Production of Recombinant Proteins in Tobacco Guttation Fluid[1]

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Guttation, the loss of water and dissolved materials from uninjured plant organs, is a common phenomenon in higher plants. By using endoplasmic reticulum signal peptides fused to the recombinant protein sequences, we have generated transgenic tobacco (*Nicotiana tabacum* L. cv Wisconsin) plants that secrete three heterologous proteins of different genetic backgrounds (bacterial xylanase, green fluorescent protein of jellyfish [*Aequorea victoria*], and human placental alkaline phosphatase) through the leaf intercellular space into tobacco guttation fluid. Production rates of 1.1 μg/g of leaf dry weight per day were achieved for alkaline phosphatase with this protein comprising almost 3% of total soluble protein in the guttation fluid. Guttation fluid can be collected throughout a plant's life, thus providing a continuous and nondestructive system for recombinant protein production. Guttation fluid has the potential of increasing the efficiency of recombinant protein production technology by increasing yield, abolishing extraction, and simplifying its downstream processing.

The expression of foreign genes is now routine for many plant species. Easy cultivation and the ability of plants to carry out numerous post-translational protein modifications make plants suitable bioreactors for the production of many valuable recombinant proteins used as pharmaceuticals, industrial enzymes, or fine chemicals. Numerous heterologous proteins have been expressed in different plant organs and plant cell compartments (Conrad and Fiedler, 1998). However, the high cost of protein extraction and purification from biochemically complex plant tissues is an important obstacle for the large-scale protein production in plants. This obstacle has been overcome partially by aseptically cultivated cell cultures or plant organs that secrete recombinant proteins into the surrounding medium (Wongsamuth and Doran, 1997). However, in vitro systems can be expensive, slow growing, unstable, and relatively low yielding. To partially overcome this problem, a rhizosecretion system for the production of recombinant proteins has been developed recently (Borisjuk et al., 1999). This system takes advantage of the ability of roots of hydroponically cultivated plants to secrete properly targeted recombinant proteins into the surrounding medium.

Guttation fluid is another easily collectable solution exuded daily by plants. In a natural environment, guttation fluid is most often observed at dawn after cool, still nights when conditions for absorption of water by roots are very favorable while transpiration is suppressed. Guttation, the phenomenon of loss of water and dissolved materials from leaves and other uninjured plant organs, has been known for over 300 years (Ivanoff, 1963) but was thought to be insignificant for plants or people (Stocking, 1956). On the leaf surface, hydathodes form the natural openings that represent a pathway of low resistance to the flow of fluid from the tracheary endings to the outside environment through the apoplastic spaces between the layers of epithem or mesophyll cells (Stocking, 1956). Hydathodes represent the main points of guttation fluid production; however, guttation fluid can be released through the cuticle (Lausberg, 1935) or stomata (Bald, 1952) as well.

It has long been recognized that some proteins are naturally secreted into plant guttation fluid. At the beginning of this century, for the first time, Wilson (1923) reported that proteins (catalase and peroxidase) were present in the guttation fluid of maize (*Zea mays*) and oats (*Avena sativa*), whereas reductase was released into the guttation fluid of timothy (*Phleum pzatense*). More recently, several peroxidases were partially characterized in the guttation fluid of strawberry (*Fragaria ananassa*), tomato (*Lycopersicon esculentum*), and cucumber (*Cucumis sativus*) (Biles and Abeles, 1991). Moreover, tomato mosaic virus and pepper mild mottle virus particles were recovered from guttation fluid of systemically infected tomato plants at the concentrations of 0.9 μg/mL and 0.5 μg/mL, respectively (French et al., 1993).

To further elucidate the mechanisms of protein release into guttation fluid of plants, we hypothesized that foreign proteins targeted to the apoplast can be recovered in the guttation fluid. To test this possibility, tobacco (*Nicotiana tabacum* L. cv Wiscon-

[1] This work was funded by the New Jersey Agricultural Experiment Station and by the New Jersey Commission for Science and Technology. Additional funding was provided by Phytomedics, Inc.

