In situ studies of sugar metabolism in *Ricinus communis* L. and *Saccharum officinarum* L.

Dissertation

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To my parents, my wife and my son

Contents

Abbreviations

AGPP	ADP-glucose pyrophosphate phosphatase
Amp	ampicillin
Anti-Dig-AP	anti-digoxigenin-AP, Fab fragments
AP	alkaline phosphatase
BCIP	5-bromo-4-chloro-3-Indolyl phosphate
bp	base pairs
BSA	bovine serum albumin
CCCP	carbonylcyanide-*m*-chlorophenylhydrazone
cDNA	complementary DNA
cRNA	complementary RNA
Ct	threshold cycle
DEPC	diethyl pyrocarbonate
Dig-dUTP	digoxigenin-11-2'-deoxy-uridine-5'-triphosphate
DMSO	dimethyl sulfoxid
DNase I	deoxyribonuclease I
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
E.coli	*Esherichia coli*
EDTA	ethylendiamintetra acetate
IPTG	isopropyl-ß-D-thiogalactopyranosid
ISH	*in situ* hybridization
Km	Michaelis Menten constant
LB	Luria Bertani broth
NBT	4-NitroBlue-tetrazoliumchlorid
OD	optical density
PCMBS	p-chloromercuribenzene sulfonic acid
PCD	programmed cell death
PCR	polymerase chain reaction
RACE	rapid amplify cDNA ends
RNase	ribonuclease
RPA	RNase protection assay
RT-PCR	reverse transcription-Polymerase chain reaction

SBE	starch branching enzyme
SCYLV	sugarcane yellow leaf virus
SE-CCC	sieve element-companion cell complex
SPP	sucrose phosphate phosphatase
SPS	sucrose phosphate synthase
T-DNA	transferred DNA
Tth	*Tth* DNA polymerase (from *Thermus thermophilus*)
UTR	untranslated region
X-gal	5-bromo-4-chloro-3-indolyl-ß-glucuronic acid

Introduction

In situ hybridization in tissue sections is one of the most powerful analytical tools available to the molecular developmental biologist. For many workers, this procedure provides the first insights into the function of newly isolated genes, allowing the formulation of hypotheses and setting the course for further research. *In situ* hybridization techniques allow specific nucleic acid sequences to be detected in morphologically preserved chromosomes, cells or tissue sections. In the late 1960s, the introduction of *in situ* hybridization (ISH) techniques (Gall and Pardue, 1969; John *et al*., 1969) opened a new era in histology and cell biology. ISH fills the gap between the detection of a specific sequence and its precise location within the tissue or the cell.

A wide variety of labels are available for *in situ* hybridization experiments, for example, radioactively labelled DNA or RNA probes, as originally used in 1969 by Gall and Pardue and John *et al*. (1969), and later, direct immunofluorescence microscopic hybridocytochemistry, applying fluorochrome-labelled DNA or RNA (Baumann, 1985), which is, however not widely used because of its relatively low sensitivity. Synthetic oligonucleotides are usually enzymatically labelled by tailing of the 3´-end with terminal deoxynucleotidyl transferase (Normand and Bloch, 1991; Guitteny *et al*., 1988). Riboprobes are generated by *in vitro* transcription using a linearized template and a promoter for RNA polymerase (Ozden *et al*., 1990; Forster *et al*., 1985).

Biotin was recently applied to detect cellular DNA or RNA. In combination with avidin, streptavidin or antibody, it can be conjugated with fluorescent dye, enzyme or gold. However, emphasis has recently been placed on the false-positive results that are obtained when this probe is used, because endogenous biotin may sometimes interfere with specific signals. Digoxigenin appears to be an interesting alternative because it is present exclusively in *Digitalis* plants as a secondary metabolite.

Non-radioactive ISH is used to detect a variety of nucleic acid sequences in mature, developing, and pathologically altered tissues. But the classical technique of *in situ* hybridization is a far less sensitive method than PCR and requires 50-100 copies of target sequence per cell (Keller and Manak, 1993). RT *in situ* PCR allows for the routine and rapid detection of low copy viral and human RNAs. The one-step *Tth* RT-PCR system

allows for the reproducible amplification and detection of low copy RNA targets within a few hours.

Transgenic plants are a powerful tool for studying plant development. Promoter-reporter gene fusion can also be used to study gene expression during development (Imlau *et al*., 1999; Truernit and Sauer, 1995). But the limit is: many plants cannot be transformed as easy as Arabidopsis or tobacco plants. In fact, no report about transgenic *Ricinus* has been mentioned. Sugarcane can be transformed, but it needs a long period of time to regenerate the new plants.

Because two different plants were used for this work (*Ricinus* and sugarcane) and there is no direct correlation, this work is divided into two parts. The first part is focused on the establishing of different *in situ* techniques (*in situ* hybridization, *in situ* RT-PCR and immunolocalization) to study the expression site of sucrose transporter genes of *Ricinus* seedlings during germination. In the second part, starch synthesis related genes and sugarcane yellow virus were also studied with the *in situ* techniques.

Part I

Expression of sucrose transporter genes, *RcSCR1* and *RcSCR2*, in the endosperm of *Ricinus communis* L. during seed germination

A. Introduction

“ All roads lead to Rome.”

In nature, many different organisms have developed different ways to achieve the same goal. For example, the tuber of potato and the root of radish came from different organs, but potato and radish use these organs for storing nutrients. On the other hand, the organs or tissues even have the same origin, but they have various functions in different organisms. For example, the stem of cactus is used for photosynthesis and storing water. The stem of tomato is the central part of the plant, where long distance transport of water and nutrients takes place and from which leaves and flowers grow.

The same holds true for small molecules. Maybe plants use different molecules to achieve the same function, but sometimes one small molecule can be used in many different biological pathways. For example *S*-adenosyl-L-methionine is a precursor of ethylene, which is a simple organic molecule with complex biological function. It can affect growth and development of higher plants. These effects include promoting fruit ripening, flower senescence and petal and leaf abscission. On the other hand, *S*-adenosyl-L-methionine is also a precursor of polyamines (putrescine, spermidine and spermine). Polyamines have anti-senescence properties (Evans and Malmberg, 1989) and can inhibit the ACC synthase activity and ethylene production (Lee *et al.*, 1997).

Plants convert CO_2 and water into carbohydrates and oxygen using the energy of sunlight and the carbohydrates can serve as energy source or the carbon skeleton for other bio-molecules. These carbohydrates can be converted into other sugars and sucrose is the most important one. In higher plants, not all cells perform photosynthesis, for example: the roots, storage tissues, young developing tissues and organs and reproductive tissues. Most carbohydrates are synthesized in the mature leaves. These synthesized carbohydrates have to be transported from mature leaves to tissues and organs, which are not photosynthetically active. The carbohydrate exporting tissues are referred to as “source tissues” and the importing tissues as “sink tissues”. Many plants use sucrose as a major form to transport the photosynthetic products to other tissues (Zimmerman and Ziegler, 1975).

Sucrose is not only a transport compound; it has many different functions in plants. In single cell plants, such as algae, sucrose and other low-molecular-weight compounds accumulate in response to salt or osmotic stress. In these cases, sucrose may play a role as an osmoprotectant rather than as a transport compound (Mikkat *et al.*, 1997). It can also be found that sucrose plays a similar role in higher plants (Ingram and Bartels, 1996).

Long-distance transport of sucrose between source and sink tissues occurs in specific cells of the phloem, the sieve elements. Sieve elements and companion cells are closely connected by many branched plasmodesmata and form the so-called sieve element-companion cell complex (SE-CCC). Higher plants use many different carbohydrates as long distance transport molecules; such as mannitol, sorbitol, raffinose, stachyose and verbascose (Richardson *et al.*, 1982), but the most important transport form of carbohydrates is sucrose (Zimmermann and Ziegler, 1975). Why do plants use disaccharides, oligosaccharides and sugar alcohols as transported molecules rather than monosaccharides? As a non-reducing sugar, sucrose is less reactive and more likely to survive the journey in the phloem. Invertase (sucrase) is the only enzyme that will touch it and this is unlikely to be present in the phloem sieve tubes. The sucrose molecule has been shown to protect membrane lipids during dehydration and freezing, and to help stabilize organelles and proteins.

There are two major hypotheses for sucrose loading into the phloem: the symplastic phloem loading and the apoplastic phloem loading. Most of the mesophyll cells are not far away from the minor veins. Sucrose may move from mesophyll cells into the sieve element-companion cell complex (SE-CCC) only via plasmodesmata. This route is known as the symplastic phloem loading. The apoplastic phloem loading is a mechanism, by which sucrose exits from mesophyll cells and is subsequently actively taken up by sucrose transporters located in the SE-CCC. Based on the plasmodesmatal frequency between the sieve element companion cell complex and the surrounding cells of minor veins, plants can be classified into three groups. The type 1: the SE-CCC is connected via multiple plasmodesmata to the phloem parenchyma, the bundle sheath or in mesophyll cells. Companion cells are often modified into intermediary cells. Type 2a: the symplasmic connectivity of the SE-CCC is low to moderate and Type 2b: the SE-CCC is symplasmically isolated from the surrounding cells.

The phloem loading of type 1 plants is thought to occur symplasmically. There are some questions existing about the symplastic phloem loading. If sucrose can diffuse freely from mesophyll cells into the SE-CCC, it should be equally moving back into mesophyll cells. How can SE-CCC then accumulate sucrose? The sucrose concentration of the phloem sap is much higher than that of the adjacent cells, how can sucrose move from the mesophyll into the SE-CCC? It is impossible to explain just by simple diffusion. Some researchers try to account for the symplastic loading by the "polymer trap" model (Oparka and Turgeon, 1999; Turgeon, 1996). The sucrose may be converted to raffinose or other sugars in specialized companion cells known as intermediary cells (ICs). The most important feature of ICs is a large amount of plasmodesmata that connect ICs and bundle sheath cells. The plasmodesmata could limit the passing through of the polymers and these polymers would remain in the intermediary cells. This model holds that sucrose diffuses from the bundle sheath cells into ICs. The sucrose concentration is lower than the bundle sheath cells because sucrose is used to synthesize raffinose family oligosaccharides (RFOs), which accumulate to high concentrations in ICs (Bachmann and Keller, 1995). Indeed, recent studies have shown that raffinose and stachyose are present in high concentrations in ICs but are almost undetectable in bordering mesophyll. In this way, the sugars themselves act as probes of molecular size, indicating that diffusion of oligosaccharides does not take place through IC plasmodesmata (Haritatos *et al.*, 1996).

On the other hand, the apoplastic phloem loading, which subsequently actively takes up sucrose into the SE-CCC, is more convincible. The apoplastic phloem loading requires two steps of membrane passage: between the cytosol of mesophyll cells and the apoplastic space and between the apoplastic space and the sieve tubes. Sucrose and other sugars are specifically loaded into the SE-CCC against the concentration gradient by carrier proteins. Treatment with p-chloro-mercuribenzene sulfonic acid (PCMBS, a non-permeant thiol reagent) or carbonylcyanide-*m*-chloro-phenylhydrazone (CCCP, a protonophore) can inhibit phloem loading (Roblin *et al.*, 1998. Shakya and Sturm, 1998; Tegeder *et al.*, 1999).

Yeast complementation is a very powerful technique for functional cloning of genes coding for sucrose transporters and other membrane bound transporters. The first sucrose transporter gene *SoSUT1* was isolated from spinach by heterologous expression in yeast (Riesmeier *et al.*, 1992). Recent studies have identified several sucrose transporter genes in different species (Table 1). These include *Arabidopsis thaliana* (*AtSUC2*; Sauer and

Stolz, 1994), barley (*HvSUT1, HvSUT2*; Weschke *et al*., 2000), castor bean (*RcSCR1*; Weig and Komor, 1996), *Pisum sativum* (*PsSUT1*; Tegeder *et al*., 1999), potato (*StSUT1*; Riesmeier *et al*., 1993), *Plantago major* (*PmSUC1*; Gahrtz *et al*., 1996) and rice (*OsSUT1*; Matsukura *et al*., 2000). So far, more than twenty sucrose transporter genes have been found. All of them belong to the large family of major facilitator proteins, with 12 membrane-spanning domains, consist of about 510 amino acids and have a molecular mass of about 54 kD. So far, no three-dimensional structure is available for any sucrose transport protein.

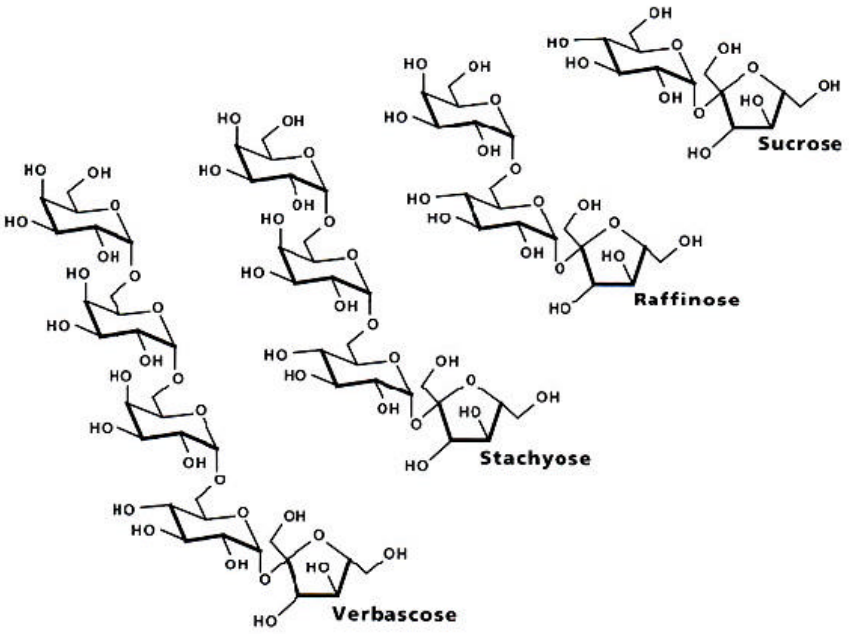


Figure 1: The raffinose sugars series. All raffinose sugars series consist of sucrose and galactose. The raffinose sugars series are nonreducing sugars.

Based on the kinetic characteristics and the site of their expression, the sucrose transporters can be divided into three different subfamilies. In general, the gene sequences show high similarity, but the *Km* values of the transporters and their site of expression are usually different (Shakya and Sturm, 1998; Wechke *et al*., 2000). The sucrose transporters can be defined as high-affinity-low-capacity (HALC) and low-affinity-high-capacity groups (LAHC). The *Km* values of HALC are between 0.2 and 2.0mM (Sauer and Stolz, 1994. Shakya and Sturm, 1998).

Table 1 : The sucrose transporter gene family in plants. The sequences are listed in alphabetical order of the gene.

Gene, accession no.	Species	Length (a.a.)	Functional expression	Site of expression	Reference
AbSUT1, AF191024	*Asarina barclaiana*	510	Yes	-	Knop *et al.* 2001
AgSUT1, AF063400	*Apium graveolens* L. (celery)	512	Yes	-	Noiraud *et al*. 2000
AgSUT2a, AF167415	*Apium graveolens* L. (celery)	512	Yes	-	Noiraud *et al.* 2000
AgSUT2b, AF167416	*Apium graveolens* L. (celery)	512	Yes	-	Noiraud *et al.* 2000
AmSUT1, AF191025	*Alonsoa meridionalis*	502	-	-	Knop *et al.* 2001
AtSUC1, X75365	*Arabidopsis thaliana*	513	Yes	-	Sauer and Stolz, 1994
AtSUC2, X75382	*Arabidopsis thaliana*	512	Yes	-	Sauer and Stolz, 1994
AtSUC3, AJ289165	*Arabidopsis thaliana*	512	Yes	-	Meyer *et al.*2000
AtSUT4, AF175321	*Arabidopsis thaliana*	510	Yes	-	Weise *et al.* 2000
BoSUT1, AY065839	*Brassica oleracea*	513	-	-	
BvSUT1, U64967	*Beta vulgaris* (sugar beet)	523	Yes	-	Chiou and Bush, 1996
DcSUT1a, Y16766	*Daucus carota* (carrot)	501	Yes	-	Shakya and Sturm, 1998
DcSUT1b, Y16767	*Daucus carota* (carrot)	501	Yes	-	Shakya and Sturm, 1998
DcSUT2, Y16768	*Daucus carota* (carrot)	515	Yes	-	Shakya and Sturm, 1998
GmSUT1, BM732362	*Glycine max* (soybean)	520	-	-	Aldape *et al*. 2003
HvSUT1, AJ272309	*Hordeum vulgare* cv. Barke (Barley)	523	Yes	-	Weschke *et al.* 2000
HvSUT2, AJ272308	*Hordeum vulgare* cv. Barke (Barley)	506	Yes	-	Weschke *et al.* 2000
LeSUT1, X82275	*Lycopersicon esculentum*	511	Yes	leaf	Barker L. *et al.* 2000
LeSUT2, AF166498	*Lycopersicon esculentum*	511	No	leaf	Barker *et al.* 2000
LeSUT4, AF176950	*Lycopersicon esculentum*	500	No	sieve element	Weise *et al.* 2000
LjSUT4, AJ538041	*Lotus japonicus*	511	-		Flemetakis *et al.* 2003

Gene, accession no.	Species	Length (a.a.)	Functional expression	Site of expression	Reference
NtSUT1, X82276	*Nicotiana tabacum* (tobacco)	507	No	leaf	Bürkle *et al.* 1998
NtSUT3, AF149981	*Nicotiana tabacum* (tobacco)	521	No	-	Lemoine *et al.*1999
OsSUT1, D87819	*Oryza sativa* (Rice)	537	Yes	leaf	Hirose *et al.* 1997
OsSUT2, AB091672	*Oryza sativa* (Rice)	502	Yes		Aoki *et al.* 2003
OsSUT3, AF419298	*Oryza sativa* (Rice)	507	?		Hirose *et al.* 1997
OsSUT4, AB091673	*Oryza sativa* (Rice)	596	?		Aoki *et al.* 2003
OsSUT5, AB091674	*Oryza sativa* (Rice)	536	?		Aoki *et al.* 2003
PmSUC1, X84379	*Plantago major*	503	Yes	phloem	Gahrtz *et al.* 1996
PmSUC2, X75764	*Plantago major*	510	Yes	phloem	Stadler *et al.* 1995
PsSUT1, AF109922	*Pisum sativum* (pea)	524	Yes	seed	Tegeder *et al.* 1999
RcSCR1, Z31561	*Ricinus communis* (ricinus)	533	Yes	cotyledon	Weig and Komor, 1996
RcSUT1, Aj224961	*Ricinus communis* (ricinus)	Partial	-	cotyledon	Bick *et al.* 1998
RcSCR2, (Not available)	*Ricinus communis* (ricinus)	509	No	endosperm	Eisenbarth, unpublished
SoSUT1, X67125	*Spinacea oleracea* (spinach)	525	Yes	leaf	Riesmeier *et al.* 1992
STSUT1, X69165	*Solanum tuberosum* (potato)	516	Yes	phloem	Riesmeier J.W. *et al.* 1993
STSUT4, AF237780	*Solanum tuberosum* (potato)	488	Yes	sieve element	Weise *et al.* 2000
TaSUT1A, AF408842	*Triticum aestivum* (wheat)	523	Yes	grain	Aoki *et al.* 2002
TaSUT1B, AF408843	*Triticum aestivum* (wheat)	523	Yes	grain	Aoki *et al.* 2002
TaSUT1D, AF408844	*Triticum aestivum* (wheat)	523	Yes	grain	Aoki *et al.* 2002
VfSUT1, Z93774	*Vicia faba* (Fava bean)	523	Yes	cotyledons	Weber *et al.* 1997
VvSUC11, AF021808	*Vitis vinifera* (grape berry)	501	Yes	grape berry	Davies *et al.* 1999
VvSUC12, AF021809	*Vitis vinifera* (grape berry)	612	No	grape berry	Davies *et al.* 1999

Gene, accession no.	Species	Length (a.a.)	Functional expression	Site of expression	Reference
VvSUC27, AF021910	*Vitis vinifera* (grape berry)	505	No	grape berry	Davies *et al.* 1999
ZmSUT1, AB008464	*Zea mays* (Maize)	521	Yes	leaf	Aoki *et al.* 1999

The Arabidopsis sucrose transporters SUC1 and SUC2, the potato sucrose transporter StSUT1 and the tomato sucrose transporter LeSUT1 belong to this group (SUT1 subfamily) (Kühn *et al.*, 1999). Data from the heterologous expression and *in situ* studies suggest a role for the HALC group for phloem loading or retrieval of sucrose escaped from the SE-CCC. It is suggested that the HALC transporters are the major transporters in the phloem. In fact, the SUT1 subfamily completely consists of high-affinity sucrose transporters from dicotyledons, whereas none of the transporters from monocotyledonous plants groups within this subfamily (Kühn, 2003).

The other two subfamilies of sucrose transporter-like proteins are either putative sucrose-sensing proteins (SUT2 subfamily) or low-affinity transporters (SUT4 subfamily) (Kühn, 2003). Most of the known sucrose transporters from monocotyledons are closely related to the SUT2 subfamily (putative sucrose-sensing proteins of dicotyledonous plants, such as AtSUC3, LeSUT2, StSUT2) and include high-affinity transporters, suggesting a different evolutionary origin of dicotyledonous and monocotyledonous sucrose transporter gene families (Aoki *et al*., 2003).

The SUT2 genes were identified by heterologous screening of cDNA libraries (Barker *et al*., 2000). The amino acid sequences of the SUT2 subfamily are different from other identified sucrose transporter proteins. Compared to the SUT1 subfamily, the SUT2 subfamily has extended domains of about 30 amino acids at the N terminus and of about 50 amino acids at the central cytoplasmic loop (Barker *et al.*, 2000). The function of the members of the SUT2 subfamily is still unknown. SUT2 genes were identified in tomato, potato, Arabidopsis and Plantago (Barker *et al*., 2000). Due to the structural features, low or no ability to take up sucrose in yeast uptake experiments, the low codon bias and the similarity to yeast sugar sensors, a sensing function has been postulated for members of the SUT2 family.

The SUT4 subfamily of sucrose transporters has known members in Arabidopsis, tomato, potato (Weise *et al.*, 2000), grape berry (Manning *et al.*, 2001), carrot (Shakya and Sturm, 1998), fava bean (Weber *et al.*, 1997), barley (Weschke *et al.*, 2000), rice (Aoki *et al.*, 2003) and *Lotus japonicus* (Flemetakis *et al.*, 2003). The mRNA and proteins of SUT4 were detected in the vasculature of sink leaves, source leaf minor veins, pistil and anthers of flowers (Weise *et al.*, 2000). The *Km* values of StSUT4 and AtSUT4 measured by yeast uptake experiments were shown to be in the range of 6 mM at optimal pH; the affinity of members of the SUT1 subfamily in relation to sucrose is higher, the *Km* value being in the range of 1 mM (Weise *et al.*, 2000). The phloem loading is thought to occur in minor veins. Thus, in Arabidopsis, expression of AtSUT4 in source leaf minor veins suggests a function in phloem loading. The SUT4 transporters' *Km* values are between 6 and 11.7mM (Schulze *et al.*, 2000). They were assigned to LAHC transporters (SUT4 subfamily). SUT4 transporters may potentially regulate the transport process or transport the sucrose into the tissues where the sucrose concentration is very high (Weise *et al.*, 2000).

To date, many different strategies such as sucrose analogs (Bornke *et al.*, 2002), heterologous expression (in yeast), antisense inhibition (in plants) or reverse genetic screening (in plants) have been used to study the function of sucrose transporters. The use of antisense RNA inhibition can reduce the amount of sucrose transporters. Antisense plants have a retarded growth phenotype. Their leaves were found to accumulate more starch and export fewer carbohydrates compared to wild type plants (Riesmeier *et al.*, 1994; Kühn *et al.*, 1996; Lemoine *et al.*, 1996). The disadvantage is that the antisense mRNA may affect other sucrose transporter genes, because these genes are very similar. By using reverse genetic screening, T-DNA insertion was performed by *Agrobacterium* infection (Gottwald *et al.*, 2000). The T-DNA inserts into different regions of sucrose transporter genes. Since T-DNA is longer than 10 kb and contains stop codons in the sequence, the T-DNA inserted sucrose transporter genes cannot produce functional sucrose transporters. The mutants are smaller than wild type plants and have yellowing, translucent cotyledons, very short primary roots and no rosette leaves. Mutant seedlings germinated without sucrose supplement are unable to develop beyond the cotyledon expansion stage, but they can be partially rescued by addition of sucrose. These studies indicate the importance of the sucrose transporters in phloem loading.

The sucrose transporter proteins can be modified with chemicals or by using site directed mutagenesis to produce mutated proteins. The kinetic characterizations of the modified proteins can be analyzed. For example, the amino acid residue at position 65 of most sucrose transporters is histidine. Mutations at His-65 result in different sucrose transport activities. This result indicates that His-65 may be involved in sucrose binding (Lu and Bush, 1998). By substitution of the different domains to form a chimera, the function of the domains can also be analyzed. For example, substitution of the central loops between two sucrose transporters, AtSUT2 and StSUT1, indicated that the central loops are not involved in sucrose binding (Schulze *et al*., 2000).

The castor bean sucrose transporter gene *RcSCR1* was cloned by Weig (Weig and Komor, 1996). In an attempt to determine the kinetic properties of *RcSCR1*, the *RcSCR1* cDNA was subcloned in a yeast expression vector and transformed to a yeast mutant, which lacks invertase. These yeast mutants can only grow in a medium containing glucose as the only energy and carbon source. The transformants can synthesize sucrose transporters, which enable the uptake of sucrose from the medium. The control transformants, which were transformed with an empty vector, were not able to synthesize sucrose transporters and therefore no uptake of sucrose from the medium takes place. After incubation in ^{14}C labelled sucrose-containing medium for a certain period of time, the amount of accumulated ^{14}C labelled sucrose in the yeast cells was measured. The yeast transformed with the sucrose transporter gene accumulated high amounts of ^{14}C labelled sucrose. In comparison with yeast transformed with an empty vector only very low radioactivity could be measured. By this study, the biological function of *RcSCR1* was identified. The *RcSCR1* is a sucrose transporter and the kinetic parameters are very similar to that of Arabidopsis sucrose transporters SUC1 and SUC2 (Weig and Komor, 1996). Due to the *Km* value and the amino acid sequence, *RcSCR1* can be grouped into the HALC transporters (SUT1 subfamily).

Later the cloning of the second sucrose transporter gene of castor bean succeeded. *RcSCR2* was cloned by Eisenbarth (Eisenbarth, Diploma thesis, Bayreuth 1999). With the same strategy, using heterologous expression in yeast, *RcSCR2* was subcloned to a yeast expression vector and expressed in the yeast mutant, but the *RcSCR2* transformants were not able to take up sucrose into the cytoplasm. To compare RcSCR2 with RcSCR1, the deduced amino acid sequences of RcSCR1 protein and RcSCR2 protein are only 50%

identical and the highest degree of sequence conservation was found in the transmembrane domains (73.4%). The physiological function of *RcSCR2* is not yet clear.

The deduced amino acid sequence of *RcSCR2* was also compared to the new subfamily SUT4 (Flemetakis *et al.*, 2003), which has high *Km* values and is expressed in the minor veins of source leaves or in sink tissues. The amino acid sequence of *RcSCR2* is closely related to that of SUT4, suggesting that these proteins may have a common function.

Figure 2 : Comparison of the amino acid sequences of RcSCR1 protein (top line), RcSCR2 protein (middle line) and StSUT4 protein (bottom line). The amino acid sequences are in the one-letter code and have been aligned by introducing gaps (-) to maximize identity. The sequences in blue color represent putative membrane-spanning domains.

Table 2 : The similarity table of the deduced amino acid sequences of SUT1 and SUT4. RcSCR2 shows high similarity to AtSUT4, LeSUT4, LjSUT4 and StSUT4.

	RcSCR2	LeSUT4	StSUT4	LjSUT4	AtSUT4	AtSUC2	RcSCR1
RcSCR2	100	73	72	71	69	50	50
LeSUT4		100	96	71	68	50	49
StSUT4			100	70	68	49	49
LjSUT4				100	68	49	49
AtSUT4					100	48	49
AtSUC2						100	66
RcSCR1							100

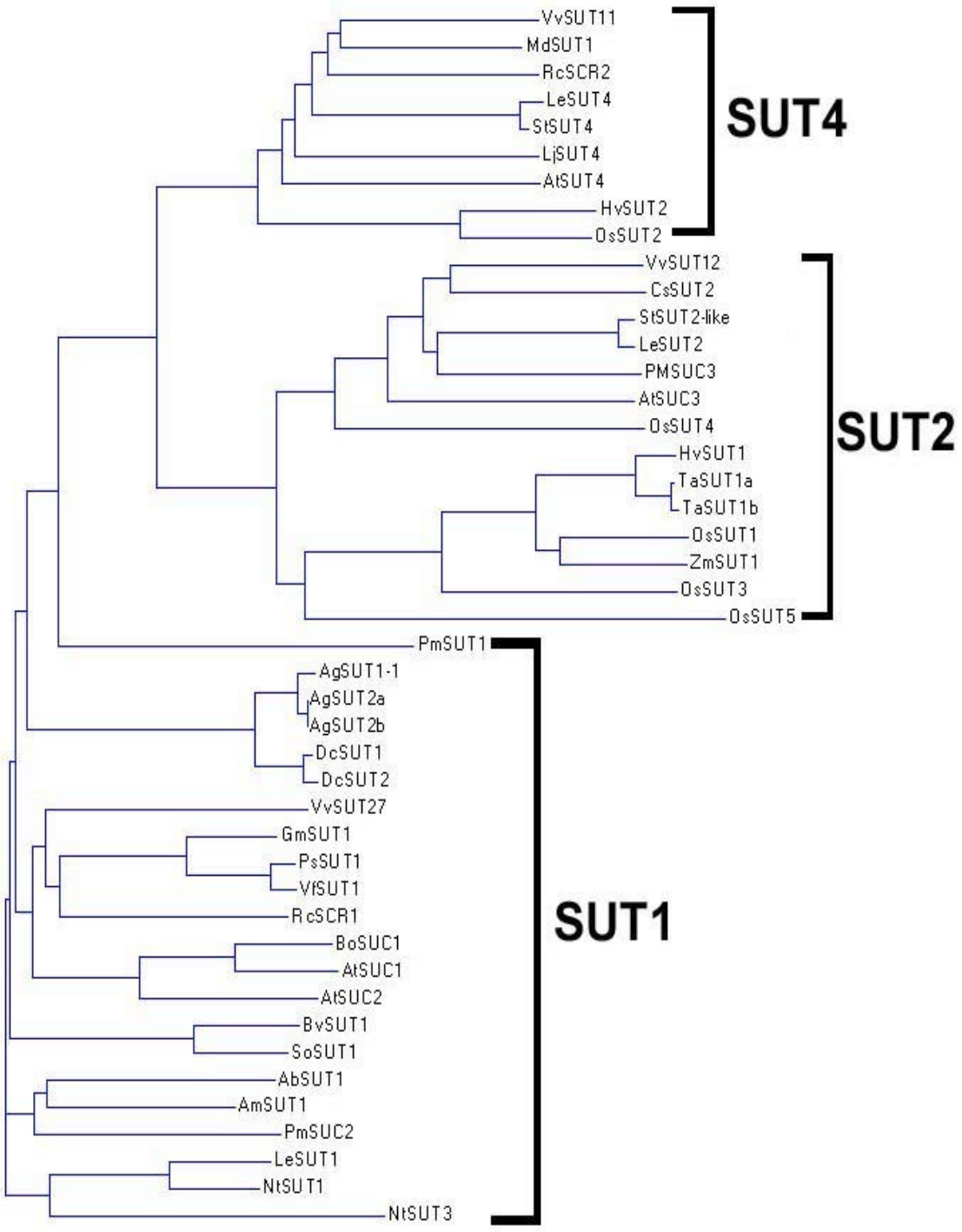


Figure 3 : Phylogenic tree of sucrose transporters. All detail information can be found in Table 2. These sucrose transporters can be divided into three subfamilies, SUT1, SUT2 and SUT4. The SUT1 subfamily is the largest sucrose transporter subfamily. The SUT2 subfamily consists of 2 parts, putative sucrose sensing proteins of dicotyledonous plants and the functional sucrose transporters of monocotyledonous plants. The SUT4 subfamily is a low-affinity sucrose transporter subfamily. The tree is based on the alignment of amino acid sequences of sucrose transporters from *Asarina bardaiana* (AbSUT1: accession AF191024), *Apium graveolens* (AgSUT1: accession AF063400,

AgSUT2a: accession AF167415, AgSUT2b: accession AF167416), *Alonsoa meridionalis* (AmSUT1: accession AF191025), *Arabidopsis thaliana* (AtSUC1: accession X75365, AtSUC2: accession X75382, AtSUC3: accession AJ289165, AtSUT4: accession AF175321), *Beta vulgaris* (BvSUT1: accession U64967), *Brassica oleracea* (BoSUC1: accession AY065839), *Citrus sinensis* (CsSUT2: accession AY98894), *Daucus carota* (DcSUT1: accession Y16766, DcSUT2: accession Y16768), *Glycin max* (GmSUT1: accession BM732362), *Hordeum vulgare* (HvSUT1: accession AJ272309, HvSUT2: accession AJ272308), *Lycopersicon esculentum* (LeSUT1: accession X82275, LeSUT2: accession AF166498, LeSUT4: accession AF176950), *Lotus janopicus* (LjSUT4: accession AJ538041), *Nicotiana tabacum* (NtSUT1: accession X82276, NtSUT3: AF149981), *Oryza sativa* (OsSUT1: accession D87819, OsSUT2: accession AB091672, OsSUT3: accession AF419298, OsSUT4: accession AB091673, OsSUT5: accession AB091674), *Plantago major* (PmSUC1: accession X84379, PmSUC2: accession X75764), *Pisum sativum* (PsSUT1: accession AF109922), *Ricinus communis* (RcSCRl: accession Z31561), *Spinacea oleracea* (SoSUT1: accession X67125), *Solanum tuberosum* (StSUT1: accession X69165, StSUT2: accession AY291289, StSUT4: accession AF23778), *Triticum aestivum* (TaSUT1A: accession AF408842; TaSUT1B: accession AF408843, TaSUT1 D: accession AF408844), *Vicia faba* (VfSUT1: accession Z93774), *Vitis vinifera* (VvSUC11: accession AF021808, VvSUC12: accession AF021809, WSUC27: accession AF021810), *Zea mays* (ZmSUT1: accession AB008464).

