previously (Palauqui and Vaucheret, 1995). Transformants carrying the *35S-Nii1* or *70enh-Nii1* construct were selected on a medium supplemented with hygromycin (25 mg/L) or kanamycin (50 mg/L), respectively. Transformants were allowed to self-fertilize in the greenhouse. Surface-sterilized seeds were sown in vitro on the hygromycin/kanamycin medium. The viability of the seedlings was monitored after 2 months of growth. Seeds were also sown in the greenhouse and grown under a natural light/dark regime and watered with a nutrient solution containing 12 mM nitrate. Transgenic lines showing a 3:1 segregation ratio on medium supplemented with the antibiotic and showing chlorotic plants in their progeny were retained for further analysis. Chlorotic plants were allowed to self-fertilize. Surface-sterilized seeds were sown in vitro on the hygromycin/kanamycin medium described above. Chlorotic plants were homozygous for the transgene locus.

Field Trial Analysis

Greenhouse/Field Conditions

Seeds corresponding to the different transgenic lines were sown directly in soil in the greenhouse in April and watered with a nutrient solution containing 12 mM nitrate. One hundred twenty plants per combination were transferred to individual vessels in May and watered with the same nutrient solution in the greenhouse. Plants were transferred to a standard nitrate-fertilized field (200 kg N/ha) in June.

In Vitro/Greenhouse/Field Conditions

Seeds corresponding to different transgenic lines were surface-sterilized and sown in vitro in April and grown at 23°C under conditions of a 16-h light/8-h dark photoperiod, 70% RH, and 120 mE m^{-2} lighting. One hundred twenty plants per combination were transferred to individual vessels in May in the greenhouse. Plants were transferred to a nitrate-fertilized field (200 kg N/ha) in June.

The number of chlorotic plants was determined every week from sowing until flowering. In accordance with French legislation, flowers were destroyed and the transgenic plants were eliminated.

Nucleic Acid Analysis

RNA was extracted from leaves. Northern blot analysis was performed with the tobacco *Nia2* or *Nii1* cDNA as described previously (Kronenberger et al., 1993).

RESULTS

Frequencies of Co-Suppression

Transgenic Tobacco Plants Carrying the 35S-Nia2 cDNA Construct

The introduction of three derivatives of a *35S-Nia2* cDNA construct into tobacco plants was described previously (Dorlhac de Borne et al., 1994; Palauqui and Vaucheret, 1995). One construct, 30, carries a full-length *Nia2* cDNA cloned between the CaMV 35S promoter and the natural termination sequences of the *Nia2* gene (Fig. 1). A second construct, 34, carries a *Nia2* cDNA devoid of its 5′ untranslated region cloned between the CaMV 35S promoter and the natural termination sequences of the *Nia2* gene. A third

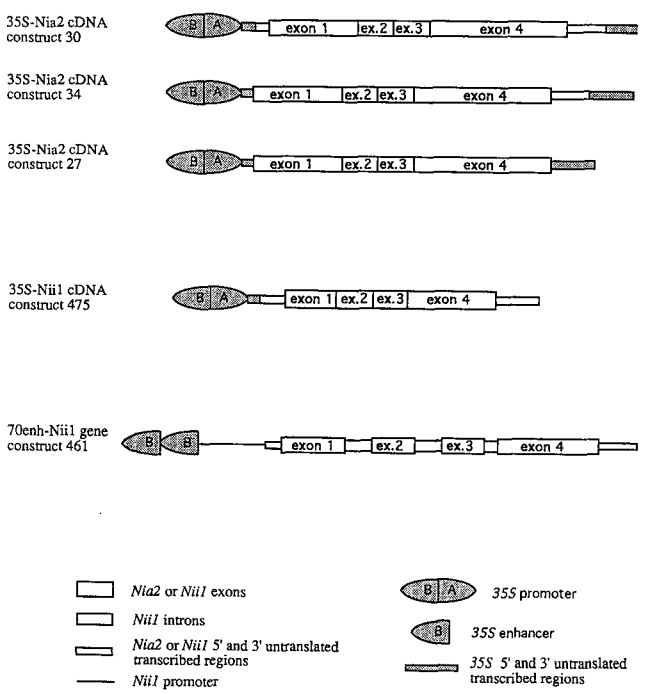

Figure 1. Physical map of the *35S-Nia2* cDNA, *35S-Nii1* cDNA, and *70enh-Nii1* gene constructs.

construct, 27, carries a *Nia2* cDNA devoid of its 5′ and 3′ untranslated regions cloned between the CaMV 35S promoter and termination sequences. Among 43 transformants carrying one of these three transgenes, three showed co-suppression of *Nia* host genes and transgenes; i.e. they were chlorotic and contained no NR activity and no detectable *Nia* mRNAs (Dorlhac de Borne et al., 1994). When seeds obtained by self-fertilization of the 40 remaining transformants were sown in the greenhouse, chlorotic individuals with no NR activity were observed at various frequencies in the progeny of 7 of them (Palauqui and Vaucheret, 1995; this work). These results indicate that 23% of the transformants carrying a *35S*-cDNA construct exhibit co-suppression, with no significant differences between constructs 27, 30, and 34 (Table I).

