Abstract  Synthesis of five different Sudan-β-D-glucuronides (I, II, III, IV, and RedB) was performed by condensation of a set of red Sudan diazo dyes with methyl (1-deoxy-2,3,4-tri-O-acetyl-1-trichloroacetimidoyl-α-D-glucopyran)uronate. After the acid and alcohol groups had been deprotected, the resulting compounds were used for histochemical localization of β-glucuronidase (GUS) activity in transgenic plants (Petunia hybrida, Arabidopsis thaliana, and Nicotiana tabacum) that contained the GUS reporter system. Because the cleavage of the β-glucuronide results in the liberation of an insoluble Sudan dye, Sudan substrates gave no diffusion artifacts as described for the commonly used 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc). A comparison of assays with different Sudan glucuronides and X-gluc demonstrated that the SudanIV variant is a valuable glucuronide substrate for the precise histochemical localization of GUS activity in transgenic plants.

Key words  β-Glucuronidase · Histochemistry · Sudan

Abbreviations  GUS β-Glucuronidase · X-gluc 5-Bromo-4-chloro-3-indolyl-β-D-glucuronide

Introduction  The gene that codes for β-glucuronidase (GUS) in Escherichia coli (uidA) was introduced as a suitable reporter gene system for plants by Jefferson et al. (1986, 1987). Since then, the system has been widely used for promoter studies in plants. The most commonly used substrate for the histochemical localization of GUS activity in plants is 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc). After enzymatic hydrolysis, the water-soluble indoxyl intermediate must undergo an oxidative dimerisation to produce a blue indigo precipitate. The diffusibility of this indoxyl intermediate sometimes results in the formation of a blue precipitate in cells that lack GUS activity (Lojda 1970). To minimize diffusion artifacts, the use of an oxidation catalyst, such as an equimolar mixture of ferri/ferrocyanide, has been proposed (Lojda 1970). Ferricyanide, however, is a strong inhibitor of the GUS enzyme and therefore may mask weak reporter activity (De Block and Debrouwer 1992; Mascarenhas and Hamilton 1992). Various GUS substrates are on the market nowadays, such as modified indigo-glucuronide X-GLuc (Magenta and Salmon; Biosynth AG, Staad, Switzerland) as well as fluorescent types of glucuronides, such as ImaGene Green and Red and the enzyme-labeled fluorescence (ELF)-based substrate (Molecular Probes,
Eugene, Ore., USA). The advantages and disadvantages of most of these substrates have been reviewed by Guivarc’h et al. (1996).

As an alternative to the commonly used X-gluc substrate, we first synthesized chemically a SudanII-glucuronide (SudanII-gluc). The major advantage of this Sudan substrate is that the Sudan moiety precipitates instantly after cleavage of the β-glucuronide moiety in a one-step reaction, without the formation of potentially diffusible intermediates. In addition, the bright red color of the Sudan precipitate might be useful for particular applications, because in bright-field observations it contrasts better with the green plant tissue than the blue-indigo precipitate of X-gluc.

Promising results on the usefulness of this SudanII substrate have been published by Terryn et al. (1993), who analyzed transgenic Arabidopsis plants that expressed the gus gene under the control of the rha1 promoter. Guivarc’h et al. (1996) tested several commercially available GUS substrates, as well as SudanII-gluc, on transgenic tobacco plants expressing gus under the control of the rolC promoter and immuno-localized the RolC protein into phloem companion cells as a control. Assays carried out with SudanII-gluc gave a very specific staining only in companion cells, whereas the other GUS substrates tested also showed staining in the neighboring phloem cells. With the traditionally used X-gluc substrate, specific staining could only be observed in the presence of a high concentration of ferricyanide. Here, we wish to present an improved Sudan-β-D-glucuronide substrate, SudanIV-gluc, as a better alternative for monitoring GUS activity in plant cells with high precision.

Because structural variations in the formula of Sudan dyes play an important role in the uptake of the substrate by plant cells and in the appearance of the final precipitate, several Sudan derivates were chemically synthesized and tested histochemically. Of five Sudan variants, SudanIV proved to meet the standards required to be a reliable histochemical GUS substrate.