With some well-studied species, our knowledge of phloem loading is limited to the influx transporters in the sieve elements or companion cells. But there is still a question, how the cells of the source tissue release sucrose to the apoplastic space. Can sucrose directly diffuse to the apoplastic space or is (are) there sucrose efflux transporter(s) in the membrane. It has been suggested that the influx sucrose transporter could function as an efflux transporter without energy of the transport, since sucrose would be transported along its concentration gradient. However, no such system has been identified so far.

During seed germination of *Ricinus communis*, the endosperm provides nutrients for the growth of the seedling. Endosperm releases sucrose and amino acids and then cotyledons take up and transport these nutrients to other parts of the seedling.

Immunolocalization and *in situ* hybridization studies of RcSUT1 protein has been reported for the cotyledons and the hypocotyl (Bick *et al*., 1998). Data from these studies suggest a role for the RcSUT1 protein during phloem loading. On the other hand, RcSCR1, another member of SUT1 in *Ricinus* plant, was found not only in the hypocotyl and the cotyledons but also in the endosperm (Weig and Komor, 1996). The task of the endosperm is to release nutrients for supplying the growth of the seedling, why should an influx transporter

be expressed in the endosperm cells? Why do the endosperm cells retrieve sucrose from the extracellular space? Or can the RcSCR1 also release sucrose?

In recent years, many papers have reported the possibility of examining mRNA in tissues, taking advantage of the amplifying potential of the polymerase chain reaction (PCR). Particularly, the *in situ* reverse transcription-polymerase chain reaction (IS-RT-PCR) has been successfully applied in detecting low copy numbers of viral RNA and animal RNA in formalin fixed and paraffin embedded material, and in evaluating the cellular location. So far, there are only a few publications about using this method with plant material. The *in situ* RT-PCR technique may be applied to study the expression of *RcSCR2* in the endosperm.

Aim of this work

One challenge is to understand how plants regulate the flow of sucrose in the whole plant and the cellular level. At the beginning of my work, no sucrose efflux transporter was known and described, but two sucrose influx transporter genes; *RcSCR1* and *RcSCR2* were found in *Ricinus communis*. In some plant species, localization of the transporters was carried out by using mRNA *in situ* hybridization. Sucrose transporters were localized also within the vascular tissue by immuno-fluorescence in some species (Gahrtz, *et al*. 1994). However, the site of expression of RcSCR1 and RcSCR2 at tissue and cellular is still unknown.

The aims of my work can be classified to three objects:
(1) To find the sucrose efflux transporter(s) in castor bean germinating seedling.
(2) To identify the expression levels of *RcSCR1* and *RcSCR2* in the endosperm of castor bean seedling.
(3) To study the spatial and time courses of the expression of *RcSCR1* and *RcSCR2* in the endosperm of castor bean seedling.

Taken all these three tasks together, the aim of this work was mainly directed to the subcellular localization of the different sucrose transporters by using *in situ* hybridization, *in situ* RT-PCR and immunological techniques. To isolate an efflux transporter is a

challenge but it is interesting and worthwhile to understand how *Ricinus* endosperm cells release sucrose.

B. Material and Methods

B.1. Material

B.1.1. Instruments

Balance	Sartorius
Digital Camera, Type	RS Photometrics
Elektroporationsapparat,	Pharmacia
Centrifuges, Typ Centrifuge 5403	Eppendorf
Centrifuges, Mikro 12-24	Hettich
Centrifuges, RC-5B	Sorvall
Microscope, Type BH-2	Olympus
Microscope, Type M3B	Wild
Microtone, Type 2050	Reichert-Jung
Thermocycler, Typ 480	Perkin Elmer
Thermocycler, Type PTC-100	MJ Research
Spectrophotometer, Type 650	Beckman
Vortexer, Type REAX-1R	Heidolph
Power pack, Type EPS 200	Pharmacia
Shaking incubator, Type 3031	GFL
Speed vac, Type SVC 100E	Savant
Heated magnetic stirrer, Type RCT B	IKA
Microwave, Type 8017	Privileg

B.1.2. Chemicals and Enzymes

All chemicals used in this study were obtained from Amerscham, Applichem, Bio Rad, Boehringer, Fluka, Gibco, Merck, Roth, Serva or Sigma, unless otherwise stated in the text. All restriction enzymes were purchased from MBI Fermentas or New England Biolabs Inc.

Various DNA polymerases were used according to the application (the polymerase used for each application is detailed in Methods) and were purchased from Stratagene, Invitrogen, or BD Bioscience. DNase I was purchased from Roche, and calf intestinal alkaline phosphatase was purchased from New England Biolabs.

Chemicals	**Company**
Aquatex	Merck GmbH
Agarose NEEO	Carl Roth GmbH
BCIP	Applichem GmbH
Blocking reagent	Roche Diagnostics GmbH
CDP Star	Roche Diagnostics GmbH
Chinosol	Sigma-Aldrich chemie Gmbh
CSPD	Roche Diagnostics GmbH
DEPC	Carl Roth GmbH
BSA	Sigma-Aldrich chemie Gmbh
RNA D ig labelling Mix	Roche Diagnostics GmbH
DNA Agarose gel extraction Kit	Roche Diagnostics GmbH
Histoclear (Roticlear)	Carl Roth GmbH
n-Lauryl-Sarcosine	Sigma-Aldrich chemie Gmbh
NBT	Applichem GmbH
Paraplast	Sigma-Aldrich chemie Gmbh
Paraffin wax	Carl Roth GmbH
Polyethylene glycol 8000	Sigma-Aldrich chemie Gmbh
Polyvinyl alcohol	Sigma-Aldrich chemie Gmbh
RNase inhibitor	MBI Fermentas
Triethanolamine	Sigma-Aldrich chemie Gmbh
Triton-X 100	Sigma-Aldrich chemie Gmbh

Tween 20	Sigma-Aldrich chemie Gmbh
YPD broth	Invitrogen
Yeast nitrogen base	Sigma-Aldrich chemie Gmbh

Enzymes	**Company**
DNase I, RNase free	Roche Diagnostics GmbH
EcoRV	MBI Fermentas
MMLV Reverse transcriptase	MBI Fermentas
Pfu DNA polymerase	Stratagene
PowerScript DNA polymerase	BD Bioscience
Proteinase K	Roche Diagnostics GmbH
RNase A	Promega GmbH
Shrimp alkaline phosphatase	Stratagene
SP6 RNA Polymerase	MBI Fermentas
T3 RNA Polymerase	MBI Fermentas
T4 DNA ligase	MBI Fermentas
T7 RNA Polymerase	MBI Fermentas
Taq DNA polymerase	Promega GmbH
Tth DNA polymerase	Promega GmbH

B.1.3. Buffers, Solutions and Media

B.1.3.1. Buffer for plasmid DNA isolation

GTE (50 mM glucose, 25 mM Tris, 10 mM EDTA, pH 8.0)
SDS 10% (10g SDS, add H_2O to 100 ml)
Sodium acetate 3M (pH 5.2)
TE (10 mM Tris, 1 mM EDTA, pH 8.0)

B.1.3.2. Buffer for gel electrophoresis

TBE 10x (108g Tris base, 55g Boric acid, 40 ml 0.5M EDTA, pH8.0, H_2O was added to final volume 1 liter.)

B.1.3.3. Buffer for RNA extraction

1M Tris-HCl [tris (hydroxymethyl) aminomethane]

DEPC-water (0.1% (v/v) diethylpyrocarbonate into demanded amount of distilled water)

EDTA 0.5M (pH 8.0) (181.6g Na_2.EDTA.2H_2O, 20g NaOH, add H_2O to 1l.)

Lauryl-Sarcosine 10% (5 g N-Lauryl-Sarcosine, DEPC H_2O was added to final volume 50 ml)

LiCl 8M (g LiCl)

NaCl 5M (292.5g NaCl, add H_2O to 1l.)

SDS 10% (10g SDS, add H_2O to 100 ml)

B.1.3.4. Buffer for Northern analysis, *in situ* hybridization and *in situ* RT-PCR

Antibody solution (1000 µl 10% Blocking reagent, 1 µl Anti-digoxigenin antibody-AP)

Blocking solution (1 g Blocking-reagent powder (Roche GmbH) in 100 ml buffer 1)

BCIP stock solution (50 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate, toluidinium salt in 100% dimethylformamide)

Buffer 1, 10X (1M Tris, 1.5M NaCl, pH 7.5)

Buffer 2, 10X (1M Tris, 1.5M NaCl, pH 9.5)

Color development solution (10ml 10X buffer 2, 5 ml 1M $MgCl_2$, 85 ml H_2O, 450µl NBT stock solution, 350µl BCIP stock solution)

$MgCl_2$ 1M (203g $MgCl_2$.6H_2O, add H_2O to 1l.)

NBT stock solution (75mg/ml nitroblue tetrazolium salt in 70%(v/v) dimethylformamide)

NTE-buffer (500 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0)

PBS buffer, 10X (2g KCl, 2.4 g KH_2PO4, 80 g NaCl, 14.4g Na_2HPO_4.7 H_2O in 800ml H_2O, adjust pH to 7.4, then add H_2O to 1l.)

PBST (0.01% Tween 20 in 1XPBS)

SSC buffer, 20X (3M NaCl, 0.3M Sodium citrate, pH 7.0)

SSPE 20x (3M NaCl, 0.2M NaH_2PO_4.7 H_2O, 0.02M EDTA, pH7.4)

TBST 10x (125 ml 1M Tris-HCl (pH 7.5), 45 g NaCl, 500 µl Tween-20 (10%) DEPC-water was added to 500 ml)

B.1.3.5. Media

LB (10g Tryptone, 10g NaCl, 5g Yeast extract, add 800 ml H_2O, then1M NaOH was added to adjust pH to 7.0. Then H_2O was added to final volume 1 liter.)

MMA (0.67 g yeast nitrogen base, 2 gglucose, add to H_2O 100 ml, autoclave 20 minutes.)
SOB (20g Tryptone, 5g yeast extract, 0.5g NaCl, 10 ml 250mM KCl, add H_2O to 990 ml, Autoclave. Add 10 ml 1M $MgCl_2$ solution before use.)
SOC (1liter SOB with 20 ml 1M glucose)
YPD (20 g glucose, 20 g Tryptone, 10 g yeast extract add H_2O to 1000 ml, autoclave 20 minutes.)

B.1.4. Softwares for Gene analysis

GCG for various sequence analyses (Wisconsin)
Chromas for sequence reading
MACAW for multiple alignment (http://www.ncbi.nlm.nih.gov/)
Blast (http://www.ncbi.nlm.nih.gov/BLAST/)
Entrez Pubmed (http://www.ncbi.nlm.nih.gov/Entrez/)

B.1.5. Oligonucleotides

General PCR reactions for screening of clones and incorporation of restriction sites were carried out with sequence-specific primers from MWG (MWG Biotech AG) or Biomers (Biomers.net GmbH). All primers used are listed in Results. General PCR reactions for the screening and sequencing of clones, and incorporation of restriction sites, were carried out with sequence-specific primers from MWG or Biomers. Sequence specific primers for 5' and 3' RACE, amplification of full-length clones were obtained from Biomers.

TS primer 5'-AGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG -3'
TS-PCR 5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCC -3'
TS-dT primer 5'-ATTCTAGAGGCCGAGGCGGCCGACATG-dT(30) -3'

B.1.6. Kits

B.1.6.1. Nucleic acids purification

Agarose Gel DNA Extraction Kit (Roche Diagnostics GmbH)

QIAprep Spin Miniprep Kit (Qiagen GmbH)

B.1.6.2. PCR cloning kits

pGEM-T and pGEM-T Easy Vector system (Promega GmbH)

B.1.6.3. RNA labeling kit

DIG RNA Labeling kit (SP6/T7) (Roche Diagnostics GmbH)

B.1.7. Plasmid vectors

B.1.7.1. *E.coli.* vectors

pBluescript II KS+ (Stratagene) (pUC origin, ampicillin resistance, *lacZ* reporter and *lacI* repressor, T7 and T3 RNA polymerase promoters)

pGEM-T vector (Promega) (pUC origin, ampicillin resistance, *lacZ* reporter and *lacI* repressor, T7 and SP6 RNA polymerase promoters)

B.1.7.2. *E.coli* Yeast Shuttle vectors

181A1NE (Riesmeier *et al.* 1992) Adh1 promoter, Adh1 terminator, Amp^r, Leu, 2µ replication origin

NEV-N (Sauer and Stolz, 1994) PMA1 promoter, PMA1 terminator, Ampr, URA3, 2µ replication origin

nSC4+ (Sauer and Stolz, 1994) Adh1 promoter, *RcSCR1*, Adh1 terminator, Ampr, URA3, 2µ replication origin

B.1.8 Yeast cell strain

SEY2102 (*MAT*α, *ura3, leu2, his4, suc2, gal2*)

B.2. Methods

B.2.1. Preparation of plant material

Ricinus communis (Carmencita rot) was used for all experiments. The seeds were treated as following: The *Ricinus communis* seeds were put into a beaker, immersed in ddH_2O and incubated at 28°C overnight. 2 % agarose was used as culture medium for seeds. 2 g of agar was added to 100 ml distilled water, autoclaved and poured into sterilized plastic plates. When the agar was solidified, they were stored in a cold room at 4 C. Next day, the seeds were washed three times with ddH_2O and then incubated in 0.3% Chinosol (Fluka) for 12 minutes. After that, the seeds were washed three times with sterile water and incubated in sterile water for 1 hour. Then the seeds were washed 2 times with sterile water and then placed on a 2% agar plate.

The agar plate was put in a 28°C incubator and kept in dark for 48 hours. After that, the seeds were transferred to a breeding pod. The pod was supplied with 0.5 mM $CaCl_2$. The compressed air was supplied with moderate air bubbles from the bottom for the growing seedlings. The breeding pod was kept in a dark, 28°C incubator. Some of the seeds were used for RNA extraction or fixed with FAA (fixation solution, see *'in situ* hybridization'). These samples were collected from two days old seedlings. Accordingly, samples of 3-, 4-, 5- and 6 days old seedlings were collected.

B.2.2 Preparation of Plasmid DNA by Alkaline Lysis

Plasmid DNA was isolated from small-scale (1-3 ml) or from large-scale (500 ml) bacterial cultures by treatment with alkali and SDS. The small-scale preparation method and large-scale method were described respectively by Birnboim (Birnboim and Doly, 1979) and Ish-Horowicz (Ish-Horowicz and Burke, 1981).

B.2.2.1. Minipreparation

A single colony of transformed *E.coli* was inoculated to 2 ml of LB medium containing the appropriate antibiotic. The culture was incubated at 37° C with vigorous shaking overnight.

Then 1.5 ml of the culture was poured into a microfuge tube and centrifuged at maximum speed for 1 min at 4°C. Afterwards the medium was removed and the pellet was resuspended in 100 µl of ice-cold GTE (50 mM glucose, 25 mM Tris, 10 mM EDTA, pH 8.0). 200 µl of freshly prepared alkaline lysis solution was added to the bacterial suspension. Then the content was mixed by inverting the tube rapidly four times. The tube was stored at room temperature for 5 minutes. Subsequently 150 µl of 3M KOAc was added to the tube. The content was mixed by inverting the tube rapidly four times. Afterwards, the tubes were stored on ice. Subsequently the bacterial lysate was centrifuged at maximum speed for 5 minutes at 4°C and the supernatant was transferred to a fresh tube. The nucleic acids were precipitated from the supernatant by adding 0.8 volumes of isopropanol at room temperature. The solution was mixed by vortexing and then stored for 5 minutes at room temperature. Then the nucleic acids were precipitated by centrifugation at maximum speed for 5 minutes at 4°C. The supernatant was removed and the tubes were put in an inverted position on a paper towel. The pellet was air-dried. Afterward 1 ml of 70% ethanol was added to the pellet and the closed tube was inverted several times. The DNA was recovered by centrifugation at maximum speed for 2 minutes at 4°C and then all of the supernatant was removed. The open tubes were put at room temperature until the ethanol had evaporated and no fluid was visible in the tube any more (5-10 minutes). The nucleic acids were dissolved in 50 µl of TE (pH 8.0) containing 20 µg/ml DNase-free RNase A (pancreatic RNase). The solution was gently vortexed for a few seconds. The DNA solution was stored at -20°C.

B.2.2.2. Maxipreparation

250 ml of LB medium containing the appropriate antibiotic in a 1-liter flask were inoculated with 10 ml of a small-scale liquid culture grown from a single colony. The culture was incubated overnight at 37°C with vigorous shaking (300 cycles/minute on a rotary shaker). Then the bacterial cells were harvested by centrifugation at 3000x*g* for 15 minutes at 4°C. The supernatant was discarded. The bacterial pellet was resuspended in 100 ml of ice-cold GTE. The bacterial cells were colleted by centrifugation at 3000x*g* for 15 minutes at 4°C. The pellet of bacteria was stored in the centrifuge bottle at -20°C. Then the frozen bacterial cell pellet was thawed at room temperature for 5-10 minutes. The pellet was then resuspended in 10 ml of ice-cold GTE and 1 ml of freshly prepared lysozyme (10 mg/ml) was added. After that 20 ml of freshly prepared alkaline lysis solution was added. The

centrifuge bottle was closed and the content was mixed thoroughly by gently inverting the bottle several times. The bottle was incubated for 5-10 minutes at room temperature. Subsequently, 20 ml (15 ml) of ice-cold 3M KOAc was added. The centrifuge bottle was closed and the content was gently mixed well by swirling the bottle several times. The bottle was placed on ice for 10 minutes. Later the bacterial lysate was centrifuged at 20,000x*g* for 30 minutes at 4° C. The clear supernatant was transferred into a graduated cylinder. The pellet remaining in the centrifuge bottle was discarded. After that the volume of the supernatant was measured. The supernatant was transferred to a fresh centrifuge bottle. 0.8 volume of isopropanol was added to the supernatant. The content was well mixed and the bottle was put for 10 minutes at room temperature. Then the nucleic acids were recovered by centrifugation at 12,000x*g* for 15 minutes at room temperature. After that the supernatant was decanted carefully and the open bottle was inverted on a paper towel to allow the last drops of supernatant to drain away. The pellet and the walls of the bottle were rinsed with 70% ethanol at room temperature. The ethanol was drained off. The inverted, open bottle was placed on a pad of paper towels for a few minutes at room temperature. Then the pellet of nucleic acid was dissolved in 3 ml of TE (pH 8.0). After that the plasmid was checked by restriction enzyme digestion followed by gel electrophoresis.

B.2.3 Agarose Gel Electrophoresis

Nucleic acids that were subjected to electrophoresis through agarose gels were detected by staining with Ethidium bromide and visualized by illumination with UV light.

Agarose gel electrophoresis was used to visualize and isolate DNA molecules following PCR amplification or restriction digestion. Agarose (0.8 %) was dissolved in TBE buffer (45 mM Tris, 45 mM borate, 1 mM EDTA) by heating in a microwave. After cooling, 1 µl of a 1 mg/ml ethidium bromide solution was added per 50 ml gel solution and the gel was poured. Gels were routinely run at 80-100 V for 1 hours, depending on the size of the examined DNA fragment or on the degree of band separation required.

B.2.4 DNA digestion with restriction enzymes

Digestions with restriction enzyme are performed by incubating double-stranded DNA molecules with an appropriate amount of restriction enzyme, in a buffer recommended by the supplier, and at the optimal temperature for the specific enzyme. The optimal buffer for the reaction varies for different enzymes. Typical digestions include one unit of enzyme per microgram of DNA, since one enzyme unit usually is defined as the amount of enzyme needed to completely digest one microgram of double-stranded DNA in one hour at the appropriate temperature. Usually the reactions are incubated for 1-3 hours at the optimal temperature for enzyme activity, typically 37°C, to ensure complete digestion.

Reagents:

Sample DNA	100 ng - 5 µg
Enzyme specific buffer 10X	2 µl
Restriction enzyme, 10 U/µl	1 µl
ddH_2O	To 20 µl

The reaction mix was incubated for 1-3 hours, depending on the required degree of digestion. The incubation temperature was dependent on the characters of the restriction enzyme.

B.2.5. DNA Extraction from agarose gel

In order to extract DNA fragments from an agarose gel, the Agarose Gel DNA Extraction Kit from Roche was used. The appropriate size of DNA fragments which can be isolatedwith the agarose gel DNA fragment isolation kit ranges from 400 bp – 5 kb with a reproducible recovery of about 80%.

The DNA of interest was separated in an agarose gel of suitable concentration. After sufficient separation the interesting DNA fragment was cut out with a sharp scalpel or razor blade. The gel slice was transferred to a pre-weighed reaction tube. Then 300 µl of agarose solubilisation buffer (vial 2) was added to per 100 mg of agarose gel. Subsequently, the 10 µl of silica suspension (vial 1) was added to the sample. Then the mixture was incubated for 10 minutes at 56-60° C and vortexed every 2-3 minutes. The mixture was centrifuged in a tabletop centrifuge for 30 seconds at maximal speed and the

supernatant was discarded. After that the matrix containing the DNA was resuspended with 500 µl of nucleic acid binding buffer (vial 3, green cap) on a vortex mixer. The mixture was centrifuged for 30 seconds at maximal speed and the supernatant was discarded as before. Then the pellet was washed with 500 µl of washing buffer (vial 4, blue cap). The mixture was centrifuged for 30 seconds at maximal speed and the supernatant was discarded. This step was repeated once. After removing all the liquid with a pipette, the tube was inverted on an adsorbent tissue and air-dried at room temperature for 15 minutes. 50 µl of ddH_2O was used for the elution of DNA. The matrix was eluted in two elution cycles with 2 × 25 µl ddH_2O. The mixture was incubated for 10 min at 56 to 60°C and vortexed every 2-3 minutes. The matrix was centrifuged at maximum speed for 30 seconds and the DNA containing solution was transferred to a new tube.

B.2.6. DNA fragments ligation

DNA ligations were performed by incubating DNA fragments with appropriately linearized cloning vector in the presence of buffer, ATP, and T4 DNA ligase.

Reagents:

Linearized pBluescriptII KS+, 100ng/µl	1 µl
DNA fragment to be inserted	
Ligase buffer, 10X	1 µl
PEG 4000, 50%	1 µl
Ligase, 1U/µl	1 µl
ddH_2O	To 10 µl

Some restriction enzymes generate fragments that have a single-stranded "tail" at both ends, called sticky-end. The tails on the fragments generated at a given restriction site are complementary to those on all other fragments generated by the same restriction enzyme. DNA ligase can insert DNA restriction fragments into replicating DNA molecules producing recombinant DNA. Sticky-end ligation of restricted pBlueScriptII vector and desired fragment was carried out using T4 DNA ligase. The standard insert to vector ratio was 3:1. The reaction mix was incubated for 2 hours 22°C. Afterwards, the reaction mix was treated at 65°C for 15 minutes to inactivate the ligase. 2 µl of the ligation product was used to transform DH5α competent cells.

B.2.7. Preparation and Transformation of Competent *E. coli* Cells

There are two main methods to prepare competent bacterial cells for transformation, the chemical method (Cohen *et al.*, 1972.) and the electroporation method (Chassy and Flickinger, 1987). The following chemical method developed by Inoue *et al.* (1990) was derived from the calcium chloride method, which was widely used before.

Transformation buffer was prepared as follows: 0.5 M PIPES (pH 6.7) (piperazine-1, 2-bis [2-ethanesulfonic acid]) was prepared by dissolving 15.1 g of PIPES in 80 ml of sterilized H_2O. The pH of the solution was adjusted to 6.7 with 5 M KOH and sterilized H_2O was added to a final volume of 100 ml. Then the transformation buffer was prepared by dissolving all the solutes listed below in 800 ml of pure H_2O and adding 20 ml of 0.5 M PIPES (pH 6.7). The volume of Inoue transformation buffer was adjusted with pure H_2O to 1 liter. The solution was sterilized by filtration through a disposable pre-rinsed filter (0.22 µm pore size).

E.coli DH5α competent cells were prepared as follows: A single bacterial colony (2-3 mm in diameter) was picked from a LB plate that had been incubated for 16-20 hours at 37° C. The colony was transferred into 3 ml of SOB medium (LB may be used instead) in a 13 ml polypropylene tube. The culture was incubated overnight at 37° C with vigorous shaking (250-300 rpm). In the evening, 1 ml of this starter culture was used to inoculate to 100 ml of SOB in a 300 ml flask. The flask was incubated overnight at 18-22° C with moderate shaking. When the OD_{600} of the culture reached 0.6, the culture was transferred to an ice-water bath for 10 minutes. Then the cells were collected by centrifugation at 2500x*g* for 10 minutes at 4° C. The medium was poured off and then the cells were gently resuspended in 32 ml of ice-cold transformation buffer. Then the cells were precipitated by centrifugation at 2500x*g* for 10 minutes at 4° C. After that the medium was poured off. The cells were gently resuspended in 8 ml of ice-cold transformation buffer. Then 0.6 ml of DMSO was added to the resuspended cells. The bacterial suspension was mixed by swirling and then put on ice for 10 minutes. After that the suspension was dispensed in 200 µl portions into chilled, sterile microfuge tubes. The competent cells were immediately frozen by immersing the tightly closed tubes in a bath of liquid nitrogen. Competent cells were stored at -80° C until needed.

After thawing competent, the plasmid DNA was added. The tubes were gently swirled several times to mix their content. Then the tubes were kept on ice for 30 minutes. After that the tubes were put on a rack and placed in a preheated 42° C water bath exactly 30 seconds and without shacking. Then the tubes were rapidly put to an ice bath for 1-2 minutes. Then 800 µl of SOC medium was added to each tube. The cultures were incubated in a 37° C shaking incubator for 45 minutes to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. Afterwards the appropriate volumes of transformed competent cells were plated onto LB agar plates containing the appropriate antibiotic. The plates were stored at room temperature until the liquid had been absorbed and then incubated at 37° C. Transformed colonies appeared within 12-16 hours.

B.2.8. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a technique, which can amplify the desired DNA fragment *in vitro*. PCR can start from even one molecule of DNA, and as a consequence many conventional analytical fractionation techniques are now many times more sensitive than before. The amplification of DNA fragments by PCR was performed in a PCR machine. The cycling protocol consisted of 25-30 cycles of three-temperatures: denaturation at 95°C, primer annealing at 55°C, and primer extension at 72°C.

Reagents:

template DNA	10-20 ng
10X PCR buffer	5 µl
10 mM dNTPs	1 µl
Forward primer, 10 pmole/µl	1 µl
Reverse primer, 10 pmole/µl	1 µl
Taq DNA polymerase, 5 U/µl	1 µl
Add ddH20 to final volume 50 µl	

PCR was performed as follows: the reagents were added to a 0.2 ml PCR tube. Then two drops of mineral oil were added to cover the reaction mix, and the tube was placed in the wells of a Thermal Cycler, which had been pre-heated to 94°C. DNA was amplified in 34

cycles of 94 °C for 30 sec, annealing at 56 °C for 30 sec and polymerization at 72 °C for 1-2 min; followed by a final extension step of 72° C for 10 min. The annealing temperature was dependent on the primers. The time for polymerization depended on the size of desired fragment. An aliquot of 10 µl was analyzed on a 1-2% agarose gel.

B.2.9. Cloning PCR Products with T-Vector

Degenerated PCR- and RACE-amplified products were cloned into T-Vectors using the TA-cloning kit (Promega). This method allows direct cloning of PCR products that were amplified by *Taq* polymerase. Normally *Taq* polymerase adds a single deoxyadenosine to the 3' ends of PCR products. As the linearized pGEM-T vector supplied with the kit has 3'deoxythymidine overhangs, ligation of insert and vector proceeds very efficiently. After PCR amplification, PCR products were checked by agarose gel electrophoresis and cut out of the gel using a clean scalpel and then extracted from the gel by using activated silica-based gel extraction kit (Roche Diagnostics GmbH, Mannheim, Germany).

Reagents:

PGEM-T, 50 ng/µl	1 µl
Purified PCR product	3 µl
Ligase buffer, 10X	1 µl
PEG 4000, 50%	1 µl
Ligase, 1 U/µl	1 µl
ddH_2O	To 10 µl

The ligation reaction was incubated for 2 hours at 22° C and then incubated at 65° C for 15 minutes. Following incubation, an aliquot from the ligation reaction was used to transform *E. coli* DH5α competent cells. To a 200-µl vial of *E. coli* DH5α competent cells, 2 µl of the ligation reaction was added and mixed by stirring with the pipette tip. The remaining ligation reaction was stored at -20° C. The vial was incubated for 30 minutes on ice. Then the cells were subjected to a heat shock at 42° C for 30 seconds, and immediately placed on ice for 1-2 minutes. After addition of 1ml of SOC medium. The vial was incubated at 37° C for 1 hour in a shaking incubator at 250 rpm. After that, 200 µl of transformed competent cells was plated on LB agar plates that were supplemented with IPTG, 0.4 % X-

gal and 50 µg/ml ampicillin, and incubated at 37° C overnight. Then the plates were stored at 4° C for 2-3 hours. Single white colonies were chosen for PCR screening.

B.2.10. Purification of total RNA from Plant Tissues

By this technique, plant tissue was homogenized and RNA was purified from the lysate by extraction with phenol-chloroform at reduced pH. Many samples were processed simultaneously and speedily. The desired tissues were isolated by dissection and placed immediately in liquid nitrogen. Approx. 200 mg of the frozen tissue was transferred to a mortar containing liquid nitrogen and pulverized by using a pestle. The tissue was kept frozen during pulverization by the addition of liquid nitrogen. The powdered tissue was transferred to a snap-cap tube containing 0.5 ml of RNA extraction buffer, 0.5 ml of acid Phenol-chloroform and about 0.3 ml of glass beads. The tube was vortexed for 2 minutes at room temperature. After that the tube was centrifuged at 15,000rpm for 5 minutes at 4° C and then the upper aqueous phase containing the extracted RNA was transferred to a fresh tube. Then equal volume of chloroform was added and the tube was capped and mixed well. Then the tube was centrifuged and the upper aqueous phase was transferred to a new tube. The solution was extracted with an equal volume of chloroform. Afterwards the upper aqueous phase was transferred to a new tube, 1/3 volume of 8M LiCl was added and stored overnight at 4° C. Then the solution was centrifuged for 1 hour at 4° C. The supernatant was poured off and the pellet was washed with cold 80% ethanol and air-dried. Then the pellet was dissolved in 400 µl of 0.3M NaOAc (pH 5.2) and an equal volume of chloroform was added to the RNA solution. The solution was mixed well and centrifuged for 5 minutes at 4° C. After that the upper aqueous phase was transferred to a new tube. 1 ml of cold 100% ethanol was added. The tube was kept for 1 hour or more at -20° C. Then the total RNA was centrifuged at 15,000 rpm for 30 minutes at 4° C. The supernatant was carefully decanted and the RNA pellet was washed with 1 ml cold 80% ethanol. The ethanol was carefully removed and the pellet was air-dried and dissolved in 50-100 µl DEPC-H_2O.

B.2.11. RNA gel electrophoresis through Agarose Gels

The first stage of northern blotting and hybridization is the separation of RNAs on agarose gels. This protocol uses formaldehyde to denature the RNA and ethidium bromide to stain RNA. RNA electrophoresis through agarose gels containing 2.2 M formamide separates the formaldehyde-RNA-ethidium adducts. RNA is labile and RNase exists everywhere; therefore RNA must be handled very carefully. All reagents used in this protocol were prepared with DEPC-treated H_2O. The method was described by Lehrach (Lehrach *et al*., 1977).

To prepare 50 ml of a 1% agarose gel, 0.5 g of agarose was added to 40 ml of DEPC-H_2O and melted in a microwave oven. After cooling down the gel to 60° C, 5 ml of 10x MOPS and 4.5 ml of formaldehyde was added and poured into a gel tray. The denaturation reaction was prepared as follows.

Reagents:

RNA (up to 20 µg)	2.0 µl
10x MOPS electrophoresis buffer	2.0 µl
Formaldehyde	4.0 µl
Formamide	10.0 µl
ethidium bromide (200 µg/ml)	1.0 µl

The RNA solutions were incubated for 10 minutes at 85° C then chilled for 10 minutes in ice water and centrifuged for 5 seconds to spin down the fluid to the bottom of the microfuge tubes. 2 µl of 10x formaldehyde gel loading buffer was added to each sample and the tubes were kept on ice. The agarose/formaldehyde gel was installed in a horizontal electrophoresis tank. 1x MOPS electrophoresis buffer was added until the gel was covered with a layer of 1 mm buffer. After pre-running the gel for 5 minutes at 50 V, the RNA samples were loaded into the wells of the gel. Then the gel was run at 50 V until the bromophenol blue had migrated approx. for 8 cm (4-5 hours). After that sample RNA was monitored by placing the gel under the UV illumination. The gel was used for Northern blot analysis. Afterwards the RNA gel was rinsed with DEPC-treated H_2O. Then the gel was soaked for 20 minutes in 5x gel volumes of 0.01 N NaOH/3 M NaCl. Without delay, the partially hydrolyzed RNA was transferred to a positively charged nylon membrane by

capillary transfer. Then a 5 to 8 cm high staple of paper towels was stacked in a glass dish. The size of paper towels was cut slightly larger than the gel. Four sheets of Whatman 3MM paper were placed on the top of the paper towels. The fifth sheet was wetted in transfer buffer prior to placing it on top of the paper stack. Then the nylon membrane was carefully placed on top of the chromatography paper without any bubbles between membrane and chromatography paper. Afterwards the gel was carefully placed onto the membrane. Three sheets of Whatman 3MM chromatography paper were wetted with alkaline transfer buffer and placed on top of the gel. A glass dish was filled with alkaline transfer buffer and placed next to the transfer pyramid. Then two layers of Whatman 3MM chromatography paper were soaked in alkaline transfer buffer and placed on the transfer pyramid and glass dish to form a bridge. A glass plate was placed on the top of the transfer pyramid (Figure 1). After 4 hours, the chromatography papers were removed and the membrane was transferred into 300 ml 6X SSC buffer and very carefully agitated for 5 minutes. Then the membrane was removed from the 6x SSC and excess fluid was drained away. It was put, RNA side upward, on a dry sheet of Whatman 3MM paper for a few minutes. The RNA samples were fixed by baking in an 80° C vacuum oven for 2 hours.