Transgenic Tobacco Plants Carrying the 35S-Nii1 cDNA Construct

A *35S-Nii1* transgene (P. Crété, M. Caboche, C. Meyer, unpublished data) consisting of a full-length *Nii1* cDNA cloned between the CaMV 35S promoter and the natural termination sequences of the *Nii1* gene (Fig. 1) was introduced into tobacco plants. Among 28 transformants, two were chlorotic and contained no NiR activity or *Nii* mRNA (data not shown). When seeds obtained by self-fertilization of the remaining 26 transformants were sown in the greenhouse, chlorotic individuals with no NiR activity were observed at various frequencies in the progeny of 5 of them. These results indicate that 25% of the transformants carrying a *35S-Nii1* cDNA construct exhibit co-suppression, a result comparable to that observed with the *35S-Nia2* cDNA construct (Table I).

Transgenic Tobacco Plants Carrying the 70enh-Nii1 Gene Construct

A *70enh-Nii1* transgene (Vaucheret et al., 1995) consisting of the native *Nii1* gene with its own regulatory signals (promoter, introns, and terminator), cloned downstream of two copies of the enhancer of the 35S promoter (Fig. 1), was previously introduced into tobacco plants. Among 9 transformants, 1 was chlorotic and showed co-suppression of *Nii* host genes and transgenes. When seeds obtained by self-fertilization of the remaining 8 transformants were sown in the greenhouse, chlorotic individuals with no NiR activity were observed in the progeny of one of them (Vaucheret et al., 1995). To analyze a larger number of transformants, another round of transformation was performed. Among 12 new transformants, 1 was chlorotic and showed co-suppression of *Nii* host genes and transgenes, and another showed co-suppression in its progeny (data not shown). Taken together, these results indicate that 19% of the transformants carrying a *70enh-Nii1* cDNA construct exhibit co-suppression, a result comparable to that observed with the *35S-Nia2* and *35S-Nii1* cDNA constructs (Table I).

Timing of Co-Suppression

Transgenic Tobacco Plants Carrying the 35S-Nia2 cDNA Construct

Co-suppression of NR was previously shown to occur during a long phenocritical period between the 1st and 4th month of growth. At each generation, the number of plants showing co-suppression increased progressively during the first 3 months of growth before reaching a plateau at flowering (Dorlhac de Borne et al., 1994). The analysis of several independent lines carrying a single transgene locus showed that the final percentage of plants affected by co-suppression differs from one transgenic line to another, but remains stable at each generation whether the seeds are harvested from a suppressed or an isogenic, nonsuppressed plant. In addition, we showed that these percentages were increased by in vitro culture (Palauqui and

Table I. *Frequencies of co-suppression*

For each construct, the frequency is defined by the percentage of transformants affected by co-suppression either as primary regenerants or as individuals of their self-progeny (average is given for the three 35S-Nia2cDNA constructs).

Construct	No. of Transformants	No. of Co-Suppressed	Percentage of Co-Suppressed
35S-Nia2 (construct 30)	28	6	
35S-Nia2 (construct 34)	5	2	23
35S-Nia2 (construct 27)	10	2	
35S-Nii1 (construct 475)	28	7	25
70enh-Nii (construct 461)	21	4	19

Vaucheret, 1995). During a new field trial performed in the summer of 1995, we reanalyzed the timing of NR co-suppression of four transgenic lines previously shown to carry multiple copies of the transgene inserted at a single locus and to give the highest percentages of co-suppression (Palauqui and Vaucheret, 1995). Two lines (30–18.1 and 30–18.2) carry construct 30. The two other lines (34–2.5 and 27–44.7) carry constructs 34 and 27, respectively. Plants were sown directly in soil in the greenhouse or grown in vitro prior to transfer to the greenhouse and to the field (see "Materials and Methods"). Figure 2 shows for each line the percentage of plants affected by co-suppression reported as a function of the number of days of culture. As shown previously, co-suppression appeared between d 15 and flowering, affecting progressively an increasing number of plants, and was higher under "in vitro" conditions. Although the percentage of plants affected by co-suppression was slightly higher than that observed in the summer of 1994 (Palauqui and Vaucheret, 1995), probably due to the exceptionally high temperature and light levels during the summer of 1995 in France, none of the four single homozygous transgenic lines showed 100% of plants affected by co-suppression, even under in vitro conditions.

Transgenic Tobacco Plants Carrying the 35S-Nii1 cDNA Construct

Among the seven transformants showing co-suppression of *Nii* host genes and *35S-Nii1* transgenes, four carried multiple copies of the *35S-Nii1* cDNA construct inserted at a single locus (data not shown). Homozygous lines were derived from these plants. The timing of NiR co-suppression of these four transgenic lines was analyzed during the same field trial in the summer of 1995. Plants were sown directly in soil in the greenhouse or grown in vitro prior to transfer to the greenhouse or the field. Figure 2 shows for each line the percentage of plants affected by co-suppression reported as a function of the number of days of culture. Co-suppression of NiR was triggered during a large phenocritical period that extended from d 15 to flowering and progressively affected an increasing number of plants. The percentage of plants affected by co-suppression reached 100% in three lines (2.1, 9.1, and 16.2), irrespective of the conditions of growth. In the remaining line (11.5), the percentage of plants affected by co-suppression reached 100% under in vitro conditions only, whereas it remained lower under "greenhouse" conditions, as found in the case of plants carrying the *35S-Nia2* cDNA construct.

