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Agrobacterium tumefaciens AK-*6b* gene modulates phenolic compound metabolism in tobacco

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Abstract

The *6b* gene (AK-*6b*) of *Agrobacterium tumefaciens* AKE10 can substitute for the requirement of tobacco tissues for auxin and cytokinin to maintain callus growth in the culture medium. To identify compounds that might be involved in this process we analyzed phenolic metabolites in transgenic tobacco tissues expressing the AK-*6b* gene. On medium containing both cytokinin and auxin (SH medium), transgenic calli accumulated higher levels of chlorogenic acid, caffeoyl putrescine, rutin and kaempferol-3-rutinoside, than did wild-type tissues. In contrast, the levels of scopolin and its aglycone, scopoletin were lower in transgenic tissues. On hormone-free medium, these phenolic compounds showed neither significant levels nor an apparent relationship with AK-*6b* transcript levels, except for the negatively correlated levels of scopoletin and AK-*6b* transcripts. Apparently, the AK-*6b* gene acts, in SH medium, to redirect the synthesis of scopolin in tobacco tissues towards the preferential synthesis of caffeic acid derivatives and flavonoids.

Keywords: *Nicotiana tabacum*; Solanaceae; Tobacco; Metabolism; Phenylpropanoids; Flavonoids; Chlorogenic acid; Caffeoylputrescine; Rutin; Kaempferol-3-rutinoside; Scopolin; Scopoletin

1. Introduction

The *6b* gene is an integral part of the T-DNA region of the *Agrobacterium tumefaciens* tumor inducing (Ti) plasmid. 6b has been shown to have weak tumorigenic capacity in a limited number of plant hosts, including *Nicotiana glauca* and *Kalanchoe tubiflora* (Hooykaas et al., 1988). Subsequent studies have provided accumulating evidence for the supplementary role of *6b* genes to the main T-DNA encoded oncogenes that are responsible for overproduction of cytokinin (by the *ipt* gene) and auxin (by the *tms1* and *tms2* genes), and thus for tumor initiation and maintenance (for review see Nester et al., 1984; Hamill, 1993). *6b* genes derived from a wide range of *A. tumefaciens* strains have been shown to reduce cytokinin activity (Ach5-*6b*, Spanier et al., 1989; C58-*6b*, Gális et al., 1999), to enhance both cytokinin and auxin effects (S4-*6b*, Canaday et al., 1992), and to modify the sensitivity of isolated protoplasts to phytohormones (Tm4-*6b*, Tinland et al., 1992) in a variety of plant systems.

The recently-isolated *6b* gene (AK-*6b*) from the *A. tumefaciens* strain AKE10 (Wabiko and Minemura, 1996) shows 52–55% amino acid sequence identity to other *6b* genes, such as C58-, T37-, Ach5- and Tm4-*6b*. The tumorigenic feature of the AK-*6b* gene is particularly pronounced, being capable of inducing shooty callus growth of tobacco tissues in the absence of exogenous plant hormones (Wabiko and Minemura, 1996). However, despite its cytokinin-like effects, the AK-*6b* gene does not increase cytokinin (zeatin and zeatin riboside) contents in hormone-autonomous tissues (Wabiko and Minemura, 1996). On the other hand, the C58-*6b* gene derived from *A. tumefaciens* strain C58 has been shown to modulate the sensitivity of tobacco seedlings to an exogenously-applied cytokinin, N-6 benzyladenine, again relating the *6b* gene to cytokinin action but not to its metabolism (Gális et al., 1999). These observations suggest an indirect effect of the *6b* gene on cytokinin action via unknown intermediates. Alternatively, interactions with a transcription factor-like gene (Kitakura et al., 2002) or with the genes involved in cytokinin signaling (Inoue et al., 2001; Hwang and Sheen, 2001) may be affected.

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As some plant phenolics have been shown to affect hormone action in vitro (Lee et al., 1982; Jacobs and Rubery, 1988; Teutonico et al., 1991; Wang and Letham, 1995) and in vivo (Mathesius et al., 1998; Murphy et al., 2000), a detailed analysis of phenolic metabolism in AK-*6b* transformed tobacco tissues was performed to identify compounds that could modulate plant hormonal metabolism and/or action. As a result, several well-pronounced changes in phenolic metabolism were observed in the AK-*6b* transformed tissues.

Table 1
Phenotypic distribution of leaf discs transformed and selected on SH medium containing kanamycin[a]

Clones (Genotype)	Phenotypes	
	Shooty	Compact callus
pGANE7 (own promoter/AK-*6b*)	3	>18
pBAK61 (CaMV35S/AK-*6b*)	16	>20

[a] Number of leaf discs showing individual phenotypes are presented. Approximately 100 leaf discs were transformed by each clone.

2. Results

2.1. Growth and AK-6b gene expression of transformed tobacco calli

Tobacco leaf discs on SH medium (containing auxin and cytokinin) were transformed and regenerants were selected for kanamycin resistance, to generate transgenic and control lines: the AK-*6b* gene under the control of the CaMV-35S promoter (35S-Ca lines; from the vector pBAK61, Table 1), the AK-*6b* gene under the control of its own promoter (NE7-Ca lines; from the pGANE7 construct, Table 1), and the luciferase-containing controls without the AK-*6b* gene (TOK lines; from the pTOK119 construct). The TOK line calli proliferated quickly on kanamycin-containing SH media and showed a characteristic fragile appearance with frequent shoot formation (TOK12/9 callus as a representative example shown in Fig. 1, upper panel), as did the untransformed calli on kanamycin-free SH medium. Subsequent maturation of TOK shoots on hormone-free (HF) medium were indistinguishable from wild-type. In contrast, tissues transformed by the AK-*6b* clones showed morphologically distinct phenotypes on SH medium (Table 1). In lines 35S-Ca/1, 35S-Ca/2, and 35S-Ca/3, shoot formation occurred occasionally within tissues (Shooty phenotype, Table 1) that appeared similar to the TOK12/9 or untransformed lines (Fig. 1, upper panel). The other phenotype was slow-growing, with mostly compact morphology (compact callus, Table 1), and displayed a strong suppression of shoot formation irrespective of the promoter used (native or CaMV-35S) (lines NE7-Ca/1 and 35S-Ca/4 1, Fig. 1, upper panel).