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sin) plants were engineered to secrete human placental secreted alkaline phosphatase (SEAP), green fluorescent protein (GFP) from jellyfish (*Aequorea victoria*), and xylanase from *Clostridium thermocellum* through the plant cell default secretion pathway (Deneke et al., 1990). Our results indicated that recombinant proteins directed to the leaf intercellular space (apoplast) are effectively released into the plant guttation fluid. Because guttation fluid contains less total protein as compared to apoplast fluid and it can be collected continuously throughout the plant's lifetime, guttation can be successfully used as a vehicle for recombinant protein production in plants by the process we termed "phyllosecretion." In addition, expression of image friendly proteins, such as GFP, in the plant guttation fluid could provide a novel tool to study various molecular and physiological aspects of guttation phenomenon in plants.

RESULTS AND DISCUSSION

The presence of several proteins (Wilson, 1923; Biles and Abeles, 1991) and virus particles (French et al., 1993) in the guttation fluid of different plant species suggested that proteins might be naturally released into the fluid or washed out from the cells' surfaces. To test the hypothesis that proteins targeted to leaf intercellular space through the default secretion pathway (Deneke et al., 1990) will be released into guttation fluid, tobacco plants were engineered to release three heterologous proteins into tobacco guttation fluid: SEAP (Berger et al., 1988), expressed under the strong constitutive cauliflower mosaic virus (CaMV) 35S promoter (Benfey et al., 1989); GFP (Reichel et al., 1996), driven by the strong modified mannopine synthase (mas2′) promoter from *Agrobacterium tumefaciens* (Ni et al., 1996); and bacterial xylanase under the control of the CaMV 35S promoter (Herbers et al., 1995).

Guttation in Tobacco

Guttation is a complex phenomenon that depends on many environmental conditions. Among the factors that favor guttation are high water absorption, high root pressure, and reduced transpiration (Stocking, 1956). The pattern of localization of guttation drops on tobacco is similar to that of potato (*Solanum tuberosum*) or bean (*Phaesoleus vulgarus*), in contrast to mustard (*Sinapis alba*), barley (*Hordeum vulgare*), or cucumber, where guttation drops form principally at the edges or tips of leaves (Takeda et al., 1991). The entire surface of younger tobacco leaf exudes the guttation fluid in plants kept under constant humid conditions (moist chamber, relative humidity [RH] $\geq$ 95%) for a 24-h period (Fig. 1A). No guttation was observed on the control plants kept under standard greenhouse conditions (Fig. 1B), on the oldest leaf of the guttating plant kept in the same moist chamber (Fig. 1C), or on the leaves of plants separated from the root system and kept under identical humid conditions (Fig. 1D). These results, in addition to the presence of characteristic proteins (data not shown) and the diurnal nature of the process (see below), suggest that the droplets observed in Figure 1A are formed by the guttation fluid and not by moisture precipitation. As in many other plants, older leaves of tobacco guttate less than younger leaves, possibly because of the accumulation of gummy material in the intercellular space, which blocks the exit of liquids (Takeda et al., 1991), or alternatively because of the possible change from sink tissue to source tissue as the leaf matures.

Guttation fluid was collected at the highest rate of 1 to 2 mL/g of leaf dry weight per day (up to 5 μL/cm^2 of leaf area) from the 2-month-old tobacco plants. The fluid contained up to 20 μg/mL (40 μg/g of leaf dry weight per day) of total soluble protein. These numbers are comparable to those published earlier for other plant species. Guttation fluid production from bean leaves was recorded at the level of 6 μL/cm^2 of leaf area (Yarwood, 1952), whereas guttation fluid of rice (*Oryza sativa*) contained 25 μg/mL of copper-Folin-positive substances (Ozaki and Tai, 1962).

Phyllosecretion of Xylanase

Tobacco plants expressing a truncated xylanase gene from *C. thermocellum*, under the control of CaMV 35S promoter and proteinase inhibitor II endoplasmic reticulum-targeting signal peptide (Herbers et al., 1995), were used for the initial study of the phyllosecretion process. Single 15-μL drops of 10-fold-concentrated guttation fluid from two independent transgenic lines were placed on the surface of Remazol Brilliant Blue (RBB)-xylan-containing agar medium and covered with paper (Whatman, Clifton, NJ) to prevent spreading and evaporation (Fig. 2). A clearing zone indicating an enzymatic cleavage of RBB-xylan by active bacterial xylanase released into the guttation fluid of transgenic tobacco plants developed in 3 h (Fig. 2, lanes 2 and 3). No clearing area was detected around the control sample (Fig. 2, lane 1).