Transgenic Tobacco Plants Carrying the 70-Nii1 Gene Construct

Among the four transformants showing co-suppression of *Nii* host genes and *70enh-Nii1* transgenes, two carried multiple copies of the transgene construct inserted at a single locus (data not shown). Homozygous lines were derived from these plants. The timing of NiR co-

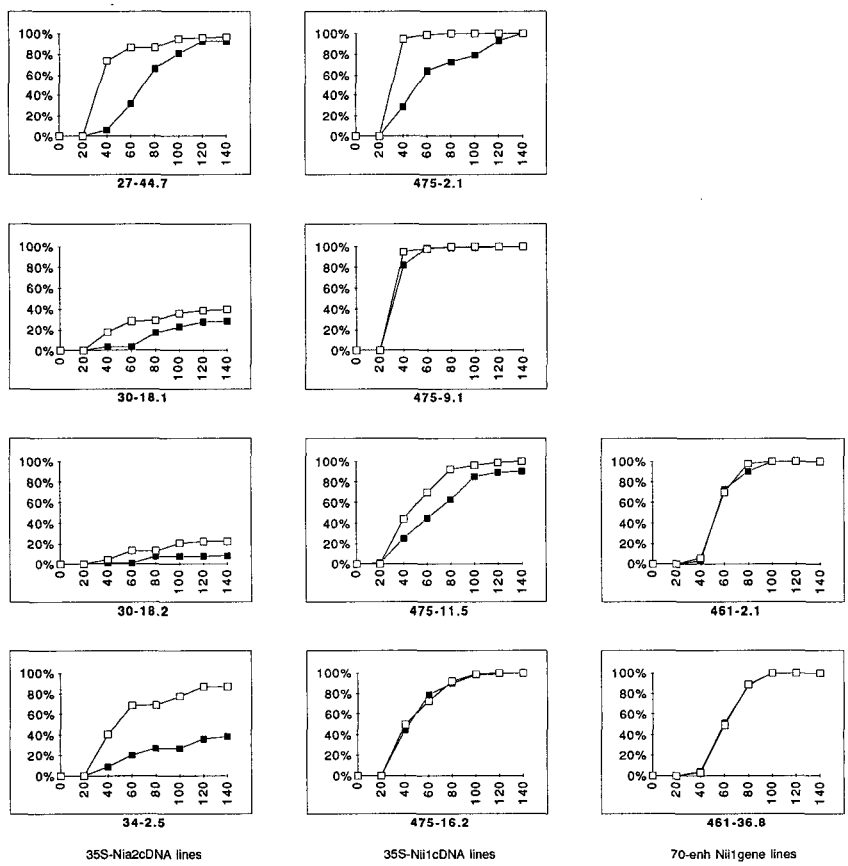

Figure 2. Time course of the appearance of co-suppression during a field trial. Plants were grown under greenhouse/field (■) or in vitro/greenhouse/field (□) conditions (see "Materials and Methods"). The number of plants becoming chlorotic was determined every week from germination to flowering. One hundred twenty plants per transgenic line were analyzed. The percentage of chlorotic plants is reported as a function of the number of days of culture. Plants remaining partially chlorotic at the end of the culture period were counted as co-suppressed.

suppression of these two transgenic lines (461–2.1 and 461–36.8) was analyzed during the same field trial in the summer of 1995. Plants were sown directly in soil in the greenhouse or grown in vitro prior to transfer to the greenhouse and to the field. Figure 2 shows for each line the percentage of plants affected by co-suppression reported as a function of the number of days of culture. As found previously in plant 461–2.1, co-suppression appeared during a very short time between d 25 and 40, and reached 100% in the two lines, irrespective of the conditions of growth.

Spatial Patterns of Co-Suppression

Transgenic Tobacco Plants Carrying the 35S-Nia2 cDNA Construct

A deficiency in NR activity leads to a loss of chloroplast function and to leaf chlorosis, which is a cell-autonomous process (Gabard et al., 1987; Saux et al., 1987). This allowed us to determine that *Nia* co-suppression was triggered in a single leaf and subsequently propagated to the rest of the plant. Chlorosis first appeared on a single leaf situated at the bottom of the plant, irrespective of the transgenic lines analyzed. Chlorotic tissues were in the form of interveinal yellow spots (Fig. 3a) or a vein-localized yellow area (Fig. 3b). Northern blot analysis of these tissues confirmed that they were being co-suppressed, since no *Nia* mRNAs were detected, whereas surrounding green tissues accumulated *Nia* mRNAs (data not shown). Although the simultaneous appearance of interveinal chlorotic spots on different leaves was observed rarely, we never detected the simultaneous appearance of vein-localized yellow areas on different leaves among thousands of plants grown during successive field trials. We also noticed that the first leaf showing co-suppression was not determined by its relative position to the sun or within the field, thus suggesting a completely stochastic triggering.

From the first chlorotic symptom, co-suppression seemed to spread progressively to the rest of the plant. Depending on the stage of development of the plant, the kinetics of propagation was slightly different. When co-suppression was initiated at an early stage of development (between d 20 and 40), chlorosis propagated quickly to the whole plant within 4 to 5 d. When it occurred later (i.e. during the 2nd or 3rd month of the phenocritical period), co-suppression propagated progressively more and more slowly, and transient intermediate states could be observed. After being triggered as a vein-localized yellow area on a leaf at the bottom of the plant, chlorosis spread progressively from the veinal zone to the interveinal zone until the whole leaf was affected. Simultaneously, chlorosis affected the top leaves in a particular way; the leaves located directly above and on the same side of the plant as the first affected leaf were first touched by chlorosis and only later were the leaves on the other side affected. At an intermediate, transient stage of this process, the leaves at the border of the affected and unaffected sides had a chlorotic half on the side of the first affected leaf and a fully green half on the opposite side (Fig. 3c). Finally, plants became completely chlorotic within 10 to 25 d (Fig. 3d), at which time none of the leaves contained *Nia* mRNAs.