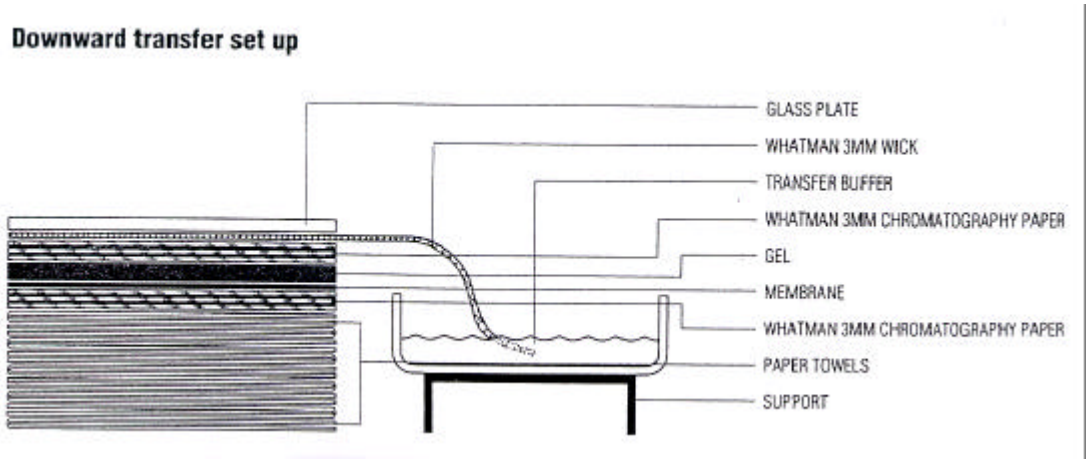


Figure 4 : The structure of downward capillary transfer pyramid. In this arrangement, capillary transfer of nucleic acids from an agarose gel to solid support is achieved by drawing the transfer buffer from the reservoir downward through the gel into a stack of paper towels.

B.2.12. Synthesis of digoxigenin-labelled RNA probe

The synthesis of digoxigenin labelled probe requires approximately 1 µg of DNA template. The DNA fragment of interest was subcloned into an appropriate vector (e.g., pBluescriptII

KS+, pGEM). The template DNA was linearized by cutting with a single restriction enzyme. The appropriate RNA polymerase was used to generate complementary RNA (cRNA) transcripts that can be used as RNA probes for the transcribed gene. The method was described by Krieg (Krieg and Melton, 1987).

The reaction mixture for the synthesis of riboprobe consisted of unlabelled mono-nucleotides, digoxigenin-labelled UTP, DNA transcript, plus the appropriate RNA polymerase. The reagents were added to a microcentrifuge tube:

Reagents:

DNA template: PCR amplified, 0.2 µg or Plasmid 1.0 µg in 1µl	1.0 µl
10x Transcription buffer	2.0 µl
10x DIG Label Mix	2.0 µl
RNase Block, 40 U/µl	1.0 µl
T7, T3, or SP6 RNA polymerase, 40 U/µl	2.0 µl
TE DEPC-H_2O	To 20 µl

Then the reaction mix was incubated for 2 h at 37°C. Afterwards 1 µl of 10 U/µl DNase I (RNase-free) was added to the reaction mix and incubated at 37°C for 15-30 minutes. After that, 2 µl of EDTA (0.5 M pH 8.0), 2.5 ml of 3M NaOAc (pH 5.2), 75 µl of 100% cold ethanol was added to the reaction mix and the cRNA probe was precipitated at -80°C for 30 minutes. Afterwards the RNA probe was centrifuged at 4°C for 15 min at 15,000x*g*. The supernatant was removed and 100 µl of 80% EtOH was added. The RNA probe was centrifuged at 4°C for 2 min at 15,000x*g*. The pellet was centrifuged for 30 s after the supernatant was removed. After that, the remaining liquid was removed and the pellet was resuspended in 50 µl of DEPC-H_2O (2 µl were removed for gel analysis). Then an equal volume of formamide was added to the RNA probe and mixed well. The RNA probe was kept at –20°C.

B.2.13. Northern Blot Analysis

The non-radioactive DIG system allows specific detection of DNA or RNA with colorimetric (NBT/BCIP) or chemiluminescent (CDP-Star or CSPD) detection. The advantages of non-radioactive labeling and detection of DNA and RNA with the DIG system, which unifies

high sensitivity and low background, have been utilized in a variety of applications. Hybridized membranes can be either detected immediately or stored for later detection. The fixed membrane was put into a hybridization tube, to which 20 ml of prehybridization solution was added. The prehybridization solution was prepared as followed:

Reagent

Formamide	25 ml
Blocking reagent	2 g
20X SSC	12.5 ml
10% Lauryl-Sarcosine	0.5 ml
10% SDS	100 µl

DEPC-H_2O was added to a final volume of 50 ml. The solution was warmed at 80°C and mixed until the entire blocking reagent was dissolved.

The prehybridization was performed in a hybridization chamber at 60-65°C for 3 hours. After prehybridization, the prehybridization solution was poured off and 10 ml of warmed new prehybridization solution and digoxigenin-labelled probe was added. The hybridization was performed in a hybridization chamber at 60-65°C overnight. After that, the membrane was removed from the hybridization tube and washed in of 200 ml 2xSSC 0.1% SDS at room temperature for 15 minutes. After washing with 2xSSC, the membrane was washed twice in 200 ml of 0.1xSSC 0.1% SDS at 65°C for 15 minutes. Subsequently, the membrane was put into washing buffer for 5 minutes and then incubated in 50 ml of blocking solution for 60 minutes. Then 1:10000 Anti-Dig-AP was added to the blocking solution. After that, the membrane was incubated with Anti-Dig-AP/blocking solution for 60 minutes. Afterwards the membrane was washed twice in 200 ml of washing buffer for 15 minutes and then put into 50 ml of detection buffer for 5 minutes. After addition 2 µl of CDP Star, the membrane was incubated in the CDP Star/detection buffer at 30°C for 5 minutes. Afterwards the membrane was removed from the CDP Star/detection buffer and the excess detection buffer was drained away. The membrane was put on a Whatman chromatography paper and wrapped with a sheet of Saran Wrap and exposed to KODAK Biomax MR film for 5 minutes to 24 hours at 30°C to obtain an image.

B.2.14. RT-PCR

Traditional hybridization techniques are not sensitive enough to detect some products of genes that are expressed at a low level. By applying the reverse transcription-polymerase chain reaction technique this problem can be overcome. Reverse transcriptase can convert RNA to a first strand of complementary DNA (cDNA). The sequence is amplified by PCR using cDNA as template. Consequently, the product's copy number is much higher than that of the original RNA. The result can be easily checked by agarose gel electrophoresis or southern hybridization. The genomic DNA was eliminated by treatment with DNAse I. The DNAse I reaction mix was prepared as follows.

Reagents:

Total RNA	10 µg
DNAse I, 10 U/µl	2 µl
10 X DNAse I buffer	4 µl
Add DEPC-H_2O to 40 µl	

The reaction mix was incubated at 37°C for 20 minutes. 5 µl of 3M NaOAc and 5 µl of DEPC-H_2O were added to the reaction mix. 50 µl of phenol/chloroform was added to the reaction mix. After mixing the solution was centrifuged at 15,000xg at 4°C for 5 minutes. Then the upper aqueous phase was transferred to a new tube and 50 µl of chloroform was added. The reaction mix was vortexed and centrifuged at 15,000xg at 4°C for 5 minutes. The upper aqueous phase was transferred to a new tube and 150 µl of cold absolute ethanol was added. Then the reaction mix was stored at -20°C for 1 hour. Afterwards the reaction mix was centrifuged at 15,000xg, 4°C for 15 minutes. The supernatant was carefully poured off. The RNA pellet was washed with 200 µl of 80% ethanol and centrifuged at 15,000xg, 4°C for 5 minutes. The supernatant was poured off and the RNA pellet was air-dried. Then the pellet was dissolved in 40 µl of DEPC-H_2O. After that, 4 µl of oligo-dT primer (10 pmol/µl) was added into 20 µl of RNA and warmed at 65°C for 5 minutes. Then the RNA/Oligo-dT primer mix was kept at room temperature for 10 minutes. The reverse transcription mix was prepared as listed.

Reagents:

5X reverse transcription buffer	8 µl
10 mM dNTP mix	4 µl
RNase inhibitor 40 U/ µl	2 µl
MMLV Reverse transcriptase 200 U/ µl	2 µl

The reverse transcription mix was added to the RNA/Oligo-dT primer mix and first incubated at 37°C for 10 minutes, then at 42°C for 90 minutes. Afterwards the reverse transcription was stopped by heating at 65°C for 20 minutes. Then 60 µl of ddH_2O was added to the cDNA. The cDNA was stored at -20°C. The PCR mix was prepared as listed.

Reagents:

First strand cDNA	1 µl
10 X PCR buffer	5 µl
10 mM dNTPs	1 µl
Forward primer, 10 pmol/µl	1 µl
Reverse primer, 10 pmol/µl	1 µl
Taq DNA polymerase 5 U/µl	1 µl
ddH_2O	40 µl
Total volume	50 µl

The following thermal profile was used:

94°C	30 seconds	30 PCR cycles
x-°C*	30 seconds	
72°C	Y minutes**	
72°C	7 minutes	

*The annealing temperature depends on the primers.

**The extension time depends on the size of the DNA fragment. As a rule of thumb, 1 min is sufficient for the extension of a 1kb DNA fragment.

B.2.15. Rapid amplification of cDNA ends (RACE)

In order to clone the full length of sucrose phosphate phosphatase gene of *Ricinus communis*, 5'- and 3'-RACE were performed. RACE uses the reverse transcription product as template to amplify the 5'-and 3'-end of cDNA.

Reagents:

Endosperm total RNA	1 µg
10 mM Oligo-dT	1 µl
10 mM TS primer	1 µl
5X First-Strand buffer	2 µl
DTT (20 mM)	1 µl
10mM dNTPs Mix	1 µl
BD PowerScript Reverse Transcriptase	1 µl

For reverse transcription, 10 pmole of Oligo-dT(30) and 10 pmole of TS primer were added to 1 µg of endosperm total RNA. DEPC H_2O was added to the mix to a final volume of 5 µl. Then the RNA/primer mixture was incubated at 70° C for 2 min. It was put on ice for 2 min and then briefly spun to collect the samples. Afterwards, 2 µl of 5X First-Strand buffer, 1 µl of DTT (20 mM), 1 µl of 10mM dNTPs Mix and 1 µl of BD PowerScript Reverse Transcriptase were added to RNA/primer mix. All components were mixed by gently pipetting and then spun down to the bottom of the tube. After that the reaction mix was incubated at 42° C for 1.5 hr in an air incubator. After that, 100 µl of Tricine-EDTA Buffer was added to the reverse transcription product. Afterwards the mix was heated at 72° C for 7 min. The diluted cDNA can be used as the template for subsequent PCR.

B.2.15.1. 5'-RACE

In order to obtain the 5'-end sequence of the SPP gene, 5'-RACE was performed. The PCR was prepared as follows:

Reagent:

cDNA (for RACE)	2.5 µl
10X BD Advantage 2 PCR Buffer	5 µl
dNTPs Mix (10 mM)	1 µl
10 mM Gene specific primer	1µl
10 mM TS-PCR prime	1µl
50X BD Advantage 2 Polymerase Mix	1 µl
PCR-Grade Water	38.5 µl

The components were mixed in a 0.2-ml PCR tube. The reaction mixture was overlaid with 2 drops of mineral oil and the cap was firmly closed. Then a 3-stage touch down PCR program was carried out. The fist stage of this PCR program is a 5 cycle PCR as follows: 94° C for 30s, 72° C for 3 min; the second stage is a 5 cycle PCR: 94° C for 30s, 70° C for 30s, 72° C for 3 min; the last stage is a 25 cycle PCR: 94°C for 30s, 68° C for 30s, 72° C for 3 min. The PCR product was checked by agarose gel electrophoresis.

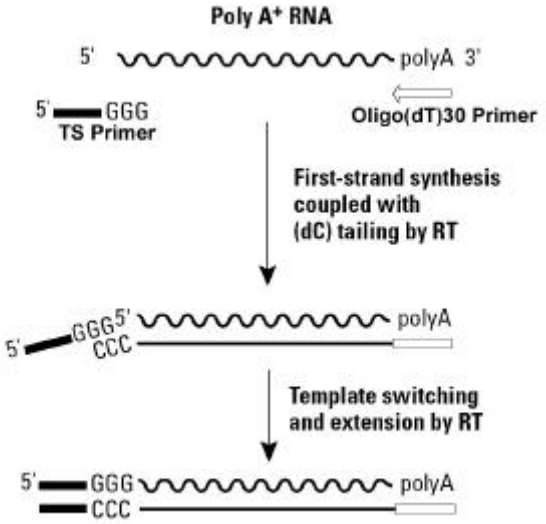


Figure 5 : Mechanism of BD SMART™ cDNA synthesis. First-strand synthesis is primed using a modified oligo (dT)30 primer. After reverse transcriptase reaches the end of the mRNA template, it adds several dC residues. Then the Template switch (TS) primer anneals to th e tail of the cDNA and serves as an extended template for BD PowerScript RT.

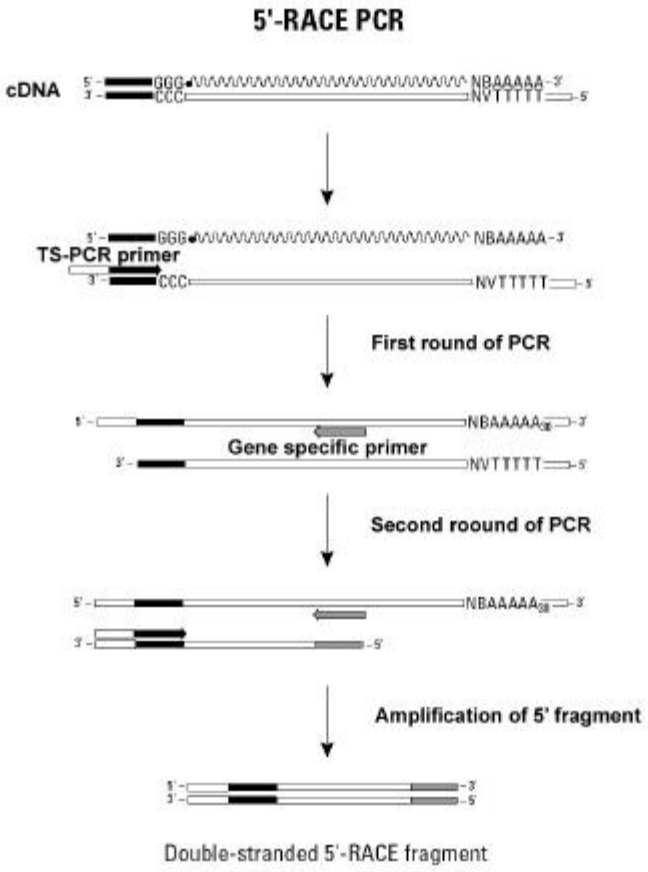


Figure 6 : Detailed mechanism of the 5'-RACE reactions.

B.2.15.2. 3'-RACE

In order to obtain the 3'-end sequence of the SPP gene, 3'-RACE was performed. The PCR of a final volume of 50 µl consisted of the following components:

cDNA (for RACE)	2.5 µl
10X BD Advantage 2 PCR Buffer	5 µl
dNTPs Mix (10 mM)	1 µl
10 mM Gene specific primer	1µl
10 mM TS-dT prime	1µl
50X BD Advantage 2 Polymerase Mix	1 µl
PCR-Grade Water	38.5 µl

The components were mixed in a 0.2-ml PCR tube. Then 2 drops of mineral oil were added to the PCR tube and the cap was firmly closed. Then a 3-stage touch down PCR program was carried out. The fist stage of this PCR program is a 5 cycle PCR as follows: 94°C for 30s, 72°C for 3 min; the second stage is a 5 cycle PCR: 94°C for 30s, 70°C for 30s, 72°C for 3 min; the last stage is a 25 cycle PCR: 94°C for 30s, 68°C for 30s, 72°C for 3 min. The PCR product was checked by agarose gel electrophoresis.

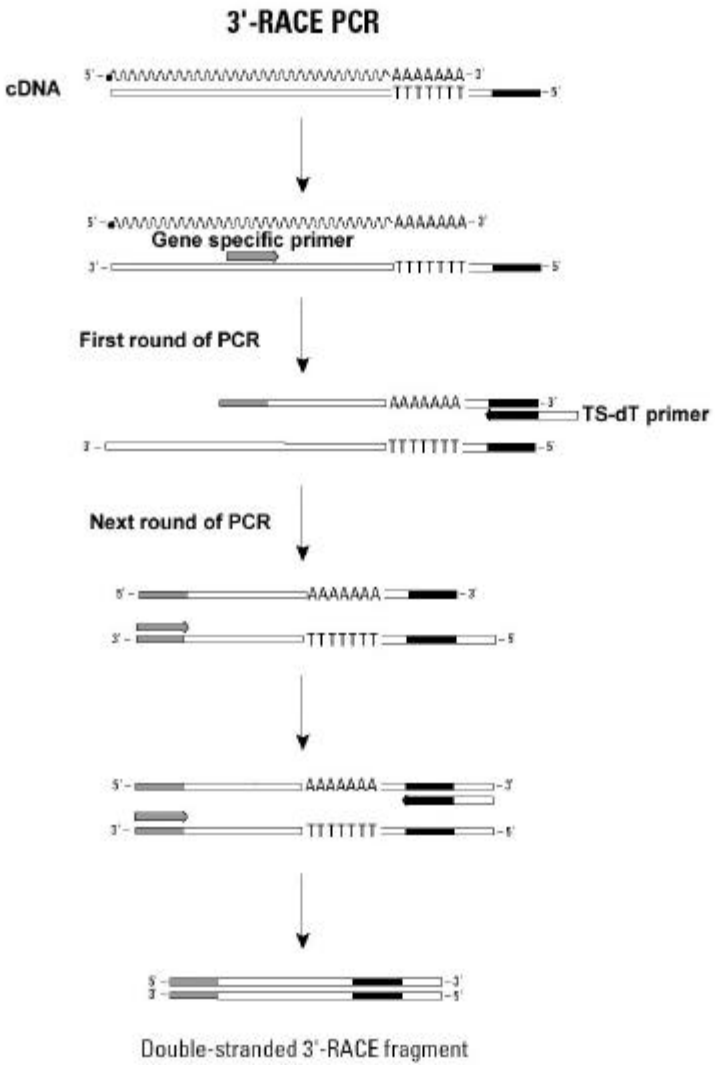


Figure 7 : Detailed mechanism of the 3'-RACE reactions.

B.2.16. *In Situ* Hybridization

By *in situ* hybridization (ISH) a target DNA or RNA sequence in a tissue section can be detected using a labelled nucleic acid probe. Since the method utilizes the sequence-specific formation of double strand target/probe duplexes, performing an *in situ* hybridization is fundamentally the same as doing Northern or Southern blots. ISH is still the only hybridization technique, which allows cellular and subcellular localisation of the target, since other methods destroy cellular integrity (for example, normal southern or northern hybridization) or retain anatomical details only at a low resolution (for example, tissue prints).

After removal from the plant, tissues must be 'fixed' to keep the structure as naturally as possible. The ideal fixative prevents tissue autolysis and makes the tissue resistant to damage during subsequent processing, embedding and sectioning stages. The optimal fixation does not dissolve tissue components and is not detrimental to the tissue component being studied.

Fixatives are divided into two main groups, coagulant and non-coagulant fixatives. Coagulant fixatives, such as ethanol, are of very limited use in ISH. Ethanol dehydrates, coagulates and precipitates cellular proteins, nucleic acids and carbohydrates. No covalent bonding occurs between the ethanol fixative and tissue components. Consequently, macromolecules such as mRNA and DNA are not firmly anchored within the tissue and maybe lost during later processing procedures.

The most commonly used fixatives for ISH are the non-coagulant, cross-linking aldehydes formaldehyde, paraformaldehyde and glutaraldehyde. These compounds fix tissue by introducing cross-links between different tissue components, such as proteins, nucleic acids and lipids. Cross-linked mRNAs are stable and securely retained during subsequent tissue processing.

Formaldehyde is the most common fixative. It is easy to prepare and can be stored for months at room temperature. Formaldehyde is water soluble and at low concentrations (4%) exists mainly as the monomer HO-(H_2CO)-H. Formaldehyde causes only small changes in tissue volume during fixation and the resulting morphological structures are

clearly visible in the light microscope. It penetrates small tissue samples rapidly, but large tissue pieces may not be fully fixed.

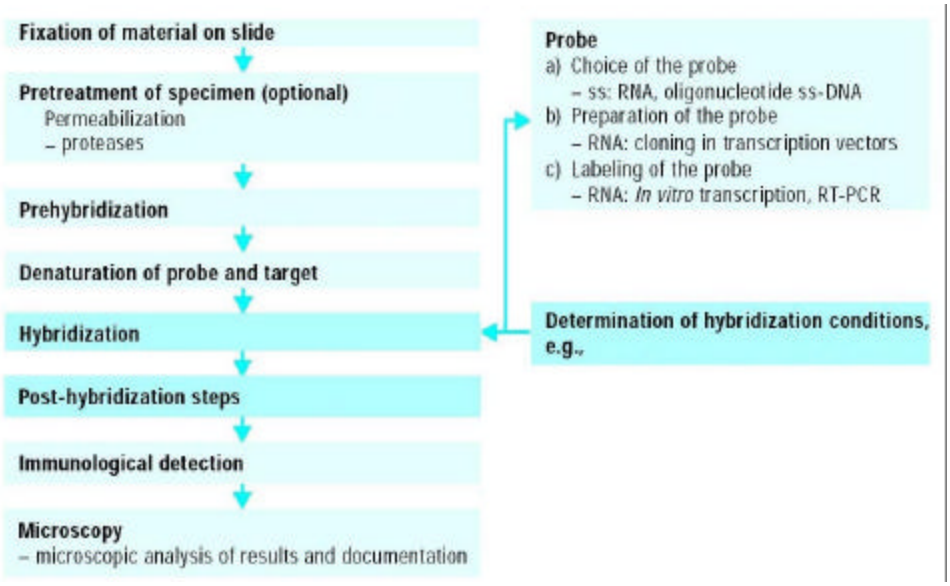


Figure 8: The flowchart of *in situ* hybridization.

Fixed tissues need to be embedded in a solid medium before section (2 - 20 µm). During sectioning the sample can be supported by embedding in paraffin wax. Tissues are first dehydrated through a serious of treatment with water/alcohols and then 'cleared' with an antemedium such as xylene or Histoclear. Paraffin and ethanol are immiscible, whereas antemedia are miscible with both compounds. After clearing, the tissues are infiltrated with paraffin wax. Thin sections can be cut from tissues infiltrated with paraffin wax.

The advantages of paraffin sections are: thin serial sections can be easily cut and afterwards stored the sections and the morphology is often better than the equivalent frozen section. This method also has some disadvantages: It requires special equipment and it takes much longer to embed tissue, since the tissue must be fixed before paraffin-infiltration.

Probes for *in situ* hybridization may be either DNA or RNA. Most researchers now work with RNA probes ("riboprobes"). Working with riboprobes is somewhat more difficult, because of the labile nature of RNA itself and the ubiquitous presence of RNases. Most of the work of ISH must be performed in an RNase-free environment. Glass and stainless

steel slide holders for dipping slides into solutions can be wrapped in aluminium foil and baked to deactivate RNase. Slide staining dishes and Coplin jars were baked at 180° C and put a long heat-up and cool-down period to prevent the form cracking.

The mRNA was chemically cross-linked or 'fixed' in place prior to hybridizatio n. It was fixed in its undenatured state, still intimately associated with normal tissue components. Consequently, not all of the mRNA within the tissue section is available to form probe/target hybrids. This effectively reduces the maximum hybridization signal that can be obtained. For standard histology using paraffin embedding tissues were fixed in fixation solution overnight at 4°C.

Reagents:

Fixation solution (for 100ml)	
Ethanol, 100%	50 ml
Formalin, containing 37% formaldehyde	10 ml
Acetic acid	5 ml
DEPC-H_2O	35 ml

The tissue was cut from plants, pieces smaller than 5mm^3 immediately and put into the fixation solution. The tissue must be smaller than. Then the tissue was fixed within fixation solution solution for 4 h at room temperature. After ad ding the tissue to the fixation solution, the tissue was vacuum infiltrated (with a water aspirator) for 10 min then incubated in the fixation solution for 50 min. Finally, the fixation solution was renewed. The procedure of vacuum infiltration, incubation and renewal of fixation solution was repeated for three times. After 4 hr fixation, the fixation solution was renewed and the samples were kept at 4°C overnight. The dehydration and infiltration processes are listed in Table 3. Then 3:1, 1:1 and 1:3 (v/v) solution of histoclear/ paraffin were prepared for the substitution. The histoclear/paraffin mixture was kept in 57°C oven. The paraffin substitution procedures were listed in the Table 4.

Table 3 . The detail of dehydration and infiltration.

1.	50% Ethanol	60 min
2.	50% Ethanol	60 min
3.	70% Ethanol	60 min
4.	70% Ethanol	60 min
5.	70% Ethanol	60 min
6.	85% Ethanol	Overnight
7.	95% Ethanol	60 min
8.	95% Ethanol	60 min
9.	95% Ethanol	60 min
10.	100% Ethanol	90 min
11.	100% Ethanol	90 min
12.	100% Ethanol	Overnight

Sectioning and mounting to slide

The paraffin block was trimmed and sectioned at 10 µm. Tissue sections were mounted on Probe-On Plus microscope slides, and incubated at 42° C for at least 24 hours. Paraffin blocks were cut into pieces containing one tissue sample each. Tissue blocks were mounted to a plastic frame and the blocks were trimmed to a rectangular cutting face. The top and bottom edges (edges facing the knife) were parallel to get a straight ribbon. Then the paraffin block was sectioned at 10 µm. A few drops of DEPC-H_2O were dropped on the slide. Then the ribbons were floated on water on subbed microscope slides. Afterwards the Slides were heated at 42° C for 5 min on a warming tray to relax ribbons or sections; then the water was removed with a pipette or by wicking it away with a paper towel. Then the slides were dried 24 to 48 hours in an oven or on a warming tray at 42° C to ensure adherence.

Table 4. The paraffin substitution.

1.	3 EtOH: 1 Histoclear	90 min
2.	3 EtOH: 1 Histoclear	90 min
3.	1 EtOH: 1 Histoclear	90 min
4.	1 EtOH: 1 Histoclear	90 min
5.	Histoclear	90 min
6.	Histoclear	90 min
7.	Histoclear	90 min
8.	3 Histoclear: 1 Paraffin	3 hours
9.	1 Histoclear: 1Paraffin	Overnight
10.	Pure paraffin	3 days

Synthesis of riboprobe

RNA probe can be labelled with radioactive nucleotides or other non-radioactive modified nucleotides. In order to synthesize non-radioactive riboprobe, digoxigenin-UTP was used. The detail method can be found in Materials and Methods B.2.12.

Hydrolysis of riboprobe

RNA probes longer than 300 bases are not suitable for *in situ* hybridization. The length of RNA probes was reduced by hydrolysis to approximately 200 bases.

Reagents:

200 mM Na_2CO_3

200 mM $NaHCO_3$

3 M sodium acetate

Ethanol

50 µl of labelled probe RNA was transferred into a microcentrifuge tube and 30 µl of 200 mM Na_2CO_3 and 20 µl of 200 mM $NaHCO_3$ were added. The incubation time was calculated by the following formula:

$T = (L0-Lf) / (K*L0*Lf)$

L0 = starting length of probe RNA (in kb)

Lf = length of probe RNA (in kb)

K = rate constant (In this case, K = 0.11 kb/min.)

T= hydrolysis time in minute

After incubation, 10 µl of 10% acetic acid, 11 µl of 3 M sodium acetate (pH 6.0) and 300 µl of (about 2.5 volumes) cold ethanol were added to the hydrolysed RNA probe solution. Then RNA probe was incubated 4 to 16 h at -20°C. Afterwards the RNA probe was centrifuged at 4°C for 15 minutes, the supernatant was discarded and the RNA pellet was air-dried in a dust free chamber. Labelled RNA probe was resuspended in DEPC-H_2O. To

check the quality and quantity of the RNA probe, the RNA probe was run in an agarose/formaldehyde gel.

Prehybridization

After sectioning, the paraffin wax was no longer needed for support. The paraffin was removed with twice treatments of Histoclear for 10 minutes without agitation. A fresh solution was used for each dip. The sections were hydrated through a series of EtOH solutions. Sections were incubated two times for 2 minutes in 100% EtOH. Then they were treated briefly in each of the following solutions (Table 5).

Table 5. Rehydration of the paraffin sections.

1.	Histoclear	10 minute
2.	Histoclear	10 minute
3.	100% EtOH	2 minute
4.	100% EtOH	2 minute
5.	95% EtOH	1 minute
6.	85% EtOH/0.85% NaCl	1 minute
7.	70% EtOH/0.85% NaCl	1 minute
8.	50% EtOH/0.85% NaCl	1 minute
9.	30% EtOH/0.85% NaCl	1 minute
10.	0.85% NaCl	2 minutes
11.	PBS	2 minutes

The sections were incubated with a Proteinase K solution (100 mM Tris, pH 7.5; 50mM EDTA; 2 µg/ml Proteinase K) for 30 minute at 37° C. Then the slides were rinsed at room temperature in 0.2% glycine in PBS for 2 minutes to block residual proteases. Then the sections were rinsed in PBS for 2 minutes. After that, the slides were dipped in a 4% formalin (not formaldehyde) PBS solution for 10 minutes. Then the slides were washed two times with fresh PBS for 2 minutes. Afterwards Slides were dipped in 200 ml of 100 mM triethanolamine (in DEPC-H_2O). Then the slides were removed (temporarily) and 1 ml acetic anhydride (reagent grade) was added to the triethanolamine and mixed well. The slides were put to the liquid and incubated for 10 minute with gentle agitation. Then slides were washed twice in PBS for 2 minutes. After that, the sections were dehydrated by a graded ethanol series ending with two times 100% EtOH treatments (Table 6). Then the tissue sections were air-dried in a dust-free environment.

Table 6. Dehydration of tissue sections on the slides.

1.	PBS	2 minutes
2.	0.85% NaCl	2 minutes
3.	30% EtOH/0.85% NaCl	1 minute
4.	50% EtOH/0.85% NaCl	1 minute
5.	70% EtOH/0.85% NaCl	1 minute
6.	80% EtOH/0.85% NaCl	1 minute
7.	95% EtOH/0.85% NaCl	1 minute
8.	100% EtOH	1 minute
9.	100% EtOH	1 minute

Then the prehybridization solution containing the following was prepared:

Reagents:

Formamide	1 ml
20X SSPE	225 µl
50% dextran sulfate	400 µl
50X Denhardt's solution	100 µl
Denatured herring sperm DNA, 100mg/ml	2 µl
Yeast tRNA, 100mg/ml	2 µl
100 mM DTT	100 µl
40 units/µl RNase inhibitor	2 µl
DEPC-H_2O	169 µl
Total volume	2 ml

Each section was covered with 150-250 µl of prehybridization solution (depending on the size of the section) and incubated in a humidified chamber at 42°C for 1 hour. After that, the slides were washed twice in PBS for 2 minutes and incubated twice in 70% ethanol for 15 seconds. Then the slides were put in 100 % ethanol for 1 minute and afterwards air dried in a dust free chamber.

Hybridization and post-hybridization washes

To make hybridization solution, 200 ng - 1 µg of cRNA probes was added to 1 ml of prehybridization solution. Then 150-250 µl of hybridization solutions was added to each slide. Then the slides were incubated in a humidified chamber at 42-50°C overnight. After the hybridization, the slides were washed in 3X SSC at room temperature for 5 minutes. Then the slides were washed with NTE buffer at room temperature for 5 minutes.

Subsequently, the slides were treated with 50 ng/ml RNase A in NTE buffer at 37^{o}C for 30 minutes to remove excess unhybridized probe. Then the slides were washed three times with NTE buffer at room temperature for 5 minutes each. After transferring the slides to 2X SSC they were kept at room temperature for 30 minutes. Then the slides were washed with 0.2X SSC at 57^{o}C for an hour and twice in PBS buffer at room temperature for 5 minutes to eliminate the non-specific binding of probe.

Detection

Digoxigenin-labelled nucleic acid probes can be detected using an indirect immunolocalization technique. In this procedure alkaline phosphatase enzyme conjugated to an antibody against digoxigenin (anti-dig FAB) localizes the nucleic acid probe sites via the chromogenic reaction of nitroblue tetrazolium (NBT) with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) Substrate. The colourless tetrazolium salt is converted to a blue/brown precipitate. The RNA *in situ* using anti-digoxigenin alkaline phosphatase (anti-dig FAB) carries out hybridizations and NBT/BCIP creates a blue/brown precipitates in the region of detected RNA.

The slides were placed into Coplin jars and incubated in buffer1 for 5 minutes. Then the buffer 1 was poured off and blocking solution was added. Slides were incubated in blocking solution at room temperature for an hour. Then slides were transferred to a humidified chamber. 100 µl of Anti-Digoxigenin-AP/ blocking solution was added to the top of each slide. All slides were kept in a humidified chamber at 37° C for 2 hours. After the treatment with anti-dig FAB, slides were dipped in Buffer 1 then washed twice with Buffer 1 for 15 minutes. Then the slides were dipped with Buffer 2 and washed three times in Buffer 2 for 5 minutes. After that, the slides were incubated in color development solution at 30° C in dark and observed every 30 minutes. When brown or blue colouring could be seen, the slides were washed in water three times for 5 minutes each. Then the slides were washed two times in 70% ethanol for 15 seconds. Then the slides were dipped in 100% ethanol for 15 seconds and then air-dried in a dust free chamber. Then 200 µl of Aquatex mounting medium was added to one end of the slide. One clean cover glass was taken and placed very slowly on slide. The extra mounting medium was removed by wiping with 3MM paper. Then the slides were placed in a chamber for one day. Then the slides were microscopically examined.