Phyllosecretion of GFP

To ensure that the synthesized GFP was targeted to the secretory pathway, a modified GFP variant, S65C (Reichel et al., 1996), was fused to the signal peptide from calreticulin, a resident endoplasmic reticulum protein of *Nicotiana plumbaginifolia* (Borisjuk et al., 1998) and placed under the control of the mas2′ promoter (Fig. 3A). Transgenic plants were generated by *A. tumefaciens*-mediated leaf-disc transformation (Horsch et al., 1985). The presence of mRNA transcripts encoding GFP was verified by northern-blot analysis (Fig. 3B). Transgenic lines producing

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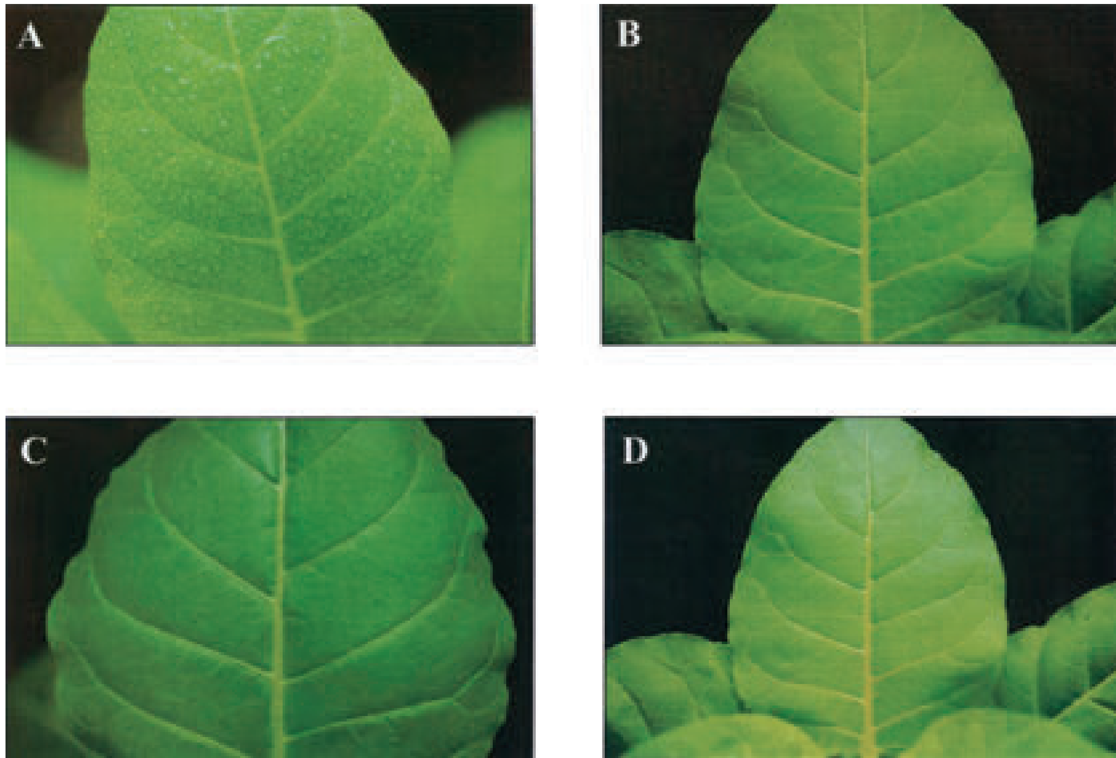

Figure 1. Guttation in tobacco. A, Guttation over the entire surface of young tobacco leaf after the plant was kept for 24 h under constant humidity. B, No guttation fluid was observed on a control plant under standard greenhouse conditions. C, Predown old leaf of the guttating plant showed no guttation. D, No guttation fluid was released by the plant with the severed root system that was kept and photographed under the same conditions as in A.