Conversely, when co-suppression was triggered at the very end of the phenocritical period (just before flowering), it did not propagate to the entire plant, and the plant therefore became variegated. Indeed, after blooming, plants remained in an intermediate state, with chlorotic and green leaves delimited by a vertical plane (Fig. 3e). Northern blot analysis of such a plant revealed that none of the chlorotic areas contained *Nia* mRNAs, whereas all of the green areas, even those on the same leaf, accumulated *Nia* mRNAs (Fig. 4A). The accumulation of *Nii* mRNAs was not impaired in the chlorotic areas (Fig. 4A). Indeed, an increase in *Nii* mRNA accumulation was observed in these chlorotic areas due to the lack of transcriptional repression of *Nii* host genes by reduced nitrogen metabolites as described previously (Kronenberger et al., 1993). Therefore, these non-clonal, spatial patterns of silencing are (trans)gene-specific and do not result from metabolic control of the nitrate assimilation pathway. This particular way of spreading of co-suppression within the plants and these spatial patterns were observed in all transgenic lines showing *Nia* co-suppression irrespective of the constructs used, the combination of loci, or the allelic state of the trangene (Palauqui and Vaucheret, 1995).

Transgenic Tobacco Plants Carrying the 35S-Nii1 cDNA Construct

As was found in transgenic plants carrying the *35S-Nia2* cDNA construct, the first chlorotic symptoms corresponded either to interveinal yellow spots on one or, rarely, two leaves (Fig. 3f), or a vein-localized yellow area on a single leaf (Fig. 3g). These chlorotic areas did not contain *Nii* mRNAs, whereas surrounding green tissues accumulated them (data not shown). Once again, the position of the leaf on which co-suppression was triggered was not dependent on the orientation of the plant within the field. When it occurred at a very early stage of development (during the 1st month of the phenocritical period), chlorosis appeared on a lower leaf of the plants in the same way as was described for *Nia* co-suppression, and then propagated to the whole plant (not shown). When it occurred at a later stage of development, the initiation site and the pattern of transmission differed from *Nia* co-suppression. Indeed, chlorosis appeared first on a medium leaf situated approximately five to seven leaves from the top of the plant, and then affected all of the upper leaves irrespective of their position relative to the first chlorotic leaf. Finally, all of the leaves situated above the medium initial leaf were chlorotic, whereas lower leaves remained green, delimiting a horizontal plane with distinct patterns from one side to the other (Fig. 3h). None of the chlorotic areas contained *Nii* mRNAs, whereas all of the green areas accumulated them (Fig. 4B). The accumulation of *Nia* mRNAs was not impaired in the chlorotic areas (Fig. 4B); in fact, an increase was observed due to the lack of transcriptional repression of *Nia* host genes by reduced nitrogen metabolites described previously (Vaucheret et al., 1992). These results confirm that these spatial patterns of silenc-