B.2.17. *In situ* RT-PCR

The basic approach that is used to analyse the temporal and spatial gene expression is the *in situ* hybridization method. Since this method is not sensitive enough, the detection of low-abundant nucleic acid targets is very hard. If the target RNA copies are less than 20 copies per cell, it is difficult to find the signals in the tissue sections. In order to overcome this limitation, the "*in situ* PCR" technique was used. This technique can detect low-abundance target DNA or RNA in nuclear and cytoplasmic regions by the amplification of the specific target sequences. Since the first report on this new PCR technique, great progress has been made in the development of the *in situ* PCR technology.

In situ RT-PCR is a variation of *in situ* PCR. It is used to identify the localization of mRNA in tissues. Originally, *in situ* RT-PCR was used for the localization of gene expression and for detection of viruses in animal cells. The first report about the use of *in situ* RT-PCR in plants was presented by Woo *et al*. To date there are only a few publications applying this method to plant tissues.

The sucrose carrier genes, *RcSCR1r* and *RcSCR2*, in endosperm could not be detected by *in situ* hybridization since their expression was very low. *In situ* RT-PCR was applied to find the localization of *Rcscr1* and *Rcscr2* in the *Ricinus* endosperm.

Fixation, dehydration, and embedding

The tissues were fixed in a modified fixation solution. The plant tissues were cut immediately into pieces in the fixation solution. Then the tissues were put into fixation solution solution. After vacuum infiltration for 10 minutes with a water aspirator, the FAA solution was renewed. This procedure was repeated 4 times. Then the tissues were kept at 4^{o}C for 2 days. After that, the tissues were washed with 63 % cold ethanol two times and dehydrated through a series of ethanol in Table 7. Then 3:1, 1:1 and 1:3 (v/v) of histoclear/ paraffin were prepared for the substitution. The substitution was carried out as listed describe in (Table 4). The histoclear/paraffin mixture was kept in an oven at 57^{o}C. Then the tissues were embedded in a paraffin block and placed in a jet-cassette.

FAA solution, modified (for 100ml)	
Ethanol, 100%	63 ml
Formalin, containing 37% formaldehyde	2 ml
Acetic acid, 100%	5 ml
DEPC-H_2O	30 ml

Table 7. Dehydration of *in situ* RT-PCR samples

1.	63 % ethanol, room temperature	60 min
2.	63 % ethanol, room temperature	60 min
3.	70% ethanol, room temperature	60 min
4.	70% ethanol, room temperature	60 min
5.	70% ethanol, room temperature	60 min
6.	85% ethanol, room temperature	60 min
7.	85% ethanol, room temperature	60 min
8.	85% ethanol, room temperature	60 min
9.	85% ethanol, 4°C	Overnight
10.	95% ethanol, room temperature	90 min
11.	95% ethanol, room temperature	90 min
12.	95% ethanol, room temperature	90 min
13.	100% ethanol, room temperature	90 min
14.	100% ethanol, room temperature	90 min

The paraffin block was trimmed and sectioned at 10 µm. The tissue sections were mounted on Probe-On Plus microscope slides and incubated at 42° C for at least 48 hours. After that, the paraffin was removed by twice treatments with Histoclear for 10 minutes without agitation. For each dip a fresh solution was used. Then the sections were incubated two times for two minutes in 100% EtOH. Then the slides were air-dried in a dust free chamber. Then the sections were treated with 200 ml of Proteinase K solution (4 µg Proteinase K in 200 ml of DEPC water) at 37° C for 5 - 90 min depending on the tissue.

Then the slides were rinsed twice in DEPC water for 5 min, and then put at 95° C for 2 minutes to inactivate Proteinase K activity. After that, the slides were acetylated to reduce the non-specific binding of digoxigenin-dUTP. The slides were submerged in 200 ml of 0.1 M triethanolamine solution; subsequently, 1.0 ml of acetic anhydride was added to the triethanolamine solution. The slides were incubated in Triethanolamine solution for 10 min then rinsed for 5 min in DEPC water. After incubating the slides in 100% EtOH for 5 min they were air-dried in a dust-free chamber. Then 40 µl of DNase I solution were applied to each slide. DNase I digestion was prepared as listed in Table 8. The slides were covered with cover glasses and placed in a humid incubator at 37° C overnight to eliminate genomic

DNA in the tissue sections. Subsequently, the slides were washed twice for 5 min in DEPC treated water, and then incubated at 95°C for 2 min to wash out and inactivate DNase I activity.

Table 8 : The treatment of DNase I and DNase I/RNase A.

	DNase I treatment	DNase I / RNase A treatment
10x DNase I buffer	4 µl	4 µl
DNase I, 10 U/µl	2 µl	2 µl
RNase inhibitor 40 U/µl	1 µl	0 µl
RNase A, 100 µg/ml	0 µl	10 µl
DEPC water	33 µl	24 µl

One-step RT-PCR

The *Tth* DNA polymerase reaction mix solution of was prepared as follows.

Reagents:

$MnCl_2$	10 µl
RT buffer	10 µl
$MgCl_2$	8 µl
Cheater buffer	8 µl
dNTPs Mix (10 mM)	2 µl
Reverse primer (10 pmol/µl)	2 µl
Forward primer (10 pmol/µl)	2 µl
2x Self seal Reagent	50 µl
DEPC water	6.5 µl
Tth DNA polymerase (5U/µl)	1 µl
1mM dig-dUTP	0.5 µl
Total	100 µl

Then 40 µl of the reaction mix was applied to each section, and the slides covered with cover glass. The *in situ* RT-PCR was performed in a MJ-PTC 100 PCR machine. The RT-PCR program was performed as follows:

65°C	30 min	Reverse Transcription
94°C	3 min	Denaturation
94°C 55°C 72°C	45 sec 1 min 1 min	30 PCR cycles

Then the slides were washed in 0.2xSSC/0.2% BSA at 60°C for 15 min. Subsequently, the slides were submerged in blocking buffer for 30 min. Monoclonal anti-dig-FAB fragment-Alkaline Phosphatase was diluted 1:1000 in blocking buffer. 200 µl of anti-dig antibody/blocking buffer was added to each slide. The slides were put in a humid chamber at room temperature and incubated overnight. The slides were rinsed twice for 10 min in Buffer 1 and three times for 5 min in Buffer 2. The slides were incubated in colour solution at 30°C in the dark and observed every 30 minutes. When brown or blue colour was seen, the slides were washed in water three times for 5 minutes each. Then the slides were put two times in 70% ethanol for 15 seconds and the slides were put in 100% ethanol for 10 minutes. Subsequently the slides were air-dried in a dust-free chamber. Then 150 µl of Aquatex mounting medium was added to one end of each slide. The clean cover glasses were placed very slowly on each slide. The extra mounting medium was removed by wiping with 3MM paper. Then the slides were placed on desktop for one day. Then the slides were examined microscopically.

B.2.18 TUNEL

TUNEL (Terminal deoytransferase (TdT)- mediated dUTP nick end labeling) is a molecular technique for labeling DNA breaks associated with programmed cell death. It is based on specific binding of TdT to 3´-OH ends of DNA, mediating a synthesis of polydeoxynucleotide polymer incorporating labelled deoxyuridine at the site of DNA breaks (Gavriele *et al*., 1992). At the cellular level, nuclear fragments always accompany with senescence by increased activity of RNases and proteinases. In order to detect DNA

fragmentation, the TUNEL procedure was applied. TUNEL identifies DNA fragmentation *in situ* by using TdT to transfer digoxigenin-dUTP to the free 3'-OH of cleaved DNA. The digoxigenin-labelled cleavage sites are then visualized by reaction with an alkline phosphatase conjugated antibody (Anti-Dig AP) followed by incubation in coloring buffer. In the following table the pretreatment of paraffin-embedded tissue with Proteinase K treatment.

Reagents:

dNTP mix, 10 mM	10 µl
digoxigenin-dUTP 0.2 mM	2 µl
5x TdTbuffer	200 µl
TdT	5 µl
ddH2O	783 µl

The tissue sections were dewaxed and rehydrated according to standard protocols (see *in situ* hybridization). Then tissue sections were incubated for 30 minutes at 37° C with 2 µg/ml Proteinase K. Afterwards the slides were rinsed twice with PBS. Then the area around sample was wiped with Whatman 3MM paper. After that, 50 µl of TUNEL reaction mixture was added on the sample. For the negative control 50 µl of label solution were added on each. Then the slides were put in a humidified chamber and incubated at 37° C for 60 minutes. Then the slides were rinsed 3 times with PBS. After that, the slides were incubated in blocking solution for 1 hour at room temperature. Then 50 µl of 1:1000 Anti-Dig AP conjugated antibody was added to each tissue section. Afterwards the slides were incubated at room temperature for 60 minutes. Then the slides were washed 3 times with buffer 1 for 15 minutes each and subsequently with 3 times buffer 2 for 5 minutes each. Then the slides were incubated in color development solution and incubated in a dark incubator at 30° C for 30 minutes.

B.2.19. Immunolocalization

Tissue preparation

Tissues can be manipulated in the subsequent steps either in separate vials or put into tissue cassettes and then incubated in a large jar or beaker. One square cm of endosperm

(or cotyledon) was fixed in freshly made FAA for 30 min at room temperature. At the mean time, a large jar or beaker was filled with paraplast chips and put into a 55 – 60°C oven to melt over night. Then tissue was dehydrated through a graded ethanol series (Table 9).

Table 9. The dehydration of immunolocalization samples.

1	50% EtOH	30 minutes
2	70% EtOH	30 minutes
3	70% EtOH	30 minutes
4	80% EtOH	30 minutes
5	80% EtOH	30 minutes
6	95% EtOH	30 minutes
7	95% EtOH	30 minutes
8	100% EtOH	30 minutes
9	100% EtOH	30 minutes
10	3 EtOH: 1 Histodear	30 minutes
11	1 EtOH: 1 Histoclear	30 minutes
12	1 EtOH: 3 Histoclear	30 minutes
13	Histoclear	30 minutes
14	Histoclear	30 minutes

Then paraplast chips were thrown into the tissue in Histoclear, 10-20 chips every 1-2 hour in a 55°C oven. When all the chips were melted, half the paraplast Histoclear mix was poured out and fresh molten paraplast was poured in. The tissue was incubated for 2-3 hours and then all the Histoclear paraplast mix was poured out and new fresh molten paraffin was poured in. Then the tissue was incubated overnight. Next day the paraplast was replaced with fresh paraplast and incubated for 4 hours. Afterwards the tissue was gradually embedded in paraplast over a period of two days. The paraplast was changed 3 times a day. After that, tissue blocks were cast in plastic disposable frames. A heating block was used to keep the paraplast molten while the tissue was correctly oriented. For cutting 8-10 µm sections, a rotary microtome and a freshly sharpened knife blade were used. Tissue ribbons were mounted in water on Probe On Plus slides (Fisher Scientific) and kept at 42°C overnight on a slide warmer. Then the slides were placed in Coplin jars and deparaffinized in Histoclear for 20 minutes. Then the slides were rehydrated through a graded ethanol series (Table 10).

Table 10. The rehydration of the immunolocalization tissue sections.

100% EtOH	1 minute
95% EtOH	1 minute
85% EtOH	1 minute
70% EtOH	1 minute
50% EtOH	1 minute
30% EtOH	1 minute
PBS	10 minutes

The slides were incubated in PBS for 10 minutes. The slides were then blocked in about 100 µl of 1 % BSA in PBS for 15-45 minutes in a moist chamber. Then the slides were washed in PBS for 10 minutes. Slides were then incubated in antibody solution or pre-immune serum for 90 minutes. After that, the slides were washed 3 x for 15 minutes each in PBS with 0.1% Tween20 (PBST). The slides were then incubated in an anti-rabbit alkaline phosphatase conjugated secondary antibody at a dilution of 1:500 for 1 hour and washed 3 x in PBST for 5 minutes each. Subsequently, the slides were washed with Buffer 2 (Tris-HCl pH 9.5 100mM, 100 mM NaCl, 50 mM $MgCl_2$) for 15 minutes, and incubated in the colouring solution (35 µl of 50mg/ml BCIP and 33 µl of 100mg/ml NBT in 10ml of Buffer 2). Afterwards slides were kept in a dark incubator at 30°C. The colour reaction was stopped with ddH_2O. Slides were rapidly dehydrated by immersing in 70% ethanol for 30 seconds and then 100% ethanol for 1 minute. Then 200 µl of Aquatex mounting medium was added to one end of the slide. One clean coverslip was taken and placed very slowly on the slide. The extra mounting medium was removed by wiping with 3MM paper. Then the slides were placed in a chamber for one day at room temperature. Then the slides were examined microscopically.

B.2.20. Starch and protein staining

Paraffin embedded tissue sections were used for starch staining. All sections were cut in a thickness of 10 µm. The same rehydration steps as in immunolocalization were used. Then the tissue sections were incubated in IKI solution (1 g KI, 1 g Iodine in 100 ml of ddH_2O) for 10 min. After that, the sections were incubated in ddH_2O for 5 seconds and then Whatman 3MM paper was used to wipe the water and slides were air-dried. After adding 200 µl of Aquatex the slides were covered with cover glasses.

In order to stain proteins, deparaffined and air-dried slides were put into Fast green staining solution (0.05g Fast green FCF in 33 ml methyl cello solve, 33ml methyl salicylate, 33ml absolute ethanol) for 1 minute. Then the slides were washed with absolute ethanol for 15 seconds. Then slides were air-dried. Afterwards 200 µl of Aquatex was added to the slides and then the slides were covered with cover glasses.

B.2.21. Real time PCR

There are many different methods to detect the specific mRNA expression. The oldest method for detecting specific mRNA relies on hybridization to specific labelled probes. A common manifestation of this is known as Northern blotting. However, there are some limitations to this technique. First, a relative quantitative estimation of the strength of the signals can be obtained, but it is generally not possible to get an absolute measurement of the amount of specific mRNA. Secondly, the technique is not very sensitive and requires a large amount of RNA.

A much more sensitive way of detecting specific mRNA is an adaptation to the reverse transcription and polymerase chain reaction (PCR). This is described as reverse transcription PCR (RT-PCR). RT-PCR has obvious advantages: it is able to detect mRNA of low abundance or to analyse gene expression in cells that are difficult to obtain in large numbers. RT-PCR still has limitations, namely quantification. It is difficult to be sure that the result is reliable by simply amplifying the template and measuring the amount of product. There is no simple and reliable relationship between the amount of template and PCR product, unless the PCR reaction is truly proceeding exponentially.

Real time PCR is a new developed technique for detecting specific mRNA. It is based on the RT-PCR technique. Real time reverse-transcription (RT) PCR quantifies the initial amount of the template very specifically, sensitively and reproducibly, and therefore it is a preferable alternative to other forms of quantitative RT-PCR, which detect the amount of final amplified products. For real time PCR, the PCR reactions can proceed without stopping and it is not necessary to run the PCR products on a gel. Real time PCR monitors the fluorescence emitted during the reaction as an indicator of PCR amplification products during each cycle as opposed to the endpoint detection by conventional quantitative PCR methods. The real time PCR system is based on the detection and quantification of a fluorescent reporter.

There are two general methods for quantitative detection of PCR amplification products: (1) fluorescent probes and (2) DNA-binding agents. The fluorescent probes, such as TaqMan probes, are designed to anneal to an internal region of PCR amplification products.

TaqMan probes are oligonucleotides that contain two different dyes. Usually the fluorescent dye is on the 5' base, and the quenching dye is on the 3' base. When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing (this is called FRET = fluorescence resonance energy transfer). Thus, the close proximity of the reporter and quencher prevents emission of any fluorescence when the probe is hybridized to the template DNA. When the polymerase replicates a template on which a TaqMan probe is hybridized, the 5' exonuclease activity of *Taq* polymerase cleaves the TaqMan probe. This stops the activity of quencher (no FRET) and the reporter dye starts to emit fluorescence. The accumulation of PCR products is detected by monitoring the increase in fluorescence of the reporter dye (Figure 9).

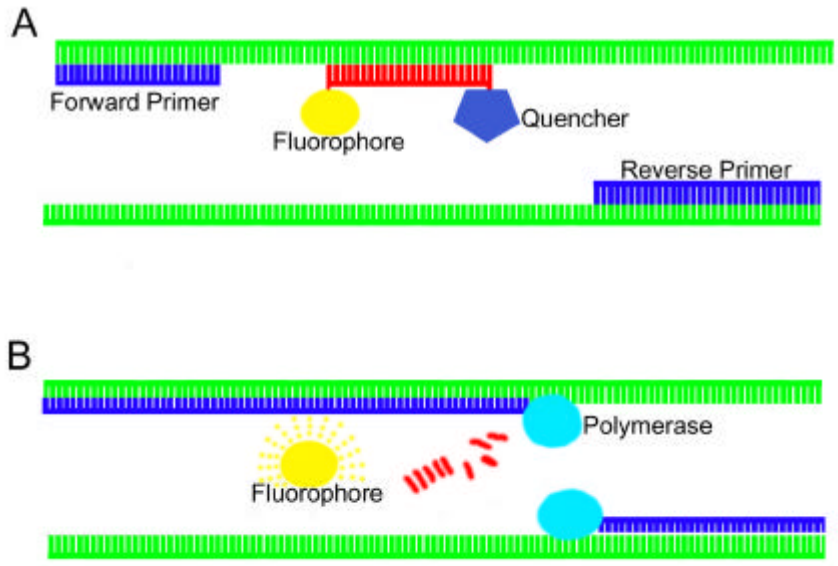


Figure 9: TaqMan probe system. (A) During the annealing phase, primers and probe anneal to the DNA target. At this moment, fluorescence cannot be detected because of the proximity between fluorophore and quencher. (B) In the extension phase, The TaqMan probe is cleaved by the *Taq* Polymerase. The quencher and fluorophore are then separated and allow fluorescence emission from the reporter dye.

Another simple method is the addition of a DNA binding reagent to the PCR mixture that fluoresces when it binds to double-stranded DNA. Usually SYBR Green I is used for detection double strand DNA. SYBR Green I is a minor groove binding dye, it does not bind to single strand DNA. Initially, the template is single-stranded, so there is no signal. As PCR proceeds, double-stranded product is amplified, and, consequently, the incoming fluoresence can be detected by the machine (Figure 10). The major problem with SYBR

Green-based detection is that non-specific amplification cannot be distinguished from specific amplifications. With some modifications, the use of SYBR green I can also obtain reliable results.

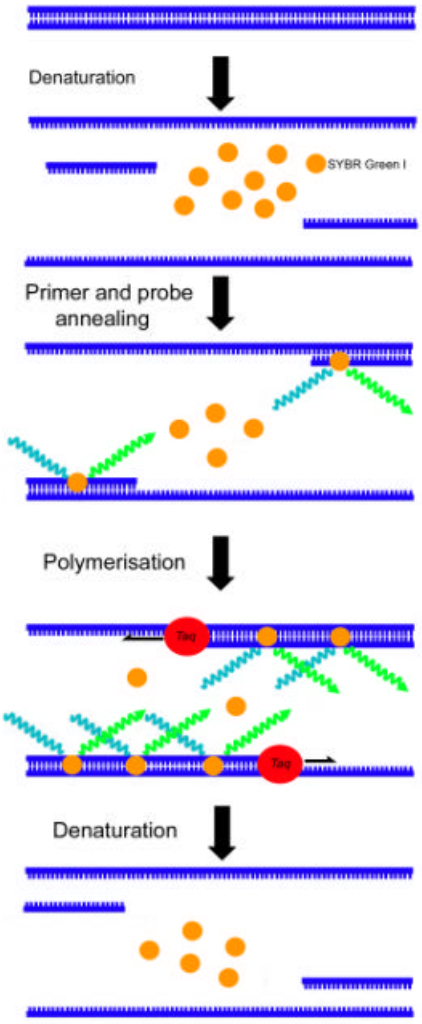


Figure 10 : During denaturation, SYBR Green I dye exhibits little fluorescence. When the primer anneals to the template DNA, some SYBR Green I dye molecules bind to the primer/target complex and resulting in light emission upon excitation. During the polymerization step, more and more dye molecules bind to the double-stranded DNA, and the increase in fluorescence can be monitored. On denaturation, the dye molecules are released and the fluorescence signal returns to background.

The most important part of accurate and reproducible quantification using fluorescence-based RT-PCR is the concept of the threshold cycle (*Ct*). The fluorescence values are recorded during every PCR cycle. They represent the amount of amplification product at a specific point of the PCR reaction. At the beginning, the more DNA templates exist in the reaction, the fewer number of PCR cycles it takes until the fluorescent signal is first

recorded as statistically significantly stronger than background (Gibson *et al.*, 1996). This point is defined as the *Ct*, which always occurs during the exponential phase of amplification. Therefore, the amount of template DNA in the PCR is the most important factor of quantification by real time PCR reaction, rather than other reaction components. The other components will become limited in the plateau phase and they make any quantification based on measurements of overall product yield intrinsically unreliable.

The standard curve is generated by performing three independent serial dilutions of the standard. In order to obtain the best accuracy, the standard dilutions should be prepared over the range of copy numbers that include the amount of target mRNA expected in the experimental samples. To correct the pipetting error, the high and low *Ct* values are discarded and the remaining values are averaged to give the final *Ct* value for that dilution. The *Ct* value is inversely proportional to the *log* of the initial copy number (Higuchi *et al*., 1993). The copy numbers of the template can be calculated after real time amplification by the linear regression of the standard curve (Figure 11).

The *nsLTPc1* was used as an artificial internal control. The 3'-end non-translated region of *nsLTPc1* was subcloned into a pGEM-T vector and used as a template for the *in vitro* transcription. The *in vitro* transcription RNA product was added to the total RNA from different days old endosperm cells. In order to eliminate the contamination of genomic DNA, total RNA from the endosperm was treated with RNase free DNase I. Afterwards the reverse transcription was performed.

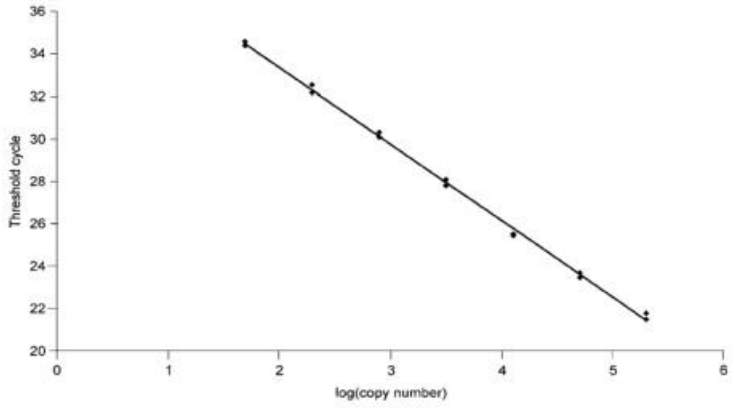


Figure 11 : The Standard curve. The Threshold cycle values (*Ct*) are plotted against input of cDNA copy number.

Some of the reverse transcription products were used to optimise the PCR procedure. After PCR, the products were checked in a 2% agarose gel. Real time RT-PCR could be performed when no non-specific amplification could be found in the PCR products.

Reverse Transcription

Genomic DNA in the RNA samples was eliminated with DNase I treatment. The DNAse I reaction mix was prepared as follows.

Reagents:

Total RNA	10 µg
DNAse I, 10 U/µl	2 µl
10 X DNAse I buffer	4 µl
Add DEPC-H_2O to 40 µl	

The reaction mix was incubated at 37°C for 30 minutes. 5 µl of 3M NaOAc and 5 µl of DEPC-H_2O were added to the reaction mix. 50 µl of Phenol/chloroform was added to the reaction mix and mixed. Subsequently, the reaction mix was centrifuged 15,000xg at 4°C for 5 minutes. The upper aqueous phase was transferred into a new tube and 50 µl of chloroform was added, then the reaction mix was vortexed and centrifuged 15,000xg at 4°C for 5 minutes. The upper aqueous phase was transferred into a new tube and 150 µl of cold absolute ethanol was added. Then the reaction mix was stored at -20°C for 1 hour. The reaction mix was centrifuged at 15,000xg, 4°C for 15 minutes. The supernatant was carefully poured off. The RNA pellet was washed with 80% ethanol and centrifuged at 15,000xg, 4°C for 5 minutes. The supernatant was poured off and the RNA pellet was air-dried. The pellet was dissolved in 40 µl DEPC-H_2O. 4 µl of oligo-(dT)20 primer (10 pmol/µl) was added to 20 µl RNA and warmed at 65°C for 5 minutes. The RNA/Oligo-dT primer mix was kept at room temperature for 10 minutes. The reverse transcription mix was prepared as listed.

Reagents :

5X reverse transcription buffer	8 µl
10 mM dNTP mix	4 µl
RNase inhibitor 40U/µl	2 µl
MMLV Reverse transcriptase 200U/µl	2 µl

The reverse transcription mix was added to the RNA/Oligo-dT primer mix and incubated at 37°C for 10 minutes, subsequently 42°C for 90 minutes. After that, the reverse transcription was stopped by heating the reaction mix at 95°C for 5 minutes. Then 60 µl of ddH_2O was added to the cDNA. The cDNA was stored at -20°C.

Optimisation of Primer Concentrations for RT-PCR

The purpose of this procedure is to minimize the non-specific amplification. The PCR reaction mix was prepared as listed.

Sample	1	2	3	4	5	6	7	8	9
cDNA	1µl	1µl	1µl	1µl	1µl	1µl	1µl	1µl	1µl
10XPCR Buffer	5µl	5µl	5µl	5µl	5µl	5µl	5µl	5µl	5µl
dNTPs mix, 10mM	1µl	1µl	1µl	1µl	1µl	1µl	1µl	1µl	1µl
Taq polymerase	1µl	1µl	1µl	1µl	1µl	1µl	1µl	1µl	1µl
Forward Primer	0.4µl	0.4µl	0.4µl	1.5µl	1.5µl	1.5µl	4.5µl	4.5µl	4.5µl
Reverse Primer	0.4µl	1.5µl	4.5µl	0.4µl	1.5µl	4.5µl	0.4µl	1.5µl	4.5µl
ddH_2O	41.2µl	40.1µl	37.1µl	40.1µl	39µl	36µl	37.1µl	36µl	33µl

The PCR was processed as follows. Then 10 µl of PCR product was analysed by agarose gel electrophoresis to confirm the absence of non-specific amplification.

50°C	2 minutes	
95°C	10 minutes	
95°C	15 seconds	40 PCR cycles
60°C	1 minutes	

Real time quantitative PCR and Data analyses

The PCR reaction was performed at ABI Prism 7000 Sequence Detection system. The PCR program was processed as listed before. The quantitative real time PCR reaction mix was prepared as follows.

Reagents:

cDNA	1 µl
10XPCR Buffer	5 µl
dNTPs mix, 10mM	1 µl
Taq polymerase 5 U/µl	1 µl
Forward Primer, 10 pmol/µl	1.5 µl
Reverse Primer, 10 pmol/µl	1.5 µl
1:1000 SYBR Green I	1 µl
ddH_2O	38 µl

After that, the PCR products were analysed by running a 2% agarose gel. If non-specific amplification products were found, the results were discarded. The optimised PCR condition must be found. Then the data analyses were done using the SDS program. The PCR reaction mix was prepared as listed.

B.2.21 *Ricinus* cDNA library screening

B.2.21.1. Yeast Transformation

5 ml of YPD liquid medium was inoculated with the invertase deficient yeast strain SEY2102and incubated overnight on a rotary shaker at 200 rpm at 30° C. After that, 2.5 x 10^8 cells were added to pre-warmed YPD to give 5 x 10^6 cells/ml. Then the flask was incubated on a rotary or reciprocating shaker at 30° C and 200 rpm. When the cell titer was at least 2 x 10^7 cells/ml, the cells were harvested by centrifugation at 3000 *g* for 5 min. Afterwards the cells were washed in 25 ml of sterile water and resuspended in 1 ml of sterile water. The cell suspension was transferred to a 1.5 ml centrifuge tube and centrifuged for 30 sec. Sterile water was added to a final volume of 1.0 ml to the cell pellet and then the cells were resuspended in sterile water. After that, 100 µl of cell suspension was added (ca. 10^8 cells) into 1.5 ml microfuge tubes. Then the tube was centrifuged at top speed for 30 sec and the supernatant was removed. Then the reagents were added as follow:

Reagents:

PEG 3500 50% w/v	240 µl
LiAc 1.0 M	36 µl
Boiled SS-carrier DNA	50 µl
nSC4B+ DNA plus Water	34 µl
Total	360 µl

The transformation mix was mixed well and incubated in a 42°C water bath for 40 min. After that, the transformation mix was centrifuged at top speed for 30 sec. The supernatant was removed with a micropipettor. Subsequently, 1.0 ml of sterile water was added to tube to resuspend the cell pellet. Then 200 µl of cell suspension was plated on to the MMA/Leucine/Histidine plate. The plate was incubated at 30°C for 4 days. The transforment was named SEYSC1.

B.2.21.2. cDNA library screening

SEYSCR1 could produce RcSCR1 protein and transport sucrose into cell when it grew in a sucrose-containing medium, SEYNEV could not produce RcSCR1 protein and no sucrose could be transported into cell when it grew in a sucrose-containing medium. Then SEYSCR1 and SEYNEV were transformed with 181A1NE plasmid. The new double transformants, which had nSC4+/181A1NE or NEV-N/181A1NE, were used as negative control and positive control. The positive control (SEYNEV::181A1NE) could grow better than negative control (SEYSCR1::181A1NE). The negative control grew better in a MMA medium without sucrose (Figure 12). If sucrose efflux transporter can release sucrose to the medium and sucrose might be not accumulated too high in yeast. These transformants might grow better than other transformants, which could not transport sucrose to medium. That is the basic theory of screening.

In order to find sucrose efflux transporters from *Ricinus* endosperm, an expression cDNA library was used. The expression cDNA library was constructed by Dolle (Dolle, diploma thesis, Bayreuth 1994). The *Ricinus* seedling cDNA was ligated to a yeast expression vector 181A1NE, which contains Adh1 promoter and Adh1 terminator. The screening was performed as following (Figure 13): the plasmid, which contains cDNA, was transformed to SEYSC1. After transformation, the yeast cells were plated on an MMA/Histidine plate and

incubated at 30°C for 4 days. After that, 5 ml of MMA were used to wash out the yeast transforments. 4 ml of the transforments was transferred to a flask, which contains 100 ml of 2% sucrose in MMA/Histidine. The rest of the transforments were transferred to a centrifuge tube and an equal volume of sterile 1M Sorbitol was added. The tube was kept in -80°C.

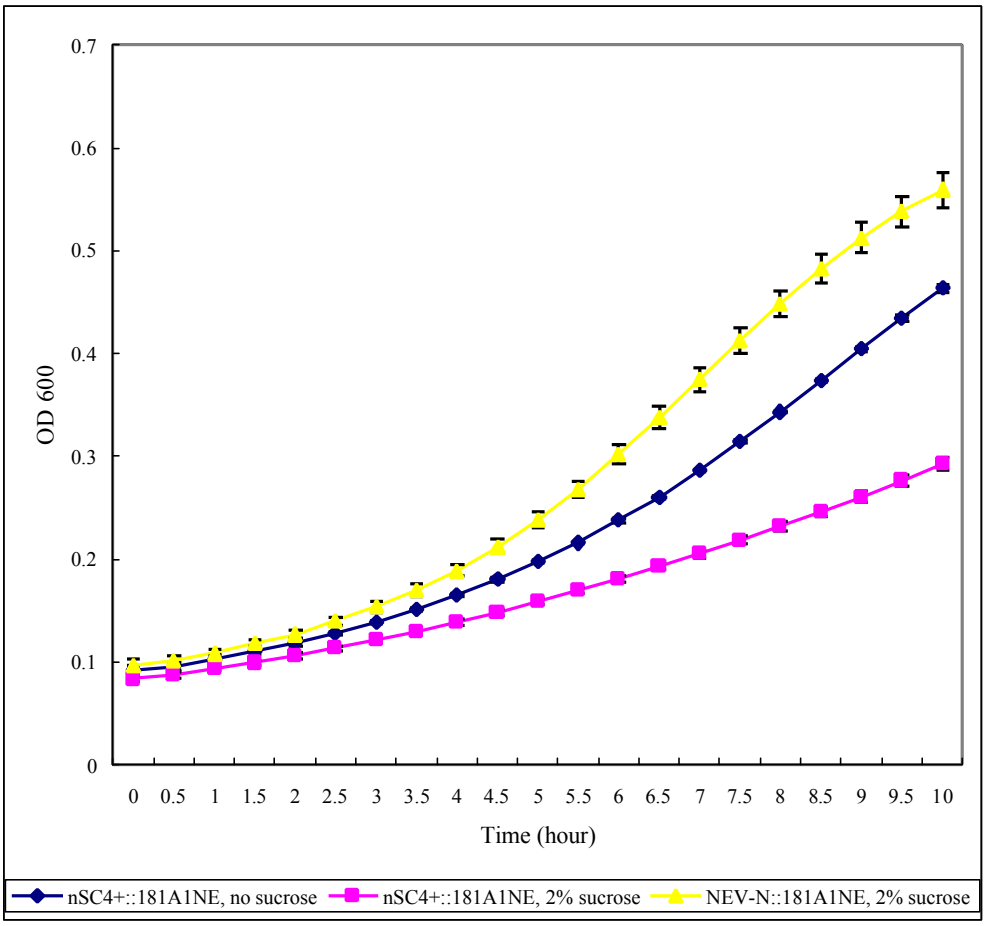


Figure 12 : The growth curves of the negative control and positive control. The positive control was cultured in a 2% sucrose-containing MMA medium. The negative control was cultured in a 2% sucrose-containing MMA medium or in a MMA medium. The positive control can grow quicker than the negative control.

The transforments in sucrose/MMA/Histidine selection medium were then incubated in a shaking incubator at 200 rpm, 30°C for 8 hours. After that, the culture was centrifuged and resuspended in 100 ml of sucrose/MMA/Histidine medium. The selection medium was changed every 8 hours for 7 days. After that, the yeast cells were collected and plated to the MMA/Histidine plate.

When the colonies could be seen on the plates, the single colonies were picked and put into 3 ml of MMA/Histidine medium. The yeast was then incubated in a shaking incubator at 200 rpm, 30°C overnight. 1ml of these overnight cultures was used to measure the OD_{600}. Then all the overnight cultures were diluted to the same concentration (OD_{600}=0.1). 200 µl of each diluted culture was transferred to a microtiter plate and put into ELISA reader to measure the growth curves.