In the absence of hormones (HF medium), callus lines 35S-Ca/3, NE7-Ca/1 and 35S-Ca/4 displayed a number of shoot-like protrusions that rarely developed into normal shoots. Furthermore, lines NE7-Ca/1 and 35S-Ca/4 grew much more rapidly than the other lines on HF medium. Conversely, lines 35S-Ca/1 and 35S-Ca/2 formed normal shoots after several passages through fresh HF medium (Fig. 1, lower panel), and these shoots grew to maturity, with only slight morphological alterations, e.g. inverse epinasty (data not shown). These two lines had to be continuously cultivated in the presence of plant hormones to maintain callus growth.

Northern blot analysis was performed to determine the accumulation of AK-*6b* transcripts in the morphologically divergent transgenic lines. On SH medium, high levels of AK-*6b* transcripts were found in compact tissues of lines 35S-Ca/4 and NE7-Ca/1, while low levels or almost no transcripts were detected in the shooty, fast-growing 35S-Ca/1 through 35S-Ca/3 calli, and no transcripts were detectable in TOK12/9 or wild-type tissues grown on the same medium (Fig. 2A, left panel). Similarly, on HF medium, transcript levels were high in

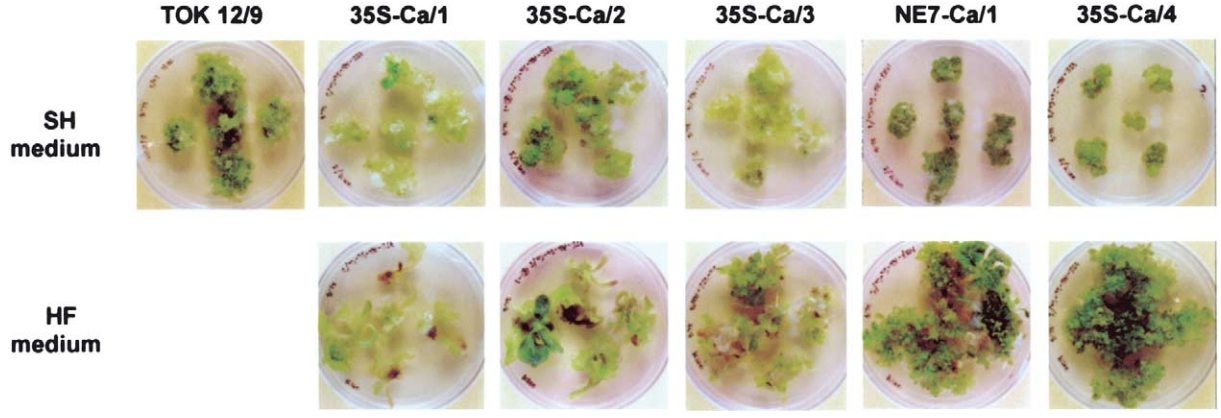

Fig. 1. Morphology of wild-type and transgenic tobacco calli. Callus tissues were cultivated on shooty (SH, upper panel) or hormone free (HF, lower panel) media for 3 weeks.

35S-Ca/4 and NE7-Ca/1, moderate in 35S-Ca/3, and almost undetectable in 35S-Ca/1 and 35S-Ca/2 (Fig. 2A, right panel). To assure detection of low levels of AK-*6b* transcripts in lines 35S-Ca/1 through 35S-Ca/3 calli, we employed RT-PCR analysis. Complementary DNA was synthesized from poly(A)$^+$ RNA which had been prepared from tissues cultivated on SH medium, and the resulting cDNA was PCR-amplified using AK-*6b*-specific primers. Agarose gel electrophoresis of the amplified DNA revealed distinct DNA bands from AK-*6b*-