significant amounts of GFP mRNA were screened for the expression of recombinant protein by western-blot analysis of the apoplast and guttation fluid, using anti-GFP monoclonal antibodies produced by mouse hybridoma cells (CLONTECH, Palo Alto, CA). A single protein band with the predicted GFP M_r of 27 kD was detected in both the apoplast (Fig. 3C, lane 4) and the guttation fluid of the transgenic tobacco (Fig. 3C, lanes 5–8). The migration position was identical to that of the commercially available GFP standard of the band (CLONTECH) (Fig. 3C, lane 3). Recombinant protein was absent from the intercellular and guttation fluid of non-transgenic plants (Fig. 3C, lanes 1 and 2). These results confirmed that GFP is efficiently secreted from the leaf cells into the intercellular fluid, and then released with the guttation fluid to the plant surface. Slightly more GFP accumulated in the apoplastic space of transgenic tobacco compared to the guttation fluid (Fig. 3C, compare lanes 4 and 5), probably because of the formation of the extracellular insoluble GFP aggregates noted earlier (Borisjuk et al., 1999). Individual six-week-old plants from the four best lines producing recombinant protein in the guttation fluid (Fig. 3C, lanes 5–8) were chosen for the quantification of GFP expression levels by sandwich ELISA. When kept under humid conditions for 24 h, the leaves of these plants released 60, 30, 30, and 15 ng GFP/g of leaf dry weight (Fig. 3D, lanes 2–5). These results demonstrated that recombinant GFP might be secreted into the apoplast space and released into guttation fluid of transgenic tobacco following the expression of GFP under the control of mas2′ promoter.

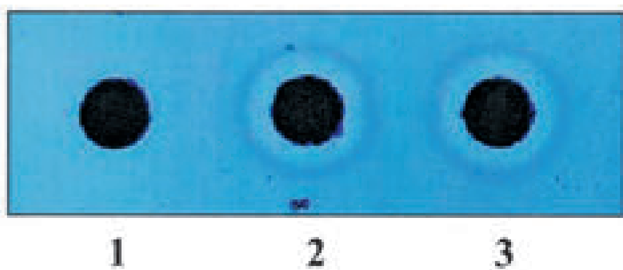

Figure 2. Phyllosecretion of bacterial xylanase into guttation fluid of tobacco plants. Fifteen μL of 10-fold concentrated samples were loaded on RBB-xylan (0.15%, w/v) containing agar medium and covered with Whatman 1 filter paper to minimize drops spreading and evaporation. Clearing zones that indicate RBB-xylan enzymatic cleavage by released recombinant xylanase developed in 3 h around guttation samples from individual transgenic lines (lanes 2 and 3), but not from the untransformed control sample (lane 1).

Phyllosecretion of SEAP

SEAP (Berger et al., 1988) was chosen as another model protein to demonstrate the ability of trans-

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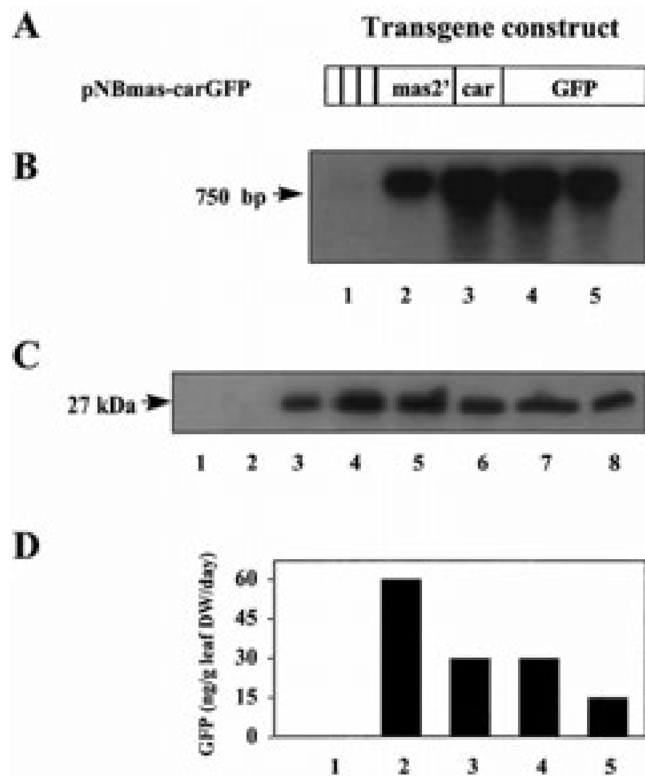