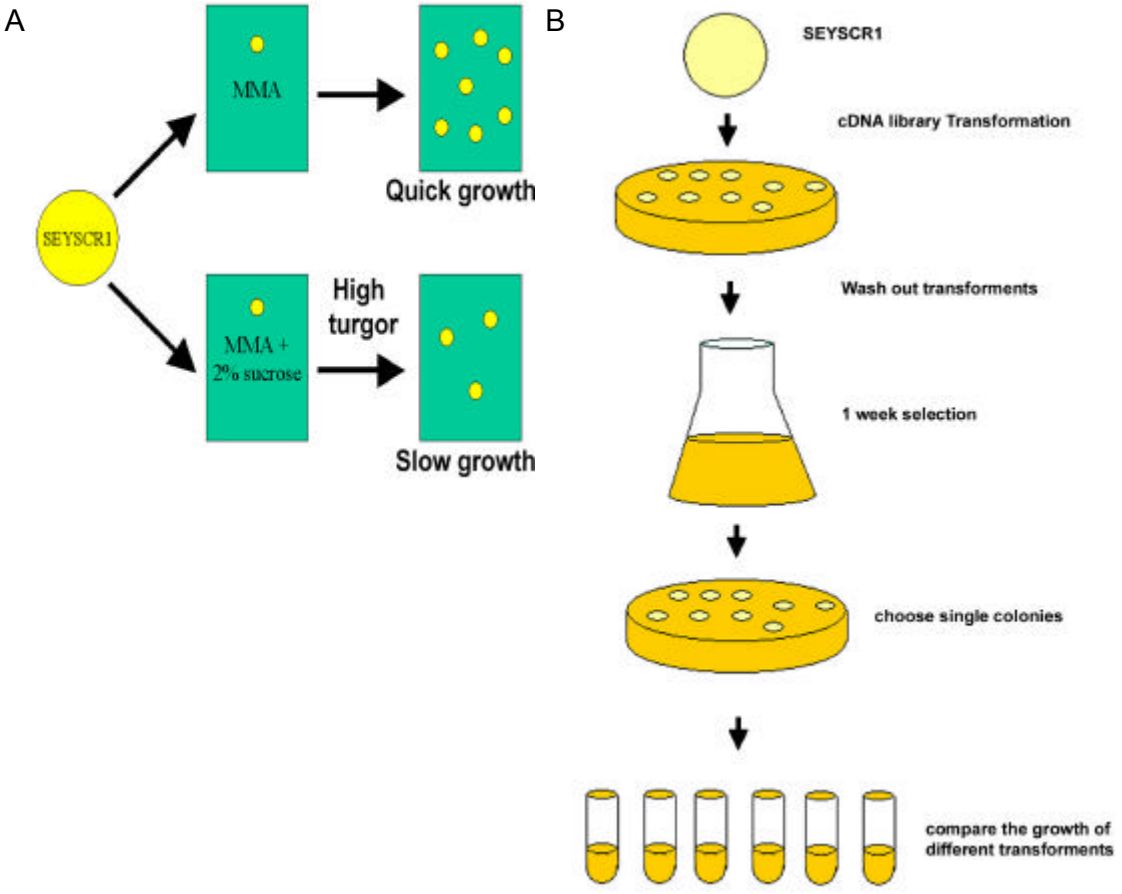


Figure 13 : The basic theory of sucrose efflux screening. A. nSC4+ transformed yeast (SEYSCR1) grows slower in a 2% sucrose containing MMA. B. The flowchart of the screening.

The growth curves of the different transforments were compared to the growth curve of the negative control. The transformants, which could growth quicker then control in a MMA/ 2% sucrose/ Histidine medium, were then cultured. The plasmids from these transforments were extracted and transformed to *E.coli* DH5α. The *E.coli* transforments were then

checked by PCR. The transforments, which did not contain nSC4+ sequence, were kept for later study.

B.2.21.3. Quick Preparation of Plasmid DNA from Yeast

2 ml of yeast liquid culture was collected and resuspend in 500 µl of lysis buffer (50mM Tris-HCl, pH 7.5, 10mM EDTA, 0.1% SDS and 10mM 2-mercaptoethanol added just before use). Sample was mixed vigorously by vortexing. An equal volume of glass beads (0.45 mm) was added and vortexed at top speed for 1 min. Then an equal volume of phenol/$CHCl_3$ was added. The aqueous phase was transferred to a new tube and extracted with $CHCl_3$ one time. DAN was precipitated by adding 50 µl of 3M NaOAc and 500 µl of isopropanol. Subsequently, DNA was washed once with 80% ETOH and air-dried. Then DNA was resuspended in 100 µl of TE and use to transform *E. coli* DH5α.

C. Results

C.1. cDNA library screening for sucrose efflux transporter

C.1.1. Growth curves of the transformants

The expression cDNA library was screened in yeast mutant SEY2102. This mutant has no functional invertase. Thus, sucrose taken up from the medium will accumulate internally and then elevate the turgor pressure in yeast cells. The *RcSCR1* cDNA was subcloned to a yeast expression vector NEV-N. The new plasmid- nSC4+ was transformed to SEY2102 to produce RcSCR1 protein. The transformant- SEYSCR1 was used for screening a cDNA library. The cDNA library was constructed in a yeast expression vector 181A1NE. The empty vector 181A1NE was transformed to yeast SEYSCR1 and then used as a negative control. The NEV-N and 181A1NE double transformant was used as a positive control. The cDNA library was then transformed to yeast SEYSCR1 for the screening of a sucrose efflux transporter. If the cDNA, which encodes the sucrose efflux transporter, was synthesized in the yeast transformant, this yeast transformant might release sucrose and reduce the turgor pressure in yeast cells. This yeast transformant may grow well and more quickly than other transformants.

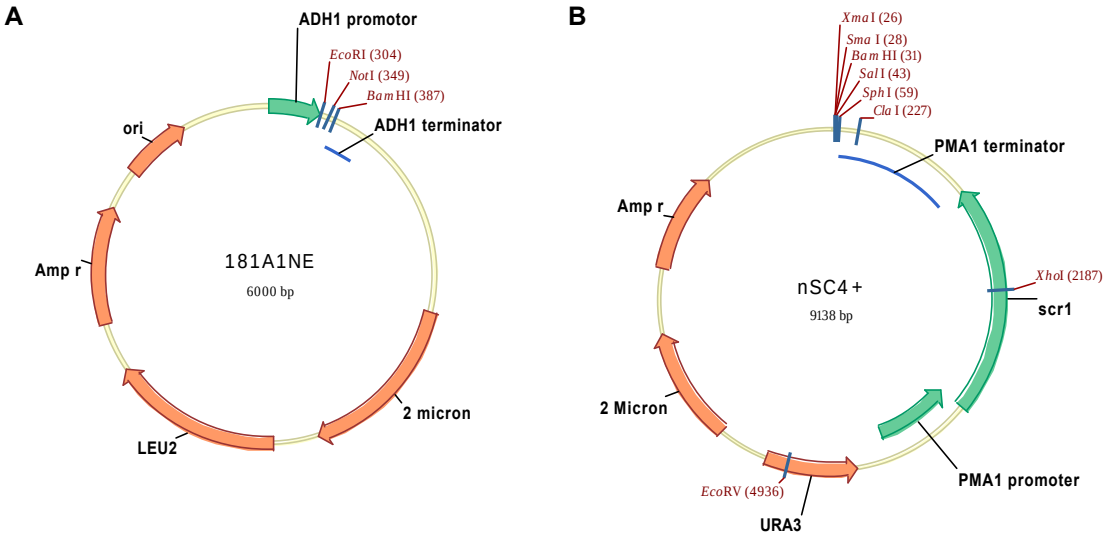


Figure 14 :181A1NE and nSC4+. (A) The expression cDNA library was constructed in the yeast expression vector 181A1NE. 181A1NE has an ADH1 promotor and LEU2 marker gene. (B) In order to produce RcSCR1 in yeast SEY2102, RcSCR1 cDNA was subcloned to a yeast expression vector NEV-N. NEV-N has a PMA1 promotor and URA3 marker gene.

The growth curves of cDNA library transformants were compared to the positive and negative controls. 12 colonies from transformants were selected and cultured overnight. The transformants were then cultured in a 2% sucrose-containing MMA medium. Positive control, negative control and transformants were dispensed into a microtiter plate and then the growth was monitored by an ELISA reader. The result showed that all the transformants could grow better than the negative control. The sucrose concentration in these transformants might be lower than that in the negative control (Figure 15).

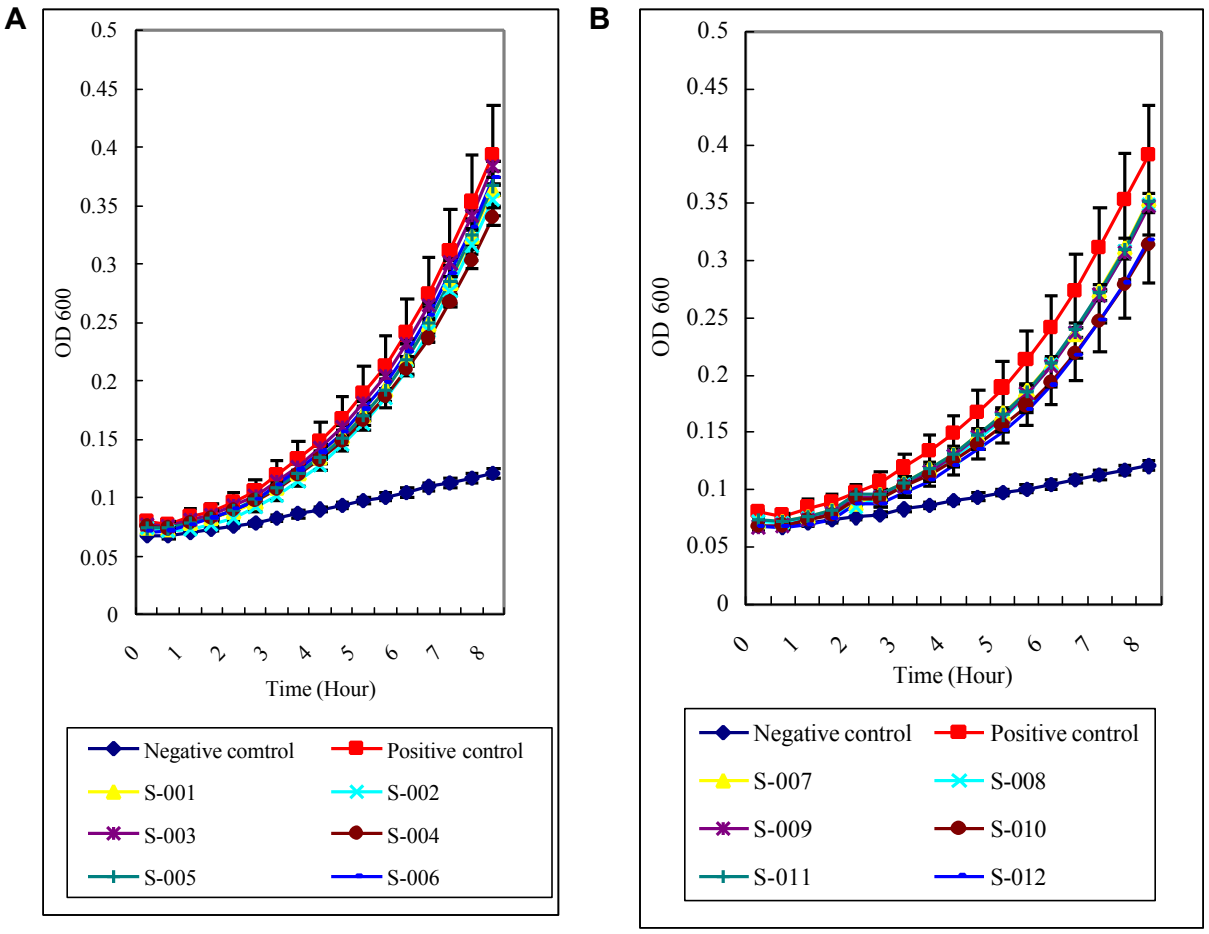


Figure 15 : Growth curves of the transformants. A: Growth curves of positive control, negative control and transformants S-001 to S-006. B: Growth curves of positive control, negative control and transformants S-007 to S-012.

The plasmids were extracted from these 12 yeast transformants and then transformed to *E.coli* DH5α competent cells. The *E.coli* transformants were checked by restriction digestion. The *E.coli* transformants, which contained a cDNA insert, were cultured (Figure

16). The plasmids, which contained the cDNA inserts, were then extracted and transformed to SEYSCR1.

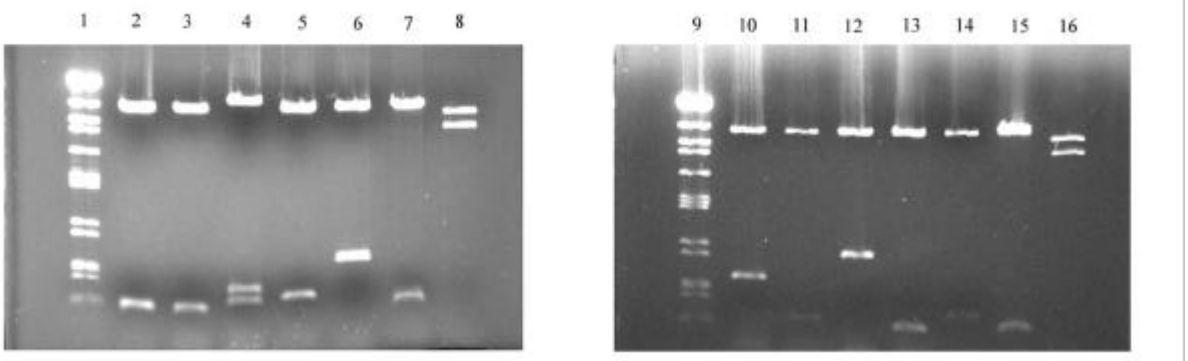


Figure 16 : Restriction digestion of the plasmids from S-001 to S-012. Plasmids were extracted from yeast transformants S-001 to S-012 and transformed to *E.coli*. The plasmids were extracted from *E.coli* overnight culture and digested with *Hind*III. The nSC4+ was also cut with *Hind*III. Lane 1 and 9: 1 /*Cla* I marker, lane 8 and 16: nSC4+/*Hind*III, land 2 to lane 7: S-001 to S-006, lane 10 to lane 15: S-007 to S-012.

C.1.2. Plasmids retransformation and growth curves of the retransformants

The retransformants were plated on MMA/Histidine plates. When the colonies could be seen, single colonies were cultured in MMA/Histidine overnight. The growth curves of these retransformants were postulated to be similar to the original transformants. They should grow better than the negative control and similar to the positive control, because the same plasmids were transformed.

But the result was disappointing. All retransformants could not grow as quickly as the positive control. In contrast, all retransformants could only grow like the negative control (Figure 17). Although they contained the same plasmids as the original transformants, the growth curves were not similar.

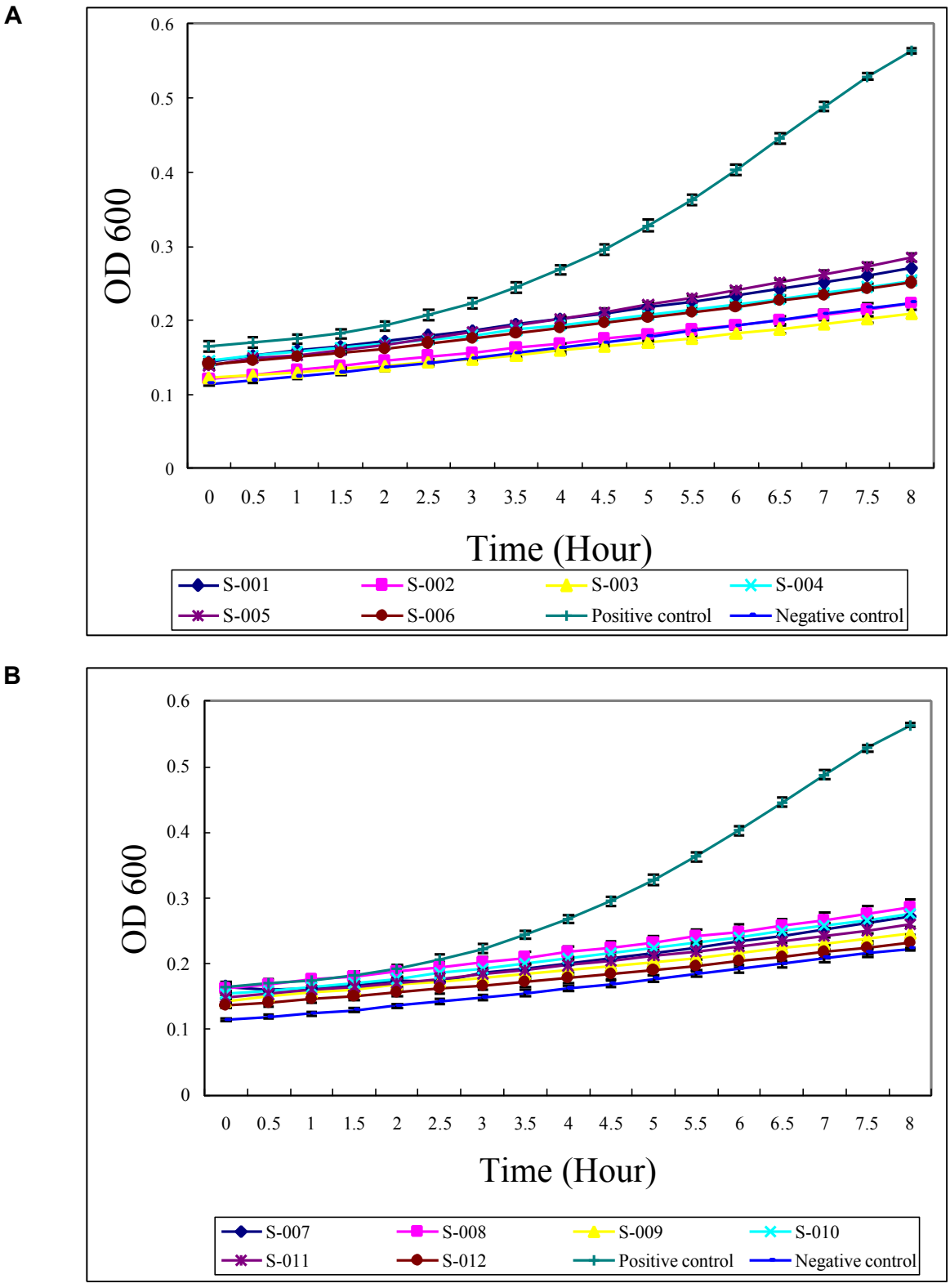


Figure 17 : Growth curves of the retransformants. A: Growth curves of positive control, negative control and retransformants S-001 to S-006. B: Growth curves of positive control, negative control and retransformants S -007 to S -012.

C.1.3. PCR of nSC4+

To find out the problem, the plasmids extracted from original yeast transformants and retransformants were used for PCR. Using URASCR-F primer (5´-ATTTCGGTTTCTTTGAAATTTTTTTGATTC-3´) and URASCR-R primer (5´-GAGGTGACTCATGAACCAAGTGTCTGTCGA-3´) and nSC4+ as template, a 5.3 kb PCR product could be amplified.

Using plasmid extracted from original yeast transformants as a template, no PCR product with the size of 5.3 kb could be amplified. The size of the major products was much smaller than 5.3 kb (Figure 18). But using plasmid extracted from yeast retransformants as a template had a different result. All plasmids, which were extracted from retransformants, could amplify a 5.3 kb PCR product (Figure 19).

The results indicated that the mutation of nSC4+ plasmid was occurred during the selection.

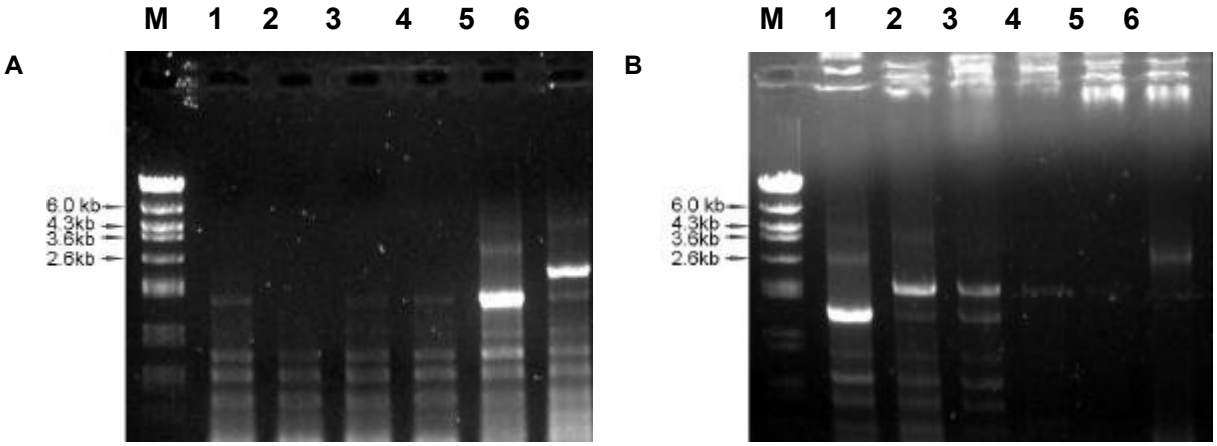


Figure 18 : PCR amplification of the plasmids from original transformants of S-001 to S-012. Plasmids were extracted from yeast transformants S-001 to S-012 and transformed to *E.coli*. Then plasmids were extracted from *E.coli* overnight culture and used as templates. A: M: λ/*Cla*I marker, land 1 to lane 6: PCR products of S-001 to S-006, B: M: λ/*Cla*I marker, lane 1 to lane 6: PCR products of S-007 to S-012.

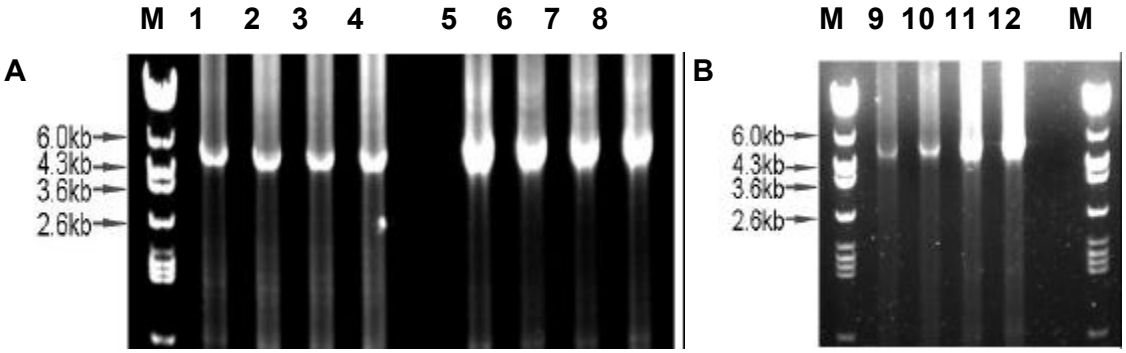


Figure 19: Gel electrophoresis of the PCR products of transformants S-001 to S-012. Plasmids were extracted from yeast transformants S-001 to S-012 and transformed to *E.coli*. Then plasmids were extracted from *E.coli* overnight culture and used as templates. A: M: l /*Cla*I marker, land 1 to lane 8: PCR products of S-001 to S-008. B: M: l/*Cla*I marker, lane 9 to lane 12: PCR products of S-009 to S-012.

C.2 Programmed cell death in *Ricinus* Endosperm

PCD is one of the procedures described as senescence. It is the final stage of vegetative and reproductive development, preceding the widespread death of cells. Senescence involves the active turnover and recapture of cellular material for use in other organs. The *Ricinus* endosperm dies during germination as soon as the storage material has been mobilized. After 6-7 days, endosperm was seperated from cotyledons.

C.2.1. Endosperm Cell layers in the germinating *Ricinus communis*

The fresh weight of *Ricinus* endosperm rises during the germination but the cell number is reduced. The cells layer of *Ricinus* endosperm were counted. The number of cell layers (cell layers between the seed coat and cotyledon) was 68 on day 2. The number of cell layers was 29 on day 6. The number of cell layers decreased dramatically between day 5 and day 6, from 53 to 29 (Table 11).

Table 11: The cell layers of the endosperm from day 2 to day 6. 10 different seeds were cut and counted. The sections were stained with Fast green. The collapsed cell layers were not counted.

Day	Day 2	Day 3	Day 4	Day 5	Day 6
Number of Cell layers (average)	67.7	62.4	57.8	52.8	29.2
Standard deviation	2.50	3.10	1.75	3.01	5.69

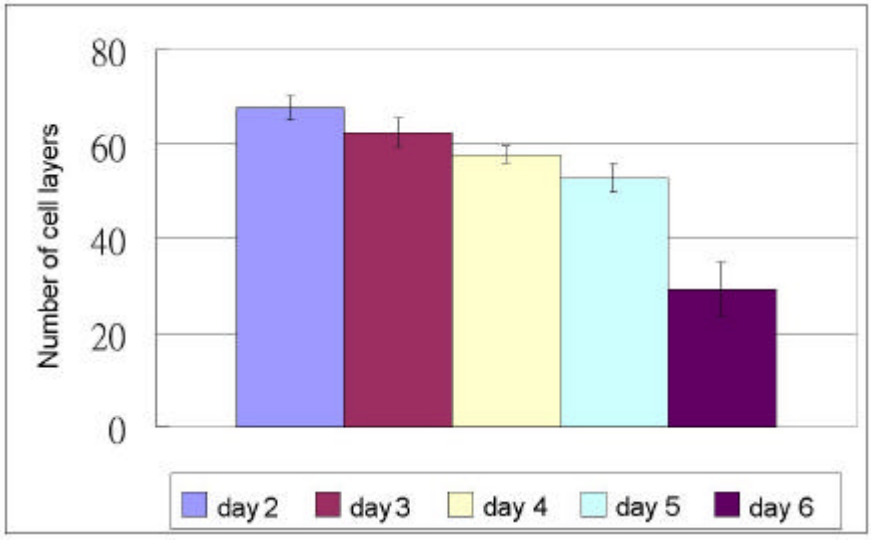


Figure 20 : The number of cell layers of from day 2 to day 6. 10 different seeds were cut and counted.

On days 2 and 3, dead and collapsed cell layers were very difficult to find in the region next to the cotyledons. The dead cell layers were observed clearly on day 4. On day 6, the collapsed cell layers increased dramatically (Figure 21).

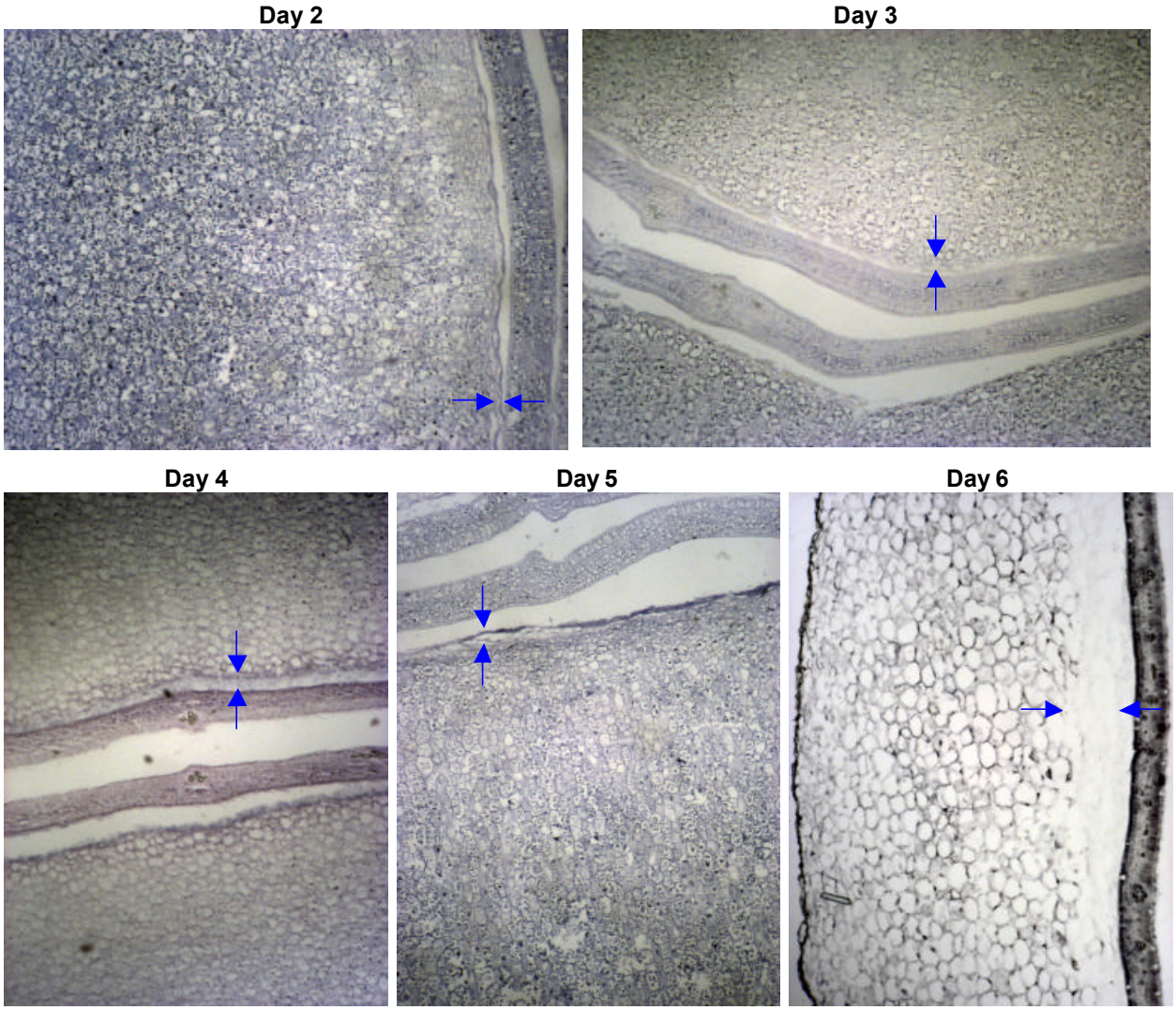


Figure 21 : The endosperm sections of germinating *Ricinus communis* seedlings. The collapsed cells were found in sections of different days. The layers between two arrows indicate the collapsed cell layers. On day 2 and day 3, there were only one or two collapsed cell layers next to the cotyledons. On day 6 a lot of cell layers between cotyledon and endosperm had collapsed.

C.2.2. Programmed cell death of *Ricinus communis* endosperm

The endosperm is a tissue specialized for storing and later for providing nutrients during germination. However, after a period of nutrient export, the endosperm's contribution to the seedling diminishes, and the endosperm undergoes its last phase: programmed cell death (PCD). Fragmentation of nuclear DNA is a feature of PCD in plants and animals. DNA nicking during PCD can be detected *in situ* by polymerizing labelled nucleotide to the exposed 3'-OH of the chromosomal DNA (Gorczyca *et al*. 1993). The result was visualized by developing in NBT/BCIP.

The fragmentation of nuclear DNA was found in the endosperm next to the collapsed cells of day 2, day 3 and day 4 samples (Figure 22, A, D and G). No fragmentation of nuclear DNA was found in cell layers next to the seed coat and in the middle layer of the same samples (Figure 22, B, C, E, F, H and I). The programmed cell death was found in the region near the cotyledons. On day 5, the fragmentation of nuclear DNA was not only found in the region next to the collapsed cells, but also in the middle and in the layers next to seed coat of endosperm (Figure 22, J, K and L). On day 6, the result was very similar to that of day 5. The fragmentation of nuclear DNA was found all over the endosperm, but the signal was very weak (Figure 22, M, N and O).

The result revealed that during germination programmed cell death in the endosperm began within the innermost cell layers closest to the cotyledon and spread across to the seed coat side of the endosperm cell layers during the next 3–4 days.

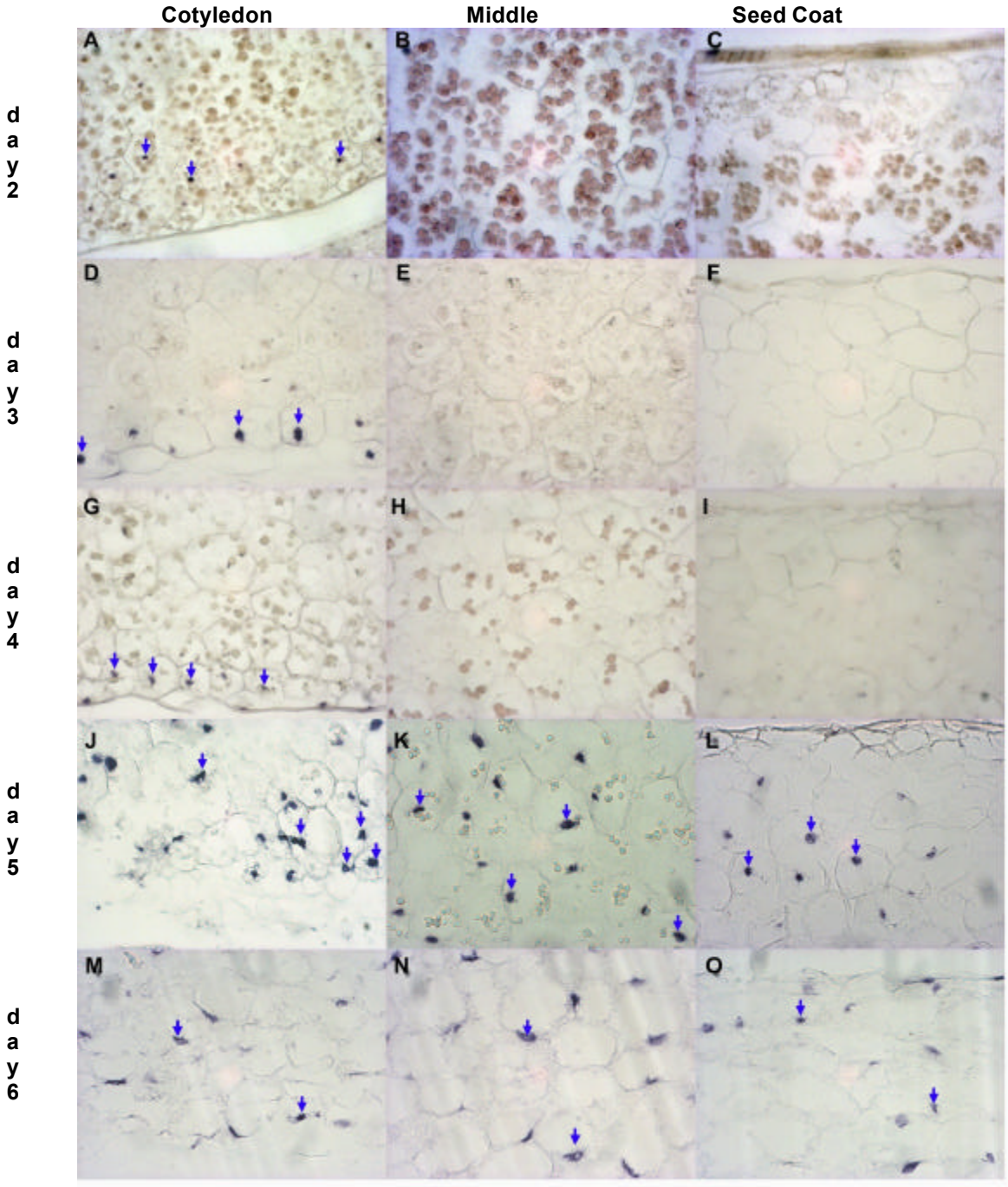


Figure 22 : The fragmentation of nuclear DNA of endosperm cells. The fragmented DNA was labelled with dig-dUTP and visualized by applying alkaline phosphatase conjugated anti-dig antibody and developing in NBT/BCIP. On Day 2, 3 and 4, DNA fragmentation was found next to cotyledons (A, D, G). No fragmentation of nuclear DNA was found in the middle or the layers near seed coat in the endosperm of day 2, day 3 and day 4 samples (B, C, E, F, H, I). The programmed cell death was found allover in the endosperm on day 5 and day 6 (J, K, L).