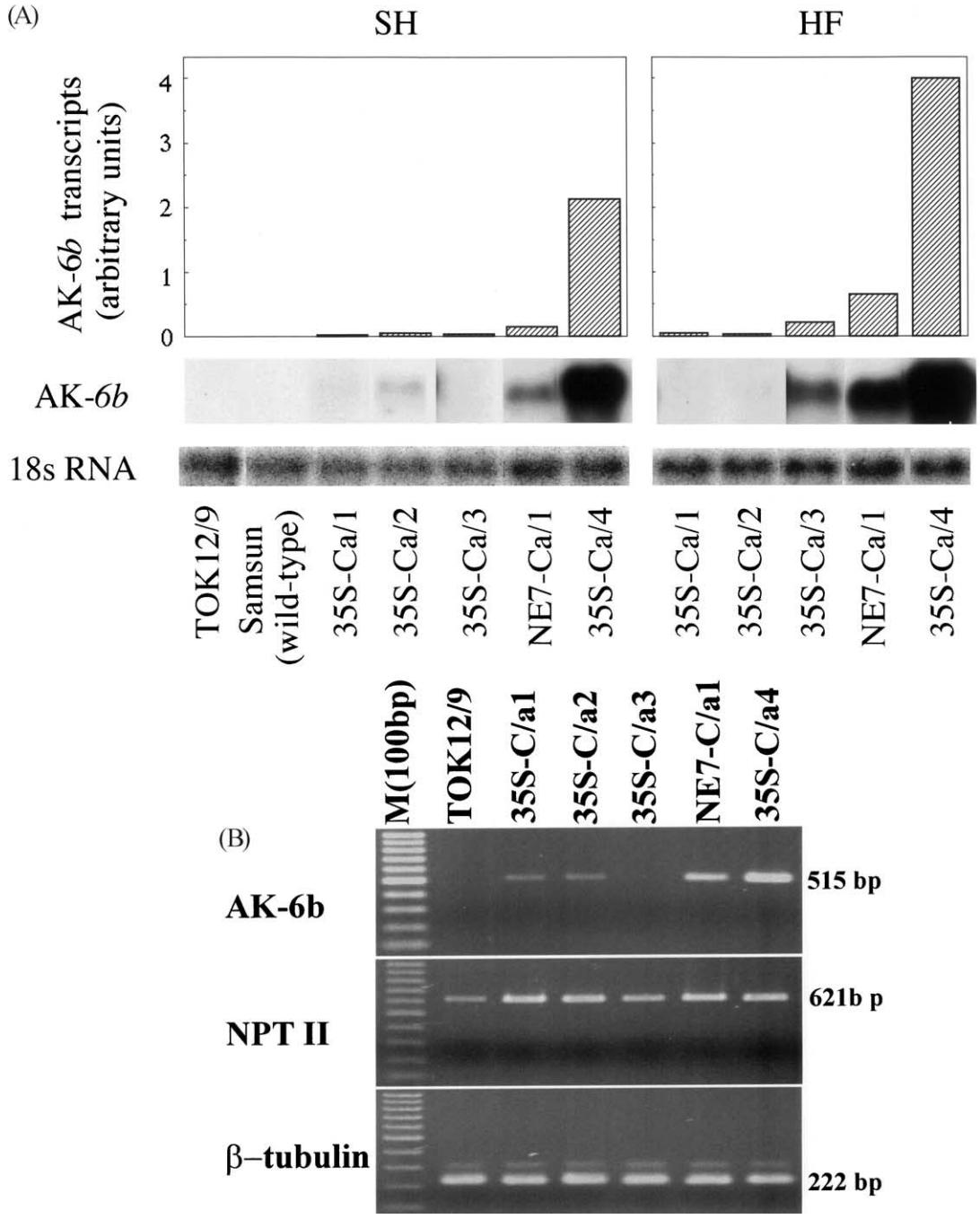

Fig. 2. Analysis of transcript accumulation in AK-*6b*-tissues. (A) Northern hybridization analysis. Accumulation of AK-*6b* transcripts. Total RNA (20 μg) was subjected to Northern hybridization using ^{32}P-labeled probes of an AK-*6b* fragment (top and middle panels), or a cDNA encoding the *Arabidopsis thaliana* 18S rRNA as loading control (bottom panel). Transcript levels, quantified by a Fuji Phosphoimager, are shown as arbitrary units in logarithmic scale (top panel), or are shown as autoradiographic exposures (Kodak X-OMAT AR5 film; middle and bottom panels). SH and HF denote SH medium and HF medium, respectively. (B) RT-PCR analysis. A poly(A)$^+$ RNA samples obtained from the AK-*6b*-tissues grown on SH medium were used for cDNA synthesis with reverse transcriptase and then subjected to PCR amplification by using AK-*6b*, NPTII (kanamycin resistance) and β-tubulin specific primers. β-Tubulin gene was employed for standardization. PCR reactions with mRNA samples prior to reverse transcription were performed similarly to exclude the contamination with genomic DNA (data not shown).

transformed tissues (Fig. 2B), but no DNA amplification was detected from RNA obtained from control TOK12/9. The extent of amplification correlated with the levels of transcripts identified by Northern blot including the clone 35S-Ca/3, in which amplified DNA was recognized only faintly (SH medium; no visible signal on Northern blot).

From these results, an inverse relationship appears to exist between AK-*6b* gene transcript accumulation and the potential of the calli to form normal shoots on either SH or HF media. This conclusion is consistent with our previous finding that AK-*6b* gene expression is required to maintain callus morphology during hormone-independent growth of transgenic tobacco tissues (Wabiko and Minemura, 1996).

2.2. *Phenolic compounds in AK-*6b *transgenic calli*

Fig. 3 depicts biosynthesis of phenolic compounds that is initiated by the activity of phenylalanine (**1**) ammonia-lyase (PAL) to generate *E*-cinnamic acid (**2**), which is further metabolized to a number of phenylpropanoids.

Visual inspection of the SH medium surrounding the wild-type and TOK12/9 tissues, after exposure of the tissues to ultraviolet light (λ 245nm), revealed that the tissues secreted large amounts of blue fluorescent substances, previously identified as plant coumarins, scopolin (**3**) and scopoletin (**4**), (Gális et al., 2002). In contrast, the AK-*6b* transgenic calli showed little fluorescence. We inferred that the differential secretion of coumarins could reflect the difference of intracellular levels of these compounds. In order to investigate the accumulation of coumarin and other phenolic compounds in more detail, the calli were subjected to RP-HPLC-based phenolic analysis. Callus tissues of an AK-*6b* transgenic (35S-Ca/4), TOK12/9 and wild-type lines, propagated on fresh SH medium, were extracted with MeOH–H_2O (4:1), the extracts were separated by HPLC, and their UV_{280} absorbance profiles were compared. As expected from the initial observation that fluorescence was absent in the AK-*6b* calli, the accumulation of scopolin (**3**) and scopoletin (**4**) was found to be strongly suppressed in the AK-*6b* line (Table 2), regardless of the various hormone combinations used to propagate the calli (data not shown).