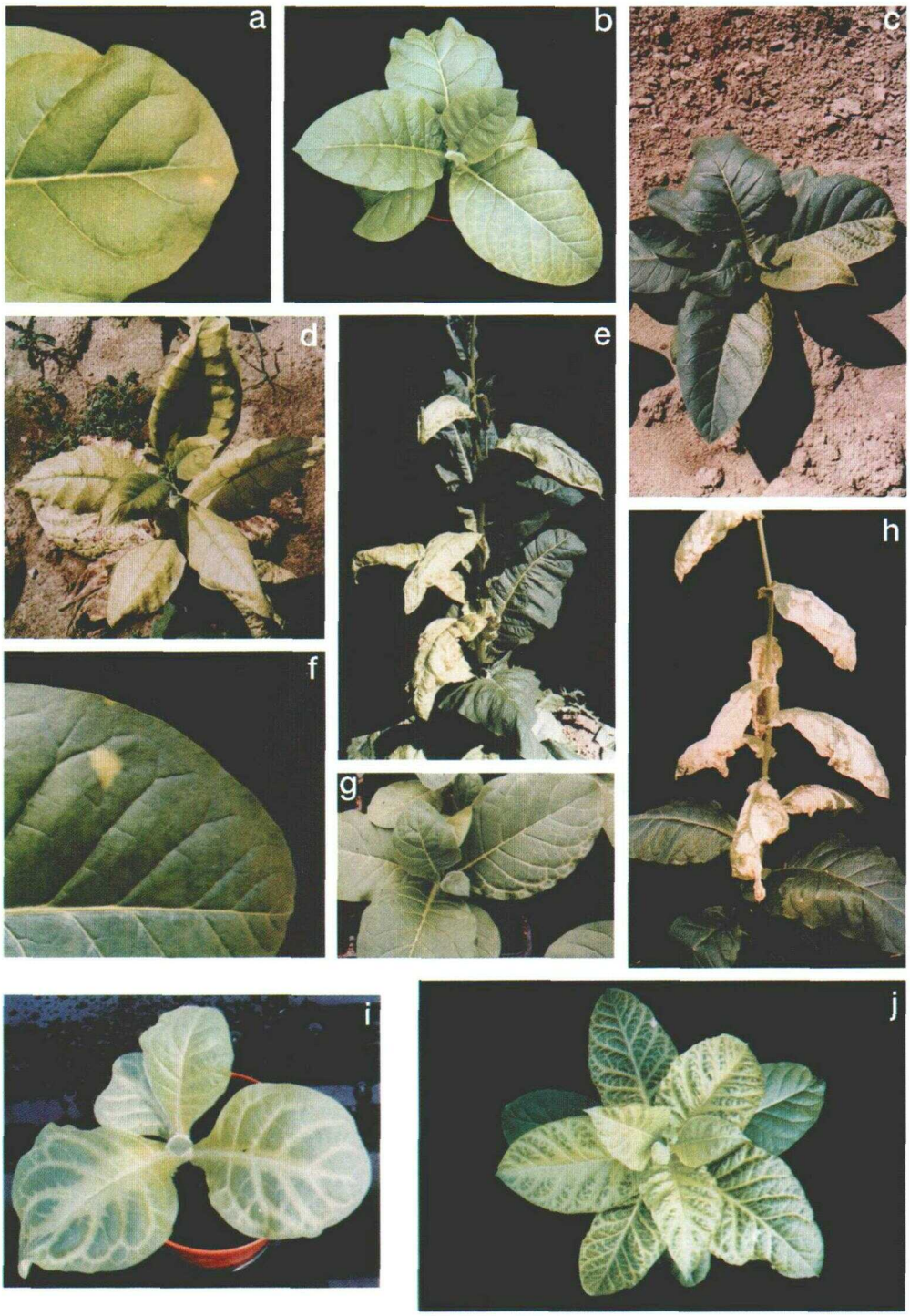

Figure 3. Spatial patterns of co-suppression at different stages of development. Representative patterns are shown for transgenic lines carrying either the *35S-Nia2* cDNA (a–e), *35S-Nii1* cDNA (f–h), or *70enh-Nii1* gene constructs (i and j). a, Early triggering of *Nia* co-suppression. Interveinal chlorotic spot on a green leaf. b, Early triggering of *Nia* co-suppression. Vein-localized chlorotic leaf. Surrounding leaves are fully green. c, Intermediate stage of *Nia* co-suppression. The plant shows fully green, fully chlorotic, and half-chlorotic, half-green leaves. d, Completed stage of *Nia* co-suppression. The plant is fully chlorotic. e, Late stage of *Nia* co-suppression. Variegated plants consist of chlorotic leaves and green leaves separated by a vertical plane going through the stem. f, Early triggering of *Nii* co-suppression (*35S-Nii1*). Interveinal chlorotic spot on a green leaf. g, Early triggering of *Nii* co-suppression (*35S-Nii1*). Vein-localized chlorosis. Surrounding leaves are fully green. h, Late stage of *Nii* co-suppression (*35S-Nii1*). Variegated plants consist of chlorotic leaves and green leaves separated by a horizontal plane perpendicular to the stem. i, Early triggering of *Nii* co-suppression (*70enh-Nii1*). Vein-localized chlorosis. j, Late stage of *Nii* co-suppression (*70enh-Nii1*). Variegated plants consist of chlorotic leaves and green leaves separated by a horizontal plane perpendicular to the stem.

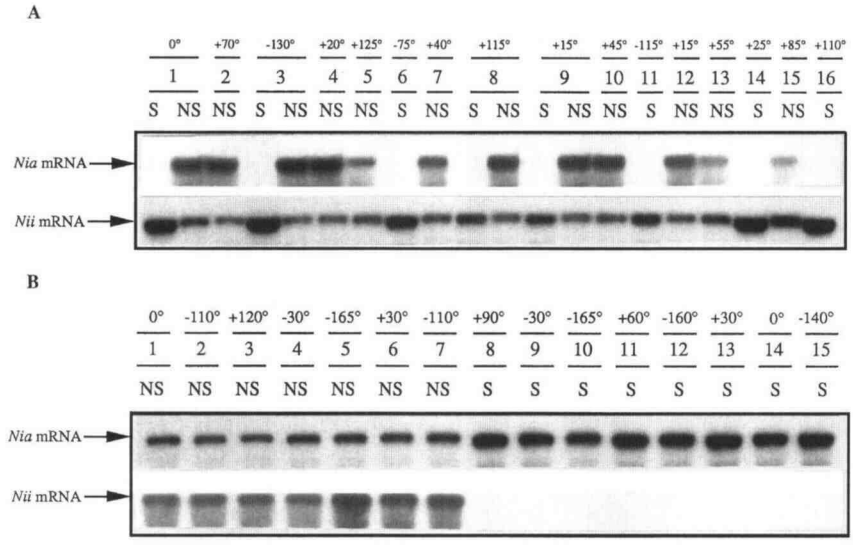

Figure 4. Expression of *Nia* or *Nii* mRNAs in green and chlorotic parts of variegated plants. Total RNA was extracted from all of the leaves of variegated plants. The numbering (1–16) refers to the position of the leaf from the bottom (1) to the top (16). The angle (from −180° to +180°) refers to the position of each leaf relative to the stem and to the bottom leaf (angle = 0 as reference). NS, Nonsuppressed green tissues; S, suppressed chlorotic tissues. When the leaf was uniform, one sample was analyzed. When the leaf was variegated, both green (NS) and chlorotic (S) parts were analyzed. Ten milligrams of RNA from each sample was probed with the tobacco *Nia2* or *Nii1* cDNA. A, Analysis of a variegated plant carrying the *35S-Nia2* cDNA; B, analysis of a variegated plant carrying the *35S-Nii1* cDNA.

ing are (trans)gene-specific and do not result from metabolic control of the nitrate assimilation pathway. This particular way of spreading co-suppression within the plants and these spatial patterns were observed in all transgenic lines carrying the *35S-Nii1* cDNA construct and showing *Nii* co-suppression.