Figure 3. Phyllosecretion of GFP in transgenic tobacco. A, Schematic representation of the expression cassette. Empty boxes indicate three *osc* elements of mas2′ promoter. B, Northern-blot analysis of GFP mRNA expression in the untransformed control plant (lane 1) and four individual pNBmas-carGFP lines (lanes 2–5). C, Western-blot detection of recombinant GFP in the apoplast (lane 1) and guttation fluid (lane 2) of a control plant, the apoplast (lane 4) and guttation fluid (lane 5) of the GFP #16 transgenic line, and the guttation fluid of the other three transgenic lines (lanes 6–8). Fifty-microgram fractions of the total soluble proteins from leaf intercellular space and guttation fluid were separated by 12% (w/v) SDS-PAGE, transferred to the polyvinylidene difluoride membrane, and incubated with the GFP-specific monoclonal antibodies. Ten nanograms of standard GFP (CLONTECH) were loaded in lane 3. D, ELISA quantification of recombinant GFP released into guttation fluid of the control sample (lane 1) and same as above individual transgenic lines (lanes 2–5).

genic tobacco plants to release heterologous recombinant proteins into guttation fluid. It was previously shown that this protein is successfully expressed in plants and rhizosecreted into surrounding medium in larger quantities than GFP (Borisjuk et al., 1999). The SEAP reporter construct contained a truncated form of alkaline phosphatase (AP) lacking the membrane-anchoring domain, and was controlled by the CaMV 35S promoter. This recombinant protein was targeted to the secretion pathway of plants by its own signal sequence (Fig. 4A). Obtained transgenic plants were screened by northern-blot analysis and by the detection of AP activity in the apoplast fluid. A single plant of the A1 transgenic line that showed the highest expression level of SEAP was allowed to self-pollinate and the produced seeds were germinated on the kanamycin-containing medium. Of the first nine individuals positive for SEAP mRNA, five lines accumulated significant amounts of the transcript (Fig. 4B, lanes 1–5). No SEAP transcript was found in the wild-type plants (Fig. 4B, lane 6). SEAP secretion into the leaf intercellular fluid and its release into the guttation fluid were determined by a