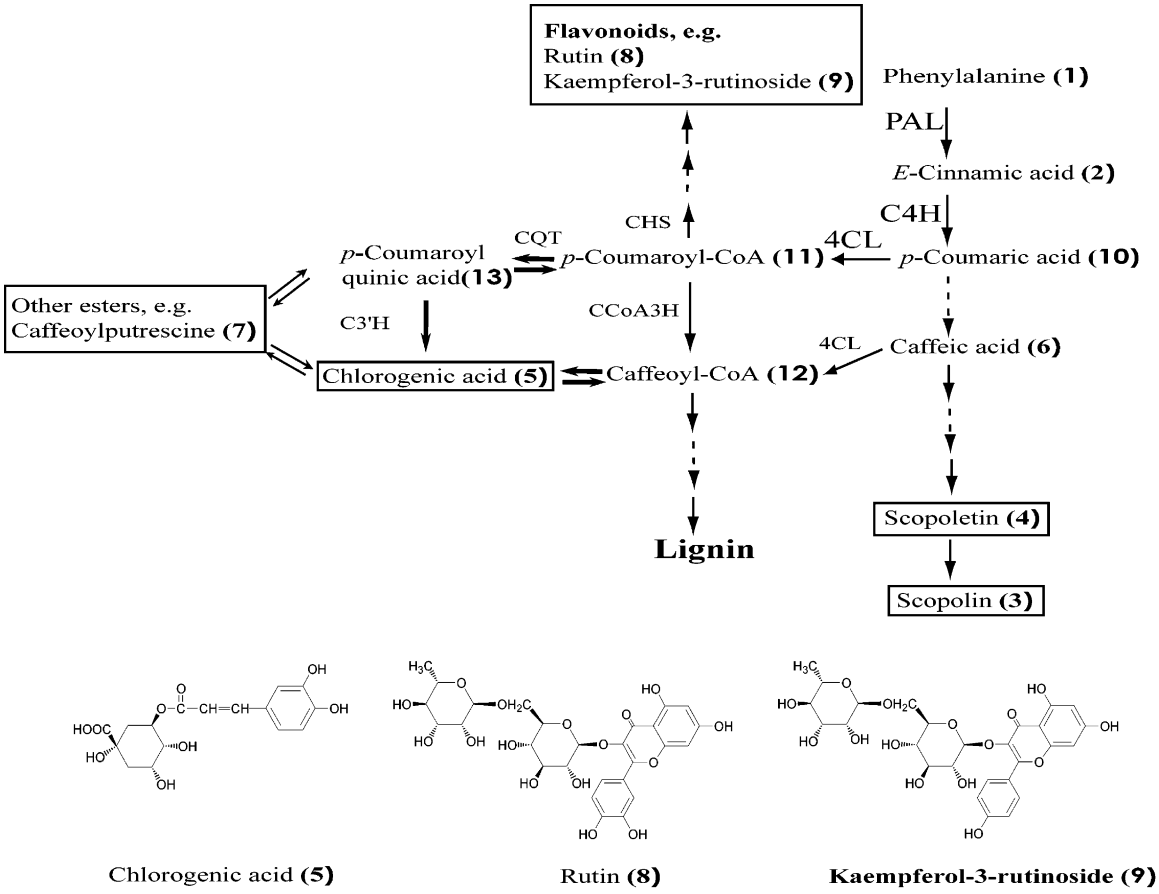

Fig. 3. Biosynthesis pathway of phenolic compounds. Pathway is according to, and modified from Steck (1967), Fritig et al. (1970), Harborne (1980) and Schoch et al. (2001). Numbers in parantheses associated with phenolic compounds are referred to in the text. Structural formula of chlorogenic acid (**5**), rutin (**8**), and kaempferol-3-rutinoside (**9**) are presented. Phenolic compounds analyzed in the present study are boxed. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase.

Chlorogenic acid (**5**) (CGA, Fig. 4, peak A) comprised the major phenolic fraction in both transgenic and wild-type tissues, with more CGA (**5**) being present in AK-*6b* transgenic compared to TOK12/9 or wild-type callus (Table 2). Another major peak (Fig. 4, peak B), with a retention time close to CGA (5), was also more abundant in transgenic calli. Furthermore, two other minor peaks (Fig. 4C and D) appeared to be specific to the AK-*6b* callus tissues. The peak fractions B, C and D possessed UV spectra that were characteristic of phenolic structures (data not shown), and so were subjected to further analysis.

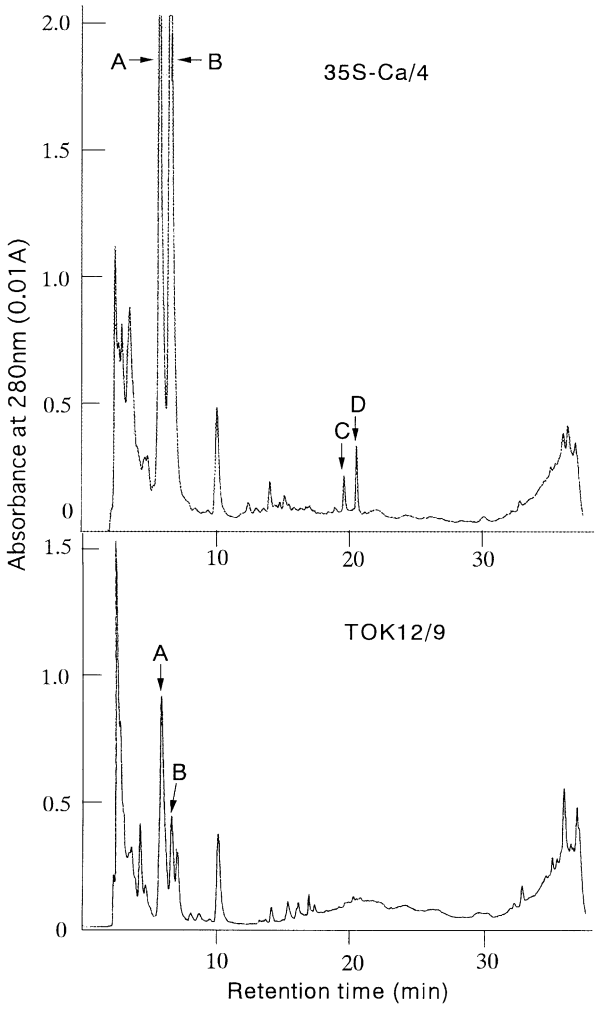

Fig. 4. HPLC chromatographic profiles of extracts from tobacco calli. Methanol-extracted samples, obtained from the transgenic callus line (35S-Ca/4, with a high level of AK-*6b* expression), and from the control callus (pTOK12/9, without the AK-*6b* gene but containing luciferase and the kanamycin resistance genes), were subjected to HPLC and subsequent detection by UV absorbance at 280 nm. Individual peaks are: A, chlorogenic acid (**5**); B, caffeoylputrescine (**7**); C, rutin (**8**); and D, kaempferol-3-rutinoside (**9**).

Peak B showed a UV absorbance spectrum very similar to CGA (**5**) and to its basic structural component, caffeic acid (**6**) (data not shown). Mass spectrometric (MS) analysis revealed that the compound has $[M+H]^+ = m/z$ 251.2 in the full scan ES spectrum, while collision induced (CID) MS^2 spectrum yielded m/z 234 which was formed by the loss of NH_3. The fragments at m/z 163 and m/z 89 are characteristic for the caffeoyl and putrescine $[amine+H]^+$ moieties, respectively (Baumert et al., 2001). Thus the compound corresponding to peak B was tentatively identified to be the conjugate of caffeic acid (**6**) and putrescine, caffeoylputrescine (**7**) (CaPu), (also see spectral data in Keinanen et al., 2001).

The UV spectral characteristics of peaks C and D were found to be similar, and MS analysis further identified these peaks as the flavonoids, rutin (**8**) and kaempferol-3-rutinoside (**9**) (K-3-R; ($[M+H]^+ = m/z$ 595.3, Fig. 5B), respectively by comparison with authentic standards. Although rutin (**8**) is reported to be a typical flavonoid constituent of tobacco plants (Elkind et al., 1990; Bate et al., 1994; Howles et al., 1996; Tamagnone et al., 1998), we were unable to identify larger amounts of rutin (**8**) in either TOK12/9 or wild-type calli (Table 2) under any of the various hormone combinations tested (data not shown).

From the data summarized in Table 2, the AK-*6b* transgenic tissue contained elevated levels of the caffeic acid derivatives, CGA (**5**) and CaPu (**7**); and the flavonoids, rutin (**8**) and K-3-R (9); and significantly reduced levels of scopolin (**3**) and scopoletin (**4**) in comparison with the wild-type tissues.