C.2.3. Mobilization of the stored macromolecules in the endosperm Starch accumulation in *Ricinus communis* cotyledons

During germination, the sucrose, which is released from *Ricinus* endosperm, is taken up by the cotyledons and then transported to other tissues. Sucrose is then used as the energy source and the carbon skeleton for other macromolecules.

The programmed cell death of endosperm cells was accelerated at day 5. During day 5 and 6, a large amount of endosperm cells collapsed. Before or at the same time, sucrose and other nutrients are released and then taken up by the cotyledons. Sucrose is used for the growth of seedling and as an energy source. A part of sucrose is converted to starch and stored in the cotyledons (Figure 23).

The starch was stained with KI. The colour of the stained starch was dark brown to black. The result indicated that starch was found in cotyledons at the day 6. On sections of day 4 and day 5 cotyledons, no staining signal was found (Figure 23, A, B). In endosperm cells starch was never found (Figure 23). The starch was accumulated not only in the parenchyma cells, but also in epidermis and companion cells (Figure 23, C). The whole cotyledons were also stained with KI. The starch was stained and found only in the cotyledons of day 6 (Figure 24).

Up to day 5, sucrose released from the endosperm seems to be used only for the growth of the whole seedling. Almost no sucrose was converted to starch and accumulated in the cotyledons. After acceleration of programmed cell death in endosperm, a larger amount of sucrose may be released and taken up by the cotyledons. Sucrose may not only be used for growth of the seedling but is also converted to starch and stored in cotyledons for later use.

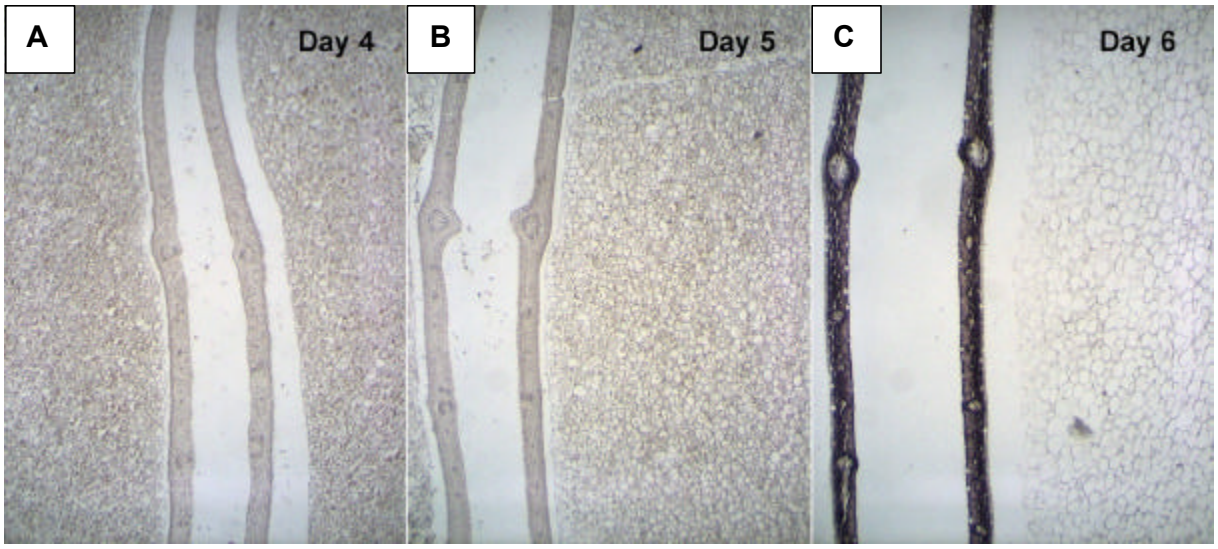


Figure 23 : Starch staining of *Ricinus communis* seeds. The paraffin sections were dewaxed and then stained with KI (A, B and C). No starch was found in the cotyledons of day 4 and day 5 (A, B). Starch could only be detected in the cotyledons of day 6 (C).

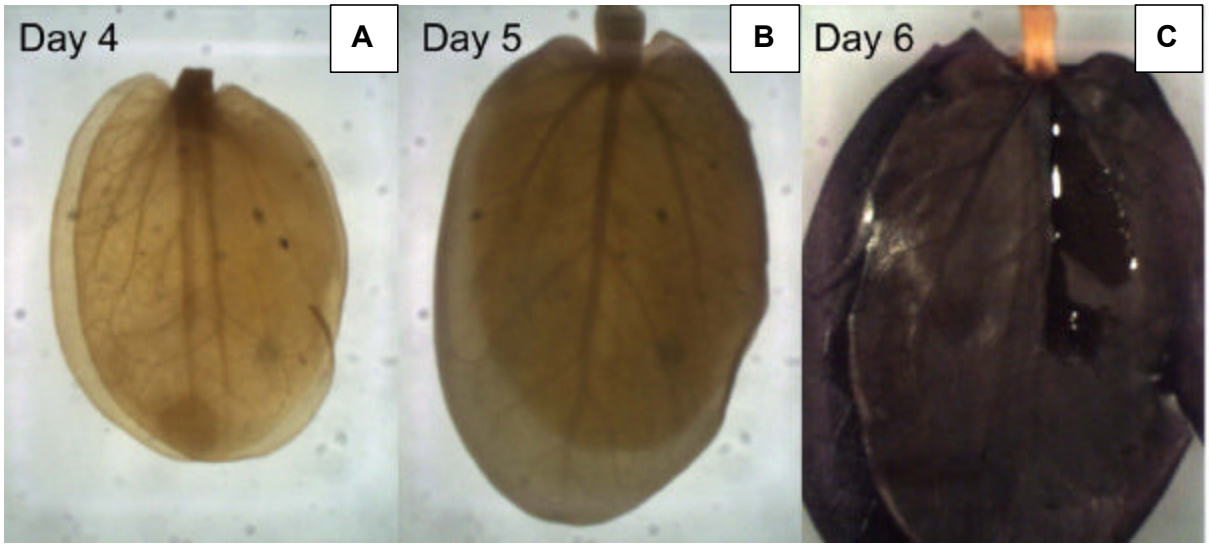


Figure 24 : Starch staining of *Ricinus communis* whole cotyledons. (A) (B) and (C), the fresh cotyledons were removed from the seedling. No starch was found in the cotyledons of day 4 and day 5 (A, B). Starch could only be detected in the cotyledons of day 6 (C).

C.3. Gene expression and localization of sucrose transporters

C.3.1. Gene expression and localization of *RcSCR1* in endosperm of *Ricinus* seedlings

C.3.1.1. Northern analysis of *RcSCR1*

The *RcSCR1* is a SUT1 type sucrose transporter, which was cloned by Weig (Weig and Komor, 1996). The *RcSCR1* full-length cDNA fragment was cloned into a pUC19 vector. The new plasmid pSC4N was used as an *RcSCR1* cDNA fragment donor.

In order to synthesize a digoxigenin labelled cRNA probe, the *RcSCR1* cDNA fragment was subcloned into a pBluescriptII KS+ vector. pSC4N was treated with *Not*I and then checked in a 0.8% agarose gel (Figure 25 a). The cDNA fragment was cut from the agarose gel and eluted. Subsequently the cDNA fragment was ligated with a pBluescriptII KS+ vector. Then the ligation product was transformed to DH5α competent cells. The transformants were picked and cultured overnight in LB+ ampicillin. The plasmid DNA was extracted from *E.coli*. and treated with *Nco*I and *Cla*I and then checked in an agarose gel. In the agarose gel two DNA fragments, 490bp and 4.4kp were observed (Figure 25 b).

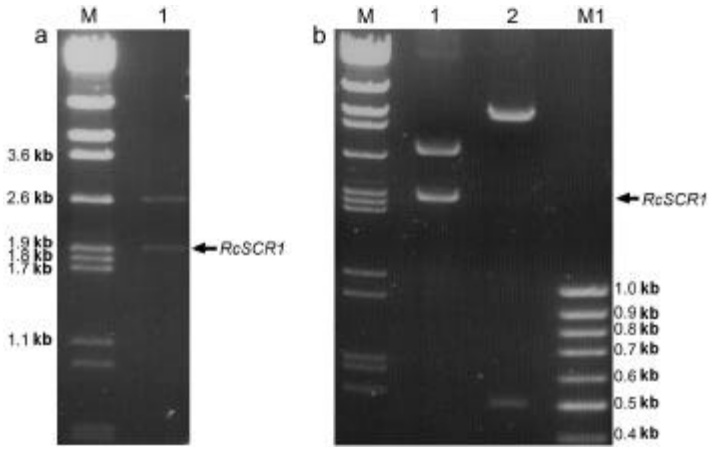


Figure 25: Gel electrophoresis of pSC4N and pSC4B. pSC4N was used as *RcSCR1* fragment donor. The cDNA fragment was then subcloned to a pBluescriptII KS+ plasmid. (a) M: l/*Cla*I marker; lane 1: pSC4N/*Not*I. (b) M: l/*Cla*I marker; M1: 100bp DNA marker. lane 1: pSC4B / *Not*I; pBluescriptII KS+ fragment = 2.95 kb; *RcSCR1* full length cDNA fragment= 1.9 kb; lane 2: pSC4B / *Nco*I + *Cla*I. Two bands (4.4 kb and 490 bp) are seen.

The new plasmid, pSC4B was constructed. The restriction map is shown in Figure 26. T3 RNA polymerase was used to synthesize a cRNA probe. The cRNA probe was treated with DNase I and then formamide was added. Afterwards it was stored at –80°C.

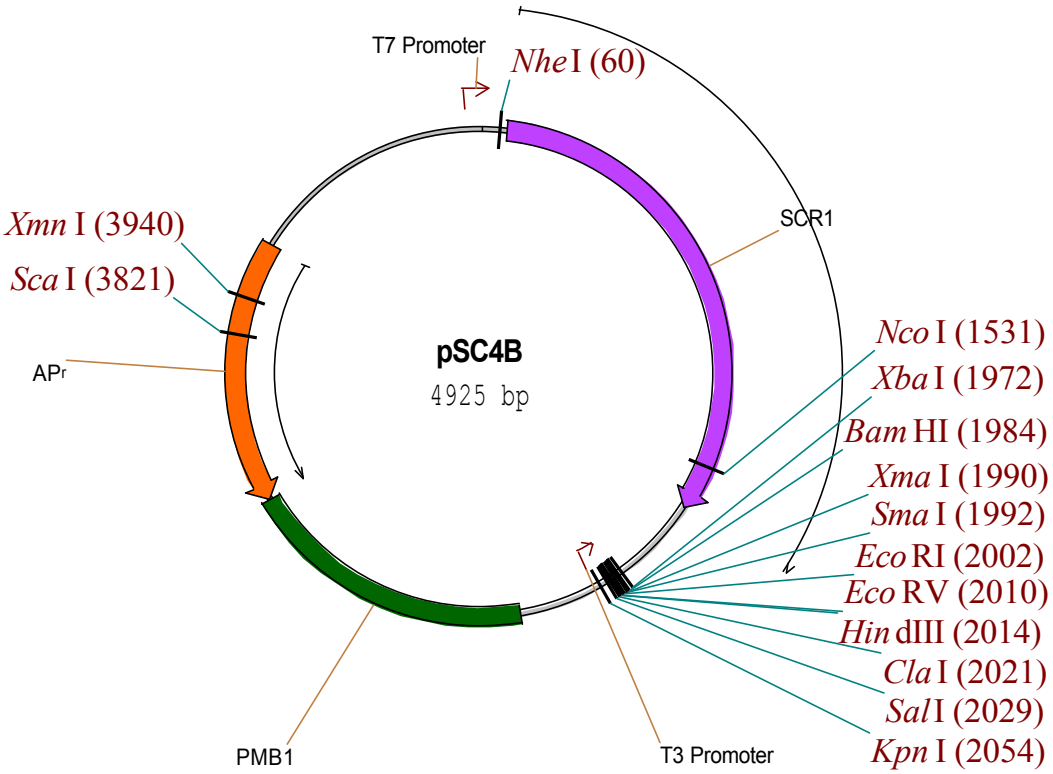


Figure 26: The restriction map of pSC4B. The *RcSCR1* cDNA fragment was subcloned to a pBluescriptII KS+ plasmid, which contains T3 promoter and T7 promoter. pSC4B can be used for *in vitro* transcription to generate digoxigenin-labelled RNA probes.

In order to analyze the *RcSCR1* expression in the endosperm, the total RNA was extracted from different days old *Ricinus* endosperms. Total RNA of day 2, 3, 4, 5, 6 was used for RNA gel electrophoresis. 20 µg of the total RNA was loaded in each lane (Figure 27 a).

After gel electrophoresis, the gel was transferred to a nylon membrane and hybridized with digoxigenin labelled RNA probe as described in materials and methods. The intensity of the signals showed that *RcSCR1* expression is increasing from day 2 to day 5. The *RcSCR1* expression is very weak on day 2. The day 5 sample has the highest expression and the expression decreased at day 6 (Figure 27 b).

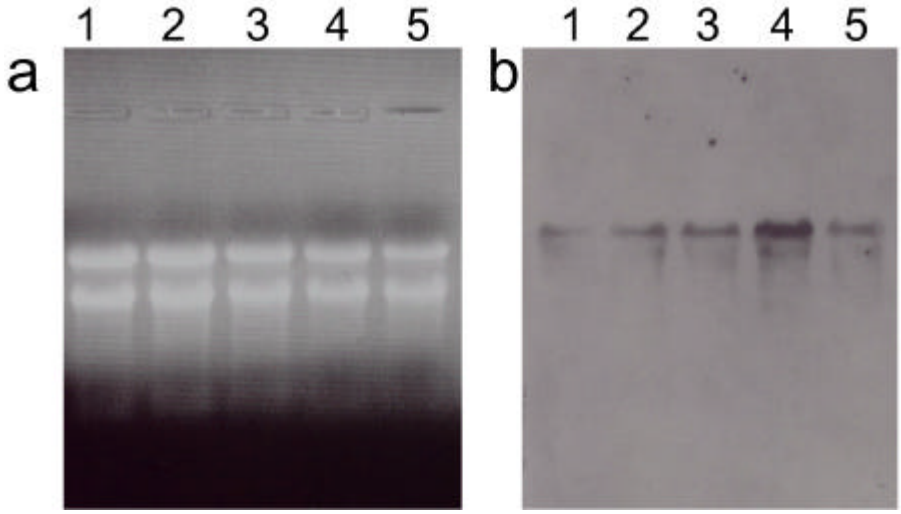


Figure 27: RNA Gel Blot Analysis of *RcSCR1*. 20 µg of total RNA was loaded to each lane.(a) RNA gel electrophoresis of total RNA endosperm; (b) The membrane was hybridized with digoxigenin labelled cRNA probe and exposed for 16 hours. Lane 1: Day 2, Lane 2: Day 3, Lane 3: Day 4, Lane 4: Day 5, Lane 5: Day 6.

C.3.1.2. *In Situ* Hybridization of *RcSCR1*

C.3.1.2.1. Establishing of the *in situ* hybridization technique

In situ hybridization is the basic method to detect mRNA on the cellular or subcellular level. To perform *in situ* hybridization, several controls are required and a highly expressed and organ or tissue specific gene is a good choice for a control. The hybridization signal can easily be distinguished from the background. We used the gene of non-specific lipid transfer protein (nsLTPc1) as a control, since it is expressed organ-specific in the cotyledons at a high level (Weig and Komor, 1992). This is why it is suitable to establish the *in situ* hybridization technique and to use it as a control for *in situ* hybridization of the *Ricinus* seedling.

C.3.1.2.2. *In Situ* Hybridization of *nsLTPc1*

In order to synthesize the digoxigenin labelled cRNA probe for *in situ* hybridization, a plasmid, which contains the 3'-NTR of nsLTPc1, was constructed. The 3'-NTR of nsLTPc1 was obtained by RT-PCR. The Oligo-(dT)15 primer and LTPc1-3NTR-F primer were used for the RT-PCR. The PCR product was purified from agarose gel and then ligated to a pGEM-T vector. Afterwards the purified PCR fragment was then transformed to DH5α competent cells. The pLTPC1NTP plasmid, which contained the 3'-NTR of nsLTPc1, was used for in vitro transcription of sense and anti-sense probes (Figure 28).

The plasmid pLTPC1NTP was cut with *Nco*I and then SP6 RNA polymerase was used to obtain a digoxigenin labelled anti-sense probe. Furthermore, pLTPC1NTP was cut with *Not*I and T7 RNA polymerase was used to obtain a digoxigenin labelled sense probe.

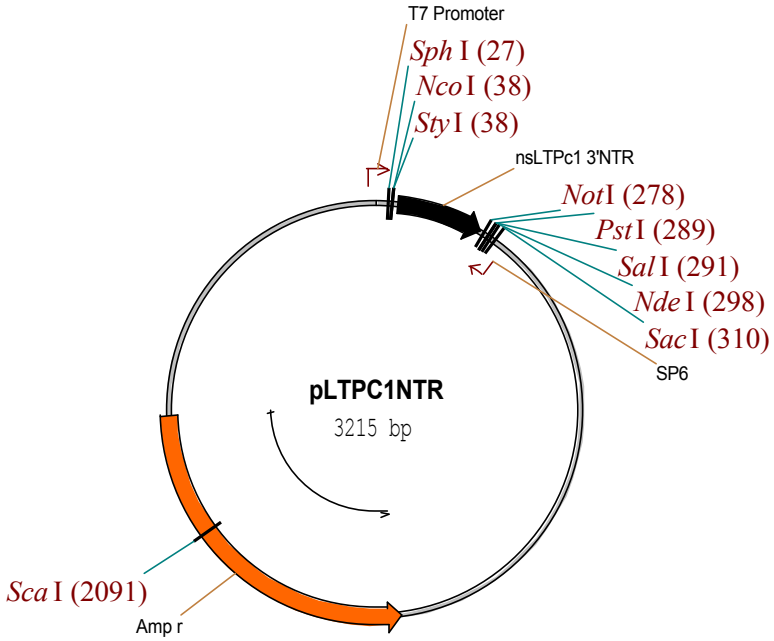


Figure 28: The restriction map of pLTPC1NTR. The 3´-NTR of *nsLTPc1* was cloned to a pGEM-T vector. This plasmid was used for *in vitro* transcription to synthesize digoxigenin labelled RNA probes.

The *in situ* hybridization was performed with the digoxigenin labelled sense and anti-sense probes. 200 ng of the probes were added to 1 ml hybridization solution and then hybridization was carried out. The result was checked under an Olympus microscope.

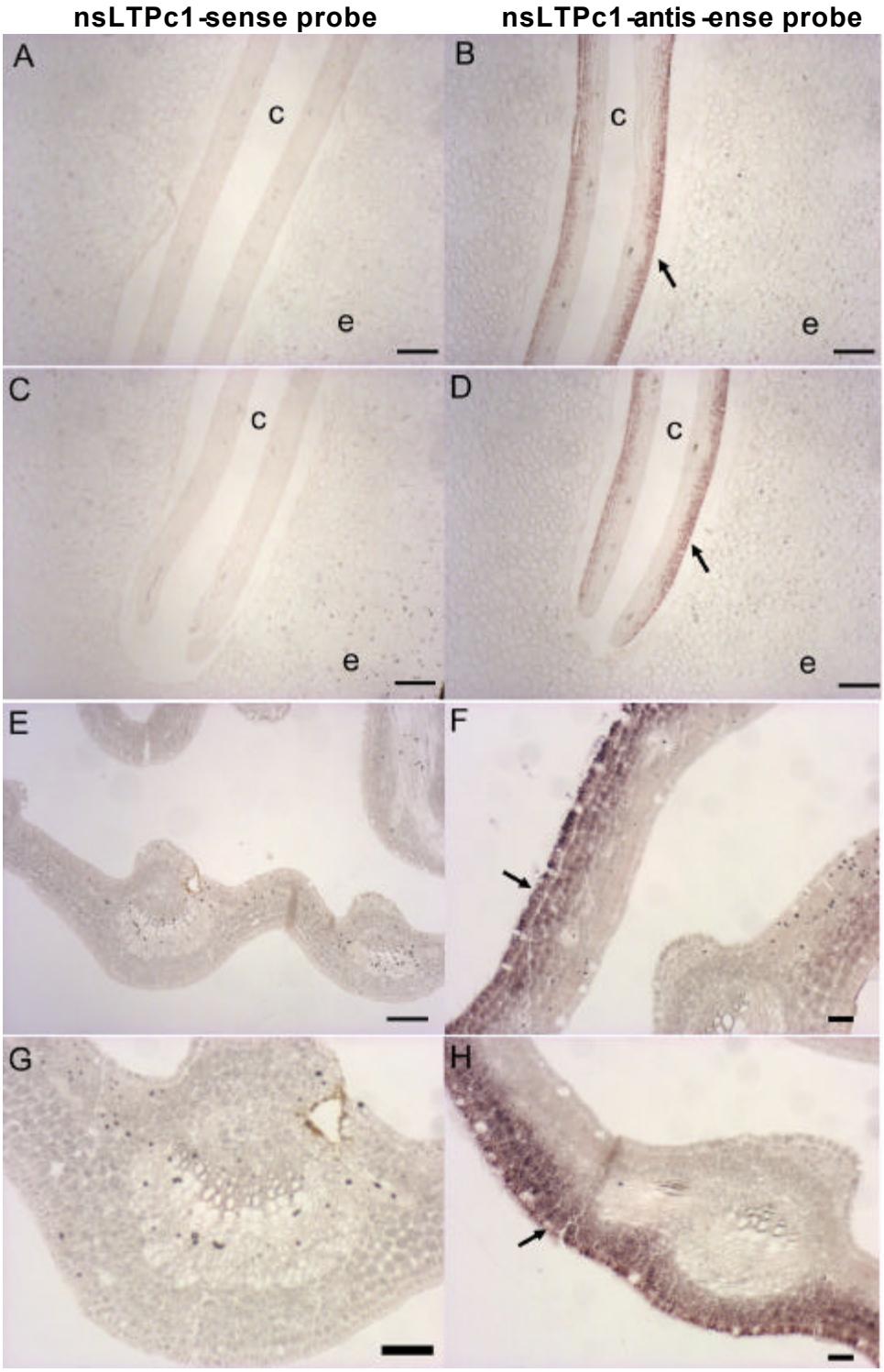


Figure 29: *In situ* hybridization of *nsLTPc1*. Sections from *Ricinus* seedlings were hybridized with digoxigenin-labelled *nsLTPc1* sense and antisense RNA probes. (A), (C), (E) and (G) sections were hybridized with digoxigenin-labelled sense RNA probe. (B), (D), (F) and (H) sections were hybridized with digoxigenin-labelled antisense RNA probe. c, cotyledon. e, endosperm. Bar in (A), (B), (C), (D) and (E) = 200 µm. Bar in (F), (G) and (H) = 25 µm.

No hybridization signal could be seen in the sections that were hybridized with the sense probe (Figure 29, A, C, E and G). In the sections, which were hybridized with the anti-sense probe, however, the *nsLTPc1* transcript could be detected specifically in the lower half of the cotyledon. About 4 to 5 layers of cells showed strong *nsLTPc1* expression (Figure 29, F and G). No hybridization signal was detected in the endosperm (Figure 29, B and D). Thus, the organ-specific expression of *nsLTPc1* could be confirmed by this technique and in addition a cell-specific expression was found.

C.3.1.2.3. *In Situ* Hybridization of *RcSCR1*

In an attempt to examine the expression site of *RcSCR1*, digoxigenin labelled sense and anti-sense probes were synthesized. Two different methods were used to synthesize these probes, (1) the full-length probe was synthesized and then partially hydrolyzed to about 200 bases and (2) a new plasmid was constructed, which contains the 3'-NTR of the *RcSCR1*.

To construct the new plasmid, a PCR was performed to amplify the *RcSCR1* 3'-NTR fragment. Afterwards the PCR product was checked in an agarose gel and then the DNA fragment of *RcSCR1* 3'-NTR was ligated to pGEM-T vector. The pSCR1NTR (Figure 30), which contains the *RcSCR1* 3'-NTR, was cut by *Not*I or *Nco*I. The sense probe and the anti-sense probe were obtained by *in vitro* transcription using SP6 RNA polymerase or T7 RNA polymerase. Because the sense and anti-sense probes were short enough, they need not be hydrolyzed. Then the *in situ* hybridization was performed with different probes. 200 ng of probe were added to 1 ml hybridization solution.

An optimal sucrose transport rate was found in 5-day-old *Ricinus* seedlings (Komor, 1977). Using tissues sections of 5-day-old seedlings to perform *in situ* hybridization and strong hybridization signal should be found in cotyledons. The results indicated that no hybridization signal was detected in the sections, which were hybridized with the sense probe. But a strong hybridization signal was detected in the sections, which were hybridized with the anti-sense probe (Figure 31).

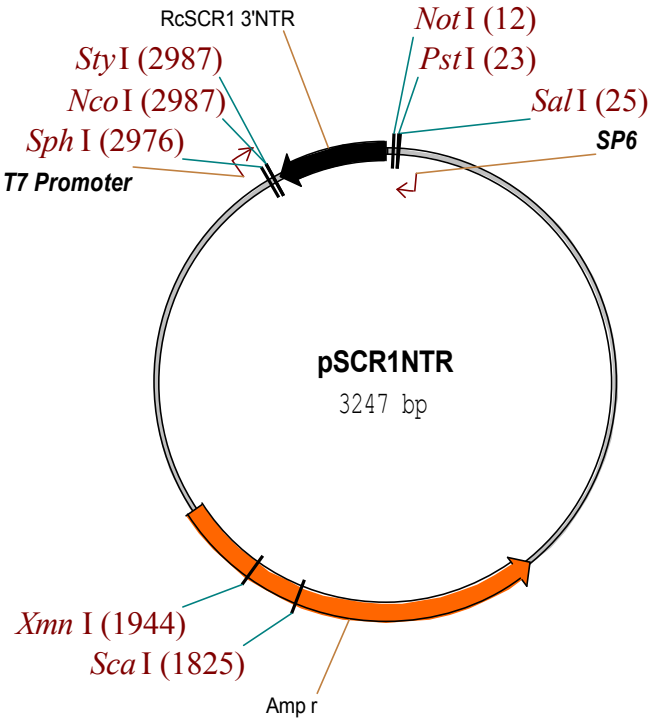


Figure 30: The restriction map of pSCR1NTR. The 3´-NTR of *RcSCR1* was cloned to pGEM-T vector. This plasmid was used to generate RNA probe encoded to 3´-NTR of *RcSCR1*.

Using hydrolyzed probes to perform *in situ* hybridization led to the similar results compared to using non-hydrolyzed digoxigenin-labelled probes. To examine the expression of *RcSCR1* during the development of the seedling, *in situ* hybridization was performed with the endosperm and the cotyledons of 2 - 6 days old seedlings. No hybridization signals were detected in all sections of day 2 to day 6 samples that were incubated with the hybridization buffer and sense probe (Figure 32, A, D, G, J and M).

Day 2 cotyledons showed only very weak hybridization signals with the anti-sense probe(Figure 32, B and C). In the day 3 - 5 samples, the signals in the cotyledons were relatively stronger and were detected mainly in the lower epidermis (Figure 32, E, F, H, I, K and L). In the day 6 sample, the highest expression of *RcSCR1* was foound in the palisade parenchyma cell layer (Figure 32, N and O). Under low magnification, some dark staining was seen in the endosperm sections of day 4, 5 and 6 samples, but the hybridization signal was hardly seen under high magnification, where it was clearly detected in the cells of the cotyledons. No hybridization signal was found in the phloem tissue of the cotyledons at any age of the seedling.

Different tissue sections of 6 days old seedlings were used for *RcSCR1 in situ* hybridization (Figure 33). The results indicated that no hybridization signal was detected in the sections, which were hybridized with the sense probe (Figure 33, A, C). But a strong hybridization signal was detected in the sections, which were hybridized with the anti-sense probe (Figure 33, B, D). In these sections, the hybridization signals were localized in the cotyledons and the hypocotyl but not in the endosperm (Figure 33, B). In the cotyledons, the *RcSCR1* transcript was detected mainly in the palisade parenchyma cell layer (Figure 33, B), in the hypocotyl, it was detected in the phloem area (Figure 33, D).

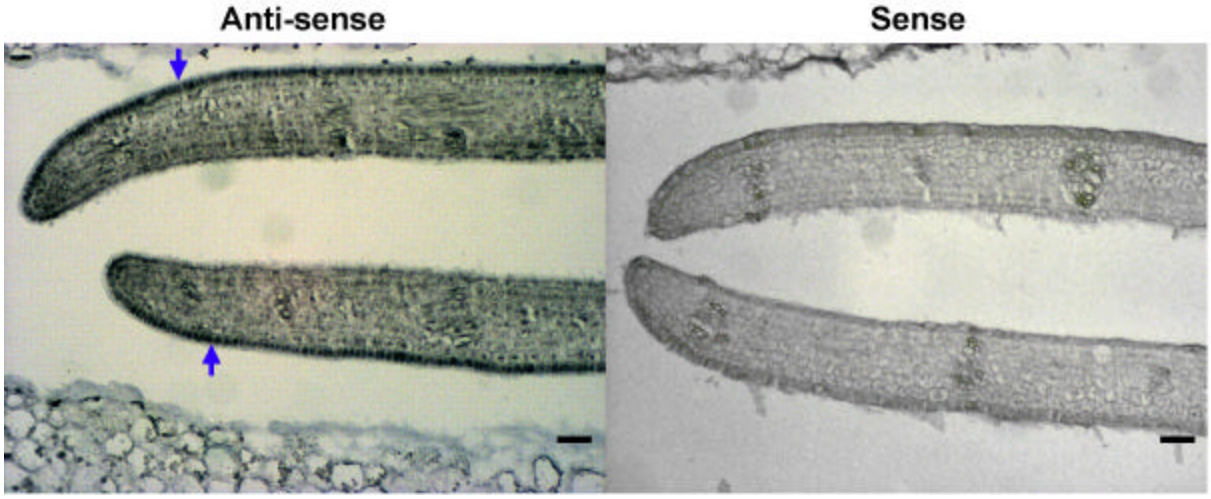
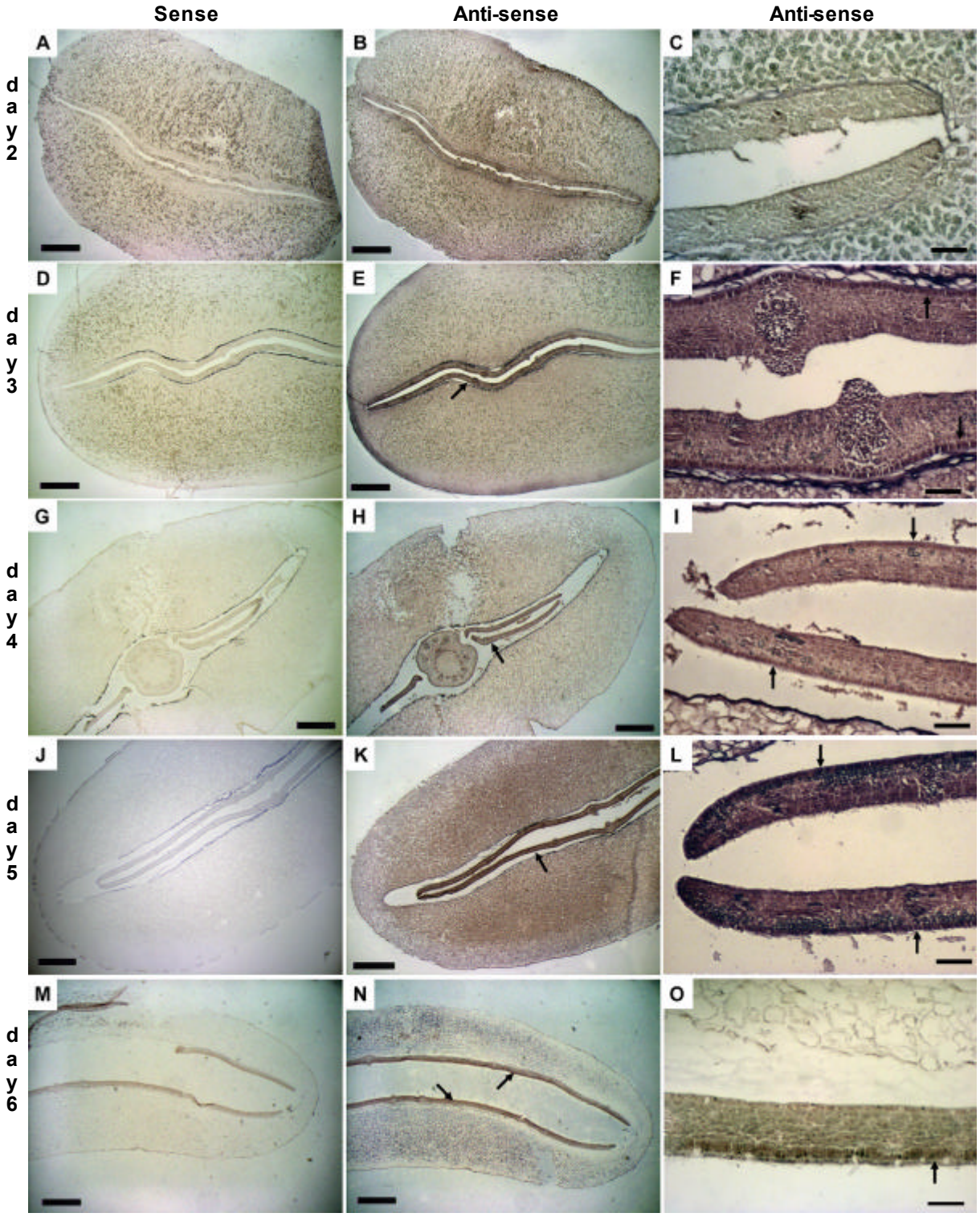


Figure 31 : *In situ* hybridization of *RcSCR1*. Cross sections of cotyledons of day 5 seedling were used. They were hybridized with digoxigenin-labelled anti-sense probe or sense probes. *RcSCR1* mRNA could be found in the lower epidermis of the cotyledons. The arrows indicate the hybridization signal. Bar = 50 µm.