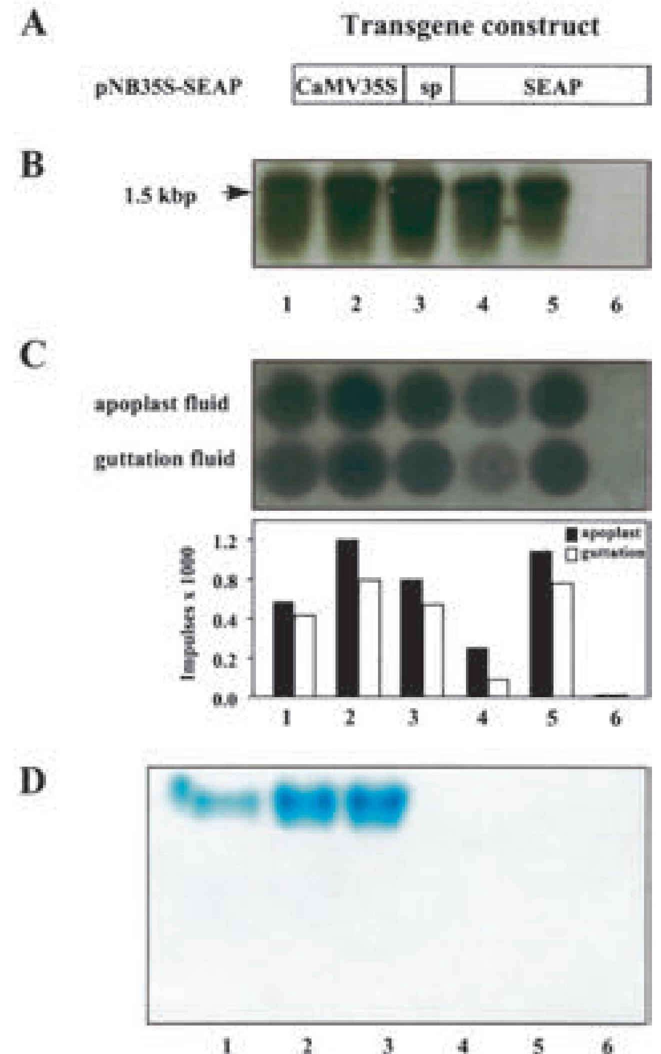

Figure 4. Phyllosecretion of SEAP in transgenic tobacco. A, Schematic representation of the expression cassette. B, Northern-blot analysis of SEAP mRNA expression in five T1 pNB35S-SEAP lines (lanes 1–5) as compared to the untransformed control plant (lane 6). C, Chemiluminescent detection of SEAP enzymatic activity in apoplast and guttation fluid of the same as above individual transgenic lines (lanes 1–5), with the untransformed control in lane 6. Top, Qualitative detection by exposure to x-ray film; bottom, quantitative detection with the Great EscAPe SEAP Chemiluminescence Detection kit (CLONTECH) and a Turner TD-20e Luminometer (Turner Designs, Inc., Mt. View, CA). D, Visualization of SEAP activity in native gel. Ten micrograms of total protein from leaf extract (lane 1), intercellular fluid (lane 2), guttation fluid (lane 3) of transgenic plant, and leaf extract (lane 4), intercellular fluid (lane 5), guttation fluid (lane 6) of the untransformed control plant were separated on native PAGE and SEAP activity was visualized by the AP isoenzyme procedure (Sigma, St. Louis).

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chemiluminescence-based activity assay (CLONTECH). SEAP activity in the guttation fluid closely paralleled and was only slightly lower than that of the apoplast, consistent with the hypothesis that the guttation and intercellular fluid are closely related (Fig. 4C). SEAP activity staining in a non-denaturing gel (Fig. 4D) confirmed recombinant protein secretion from the leaf cells into the apoplast (Fig. 4D, lanes 1–3) as well as almost equal distribution of SEAP activity between apoplast and guttation fluid (Fig. 4D, compare lanes 2 and 3). These results suggest that the guttation process did not change the activity of the recombinant enzyme. No SEAP enzymatic activity was observed in the control (nontransformed) plants (Fig. 4D, lanes 4–6).

Daily phyllosecretion of SEAP into guttation fluid was determined by measuring SEAP concentrations in the guttation fluid by chemiluminescence-based assay (CLONTECH). Two-month-old tobacco plants were used for these experiments. Guttation fluid released by all guttating leaves of a single plant was collected as a single sample, and the recombinant protein production was estimated for the unit of dry weight of all guttating leaves of that single plant. Although no background AP activity was detected in the guttation fluid of control plants, guttation fluid of SEAP-transgenic tobacco contained the recombinant protein at concentrations from 0.15 μg/g to 1.1 μg/g of leaf dry weight per day. The guttated SEAP in five studied T1 lines expressed as a percentage of total soluble protein ranged from 0.3% to 2.8%. These numbers are somewhat below the levels of SEAP production observed in the rhizosecretion system (Borisjuk et al., 1999). However, the tobacco phyllosecretion reported here was not optimized for the maximum protein recovery or yield.

The guttation-based system of recombinant protein production can be operated continuously throughout the life of the plant, capturing its total capacity to synthesize recombinant proteins. It is nondestructive and abolishes the need for tissue extraction and simplifies complex protein purification procedures. Phyllosecretion can be further optimized when additional parameters that affect the plant guttation process are studied in more detail. For example, it is believed that guttation in vascular plants is related to root pressure (Stocking, 1956). Root pressure often shows periodicity with a maximum in the daytime, but guttation is observed rarely during the day under field conditions when water loss exceeds water absorption. However, under continuously maintained high humidity (RH ≥ 95%), 14-h photoperiod, and 26°C-day/18°C-night cycle, we were able to study the rhythm of guttation fluid production in tobacco. Plants were sealed inside a humid chamber for 3-h intervals at the time of the day indicated on the *x* axes (Fig. 5A) and scored for guttation production after 24 h of the same photoperiod. Six-week-old tobacco plants released guttation fluid in a time-dependent