2.3. Correlation between levels of phenolics and AK-6b transcripts

To further elucidate the role of the AK-*6b* gene in phenolic metabolism in tobacco tissue cultures, we

Table 2
Levels of phenolic compounds[a] (n mol / g tissue) in wild-type and transgenic calli grown on SH medium[b]

Callus-lines	CGA (**5**)	CaPu (**7**)	SCT (**4**)	SCN (**3**)	Rutin (**8**)	K-3-R (**9**)
Wild-type	305±99	350±135	1.1±0	58.3±32.9	0.55±0.55	0.17±0.17
TOK12/9	424±180	255±91	1.3±0.2	46.5±27.3	0.55±0.55	0.52±0.3
35S-Ca/4	900±253	2170±800	1.0±0	3.7±0.21	9.5±4.1	18.9±2.9

[a] Abbreviations: CGA, chlorogenic acid (**5**); CaPu, caffeoyl putrescine (**7**); SCT, scopoletin (**4**); SCN, scopolin (**3**); K-3-R, kaempferol-3-rutinoside (**9**)

[b] Three independent experiments were performed with single calli. ± represents standard deviation.

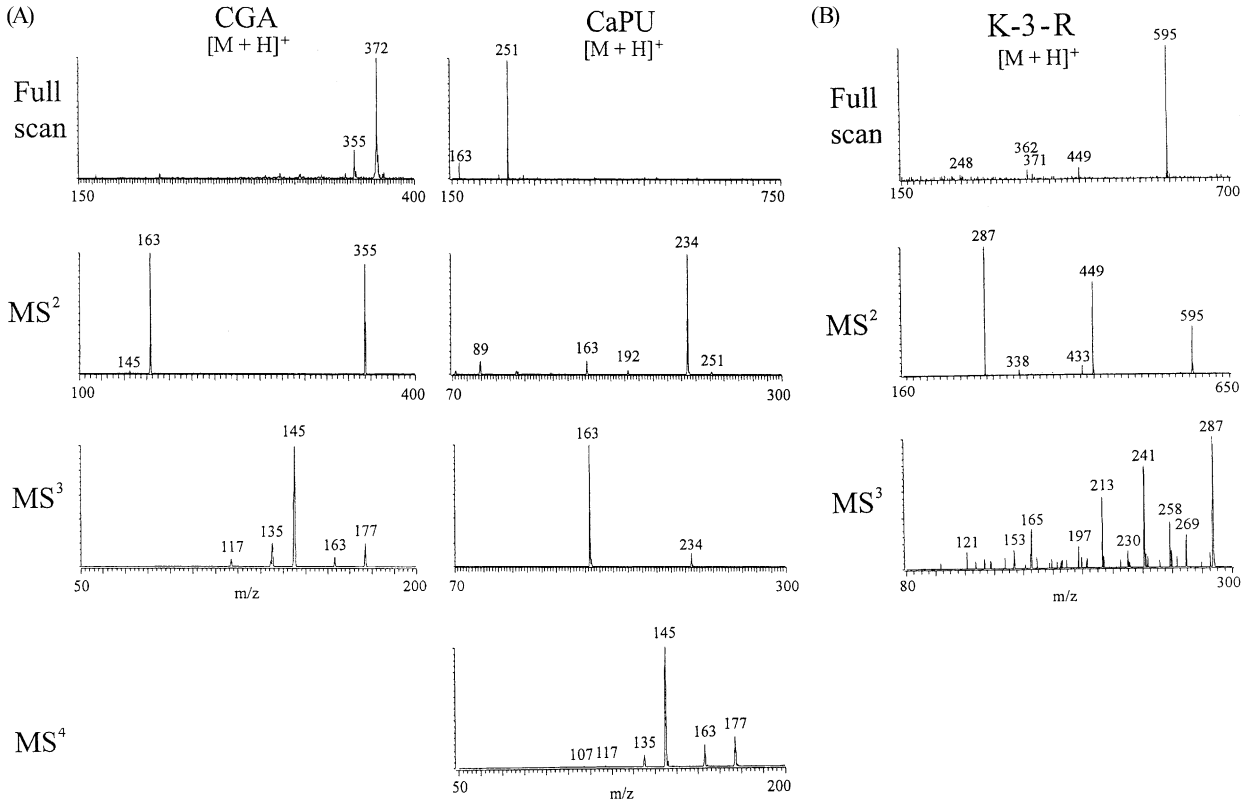

Fig. 5. ES mass spectra of phenolic substances in tobacco calli. A, Comparison of MS spectra and fragmentation data of chlorogenic acid (**5**) (CGA) and caffeoyl putrescine (**7**) (CaPu). The fragmentation of *m/z* 163 (CGA in MS^3 and CaPu in MS^4) shows an identical pattern suggesting the presence of a caffeic acid (**6**) residue in these molecules. B, Mass spectrum and characteristic fragmentation of kaempferol-3-rutinoside (**9**) (K-3-R): Full scan MS ($[M+H]^+$) at *m/z* 595, CID MS^2 of *m/z* 595, CID MS^3 of *m/z* 287.

determined the CGA (**5**), CaPu (**7**), rutin (**8**), K-3-R (**9**), scopoletin (**4**) and scopolin (**3**) contents in calli expressing various levels of AK-*6b* transcripts. Individual phenolic contents were plotted against the AK-*6b* transcript levels. Calli grown on SH medium showed positive correlative relationships between the CGA (**5**), CaPu (**7**), rutin (**8**), K-3-R (**9**) contents and AK-*6b* transcript levels (depicted by solid lines in Fig. 6A, B, E, F), whereas scopoletin (**4**) and scopolin (**3**) contents displayed inverse relationships with increased AK-*6b* transcripts (solid lines in Fig. 6C and D). On HF medium, the amounts of phenolics in the calli were much lower than on SH medium, with the exception of scopoletin (**4**) (dashed lines in Fig. 6A–F), and the correlations between phenolics and AK-*6b* transcripts were also less apparent. These results suggest that hormones are necessary for the induction and modulation of phenolic accumulation, and for the pronounced interaction between the AK-*6b* gene effects and phenolic metabolism.

3. Discussion

We have identified a novel AK-*6b* gene-specific alteration of the accumulation pattern of phenolic compounds in the tissues cultivated in the shoot-forming medium containing cytokinin and auxin.

