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SCIENTIFIC CORRESPONDENCE

Are Homeobox *Knotted*-Like Genes and Cytokinins the Leaf Architects?

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An increasing body of evidence indicates that the products of homeobox genes control the expression of specific target genes that trigger important differentiation processes in a variety of organisms (Gehring, 1987; Skott et al., 1989; Kessel and Gruss, 1990; Kerstelter et al., 1994).

In plants the *KNAT1* gene, belonging to the *knotted1* (*kn1*) homeobox gene family, has been isolated in Arabidopsis. *KNAT1* overexpression in homologous species induces modifications in leaf shape, producing lobed leaves with ectopic meristems in the margins in close vicinity to the veins (Chuck et al., 1996). Similarly, the overexpression of *kn1* genes in several heterologous species has been reported to affect the architecture of both simple and compound leaves (Hareven et al., 1996; Sinha, 1997). However, neither direct nor indirect relationships have so far been reported between the expression of *kn1* genes and the modification of plant biochemical functions that lead to processes of cell differentiation.

In our laboratory lettuce (*Lactuca sativa*) plants overexpressing *KNAT1* from Arabidopsis, driven by the pea (*Pisum sativum*) plastocyanin promoter, have been produced and characterized. Morphological analyses of the transgenic progenies showed dramatic alterations in leaf shape, consisting of a reduction in midvein elongation, an increased complexity of the vascular net, and a regular development of leaf-like structures at the serrations of leaf margins (Fig. 1, a–d). We found a positive correlation between the intensity of the phenotype and *KNAT1* expression.

To investigate a possible alteration of hormonal metabolism, preliminary in vitro tissue-culture experiments were carried out. *KNAT1* leaf discs were cultivated on hormone-free Murashige-Skoog medium and a 20-d delay in leaf bleaching was observed compared with controls. Moreover, when *KNAT1* leaf explants were cultured on auxin-containing medium (Murashige-Skoog medium plus 1 mg/L NAA), an actively growing callus was observed (which occasionally rooted), whereas controls mainly produced roots from the wounded areas. Finally, the regeneration ability of transformed callus on standard lettuce regeneration medium (Curtis et al., 1994) was higher than control callus. The behavior of in vitro cultivated *KNAT1*-transformed leaf explants strongly suggested the presence of a high content of cytokinins, affecting leaf senescence (Noodén et al., 1979), callus growth in the presence of auxin, and the ability of the callus to regenerate shoots.

It is well known from tissue-culture technology that a mere variation of the auxin-to-cytokinin ratio in favor of cytokinin switches on the cell-developmental program toward shoot differentiation (Skoog and Miller, 1957). Adventitious bud formation on leaves has been observed in plants overproducing cytokinins by ectopic expression of the *ipt* (isopentenyl transferase) gene of *Agrobacterium tumefaciens* (Estruch et al., 1991). Similar leaf phenotypes have been observed in *kn1*-overexpressing plants; therefore, these genes have been hypothesized to mimic the overproduction of cytokinins (Chuck et al., 1996).

Based on these data, immunocytolocalization of the cytokinin N^6-Δ^2-isopentenyl adenine with affinity-purified antiserum was carried out on the aldehyde-fixed leaf-margin tissues of *KNAT1*-transformed lettuce (Sossountzov et al., 1988; Dewitte et al., 1998). A relevant amount of this cytokinin base was observed in the vascular system of the transformed leaf, whereas it was only weakly represented in the untransformed leaf (Fig. 1, e–g), demonstrating that the overexpression of the *KNAT1* product is correlated with an overproduction of N^6-Δ^2-isopentenyl adenine. This cytokinin was found to be accumulated in and/or transported through the veins to leaf margins, where de novo leaf-like structures were observed.