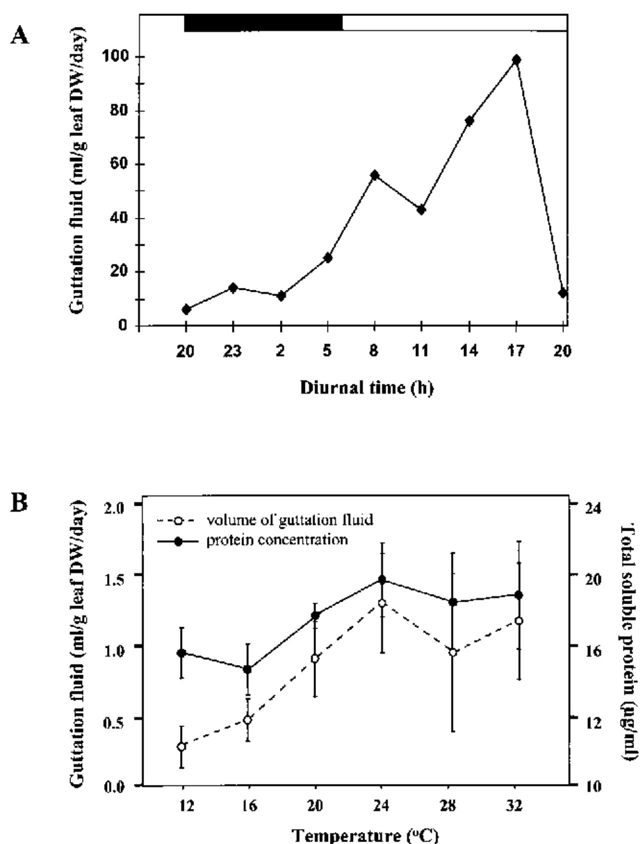

Figure 5. Environmental effects on guttation in tobacco. A, Daily production of the guttation fluid as a function of time of the day at which plants, kept at a 16-h photoperiod (indicated on the top bar) with 26°C days and 18°C nights, were shifted to constant humidity (RH ≥95%) with other environmental factors unchanged. B, Effect of temperature on the volume of the guttation fluid (solid line) and on the amount of protein released into this fluid (broken line). Plants were sealed in a humid chamber at 17 h, the approximate time of the guttation maximum (Fig. 5A). Data points in A represent the average of five replicates. Data points and error bars in B represent the mean ± SD of three replicates. Both experiments were repeated with similar results.

manner with a maximum reached late during the light period, suggesting that light increases guttation in tobacco as long as the humidity is kept high. Guttation of maize coleoptiles similarly increased in light and decreased during darkness (Engel and Friederichsen, 1954). Increasing temperature in the range of 16°C to 32°C had a generally positive effect on the guttation volume in tobacco without significantly changing the protein content of the guttation fluid (Fig. 5B).

Collection of guttation fluid might be an obstacle for scaling up phyllosecretion in tobacco. The use of other plant species could overcome this problem. In contrast to tobacco, tomato leaves exude large guttation drops at their tips and margins, which can be easily shaken off the leaves. Monocots, notably grasses, are particularly susceptible to guttation under field conditions (Stocking, 1956) and are capable

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of producing the average total surface area of guttation drops of 5 m^2 m^{-2} of ground area as it was reported for Yorkshire fog (*Holcus lanatus*; Hughes and Brimblecombe, 1994). A single uniform drop at the tip of the grass blade might represent an excellent collectable target for production of recombinant proteins by means of phyllosecretion technology. In addition, optimization of transgene expression and tissue specificity will play an important role in optimizing phyllosecretion for all plant species. Another important step in future system optimization might include assessing the ability of the simultaneous use of both the phyllosecretion and rhizosecretion systems. If successful, the combination of both techniques could significantly increase the total yield of heterologous proteins produced by plants in the easily accessible form of a water solution.

CONCLUSIONS

On the basis of the presented data it is difficult to assess the future impact of guttation fluid-based recombinant protein production. To succeed, this technology will have to compete with bacterial, cell culture, and transgenic animals technologies as well as with other methods of recombinant protein production in plants. However, the continuous and nondestructive recovery of recombinant proteins from a living plant potentially allows much higher yield than the single time harvesting, which captures only a fraction of proteins synthesized over the lifetime of a plant. An additional advantage of the guttation-based technology is the containment of the recombinant plants in the greenhouse. Most other plant-based production technologies rely on field-grown plants, which pose a potential risk to the environment. It is clear that more work will be required to develop this technology and to increase the yield of recombinant proteins. This manuscript only demonstrates the feasibility of the approach, leaving the optimization for the future. In addition, engineering solutions to the large-scale collection of guttation fluid must be developed, which might include shaking off the guttation droplets into a collection vessel or removing them from the leaf surface with a vacuum or blotting.