The main soluble fraction-associated phenolics in tobacco are derivatives of caffeic acid (**6**), with the major proportion being represented by CGA (**5**) (Elkind et al., 1990, Bate et al., 1994; Howles et al., 1996; Tamagnone et al., 1998). Another component of soluble fraction, coumarin-type scopolin (**3**) and scopoletin (**4**) are proposed to be synthesized from *p*-coumaric (**10**) or caffeic acid (**6**) through several steps including lactone-forming reactions, although coumarin lactone formation is not unequivocally determined. Another branch of the phenolic pathway in tobacco is usually represented by flavonoids and anthocyanins whose production is initiated by chalcone synthase. The four phenolic substances, CGA (**5**), CaPu (**7**), rutin (**8**), and K-3-R (**9**), share the common feature of using the CoA activated precursors, *p*-coumaroyl-CoA (**11**) (and caffeoyl-CoA, **12**), for their synthesis. *p*-Coumaroyl-CoA (**11**) is a substrate of chalcone synthase and leads to flavonoid biosynthesis (Dixon and Paiva, 1995) and caffeoyl-CoA (**12**) has originally been proposed to serve as an intermediate to caffeoyl putrescine (**7**) and CGA (**5**) formation (Fig. 3, Tamagnone et al. 1998; Harborne, 1980). Recent finding shows that CGA (5) is actively converted from *p*-cou-

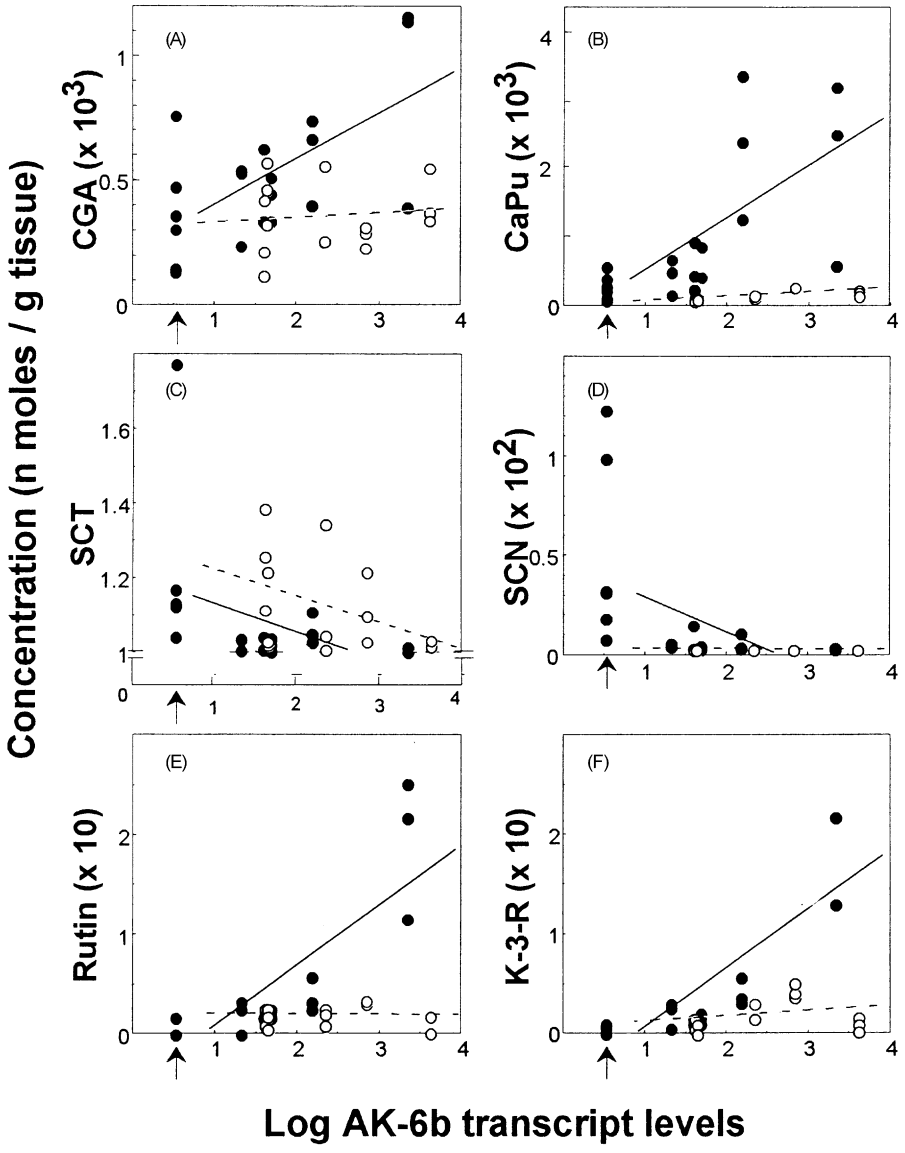

Fig. 6. Relationship between phenolic contents and AK-*6b* transcript levels in tobacco calli. A, chlorogenic acid (**5**) (CGA); B, caffeoylputrescine (**7**) (CaPu); C, scopoletin (**4**) (SCT); D, scopolin (**3**) (SCN); E, rutin (**8**); D, kaempferol-3-rutinoside (**9**) (K-3-R). Closed circles and solid lines represent values from tissues grown on SH medium. Open circles and dashed lines represent those grown on HF medium. Lines were drawn based on regression analysis and R^2 values were calculated with respect to data obtained from tissues grown on SH medium. The values are 0.386 for CGA (**5**); 0.486 for CaPu (**7**); 0.187 for SCT (**4**); 0.267 for SCN (**3**); 0.662 for rutin (**8**); and 0.709 for K-3-R (**9**),respectively. Arrows below individual graphs point to values of TOK12/9 line as a control lines. Two to three independent experiments were performed with single calli.

maroyl quinic acid (**13**), which is synthesized from a precursor, *p*-coumaroyl-CoA (**11**) (Schoch et al., 2001).

Analysis of soluble phenolic compounds in tissues cultivated on SH medium indicated that the compounds representing stable end products of the pathway accumulated differentially in transgenic AK-*6b* and control tissues. The AK-*6b* gene acts to direct increased accumulation of the caffeic acid derivatives, CGA (**5**) and caffeoyl putrescine (**7**), as well as the flavonoids rutin (**8**) and kaempferol-3-rutinoside (**9**), but decreased accumulation of scopolin (**3**) and its immediate precursor, scopoletin (**4**) in comparison with wild-type and non-*6b* transgenic TOK12/9 control calli. Our finding that normal tobacco tissues contained limited amounts of rutin (**8**) and K-3-R (**9**) is consistent with a previous study of *Citrus* cultures, in which these flavonoids were observed in leaves and greenish embryoids but not in undifferentiated calli (Moriguchi et al., 1999).