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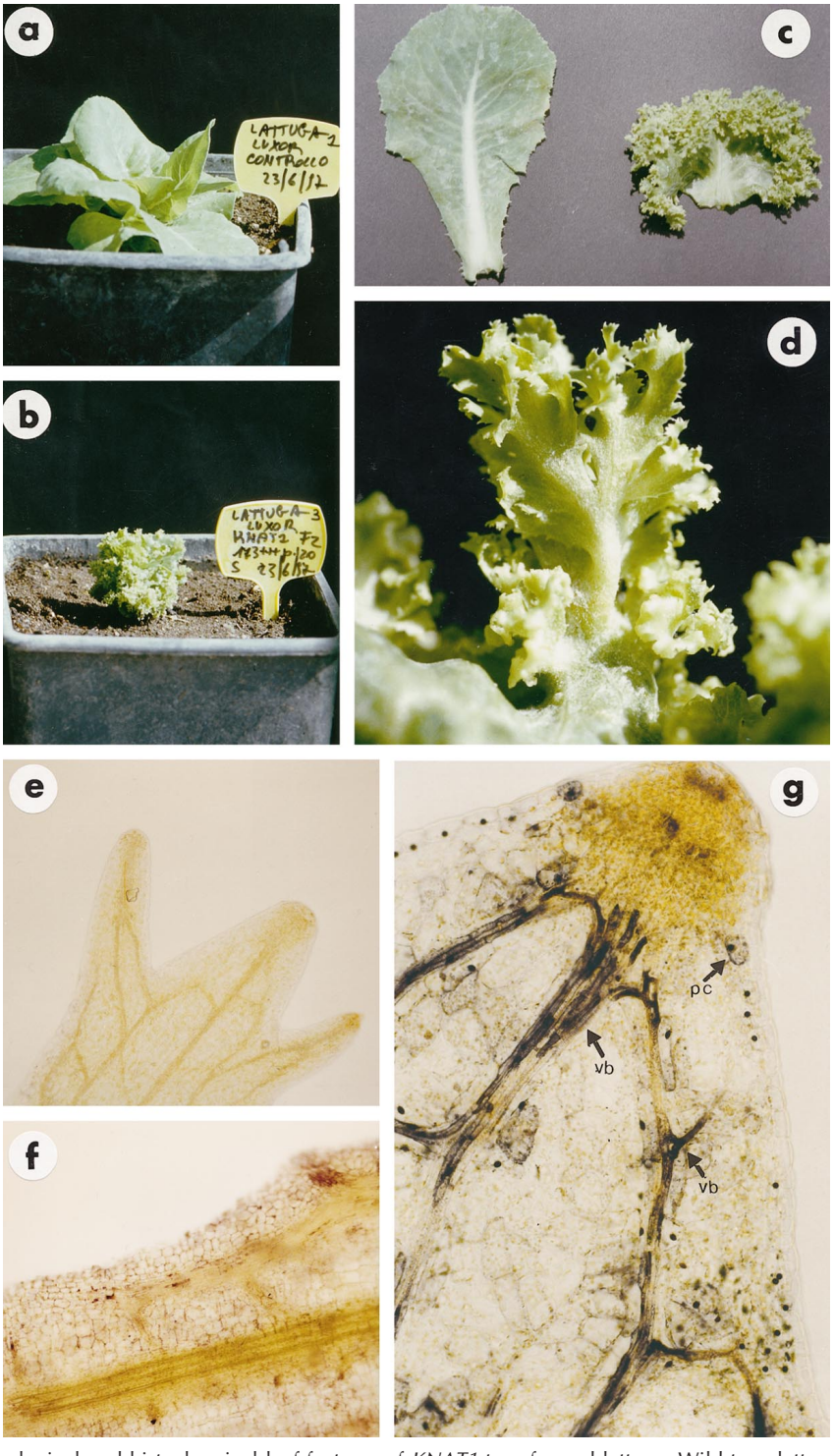

Figure 1. Morphological and histochemical leaf features of *KNAT1*-transformed lettuce. Wild-type lettuce exhibits leaves with regular margins (a and c, left), whereas *KNAT1*-transformed plants develop altered margins with leaf-like structures at the serrations (b and c, right, and d). When sections of transformed leaves were incubated with only alkaline phosphatase-conjugated secondary antibodies for a control, no deposition of the purple alkaline phosphatase-reaction product was detected (e). A faint signal was revealed when leaves of untransformed plants reacted with the anti-cytokinin antibodies (f). Leaves of plants overexpressing the *KNAT1* homeobox gene (g) showed N^6-Δ^2-isopentenyl adenine accumulation in the vascular tissue (vb) and in competent parenchyma cells (pc). Sections were cut parallel to the surface of the leaf. Magnification = ×65 (e) or ×125 (f and g).

The formation and regulation of cytokinins in plants have always been a topic of intriguing debate. A plant biosynthetic pathway based on the condensation of isopentenyl PPi with 5′ AMP to produce isopentenyl AMP (the precursor of several cytokinins) has not yet been demonstrated, because a plant *ipt* gene has never been isolated.

Might the observed cytokinin overproduction and *KNAT 1* overexpression lead us to unravel the complex pathway of cytokinin biosynthesis? Do homeobox genes regulate any of the enzymes involved in this pathway? In particular, might a plant *ipt* gene exist and be (de)regulated by *KNAT1*?

Up to now, we have observed a strict correlation among *KNAT1* overexpression, an increase of cytokinins, and the accumulation of cytokinins in competent cells, the leaf vascular system, and in the procambium extending toward the leaf margins, where initiating sites of margins and meristems were activated. Since *kn1*-like genes have been proposed to have a regulatory function in the genetic program that determines leaf morphology (Jackson, 1996), our findings strongly suggest that the cytokinins are secondary signals in this process.

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