Figure 32: (next page) *In situ* hybridization of *RcSCR1*. All sections (10 µm thick) were hybridized with digoxigenin-labelled anti-sense or sense probe. (A), (D), (G), (J) and (M) are negative controls. They were hybridized with the sense probe. (B), (C), (E), (F), (H), (I), (K), (L), (N) and (O) are samples. They were treated with the anti-sense probe. (A), (B), (D), (E), (G), (H), (J), (K), (M) and (N) were observed under low magnification. The arrows indicate the localization of *RcSCR1* mRNA. (A), (B) and (C) : cotyledons and endosperm sections of day 2; (D), (E) and (F) : cotyledons and endosperm of day 3; (G), (H) and (I) : cotyledons and endosperm sections of day 4; (J), (K) and (L) : cotyledons and endosperm sections of day 5; (M), (N) and (O) : cotyledons and endosperm sections of day 6. Bar in (A), (B), (D), (E), (G), (H), (J), (K), (L) and (M) = 1 mm; bar in (C), (F), (I), (L) and (O) = 100 µm.



Expression of *RcSCR1* was not only found in the cotyledons, but also in other tissues. *RcSCR1* was found in most of the cells of the young developing leaves of the seedling (Figure 34, A). The *RcSCR1* was also found in the phloem of the hypocotyl and the petiole

(Figure 34, B and C). In the young developing seed, *RcSCR1* was also found in the seed coat (Figure 35, A and B). The results indicated that *RcSCR1* expression is not restricted to the germinating seedling, but is also found in d eveloping seeds and other tissues.

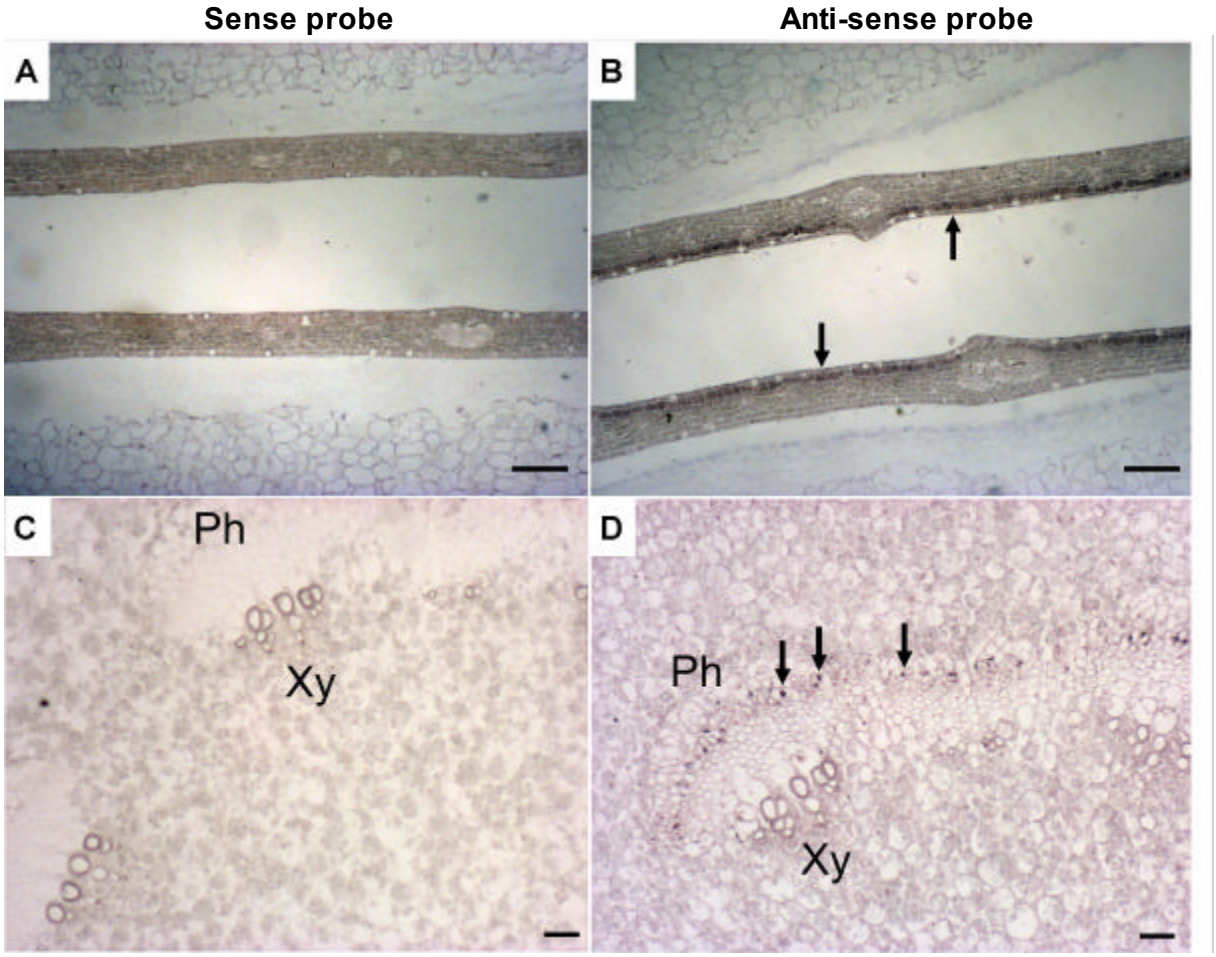


Figure 33: *In situ* hybridization of *RcSCR1*. (A) and (C) are negative controls. They were hybridized with the sense probe. (B) and (D) are samples. They were hybridized with the anti-sense probe. The arrows indicate the hybridization signal. Cross section of (A) and (B): cotyledons of day 6; (C) and (D): hypocotyl cross sections of day 6; Ph, phloem; Xy, xylem. Bar in (A) to (B) = 200 µm, bar in (C), (D) = 50 µm.

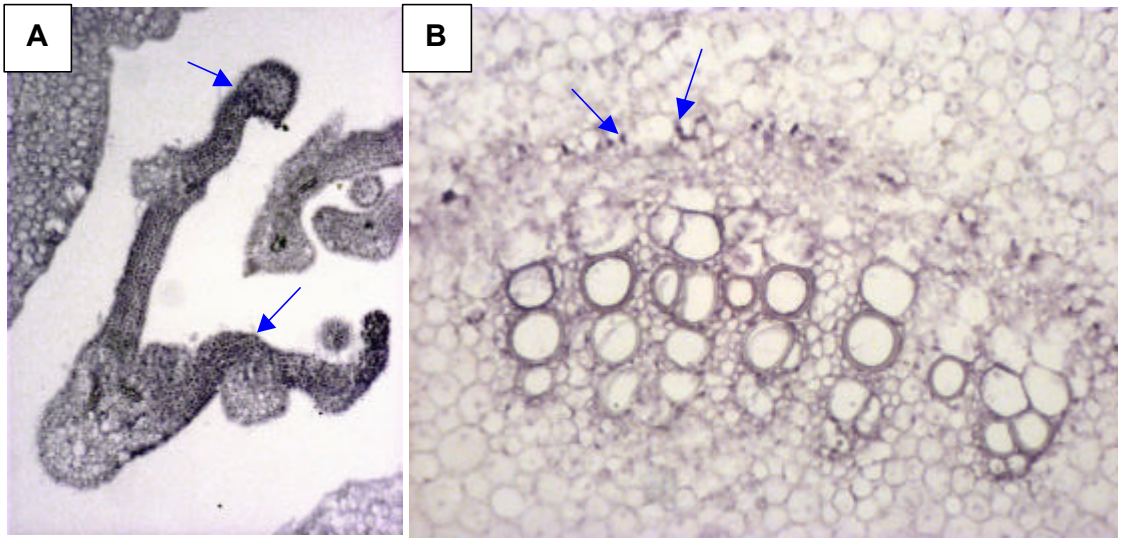


Figure 34: *RcSCR1 in situ* hybridization of different tissues of *Ricinus communis*. The sections were hybridized with anti-sense probe. (A) young developing leaf, (B) petiole. The arrows indicate the hybridization signals of *RcSCR1*.

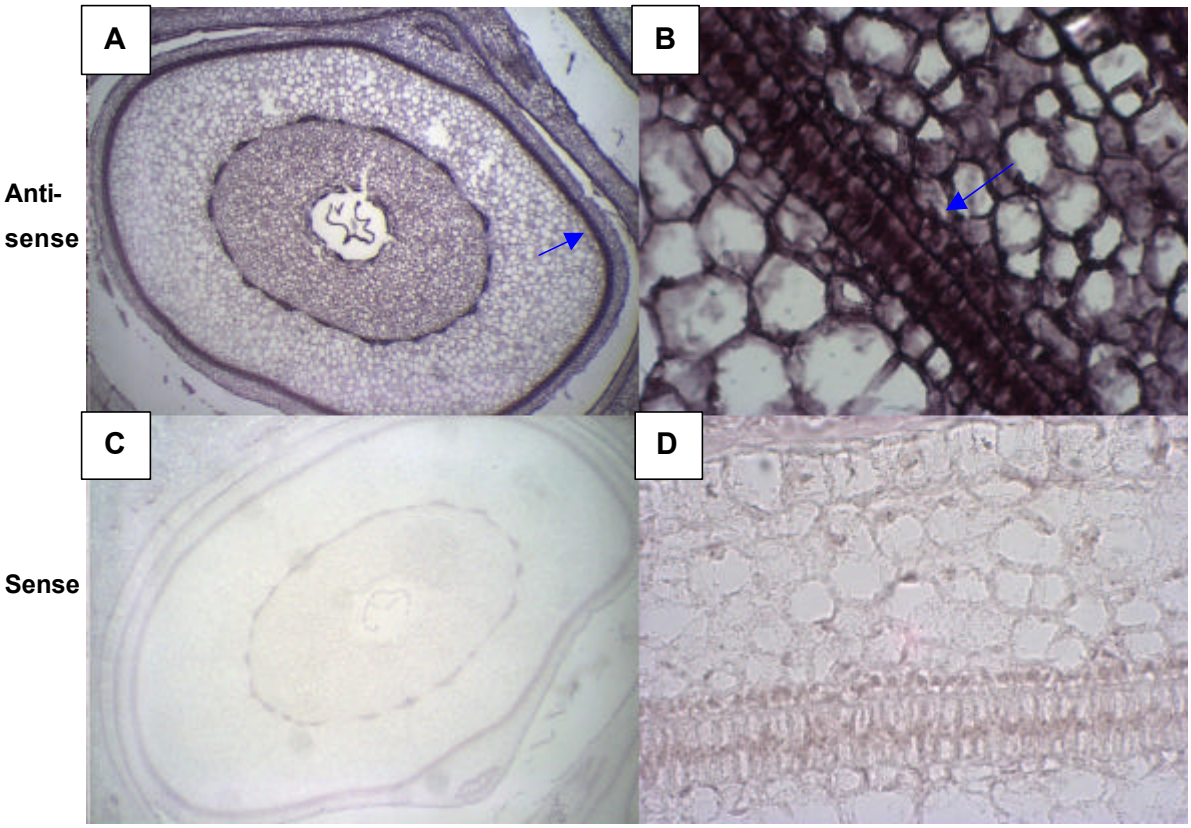


Figure 35 : *RcSCR1 in situ* hybridization of developing seeds of *Ricinus communis*. The *RcSCR1* mRNA was found in the seed coat of developing seeds. The arrows indicate the *RcSCR1* mRNA in the cross section of developing seeds. (A) and (B) were hybridized with anti-sense probe. (C) and (D) were hybridized with sense probe.

C.3.1.2.4. *In situ* RT-PCR of *RcSCR1*

To examine the overall expression of *RcSCR1* in the endosperm and to confirm the results of the *in situ* hybridization, *in situ* RT-PCR was carried out. Digoxigenin 11-dUTP was directly incorporated into *RcSCR1* cDNA fragment. To obtain reliable results, many different controls were performed.

The 3'-NTR of *RcSCR1* is about 290 bp long. By using gene specific scr1-3ntrF1 and scr1-3ntrR1 primer and the cDNA from the endosperm to perform PCR, a 245bp long PCR product could be obtained.

Table 12: Primers used for *in situ* RT-PCR of *RcSCR1*.

Primer	Sequence
scr1-3ntrF1	5'-CCCATGCATATCAGCACCATATGTGGATGT-3'
scr1-3ntrR1	5'-CTGCATATTAAACATAAGATTGTGTACAGC-3'

Two different negative controls were performed. One was treatment with RNase A and DNase I to eliminate mRNA and genomic DNA. Another negative control was RT-PCR performed without primers. The results indicated that using RNase and DNase treatment could have better result (data not shown). The samples were treated with RNase free DNase I overnight to eliminate the genomic DNA, the positive control was directly performed *in situ* RT-PCR (Figure 36).

In Figure 37 *in situ* RT-PCR of *RcSCR1* is shown with sections of the cotyledons and endosperm of 2 to 6 days old seedlings. The *RcSCR1* cDNA could be detected in the cotyledons and in the endosperm of day 2 to day 5 samples (Figure 37). In samples of day 6, the amplified *RcSCR1* cDNA was detected mainly in the cotyledons, while very weak signals were found in the endosperm of day 6 (Figure 37, N and O). In sections of day 2, the amplified *RcSCR1* cDNA was detected in all cells of the endosperm (Figure 37, B and C).

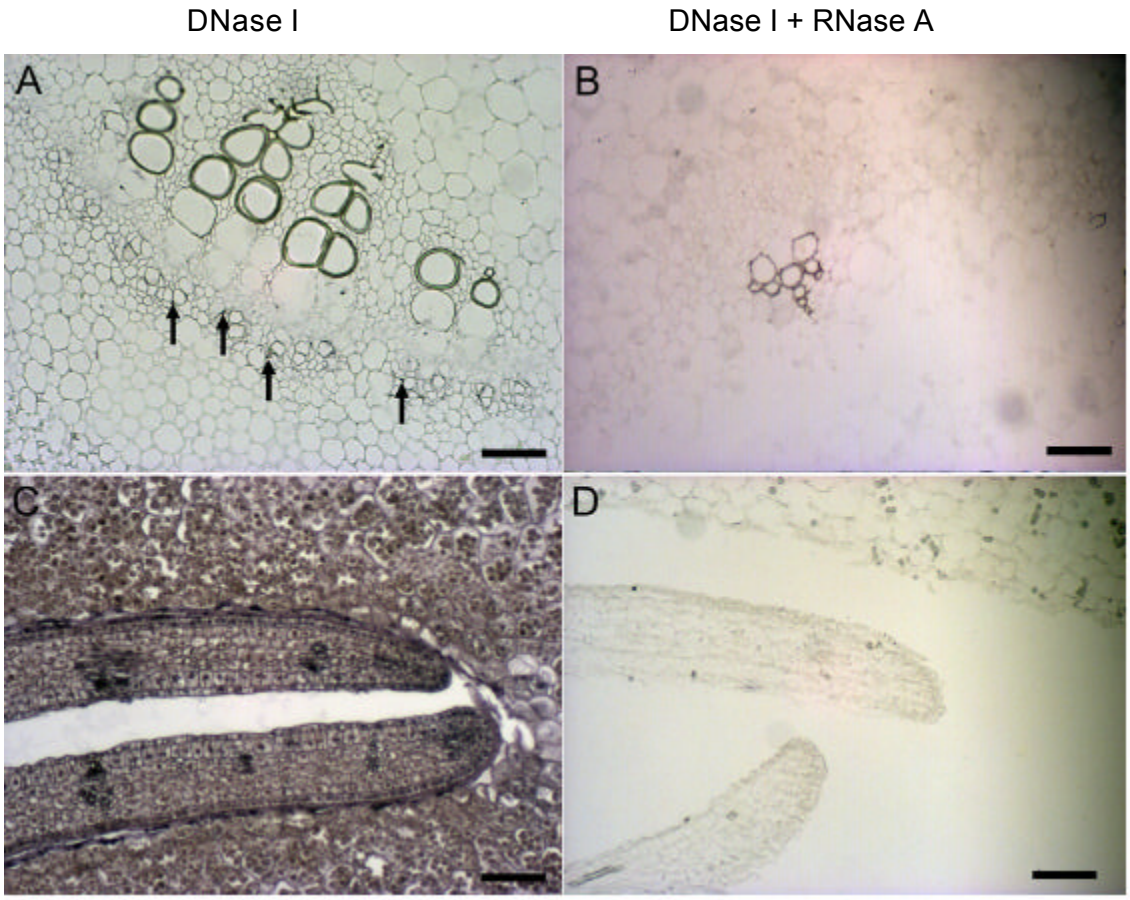


Figure 36 : *In situ* RT-PCR of *RcSCR1*. All sections (10 µm thick) were treated with proteinase K and DNase I with or without RNase A, and then RT-PCR was performed. (A) Bright-field microscopy of hypocotyl treated with DNase I. Arrows indicate the RT-PCR products of *RcSCR1* mRNA in SE-CCC. (B) Bright-field microscopy of hypocotyl that was treated with DNase I and RNase A (negative control). (C) Bright-field microscopy of cotyledons and endosperm of day 4 that were treated with DNase I. (D) Bright-field microscopy of cotyledons and endosperm of day 4 that were treated with DNase I and RNase A (negative control). Arrows indicate the signals. Bar in (A) (C) and (D) = 100 µm. Bar in (B) = 200 µm.

In sections of day 3, the amplified *RcSCR1* cDNA was detected in most of the cells of the endosperm. In the border of the endosperm (near the seed coat), in outermost 4-6 cell layers next to the seed coat did not show any signals, while the adjacent 4-6 cell layers exhibited the strongest signal. From these layers to the central of endosperm (near the cotyledons), the amplified *RcSCR1* cDNA could also be detected, but the signal was weaker than in the middle layer (Figure 37, E and F).

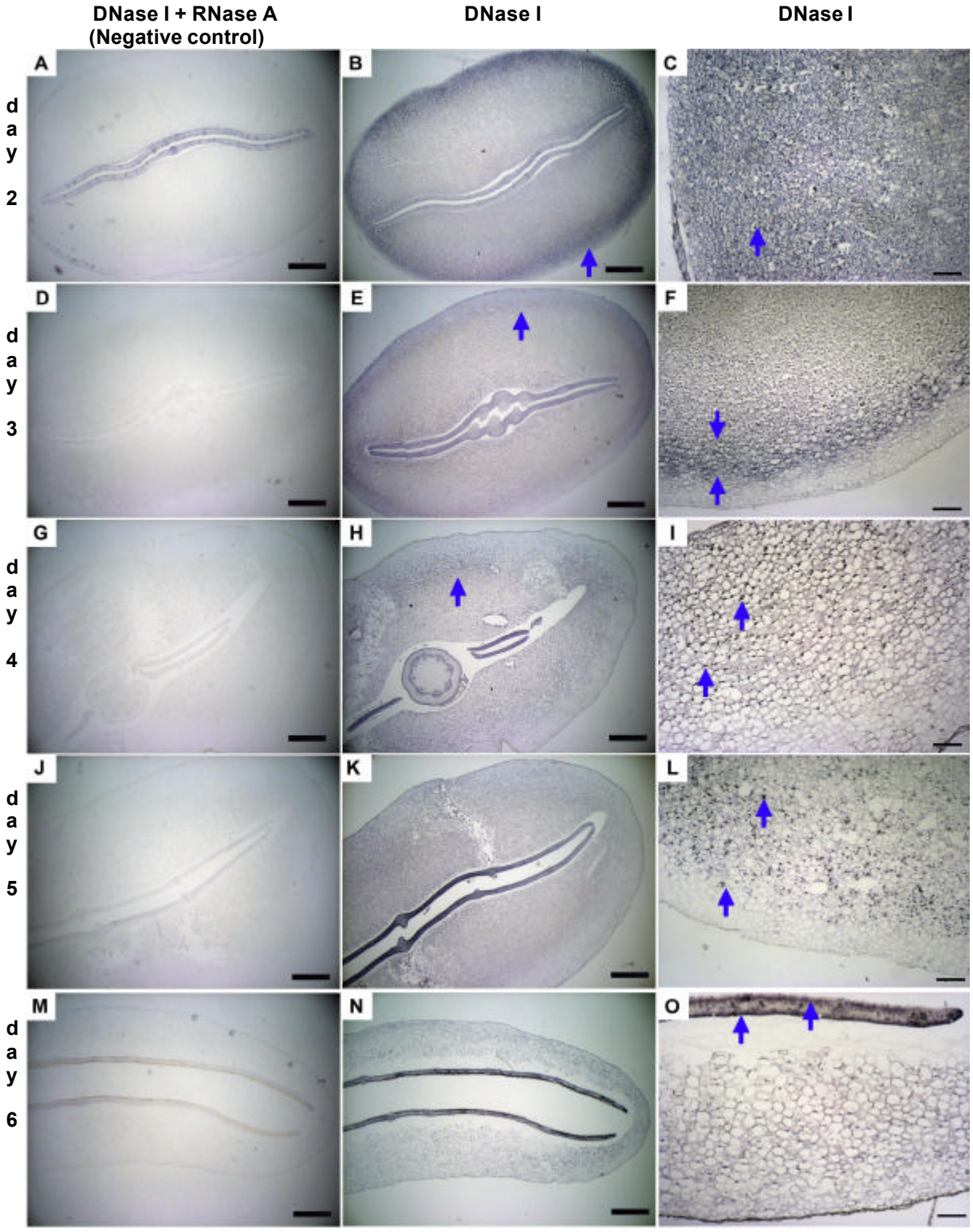
In the sections of day 4 and day 5, the *RcSCR1* signal could also be found in the inner side of the endosperm and cotyledons (Figure 37, H and K). No signal could be found in the outer layer of the endosperm (Figure 37, I and L).

In sections of day 6, the *RcSCR1* were hardly detected in the endosperm. The number of cell layers of the endosperm decreased dramatically. But the *RcSCR1* was detected in the day 6 cotyledons. The signal could be seen mainly in the upper parenchyma cell layer and the lower epidermis (Figure 37, N).

When the results of *in situ* hybridization and *in situ* RT-PCR are compared, *in situ* RT-PCR performed better than *in situ* hybridization. In day 2 and day 3 sections of *in situ* hybridization, the background is very strong. It is very difficult to find the real signals. But in day 2 and day 3 sections of *in situ* RT-PCR, the signals could be seen in the endosperm very clearly. In day 2 sections of *in situ* hybridization, the *RcSCR1* signal could not be found in the cotyledons, but in the day 2 sections of *in situ* RT-PCR, it was shown that there is *RcSCR1* expression in almost all cells in the cotyledons.

Some of the signals of *in situ* hybridization are ambiguous. To separate the signals from the noise is very difficult. By using *in situ* RT-PCR technique, a low amount of transcript in cotyledons was amplified and the signal was very clearly detected. In the *in situ* hybridization sections of day 6, the expression of *RcSCR1* was found only in the upper parenchyma cell layer of the cotyledons, but no transcript was detected in other cells of the cotyledon or in the endosperm. In the *in situ* RT-PCR sections of day 6, the *RcSCR1* transcript was detected in the upper layer of parenchyma cell layer and lower epidermis (Figure 37, O).

Figure 37 : (next page) *In situ* RT-PCR of *RcSCR1* in paraplast sections of *Ricinus communis* L. germinating seedlings. (A), (D), (G), (J) and (M) are negative controls treated with DNase I and RNase A. (B), (C), (E), (F), (H), (I), (K), (L), (N) and (O) are RT-PCR samples treated only with DNase I. (C), (F), (I), (L) and (O) show details of the *in situ* RT-PCR of day 2 to day 6 sections. The signals can be seen in the endosperm and the cotyledons. Arrows indicate the signals. All sections were cut in a thick of 10 µm thick. Bar in (A), (B), (D), (E), (G), (H), (J), (K), (M) and (N) = 1 mm, bar in (C), (F), (I), (L) and (O)= 200 µm.


DNase I + RNase A
(Negative control)
DNase I
DNase I
A
B
C
day 2
D
E
F
day 3
G
H
I
day 4
J
K
L
day 5
M
N
O
day 6

C.3.1.2.5. Immunolocalization of RcSCR1 protein

To determine the RcSCR1 protein localization is also important because the transcription and translation are independent events. To confirm localization of RcSCR1 protein in the endosperm and cotyledons, immunolocalization was carried out to find the localization of RcSCR1 protein. The anti-RcSCR1 serum is against the N-terminal (amino-MQSSTSKENKQPPSSQPHP-carboxyl) of RcSCR1 protein.

An optimal sucrose uptake was found in 5-day-old *Ricinus* seedlings (Komor, 1977). Using tissues sections of 5-day-old seedlings to perform immunolocalization and RcSCR1 protein should be found in cotyledons. The results indicated that no signal was detected in the sections, which were incubated with the pre-immune serum. But a strong signal was detected in the sections, which were incubated with the anti-RcSCR1 serum (Figure 38).

The result of immunolocalization of RcSCR1 is very similar to the result of *in situ* hybridization. Only very weak signal of RcSCR1 protein could be detected in endosperm. To separate the signal of RcSCR1 protein from the background is difficult. But a significant signal could be observed in the cotyledons.

RcSCR1 immunolocalization was performed in the cotyledon and endosperm sections and the RcSCR1 protein patterns were illustrated on Figure 39. RcSCR1 signal was observed in the endosperm of day 2, day 3 and day 4 sections (Figure 39, B, E and H). The RcSCR1 protein was detected in the endosperm of the sections of the day 2, day 3 and day 4, but the signal of RcSCR1 protein is very weak in the day 4 sections. No significant signal of RcSCR1 protein was detected in the endosperm of day 5 and day 6 sections (Figure 39, K and N).

In *Ricinus* cotyledons, the RcSCR1 protein was seen mainly in the lower epidermis (Figure 39, I, L and O). No significant signal of RcSCR1 protein was detected in the cotyledons of the day 2 sections (Figure 39, B). Only a weak signal of RcSCR1 protein was detected in the lower epidermis cells of the cotyledons of day 3 sections (Figure 39, F). The highest signal of the RcSCR1 protein was observed in the sections of the day 4 and day 5. The signal was found mainly in the lower epidermis of the cotyledons (Figure 39, K and L).

In the sections of day 6, the signal of RcSCR1 protein was found not only in the lower epidermis of the cotyledon, but was also detected in the palisade parenchyma cell layer (Figure 39, N and O). The result was very similar to the signal of *in situ* hybridization. The RcSCR1 protein was found mainly in the lower epidermis of cotyledons. No RcSCR1 protein was found in the phloem of cotyledons.

RcSCR1 was also found in the phloem of the hypocotyl of the *Ricinus communis* seedling (Figure 40, A). The RcSCR1 protein was also found in petiole (Figure 40, B), but the signal was not so strong. RcSCR1 was observed in the apical meristem, where phloem tissue was not found and the cells were small (Figure 40, C). RcSCR1 protein was observed in the seed coat of the young developing seed (Figure 40, D). The results of RcSCR1 protein immunolocalization were very similar to the result of *in situ* hybridization of *RcSCR1* in hypocotyls, petiole, and young developing tissues and seed.

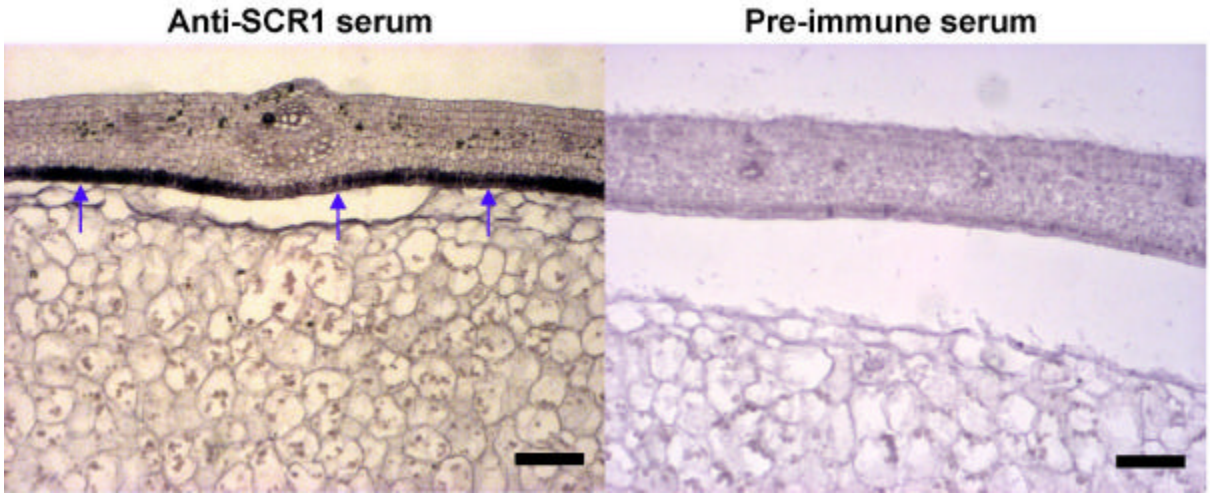


Figure 38 : Immunohistochemical localization of RcSCR1 protein of day 5 germinating *Ricinus communis* L. seedlings. All sections were cut in a thick of 10 µm. Sections were treated with pre-immune serumor Anti-RcSCR1 serum. The signals can be seen in lower epidermis of cotyledons. Arrows indicate the positive staining signals. Bar = 100 µm.

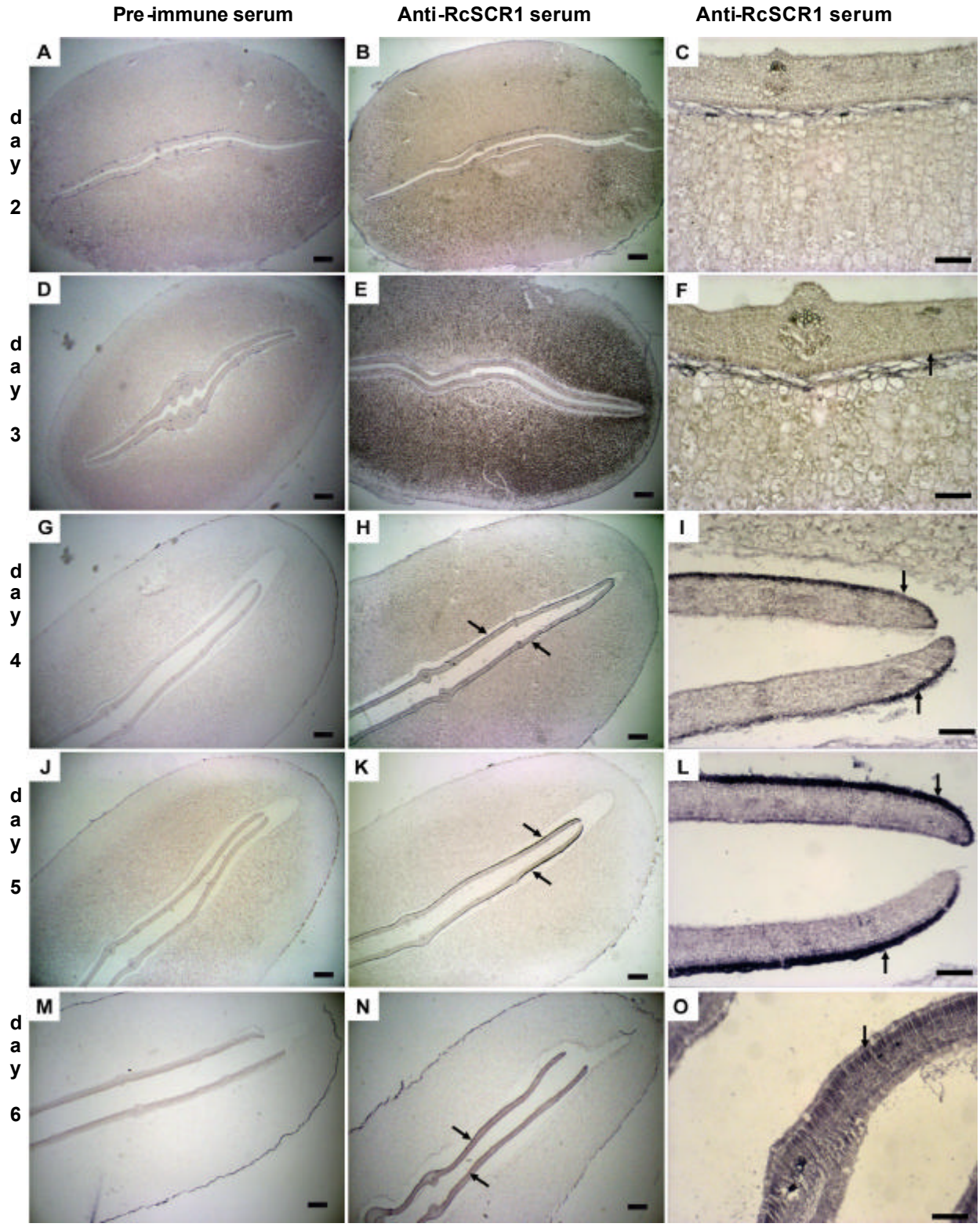


Figure 39 : Immunohistochemical localization of RcSCR1 protein in paraplast sections of germinating *Ricinus communis* L. seedlings. All sections were cut in a thick of 10 µm. (A), (D), (G), (J) and (M) were treated with pre-immune serum. (B), (C), (E), (F), (H), (I), (K), (L), (N) and (O) were treated with Anti-RcSCR1 serum. (C), (F), (I), (L) and (O) are the detail of the immunolocalization of day 2 to day 6 sections. The signals can be seen in cotyledons and endosperm. Clear signals of RcSCR1 protein

can be seen in cotyledons (H), (I), (K), (L), (N) and (O). Arrows indicate the positive staining signals. Bar in (A), (B), (D), (E), (G), (H), (J), (K), (M) and (N) = 1 mm, bar in (C), (F), (I), (L) and (O)= 50 µm.