Materials and methods

Chemical synthesis

A typical procedure to synthesize Sudan-gluc is as follows. Carefully dried methyl-1-deoxy-2,3,4,tri-O-acetyl-1-trichloroacetimidoyl-a-D-glucopyranuronate (630 mg, 1.31 mmol) and dried SudanIV (1.0 g, 2.62 mmol) were dissolved in dry chloroform (20 ml) under an inert atmosphere. Boron trifluoride diethyletherate (17 μl, 0.131 mmol) was added and the mixture was stirred for 1 day at 30 °C. Then another portion of boron trifluoride diethyletherate (17 μl, 0.131 mmol) was added and the solution was stirred for another 2 days at 30 °C. The crude product was purified by normal phase column chromatography, using dichloromethane/methanol 97:3 as eluent. A red solid was obtained (105 mg, 0.185 mmol). This product was dissolved in 17.5 ml EtOH and added to a solution of KOH in H2O (0.6 mg/ml, 17.5 ml, 0.185 mmol). The mixture was stirred for 12 h at room temperature; then ethanol was evaporated and 131 ml buffer solution (Merck Titrisol, pH 8) was added. The whole solution was filtered through a 0.20 μm PTFE 25 mm syringe filter (Alltech, Laarne, Belgium), giving 150 ml of a red 1.2-mM SudanIV-gluc solution, ready for use. All other Sudan compounds were synthesized according to the same procedure.

GUS assay: staining protocol

For the Arabidopsis thaliana (L.) Heynh. transformants, leaf, stem, and root material was collected with care from Arabidopsis grown on agar. The tissue was cut into small pieces with a razor blade and placed in a Falcon tube containing 10 ml acetone (90%). The tissue was gently rotated for 6 h at 4 °C, changing acetone (90%) twice. Acetone was removed with a pipette and the plant tissue was washed four times with 0.1 m NaHPO4 buffer (pH 7.2) for 4 h. The tissue segments were placed in a multiwell plate with 1 mm of either X-gluc (Biosynth AG) or SudanIV-gluc as a substrate. During the reaction time at 37 °C in the dark, the tissue was kept in the solution. When X-gluc was used as a substrate the assay buffer was supplemented with 0.5 mM oxidant catalyst.

After incubation, the samples were washed at least four times in 0.5 ml of 0.1 m NaHPO4 buffer. Fixation was performed in 25% glutaraldehyde overnight at 4 °C. The samples were washed again with phosphate buffer and mounted directly on microscope slides.

For the Petunia hybrida (L.) transformants, the ovary was cut into two halves and essentially treated as the Arabidopsis tissue, but without the acetone treatment. For the nuclear staining of transgenic Nicotiana tabacum (L.), 1-week-old seedlings were incubated as whole mounts in assay buffer.

Results and discussion

The chemical synthesis of Sudan-β-D-glucuronides was performed by the boron trifluoride diethyl etherate catalyzed condensation of the red diazo dye (Sudan I, II, III, IV, and RedB) with methyl (1-deoxy-2,3,4,tri-O-acetyl-1-trichloroacetimidoyl-a-D-glucopyranuronate (Bollenback et al. 1955; Schmidt 1985; Jacquetin 1990; Fig. 1). The resulting coupling product was deprotected by using potassium cyanide in methanol-tetrahydrofuran (Hertzig et al. 1986) for cleavage of the acetate groups, followed by deprotection of the acid group with potassium hydroxide in ethanol-water. To determine the specificity of the Sudan substrates, biological assays were performed on transgenic plants that expressed the gus gene under the control of different promoters.

SudanIV is larger and more hydrophobic than SudanII, resulting in smoother precipitation inside cells. Therefore, the use of SudanIV-gluc resulted in a clearly defined image without formation of large crystals as can be seen sometimes with SudanII-gluc.

Among the first transformants assayed with Sudan-gluc were transgenic Arabidopsis plants that expressed the gus gene under the control of the rha1 promoter (Terryn et al. 1993). Expression assayed with X-gluc for 3 h and 8 h at 37 °C was detected mainly in root tips and...