MATERIALS AND METHODS

Molecular Biology

To direct GFP into the secretory pathway, the *Hin*dIII fragment of the GFP coding sequence (Reichel et al., 1996) was fused to the signal peptide coding region from calreticulin (Borisjuk et al., 1998) and placed in the correct orientation between the mas2′ promoter and *nos* terminator of vector pATC940 (kindly provided by Stanton Gevin, Purdue University, West Lafayette, IN), creating the pNBmas-carGFP plant transformation vector (Fig. 3A). pNB35S-SEAP, the SEAP expression cassette, was constructed by placing a *Xho*I-*Xba*I fragment of SEAP with its own signal peptide sequence from pSEAP2-Enhancer vector (Great EscAPe SEAP system, CLONTECH) into *Kpn*I-*Xba*I-digested pBinAR-XynZ vector (Herbers et al., 1995) between the CaMV 35S promoter and *osc* terminator (Fig. 4A). Total RNA was isolated from plants (Chomczynski and Sacchi, 1987) and subjected to northern-blot analysis. DNA probes were gel purified and radiolabeled with ^{32}P by random priming (Sambrook et al., 1989).

Plant Transformation and Cultivation

The seeds of transgenic tobacco (*Nicotiana tabacum* L. cv Wisconsin) expressing a truncated xylanase gene were kindly provided by Uwe Sonnewald (Institut fuer Pflanzengenetik, Gatersleben, Germany). For plant transformation, SEAP and GFP expression cassettes were transformed into *Agrobacterium tumefaciens*. Individual *A. tumefaciens* clones were then used to inoculate sterile leaf discs of tobacco, as described previously (Horsch et al., 1985). Transgenic shoots were selected and rooted on Murashige and Skoog basal agar medium (Sigma) supplemented with 100 μg/mL kanamycin and 500 μg/mL cefatoxime. In the case of SEAP, T1 seeds of the A1 transgenic line were collected and germinated on one-half-strength Murashige and Skoog medium supplied with 100 μg/mL kanamycin. Plants were grown at 24°C under a 14-h-light/10-h-dark regimen (irradiance of 150 mE m^{-2} s^{-1} using a mixture of fluorescent and incandescent lamps).

Guttation and Apoplast Fluid Collection

Guttation fluid was collected from tobacco plants kept in a 95% RH chamber located inside of an environmental chamber (14-h photoperiod, 26°C light/18°C dark) for 24 h, by means of a handheld pipette or vacuum suction into the aspirator bottle. Care was taken not to disrupt the glandular hairs on the leaf surface. The light/dark period temperature drop was not a rapid one; therefore, no significant fluid accumulation as a result of condensation was observed on the plant surface (compare Fig. 1, A and D). Drops were combined to produce a single sample per plant. The intercellular fluid from leaves was collected after vacuum infiltration (Terry and Bonner, 1980) with an ice-cold buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8.0). Samples were transferred to cold (4°C) microcentrifuge tubes and were used immediately or stored at −20°C. The amount of the total protein in samples was quantified by the Bio-Rad (London) protein assay. Plants in Figure 1 (A, C, and D) were photographed shortly after they were removed from the humid chamber at the end of a 24-h period. The rhythm of guttation fluid production in tobacco was studied under continuous conditions of high humidity (RH ≥ 95%) when plants were placed inside a humid chamber at 3-h intervals and scored for guttation production after a 24-h period. The temperature effect (constant temperature, 12°C–32°C, step 4°C) on the guttation process in tobacco was recorded after plants were placed inside the humid chamber at the approximate peak time deduced from Figure 5A, and scored after 24 h.

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Expression Assays

Guttation fluid samples were concentrated 10-fold using ultrafiltration through a column with a 10-kD cutoff membrane (Amicon, Beverly, MA). Detection of xylanase activity was performed by loading the samples on the surface of RBB-xylan (Biely et al., 1988) containing agar medium (0.15%, w/v). Ten-fold concentrated sample drops (15 μL) were covered with Whatman 1 filter paper to minimize their spreading and evaporation, and Petri dishes were photographed after 3 h. Immunologic detection of GFP by western-blot analysis, ELISA quantification of recombinant GFP, and chemiluminescent quantification of SEAP by its activity related to standard protein were performed essentially as described previously (Borisjuk et al., 1999).

AKNOWLEDGEMENT

We thank Uwe Sonnewald (Institut fuer Pflanzengenetik, Gatersleben, Germany) for seeds of the transgenic tobacco expressing a xylanase gene.

Received July 12, 2000; accepted August 29, 2000.

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