The differential accumulation of phenolic compounds in the transgenic AK-*6b* and control tissues suggests that the preferential metabolism towards scopolin (**3**) synthesis in normal tobacco is, as a result of AK-*6b* gene action, redirected towards the synthesis of CGA (**5**) and flavonoids. The phenylpropanoid biosynthesis pathway initiated by phenylalanine ammonia-lyase (PAL) is followed by cinnamate 4-hydroxylase (C4H),

which converts *E*-cinnamic acid (**2**) to *p*-coumaric acid (**10**), and to other derivatives by steps catalyzed by 4-coumarate:CoA ligase (4CL) and other enzymes (Fig. 3). Northern hybridization analysis, using the gene segments encoding PAL, C4H, and 4CL as probes indicated that accumulation of these transcripts did not correlate with different degrees of AK-*6b* transcript levels whether the calli were grown on SH medium or HF medium (data not shown). Although transcript levels do not necessarily reflect enzymatic activities, these results suggest that modulation of phenylpropanoid accumulation by the AK-*6b* gene was not caused by modulation of gene expression of tobacco PAL/C4H/4CL.

This redirection most probably occurs at the levels of p-coumaric acid (**10**) and/or caffeic acid (**6**), since the synthesis of CGA (**5**), caffeoyl putrescine (**7**) and flavonoids requires the CoA-activated precursors, *p*-coumaroyl-CoA (**11**) and caffeoyl-CoA (**12**), whereas scopolin (**3**) and scopoletin (**4**) syntheses presumably do not. Based on these observations, we assume that the AK-*6b* gene product could be associated with the reactions generating CoA-derivatives either by directly affecting CoA transfer step or by interacting with the transcriptional machinery of various endogenous genes responsible for synthesis of CoA-derivatives. Indeed, Kitakura et al. (2002) showed that AK-*6b* protein interacts with a putative transcription factor from tobacco. Such complex might modulate phenylpropanoid synthesis gene expression.

Considering the effects of the outlined plant phenolics on plant hormones, the increased accumulation of CaPu (**7**), CGA (**5**) and flavonoids in AK-*6b* tissues is of particular interest. Wang and Letham (1995) reported that caffeic acid (**6**) showed a cofactor-like effect, stimulating the activity of partially-purified cytokinin oxidase by two-fold. In contrast, accumulating evidence also suggests that changes in auxin levels could be involved. Hence, CGA (**5**) has been shown to inhibit IAA oxidase in vitro (Lee et al., 1982), suggesting that higher levels could protect endogenous auxin in AK-*6b* tissues. Furthermore, flavonoids, including kaempferol and quercetin (rutin, **8** aglycones), have been shown to counteract normal auxin transport both in vitro (Jacobs and Rubery, 1988) and in vivo (Mathesius et al., 1998). In addition, the tt4 mutation in *Arabidopsis thaliana* seedlings, which results in a deficiency in anthocyanin and flavonoid biosynthesis, also results in an abnormal distribution of auxin (Murphy et al., 2000). Based on these reports, it is conceivable that metabolism of cytokinin and/or auxin in AK-*6b*-tissues has been influenced indirectly as a result of modulated phenolic compounds, particularly when the tissues are grown on SH medium.

We have previously shown that intracellular cytokinin levels in the AK-*6b*-tobacco tissues are normal as compared with those of wild-type plants when they are grown on HF medium (Wabiko and Minemura, 1996). However, auxin levels of tobacco seedlings expressing the C58-*6b* gene (from a strain C58), which is homologous to the AK-*6b* gene, increased in response to exogenous cytokinin addition in C58-*6b*-seedlings (Galis et al., 2002). It remains to be determined whether auxin accumulation in the AK-*6b*-tobacco tissues is also affected in SH medium.

Based on these observations, we consider it possible that the ectopic accumulation of CGA (**5**), CaPu (**7**), rutin (**8**) and K-3-R (**9**) in the AK-*6b*-transgenic tissues may be responsible for local changes in hormonal concentrations that result in a disturbance in cytokinin-auxin homeostasis, and which in turn influences the morphology of AK-*6b* calli as demonstrated in Fig. 1. While differences in phenolic metabolism may provide some explanatory basis for the morphological differences between AK-*6b* and control calli grown on hormone-containing SH medium, the question as to whether these differences are sufficient to induce hormone-independent growth of AK-*6b* calli remains unanswered, since a prominent accumulation of CGA (**5**), CaPu (**7**) and flavonoids was not evident in tissues grown on HF medium. In view of the finding that the plant phenolics, dehydrodiconiferyl alcohol glucosides (DCGs), are able to promote growth of the cytokinin-requiring tissues of tobacco in the absence of exogenous cytokinin (Binns et al., 1987; Teutonico et al., 1991), and the proposal that 7-methyl esculin may act as a regulator of locally-increased plant cell division activity in tobacco leafy galls induced by *Rhodococcus fascians* (Vereecke et al., 1997), the products of the phenylpropanoid pathway may also be considered to play significant roles in the process of cell division. To establish such a role, it will be important to determine the levels of DCGs and 7-methyl esculin in AK-*6b* transgenic tissues as a possible basis for their hormone-autonomous nature.

4. Experimental

4.1. Recombinant clones

T-DNA segment of the pTiAKE10 containing the AK-*6b* gene placed under the control of its own promoter was cloned into the vector pGA580 to generate GANE7 (Wabiko and Minemura, 1996). To construct the AK-*6b* gene under the control of a strong Cauliflower Mosaic Virus (CaMV) 35S promoter, a 3.4 kb *Eco*RI/*Hin*dIII fragment containing AK-*6b* (in the clone, pAKEH1) of pTiAKE10 T-DNA was first progressively digested with nuclease *Bal*31, then filled-in by the Klenow fragment, and subsequently cloned into pBluescript II (Stratagene). The cloned segment, extending 194 bp upstream of the initiation codon and

about 500 bp downstream of the AK-*6b* termination codon, was subsequently cloned into the *Bam*HI/*Eco*RI site of the binary vector, pBI121, to generate pBAK61, in which AK-*6b* gene is placed downstream of the CaMV 35S promoter. The pTOK119 vector with a luciferase gene, which had been widely used to transform a number of plant species, was used as a control since it did not appear to affect transformed callus growth (Komari, 1989). All these binary vectors pGA580, pBI121, and pTOK119 possess the kanamycin resistance gene as a selection marker.

4.2. Transgenic plants and growth conditions

The AK-*6b* gene in pGANE7 or pBAK61 was introduced into leaf discs of tobacco plants (*Nicotiana tabacum* L. cv. Samsun) by *Agrobacterium*-mediated gene transfer as described previously (Gális et al., 1999). The shoot-forming cultivation medium (SH medium), suitable for callus and shoot formation in wild-type tobacco, contained 1 mg/l BA and 0.1 mg/l NAA in the basal MS medium (Murashige and Skoog, 1962), while the hormone-free medium (HF medium) contained no added hormones. All media for transgenic clones were supplemented with 200 mg/l kanamycin, whereas wild-type tissues were grown in the absence of antibiotics. All calli were sub-cultured onto fresh media at four-week-intervals, and placed under 16-h light/8-h dark conditions at 25 °C.