Transgenic Tobacco Plants Carrying the 70enh-Nii1 Gene Construct

Due to the particular timing of co-suppression of the two transgenic lines 461–2.1 and 461–36.8 (Fig. 2), only early triggering events were observed. Surprisingly, interveinal chlorotic spots were never observed and chlorosis was always vein-localized (Fig. 3i). The plants became completely chlorotic within 5 to 15 d. To analyze late events of triggering we grew hybrid plants resulting either from a cross between line 461–2.1 and another homozygous transgenic line (461–7.8, which never shows co-suppression irrespective of the conditions of growth), or from the self-fertilization of one co-suppressed primary transformant (461–8) carrying at least three unlinked transgene loci (Vaucheret et al., 1995). When triggered early in the development, chlorosis appeared randomly on a single leaf at the bottom of the plants and was always vein-localized. Then, the plants became fully chlorotic. When triggered at later stages of development, chlorosis appeared first on a medium leaf situated approximately five to seven leaves from the top of the plant. The area of interveinal chlorosis increased progressively from the initial affected leaf to the upper leaves. Finally, the upper leaves became chlorotic irrespective of their orientation with regard to the first chlorotic leaf, whereas the leaves situated below remained green, thus delimiting a horizontal plane (Fig. 3j).

DISCUSSION

We report a comparative study of the frequencies, timing, and spatial patterns of co-suppression in tobacco plants carrying three distinct transgene constructs. Two constructs consist of cDNAs encoding NR and NiR, respectively, cloned between the 35S promoter and the natural termination sequences of their corresponding gene (*Nia2* and *Nii1*, respectively). These two constructs allow the constitutive overexpression of NR or NiR activity (Vincentz and Caboche, 1991; P. Crété, M. Caboche, C. Meyer, unpublished data). The third construct consists of the native *Nii1* gene with its regulatory signals (promoter, introns, and terminator) cloned downstream of two copies of the enhancer of the 35S promoter (Vaucheret et al., 1995). This construct allows the overexpression of NiR in a regulated manner, i.e. the accumulation of *Nii* mRNAs in transgenic plants carrying this construct is induced by nitrate and light, as is the accumulation of *Nii* mRNAs in wild-type plants (data not shown). The comparative analysis of co-suppression in plants carrying these three constructs allows us to discriminate between specific effects of the gene under study (common to plants carrying the *35S-Nii1* cDNA and *70enh-Nii1* gene constructs, and different for plants carrying the *35S-Nia2* cDNA construct) and specific effects of the mode of deregulation (common to plants carrying the *35S-Nia2* cDNA and *35S-Nii1* cDNA constructs, and different for plants carrying the *70enh-Nii1* gene construct).

The frequency of transformants showing co-suppression, either directly as primary transformants or as homozygous individuals of their self-progeny, was not significantly different between the three constructs, and ranged from 19 to 25%. This result suggests that *Nia* and *Nii* genes are similarly susceptible to co-suppression in transgenic tobacco plants, and that a constitutive or a regulated overexpression of the *Nii* transgene does not greatly influence the frequency of co-suppressed transformants. Conversely, we have found that the percentage of plants affected by co-suppression was clearly different from one transgenic line to another and between NR and NiR co-suppresssion. Indeed, none of the transgenic lines homozygous for a single locus carrying the *35S-Nia2* cDNA construct showed 100% co-suppression, whereas two transgenic lines homozygous for two unlinked loci showed 100% co-suppression (Palauqui and Vaucheret, 1995). Conversely, all of the transgenic lines homozygous for a single locus carrying the *35S-Nii1* cDNA or *70enh-Nii1* gene constructs showed 100% co-suppression. These results suggest that a higher dosage of transgene loci is required for an efficient triggering of NR co-suppression than is required for the triggering of NiR co-suppression.

The timing of co-suppression (i.e. the percentage of plants affected by co-suppression reported as a function of the number of days of culture) also varies from one transgenic line to another. Depending on the line considered, co-suppression can be triggered stochastically during a long phenocritical period extending from d 15 after germination to flowering, as is also reported for chitinase and SAM synthetase co-suppression (Hart et al., 1992; Boerjan et al., 1994), or it can be triggered during a shorter phenocritical period, as reported for glucanase co-suppression (de Carvalho et al., 1992; de Carvalho-Niebel et al., 1995). In addition, the timing of co-suppression in some lines is dramatically affected by the conditions under which plants are grown (Palauqui and Vaucheret, 1995; this work), a result also reported for chitinase co-suppression (Hart et al., 1992), thus confirming that environmental parameters are important for silencing.

Finally, we have analyzed the spatial patterns of *Nia* and *Nii* co-suppression in transgenic plants carrying each of the three constructs (*35S-Nia2* cDNA, *35S-Nii1* cDNA, and *70enh-Nii1* gene) and found that these patterns are not clonal, but result from the propagation of co-suppression within the plants in a particular manner. We took advantage of the fact that a deficiency in nitrate assimilation leads to a visible and cell-autonomous phenotype, chlorosis, which results from a disorganization of the chloroplasts (Saux et al., 1987). This phenotype is visible in the leaves at any stage of the development of the plant. We observed some common points but also clear differences between the three constructs. Chlorosis always appeared randomly on a single leaf, and this phenomenon was correlated with the disappearance of mRNA from the host genes and from transgenes. Chlorosis appeared as interveinal spots or vein-localized areas in *35S-Nia2* and *35S-Nii1* cDNA constructs, whereas it always appeared as a vein-localized area in the *70enh-Nii1* gene construct, suggesting some influence of the transgene regulation in the process of triggering. Chlorosis (and co-suppression) then propagated to higher parts of the plant, irrespective of the construct considered. However, the geometrical distribution of suppressed and nonsuppressed leaves in variegated plants differed between *Nia* and *Nii* constructs, but not from one transgenic line to another. *Nia* co-suppression started on a leaf situated at the bottom of the plant, invaded the other tissues of the leaf, and then spread to the upper leaves, primarily those on the same side of the plant. Conversely, *Nii* co-suppression started on one leaf situated five to seven leaves below the top of the plant and propagated to all of the upper leaves, whereas the leaves situated below remained unaffected. When it was triggered late, co-suppression did not propagate to the whole plant, and the plants remained variegated. Chlorotic and nonchlorotic tissues were separated by a vertical plane in *Nia* co-suppression and by a horizontal plane in *Nii* co-suppression. A schematic representation of the different final stages of co-suppression is shown in Figure 5.

The spatial patterns of *Nii* co-suppression resemble those reported in the case of SAM synthase silencing (Boerjan et al., 1994), whereas the spatial patterns of *Nia* co-suppression show some similarities but also some differences from those resulting from chitinase silencing (Hart et al., 1992; F. Meins, personal communication). These results suggest that different genes that do not belong to the same metabolic pathway (for example, genes encoding NiR and SAM synthetase) can exhibit similar spatial patterns of co-suppression. Conversely, two genes that belong to the

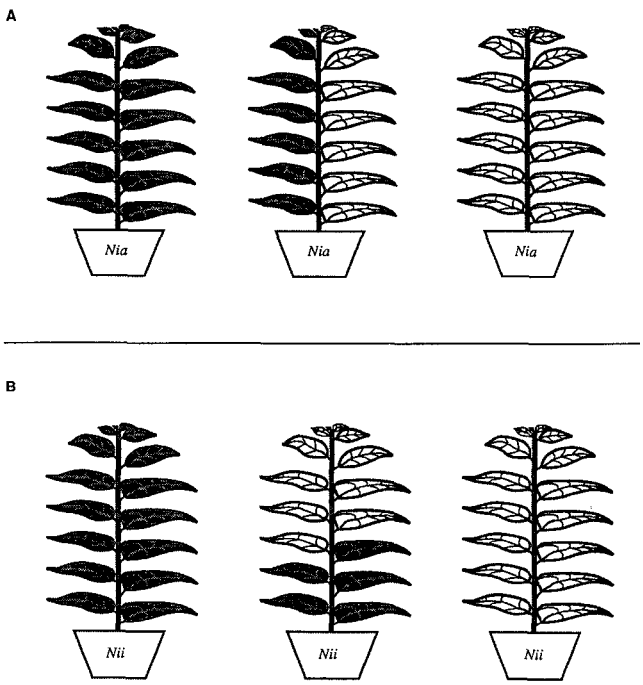

Figure 5. Schematic representation of spatial patterns of *Nia* and *Nii* co-suppresssion. The different spatial patterns observed at the end of the culture are represented. Plants may be either completely co-suppressed, completely normal, or variegated. Green leaves are represented in black. Chlorotic leaves are represented in white.

same metabolic pathway (NR and NiR) and that are naturally co-regulated at the transcriptional level (Faure et al., 1991; Vincentz et al., 1993) can exhibit distinct spatial patterns of co-suppression. In addition, the mode of deregulation of gene expression, i.e. constitutive (*35S-Nii1* cDNA construct) or regulated (*70enh-nii1* gene construct) overexpression, does not seem to influence the spatial patterns of *Nii* co-suppression. A similar result was observed for the co-suppression of chalcone synthase in transgenic petunia plants carrying either a *35S*-cDNA construct or a genomic clone with its own regulatory signals (Van der Krol et al., 1990).

Because co-suppression occurs similarly with constitutive or regulated transgenes (Van der Krol et al, 1990; this work), and because it occurs at a post-transcriptional level (Van Blockland et al, 1994; de Carvalho-Niebel et al, 1995), one might assume that the transcriptional regulation of host genes and transgenes plays a minor role in the control of co-suppression. Therefore, the distinct spatial patterns of co-suppression observed with different genes may reflect specific posttranscriptional regulations occurring at the RNA or protein level or may reflect specific metabolic controls. A heuristic model involving autoregulation by the host gene and transgene products has been invoked to account for the specific aspects of chitinase silencing (Meins and Kuncz, 1994). Similarly, Boerjan et al. (1994) proposed that high levels of gene expression trigger SAM synthase silencing, and that silenced cells act as a sink for molecules involved in the negative regulation of the endogenous gene. Since the spatial patterns of *Nia* and *Nii* co-suppression are not clonal and since neither *Nia* nor *Nii* co-suppression results from metabolic control of the nitrate assimilation pathway, one cannot exclude the possibility that molecules involved in the control of silencing diffuse through the plant in a specific manner, which may explain why each gene exhibits a different pattern of co-suppression.

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LITERATURE CITED

Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res **12:** 8711–8712

Boerjan W, Bauw G, Van Montagu M, Inze D (1994) Distinct phenotypes generated by overexpression and suppression of S-adenosyl-L-methionine synthetase reveal developmental patterns of gene silencing in tobacco. Plant Cell **6:** 1401–1414

de Carvalho F, Gheysen G, Kushnir S, Van Montagu M, Inzé D, Castresana C (1992) Suppression of beta-1,3-glucanase transgene expression in homozygous plants. EMBO J **11:** 2595–2602

de Carvalho-Niebel F, Frendo P, Van Montagu M, Cornelissen M (1995) Post-transcriptional co-suppression of beta-1,3-glucanase genes does not affect accumulation of transgene nuclear mRNA. Plant Cell **7:** 347–358

Dehio C, Schell J (1994) Identification of plant genetic loci involved in a posttranscriptional mechanism for meiotically reversible transgene silencing. Proc Natl Acad Sci USA **91:** 5538–5542