4.3. Phenolic measurements

Entire plant tissues (1 g) were homogenized in MeOH–H_2O (4:1, 4 ml), kept on ice for 60 min, and centrifuged for 20 min at 18,500 g at 4 °C. A 10 μl-aliquot of each supernatant was separated by reversed phase (RP)-HPLC (column 5 μ Symmetry Shield RP8, 4.6 × 250 mm, Waters 600E Multisolvent Delivery System, Millipore, USA) through a gradient of 100% methanol, 20 mM sodium acetate pH 5 buffer (starting at a ratio of 25:75; in 4.5–15 min linearly to 70:30; in 25–30 min linearly from 70:30 to 100:0) at a flow rate of 1 ml/min. Scopolin (**3**) and scopoletin (**4**) were detected using a fluorescence detector (Waters 474 Scanning Fluorescence Detector) with excitation (λ ex) and emission (λ em) wavelengths of 360 nm and 460 nm, respectively. Chlorogenic acid (**5**) (CGA), caffeoylputrescine (**7**) (CaPu) and the flavonoids rutin (**8**) and kaempferol-3-rutinoside (**9**) (K-3-R) were detected with a UV absorbance detector (Waters) at 280 nm. Calibration curves were constructed using the authentic standards, scopoletin (**4**), CGA (**5**) and rutin (**8**) (purchased from Sigma), and K-3-R (**9**) (from Extrasynthese, Genay Cedex, France). Since authentic scopolin (**3**) and CaPu (**7**) standards were commercially unavailable, these compounds were estimated by referring to the related compounds. To this end, the isolated scopolin (**3**) was treated with almond β-glucosidase (1 unit/sample, Sigma) to release scopoletin (**4**) (Sigma), which was found to possess 4.9-fold higher fluorescence intensity than scopolin (**3**). This value was used as a calculation factor for scopolin content. The amounts of CaPu (**7**) were expressed referring to the structurally related derivative, caffeic acid (**6**) (Keinanen et al., 2001) as follows. Absorption spectrum of caffeic acid (**6**) and tentatively assigned CaPu (**7**) was determined to be very similar (data not shown) and thus, absorption peaks of caffeic acid (**6**), were at 328 nm, 295 nm, and 218 nm respectively, and very similar to those of CaPu (**7**); 322 nm 292 nm and 218 nm (our results, Sattar et al., 1990). The molar absorption coefficients of caffeic acid (**6**) which were experimentally determined to be, ε (322 nm) $=1.36\times10^4$, ε (292 nm) $=1.12\times10^4$, ε (218 nm) $=1.37\times10^4$, are quite resembling to those of CaPu (**7**); ε (322 nm) $=1.4\times10^4$, ε (292 nm) $=1.3\times10^4$, ε (218 nm) $=1.55\times10^4$ (Sattar et al., 1990). Based on these analyses, amount of CaPu (**7**) was calculated from the ratio of ε value at 292 nm by using known amounts of caffeic acid (**6**).

Where possible, UV absorbance spectra of the corresponding peaks were determined and compared to those of the authentic standards to identify the detected compounds. The identity of the peaks was further confirmed by mass spectrometric analysis (see below).

4.4. Mass spectrometric (MS) analysis

ESI mass spectra of CGA (**5**), CaPu (**7**), rutin (**8**) and K-3-R (**9**) were recorded on a LCQ ion trap mass spectrometer (Thermoquest/Finnigan, San Jose, USA) equipped with electrospray (ESI) source. Samples were dissolved in 10 mM ammonium formate in methanol–water = 1:1 and continuously infused into the electrospray ion source held at 4.2 kV. Product ion (MS/MS) mass spectra were recorded by setting the first quadruple to transmit the parent ion of interest and scanning the second (third) quadruple. Argon was used as the collision gas for collision-induced dispersion. Collision energy of 10–30 eV was employed to achieve fragmentation.

4.5. Northern hybridization analysis

Plant tissues (1 g) were ground in liquid nitrogen, mixed with 5 ml of extraction buffer (50 mM Tris–HCl pH 8, 300 mM NaCl, 5 mM EDTA, 2% (w/v) SDS, 10 mM β-mercaptoethanol and 1 mM aurin tricarboxylic acid), and total RNA was isolated following the procedure of Gális et al. (2002). Aliquots of RNA were separated by 1% agarose-formaldehyde gel electrophoresis and blotted onto Hybond-N+ nylon membranes (Amersham, England). Northern hybridization was

performed at 42 °C in the presence of 50% aq. formamide with ^{32}P-radioactively-labeled probes (Wabiko et al., 1990). Membranes were washed under high stringency conditions (0.1 × SSC, 0.5% SDS at 65 °C). Hybridization was quantified by densitometric analysis of the autoradiograph with an imaging plate (Bio-imaging analyzer, BAS1000, FUJIFILM, Japan). The AK-*6b* gene (Wabiko and Minemura, 1996) was isolated and used as probe.

4.6. RT-PCR analysis

Total RNA samples (10 μg) were digested with RQ1 RNAse-Free DNAse (Promega) and used for poly(A)$^+$ RNA preparation with GenElute® mRNA Miniprep Kit (Sigma) as described in the manufacturers' protocols. Isolated poly(A)$^+$ fraction was subjected to reverse transcription (M-MLV reverse transcriptase; Promega) and the first strand of cDNA was used as a template for PCR. The pairwise forward and reverse primers were used to amplify individual gene segements; "GTTAGTGAGTTGAGGCAACAC" and "AACTCTCTCGTTTCCTCTTCC" primer pair for the AK-*6b* gene; "TGAGGAGGGAGATTACTATGAG" and "CACAACCAACAGAAACAAGTAC" for the β-tubulin gene, and "GCTATGACTGGGCACAACAGA" and "TATCACGGGTAGCCAACGCTA" for the NPT II gene. The products sizes were 515 bp, 222 bp and 621 bp for AK-*6b*, tubulin and NPT II amplification, respectively. PCR conditions were: denaturation (94 °C, 3 min), 32 cycles of (94 °C, 30 s; 60 °C, 30 s; 72 °C, 40 s) and final extension for 7 min at 72 °C. Aliquots from each reaction were resolved by 1.2% agarose gel electrophoresis.

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