Dorlhac de Borne F, Vincentz M, Chupeau Y, Vaucheret H (1994) Co-suppression of nitrate reductase host genes and transgenes in transgenic tobacco plants. Mol Gen Genet **243:** 613–621

Dougherty WG, Parks TD (1995) Transgenes and gene suppression: telling us something new? Current Opinion in Cell Biology **7:** 399–405

Elmayan T, Vaucheret H (1995) Expression of single copies of a strongly-expressed 35S-transgene can be silenced post-transcriptionally. Plant J **9:** 787–797

Faure J-D, Vincentz M, Kronenberger J, Caboche M (1991) Co-regulated expression of nitrate and nitrite reductases. Plant J **1:** 107–113

Flavell RB (1994) Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proc Natl Acad Sci USA **91:** 3490–3496

Gabard J, Marion-Poll A, Chérel I, Meyer C, Müller AJ, Caboche M (1987) Isolation and characterization of *Nicotiana plumbaginifolia* nitrate reductase-deficient mutants: genetic and biochemical analysis of the NIA complementation group. Mol Gen Genet **209:** 596–606

Gritz L, Davies J (1983) Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. Gene **25:** 179–188

Hart CM, Fisher B, Neuhaus J-M, Meins F (1992) Regulated inactivation of homologous gene expression in transgenic *Nicotiana sylvestris* plants containing a defense-related tobacco chitinase gene. Mol Gen Genet **235:** 179–188

Ingelbrecht I, Van Houdt H, Van Montagu M, Depicker A (1994) Posttranscriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. Proc Natl Acad Sci USA **91:** 10502–10506

Jorgensen R (1990) Altered gene expression in plants due to *trans* interactions between homologous genes. Trends Biotechnol **8:** 340–344

Kronenberger J, Lépingle A, Caboche M, Vaucheret H (1993) Cloning and expression of distinct nitrite reductases in tobacco leaves and roots. Mol Gen Genet **236:** 203–208

Linn F, Heidmann I, Saedler H, Meyer P (1990) Epigenetic changes in the expression of the maize A1 gene in *Petunia hybrida*: role of the numbers of integrated gene copies and state of methylation. Mol Gen Genet **222:** 329–336

Matzke MA, Matzke AJM (1995) How and why do plants inactivate homologous (trans)genes? Plant Physiol **107:** 679–685

Matzke MA, Neuhuber F, Matzke AJM (1993) A variety of epistatic interactions can occur between partially homologous transgene loci brought together by sexual crossing. Mol Gen Genet **236:** 379–386

Matzke MA, Primig M, Trnovsky J, Matzke AJM (1989) Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants. EMBO J **8:** 643–649

Meins F Jr (1989) A biochemical switch model for cell-heritable variation in cytokinin requirement. *In* R Goldberg, ed, Molecular Basis of Plant Development. Liss, New York, pp 13–24

Meins F Jr, Kunz C (1994) Gene silencing in transgenic plants: a heuristic autoregulation model. *In* P Meyer, ed, Current Topics in Microbiology and Immunology. Springer-Verlag, Berlin, pp 105–120

Meyer P, Heidmann I, Niedenhof I (1993) Differences in DNA-methylation are associated with a paramutation phenomenon in transgenic petunia. Plant J **4:** 89–100

Palauqui J-C, Vaucheret H (1995) Field trial analysis of nitrate reductase co-suppression: a comparative study of 38 combinations of transgene loci. Plant Mol Biol **29:** 149–159

Park Y-D, Papp I, Moscone EA, Iglesias VA, Vaucheret H, Matzke MA, Matzke AJM (1996) Gene silencing mediated by

promoter homology occurs at the level of transcription and results in meiotically heritable alterations in methylation and gene activity. Plant J **9:** 183–194

Phillips RL, Matzke MA, Oono K (1995) Treasure your exceptions. Plant Cell **10:** 1522–1527

Saux C, Lemoine Y, Marion-Poll A, Valadier MH, Deng M, Morot-Gaudry J-F (1987) Consequence of absence of nitrate reductase activity on photosynthesis in *Nicotiana plumbaginifolia* plants. Plant Physiol **84:** 67–72

Van Blokland R, Vandergeest N, Mol JNM, Kooter JM (1994) Transgene-mediated suppression of chalcone synthase expression in *Petunia hybrida* results from an increase in RNA turnover. Plant J **6:** 861–877

Van der Krol AR, Mur LA, Beld M, Mol JNM, Stuitje AR (1990) Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell **2:** 291–299

Vaucheret H (1993) Identification of a general silencer for 19S and 35S promoters in a transgenic tobacco plant: 90 pb of homology in the promoter sequences are sufficient for trans-inactivation. C R Acad Sci Paris **316:** 1471–1483

Vaucheret H, Kronenberger J, Lepingle A, Vilaine F, Boutin J-P, Caboche M (1992) Inhibition of tobacco nitrite reductase activity by expression of antisense RNA. Plant J **2:** 559–569

Vaucheret H, Palauqui J-C, Elmayan T, Moffatt B (1995) Molecular and genetic analysis of nitrite reductase co-suppression in transgenic tobacco plants. Mol Gen Genet **248:** 311–317

Vincentz M, Caboche M (1991) Constitutive expression of nitrate reductase allows normal growth and development of *Nicotiana plumbaginifolia* plants. EMBO J **10:** 1027–1035

Vincentz M, Moureaux T, Leydecker M-T, Vaucheret H, Caboche M (1993) Regulation of nitrate and nitrite reductase expression in *Nicotiana plumbaginifolia* leaves by nitrogen and carbon metabolites. Plant J **3:** 315–324