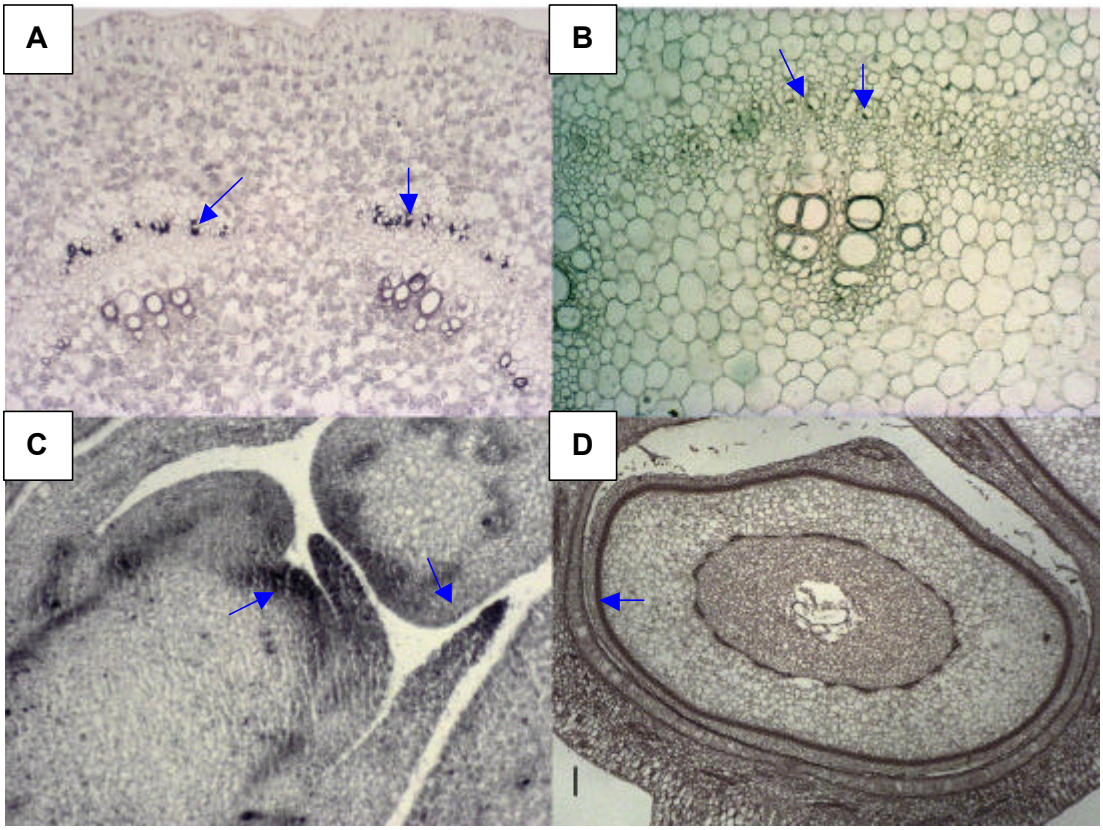


Figure 40 : Immunohistochemical localization of RcSCR1 protein in different tissues. The arrows indicate the localization of RcSCR1 protein. (A) hypocotyl; (B) petiole; (C) apical meristem and (D) young developing seed.

C.3.2. Gene expression and localization of *RcSCR2*

C.3.2.1. Northern analysis of *RcSCR2* in the endosperm of *Ricinus* seedlings

The *RcSCR2* plasmid was cloned by Eisenbarth (Eisenbarth, Diploma thesis, Bayreuth 1999). The *RcSCR2* cDNA fragment was ligated to a pBluescriptII KS+ vector. In order to synthesize the *RcSCR2* anti-sense digoxigenin labelled cRNA probe, *in vitro* transcription was performed with the pDL13 plasmid, as a template (Figure 41). The pDL13 plasmid was cut with *Sac*I and then extracted with phenol/chloroform. Then the plasmid DNA was precipitated with ethanol. The pellet was resuspended in DEPC H_2O. 1 µg of plasmid DNA and T3 RNA polymerase were used for 'run off' transcription. Subsequently, the cRNA probe was treated with DNase I and then formamide was added. The anti-sense RNA probe was stored at –80^{o}C.

The total RNA from 2, 3, 4, 5 and 6 days old endosperm was used for RNA gel electrophoresis (Figure 42, A). 20 µg of the total RNA was added to each lane. The RNA was transferred to a nylon membrane immediately after the gel electrophoresis was finished. Then the membrane was fixed and hybridized with *RcSCR2* cRNA probe at 65^{o}C overnight. Then the membrane was washed with 2xSSC, 0.2xSSC and then incubated with alkaline phosphatase conjugated anti-digoxigenin-Fab fragment. Afterwards the membrane was immersed in CDP Star and exposed with KODAK biomax MR at 30^{o}C for 16 hours to obtain the image (Figure 42, B).

The expression of *RcSCR2* could not be detected even though 20 µg of total RNA was used for northern blot analysis. This is the limitation for RNA gel electrophoresis for each lane, because a larger amount of RNA in one lane cannot be separated. Maybe the expression level of *RcSCR2* in the endosperm is very low and the hybridization technique is not sensitive enough to detect this.

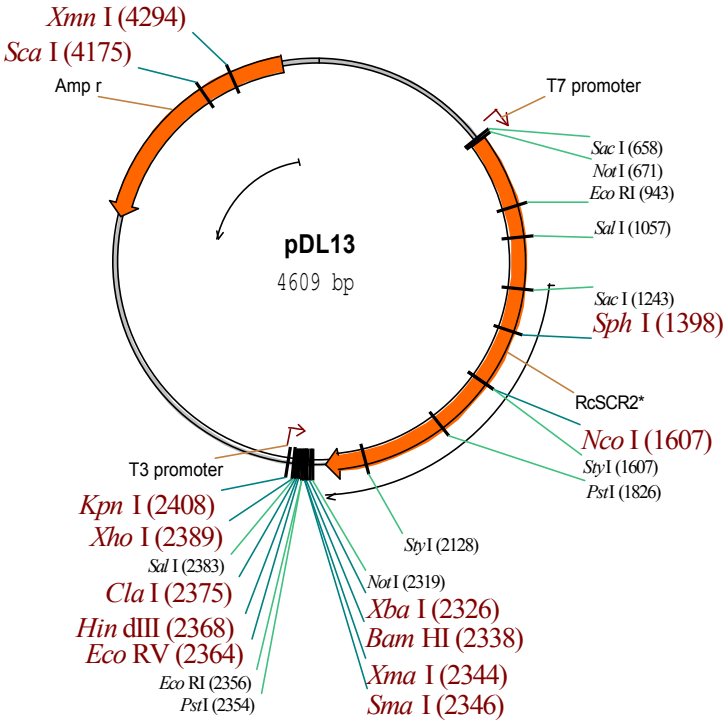


Figure 41 : The restriction map of pDL13. *RcSCR2* full-length cDNA was cloned to a pBluescriptII KS+ plasmid. This plasmid was used as template for *in vitro* transcription to synthesize the RNA probe. Sense and anti-sense probe were synthesized by using T7 and T3 RNA polymerase.

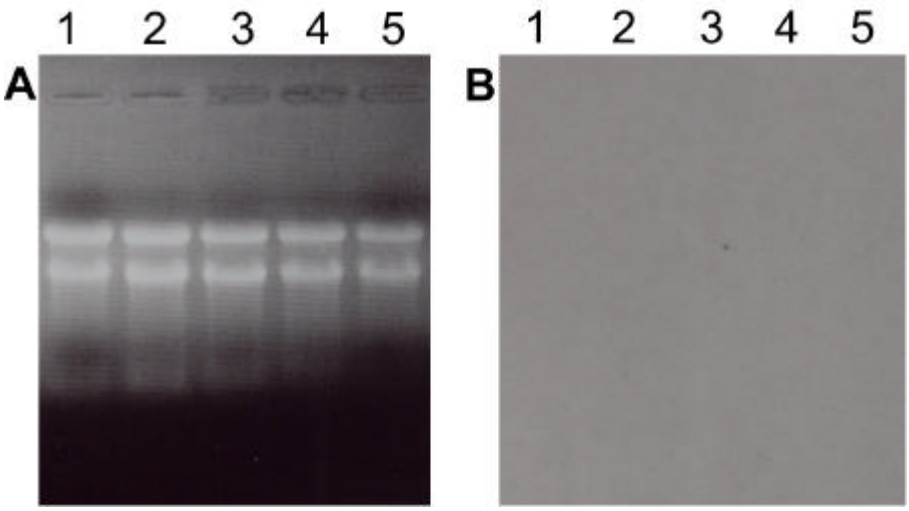


Figure 42 : RNA gel blot analysis of *RcSCR2*. 20 µg of endosperm total RNA was loaded to each lane. Lane 1: Day 2, Lane 2: Day 3, Lane 3: Day 4, Lane 4: Day 5, Lane 5: Day 6. (A) RNA gel electrophoresis through a formaldehyde-containing agarose gel. (B) Northern blot analysis of *RcSCR2*. The membrane was hybridized with digoxigenin-labelled *RcSCR2* anti-sense RNA probe and exposed for 16 hours.

C.3.2.2. RT-PCR of *RcSCR2*

Because the *RcSCR2* could not be detected in the endosperm by Northern hybridization, the more sensitive technique RT-PCR was applied. Other techniques may be applied, for example RNase protection assay or semi-quantitative RT-PCR, but compared with these techniques the real time RT-PCR is more convenient and sensitive.

C.3.2.2.1. Conventional RT-PCR of *RcSCR2*

In order to detect the *RcSCR2* expression in the endosperm, total RNA of 2, 3, 4, 5 and 6 days old endosperm was isolated. Then total RNA was treated with RNase free DNase I, and then extracted with phenol/chloroform. The RNA was precipitated with ethanol and then resuspended in DEPC H_2O. 1 µg of total RNA from the endosperm was used as a template for reverse transcription. Because of the high error rate of *Tth* polymerase during PCR, *Taq* polymerase and two-step RT-PCR were used for *RcSCR2* RT-PCR. The reverse transcription was performed with oligo-$(dT)_{15}$ primers. The cDNA was diluted to a final volume of 100 µl and 2 µl of it were used as the template in 50 µl reaction mixture.

Many different primers were tested for RT-PCR (Table 13). By using pDL13 plasmid as the template, some of the primer sets produced non-specific amplification fragments or the amount of amplification product was very low. Finally, SCR2IP1 (forward primer) and SCR2R5N (reverse primer) were used for PCR; the PCR product was 629 bp long. The PCR products were checked in an agarose gel (Figure 43) and it was ensured that the size of the PCR product was accordant to the prediction.

The RT-PCR result indicates that *RcSCR2* was indeed expressed or that the *RcSCR2* mRNA exists in the endosperm, but the amount is very low. This explains why no *RcSCR2* could be detected by Northern hybridization.

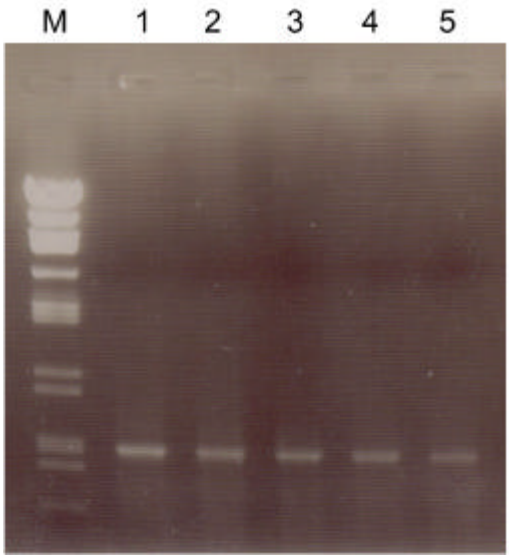


Figure 43 : RT-PCR of *RcSCR2*. The total RNA from the endosperm was isolated and used as the template of reverse transcription. The reaction was performed with oligo-$(dT)_{15}$ primers. In order to detect the cDNA synthesis from mRNA, PCR was performed. M: λ/*Cla*I marker; lane 1: Day 2 ; lane 2: Day 3; lane 3: Day 4; lane 4: Day 5; lane 5: Day 6.

Table 13 : The primers used for *RcSCR2* RT-PCR.

Primer	Sequence
SCR2F2	5'-AGCTATTAGAGATCATAGCAGG-3'
SCR2F4N	5'-AAGTAATGCCAATCCCAC-3'
SCR2IP1	5'-CCATTTCTTCTCTTTGACAC-3'
SCR2R2	5'-CTGGTTTCCATTTCTTCTC-3'
SCR2R3	5'-GCTGATGACTGTCAATTTTG-3'
SCR2R4N	5'-GCTGATGACTGTCAATTTT-3'
SCR2R5N	5'-GAGGTATTGCTTTGTGCTT-3'
Oligo-$(dT)_{15}$ primer	5'-GAATTCGCGGCCGCTTTTTTTTTTTTTTT-3'

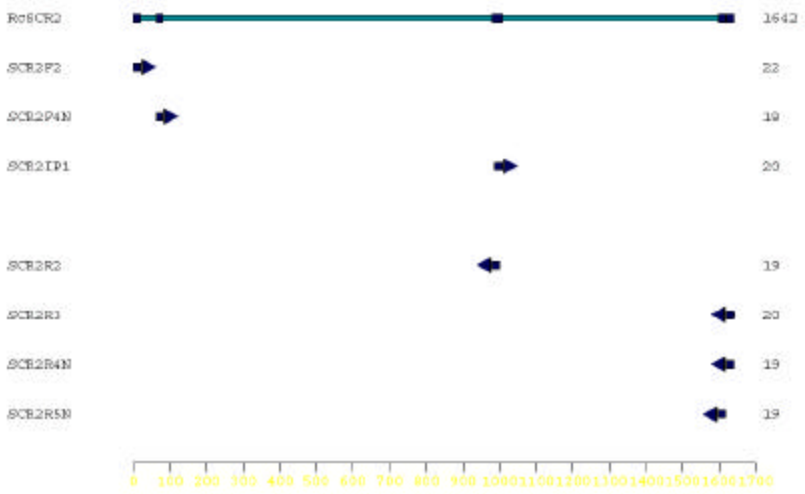


Figure 44 : The primers used for *RcSCR2* RT-PCR .

C.3.2.2.2 Quantitative RT-PCR of *RcSCR2*

From the results of the *RcSCR2* RT-PCR it was not possible to quantify the *RcSCR2* mRNA in the endosperm. It was difficult to generate a reliable result by a simple PCR. The more reliable method is real time RT-PCR.

In an attempt to investigate the *RcSCR2* expression level in the endosperm, the total RNA from different day old endosperm was isolated. Afterwards the total RNA was treated with RNase free DNase I to avoid contamination by genomic DNA.

To obtain an internal control to monitor the reverse transcription and subsequent PCR amplification, an artificial control was added to the total RNA sample from the endosperm. The *in vitro* transcription product of *nsLTPc1* was used as the artificial control. The advantage is that *nsLTPc1* is not expressed in the endosperm. 10 ng of the *nsLTPc1* transcript were added to each total RNA sample. Subsequently the reverse transcription was performed using the oligo-$(dT)_{15}$ primer. In order to obtain a reliable result, different primers were designed by program analysis. These primers were tested by RT-PCR and the products were checked in an agarose gel. The SCR2RTF1 primer and SCR2RTR1 primer were used for Real time RT-PCR, because no nonspecific amplification product was found in the agarose gel (Figure 45).

Different amounts of these two primers were used for optimization of the PCR. The result was checked in a 2% agarose gel. The best combination was 15 pmole for both primers in a 50 µl PCR reaction. Afterwards different cycles of PCR were performed. No amplification products could be found when the PCR cycle number was less than 24. But 28, 32, 36 and 40 cycles of amplification could amplify sufficient PCR product (Figure 47).

Table 14: The primes for real time RT-PCR for *RcSCR2*.

Primer	Sequence
SCR2FORH3	5’-AAGCTTATGCCAATCCCACATGCGGAGC-3’
SCR2REVXHI	5’-CTCGAGTCAAGGGAGCACCCTGGGCTT-3’
SCR2RTF1	5’-GGTTGCTTGGTGACAGAGGTGAAA-3’
SCR2RTR1	5’-CTGCGATGATCCTTTCCAGTAAGA-3’
SCR2RTF2	5’-GACTCGGGTGGCAAATGCTTATT-3’
SCR2RTR2	5’-CTCAATGTTGCATGCAGAAGTAACA-3’

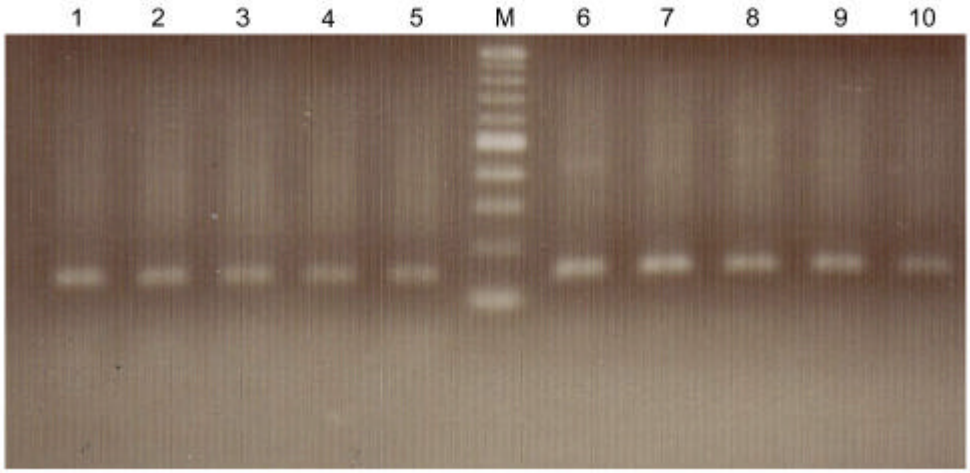


Figure 45 : RT-PCR of *RcSCR2* and *nsLTPc1*. Total RNA of the endosperm was used as a template. M: 100 bp DNA marker. 1-5: *RcSCR2* RT-PCR products. 6-10: *nsLTPc1* RT-PCR products. Lane 1 and 6: day 2; Lane 2 and 7: day 3; Lane 3 and 8: day 4; Lane 4 and 9: day 5; Lane 5 and 10: day 6.

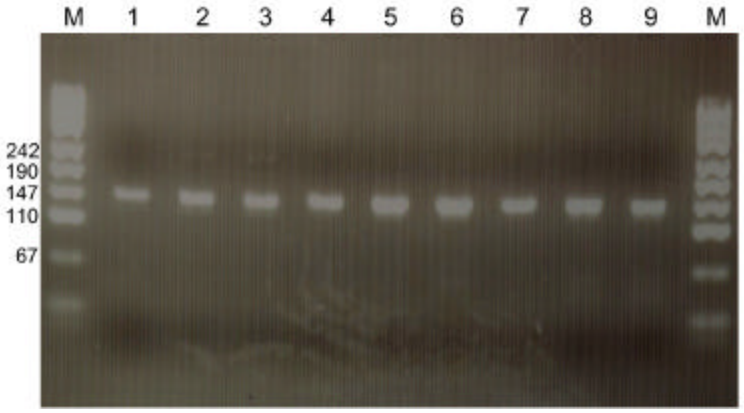


Figure 46 : The optimization of primer concentrations for real time RT-PCR of *RcSCR2*. M: pUC19/*Msc*I marker. Lane 1:SCR2RTF1: 4 pmole; SCR2RTR1: 4 pmole. Lane 2: SCR2RTF1: 4 pmole; SCR2RTR1: 15 pmole. Lane 3: SCR2RTF1: 4 pmole; SCR2RTR1: 45 pmole. Lane 4: SCR2RTF1: 15 pmole; SCR2RTR1: 4 pmole. Lane 5: SCR2RTF1: 15 pmole; SCR2RTR1: 15 pmole. Lane 6: SCR2RTF1: 15 pmole; SCR2RTR1: 45 pmole. Lane 7: SCR2RTF1: 45 pmole; SCR2RTR1: 4 pmole. Lane 8: SCR2RTF1: 45 pmole; SCR2RTR1: 15 pmole. Lane 9: SCR2RTF1: 45 pmole; SCR2RTR1: 45 pmole.

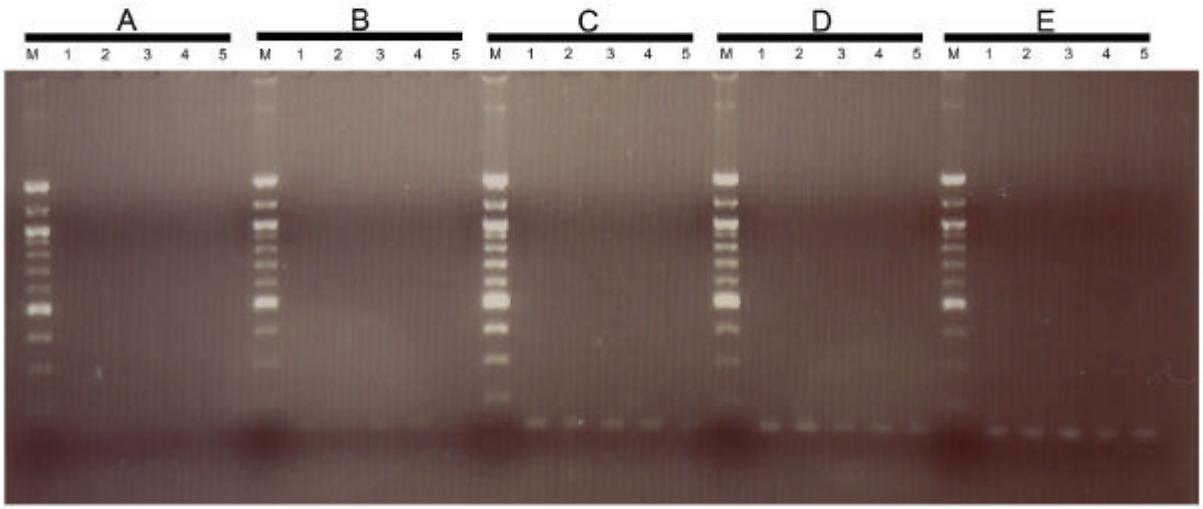


Figure 47 : Agarose gel electrophoresis of the *RcSCR2* RT-PCR product after different amplification cycles. Total RNA of the endosperm of different days old seedlings was used as template to perform RT-PCR. The *RcSCR2* was amplified and then checked in an agarose gel. A: 24 amplification cycles. B: 28 amplification cycles. C: 32 amplification cycles. D: 36 amplification cycles. E: 40 amplification cycles. M: 100bp DNA marker; 1: amplification product of day 2; 2: amplification product of day 3; 3: amplification product of day 4; 4: amplification product of day 5; 5: amplification product of day 6.

The first strand cDNA from reverse transcription was used as the template for the subsequent Real Time PCR. The PCR was performed as described in 'Material and Methods'. It was performed in ABI-7000 system and the signal was collected. Subsequently the data were analyzed and exported to Excel. The PCR product was also checked in an agarose gel. If non-specific amplification bands could be seen, the data could not be used. The experiment had then to be repeated from the reverse transcription step.

RcSCR2 transcripts were undetectable in the endosperm by RNA-blot hybridization, but low levels of RcSCR2 gene expression were detected after conventional RT-PCR and real time RT-PCR in the presence of *RcSCR2*-specific primers. Typical amplification plots for nsLTPc1 and *RcSCR2* in the endosperm during real time RT-PCR analysis are shown in Figure 48 and Figure 49.

When the *RcSCR2* transcript values in samples were normalized to the amount of the artificial control nsLTPc1 in samples, the lowest level of *RcSCR2* mRNA expression was found in day 2 endosperm. Its value was set up as 1 and the *RcSCR2* expression in other samples was calculated relative to the day 2 endosperm level (Figure 50). Expression of *RcSCR2* for other days was also very low. The highest levels of *RcSCR2* mRNA were observed in the day 3 endosperm sample, about 4-fold higher than in day 2 endosperm. The results of Northern blot hybridization, RT-PCR and real time RT-PCR clearly show that the expression of *RcSCR2* is very low.

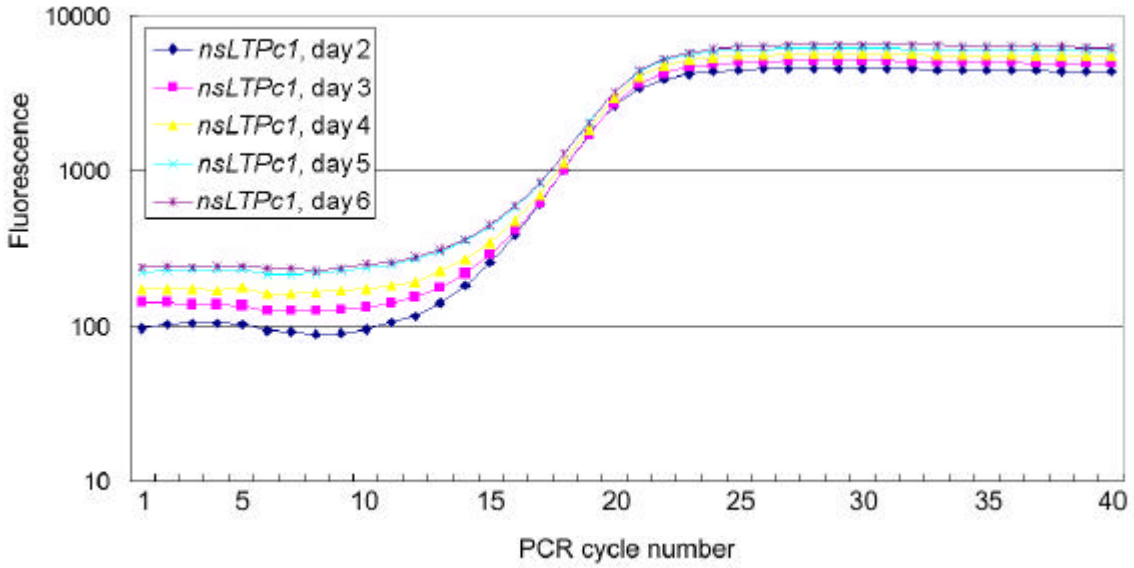


Figure 48 : The real time RT-PCR fluorescence profile of the *nsLTPc1* .

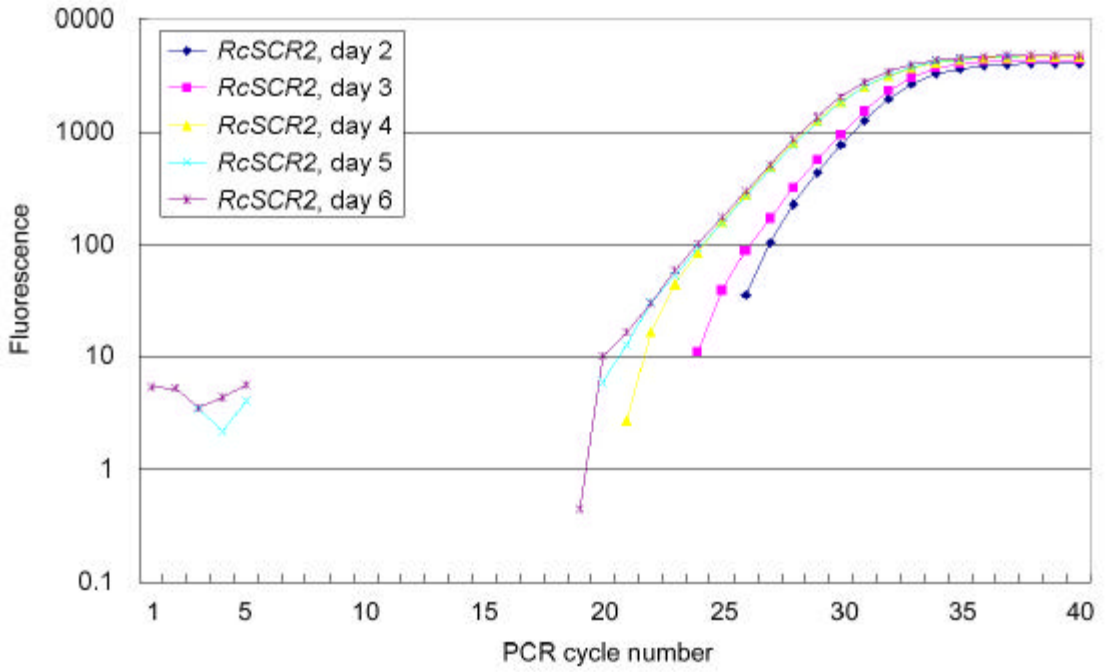


Figure 49 : Real time quantitative RT-PCR analysis of *RcSCR2* expression in endosperm. Quantitative RT-PCR was performed on total RNA isolated from different days old endosperm as described in Materials and Methods.

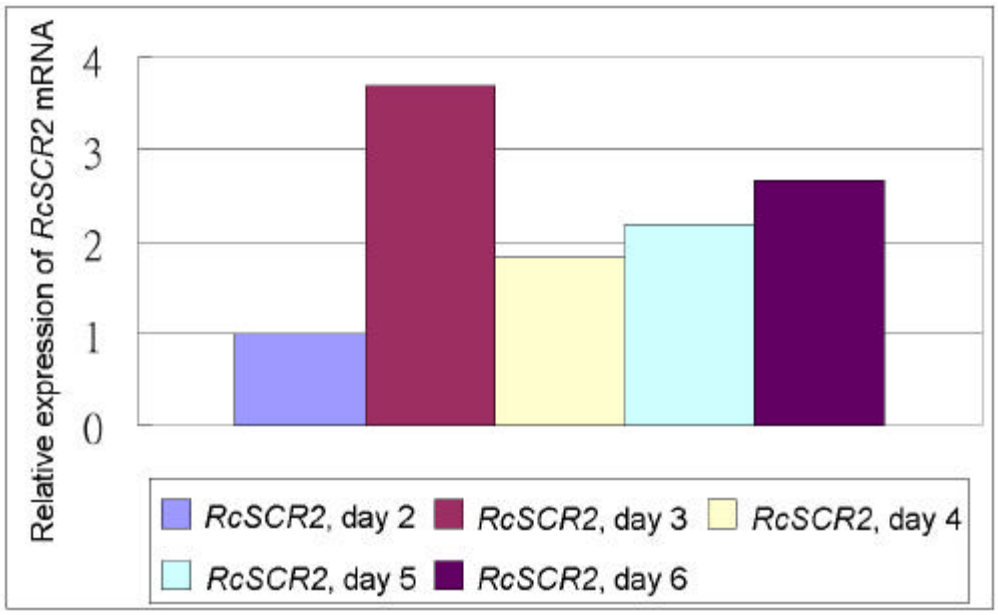


Figure 50 : Quantification of *RcSCR2* expression in different days old endosperm. *RcSCR2* expression values were normalized to the level of the amount of nsLTPc1 in different days samples. *RcSCR2* expression in day 2 endosperm was set up as 1.

C.3.2.3. *In situ* hybridization of *RcSCR2*

In order to detect the site of expression of *RcSCR2* in the endosperm and the cotyledons, digoxigenin labelled sense and anti-sense probes were synthesized. Because the coding regions of *RcSCR1* and *RcSCR2* are very similar (Figure 51), using the 3'-NTR as a template to synthesize digoxigenin labelled probes would have been a better choice. But since the 3'-NTR of *RcSCR2* is shorter than 100bp, it is not suitable for synthesizing RNA probes. Instead, two different methods were used to synthesize these probes, (1) the full-length probe was synthesized or (2) the full-length probe was synthesized and then hydrolyzed.

To synthesize the full-length RNA probe, a M13 forward primer and a reverse primer were used to amplify the *RcSCR2* cDNA fragment and pDL13 plasmid was used as a template. Afterwards the PCR product was checked in an agarose gel and then the DNA fragment of *RcSCR2* was precipitated by ethanol. 200 ng of the PCR product were used as a template to synthesize the sense probe and the anti-sense probe. The sense probe and the anti-sense probe were obtained by *in vitro* transcription using T3 RNA polymerase or T7 RNA polymerase. In order to obtain 150 to 200 bases long probes, the full-length probes were subsequently hydrolyzed. Then the *in situ* hybridization was performed with full-length probes and hydrolyzed probes. 200 ng of probe were added to 1 ml hybridization solution.

Section of cotyledons and hypocotyls were used for *RcSCR2 in situ* hybridization. With the sense probes no signals were detected (Figure 52, A and C). In the section of the endosperm, which was hybridized with the anti-sense probe, no signal was found (Figure 52, B). In the section of the hypocotyls, which were hybridized with the anti-sense probes, the signals were found in almost every cell and most of the signals were very strong (Figure 52, D). This result is contrast to the quantitative RT-PCR of *RcSCR2*, where low mRNA of *RcSCR2* was detected (Eisenbarth, Diploma thesis, Bayreuth 1999). One reason for this difference may be that the full-length or partial sequence of *RcSCR2* is very similar to another (transporter) mRNA sequence, so that the anti-sense probes could bind to it and produce a false signal.

Figure 51: The alignment of *RcSCR1* and *RcSCR2*.

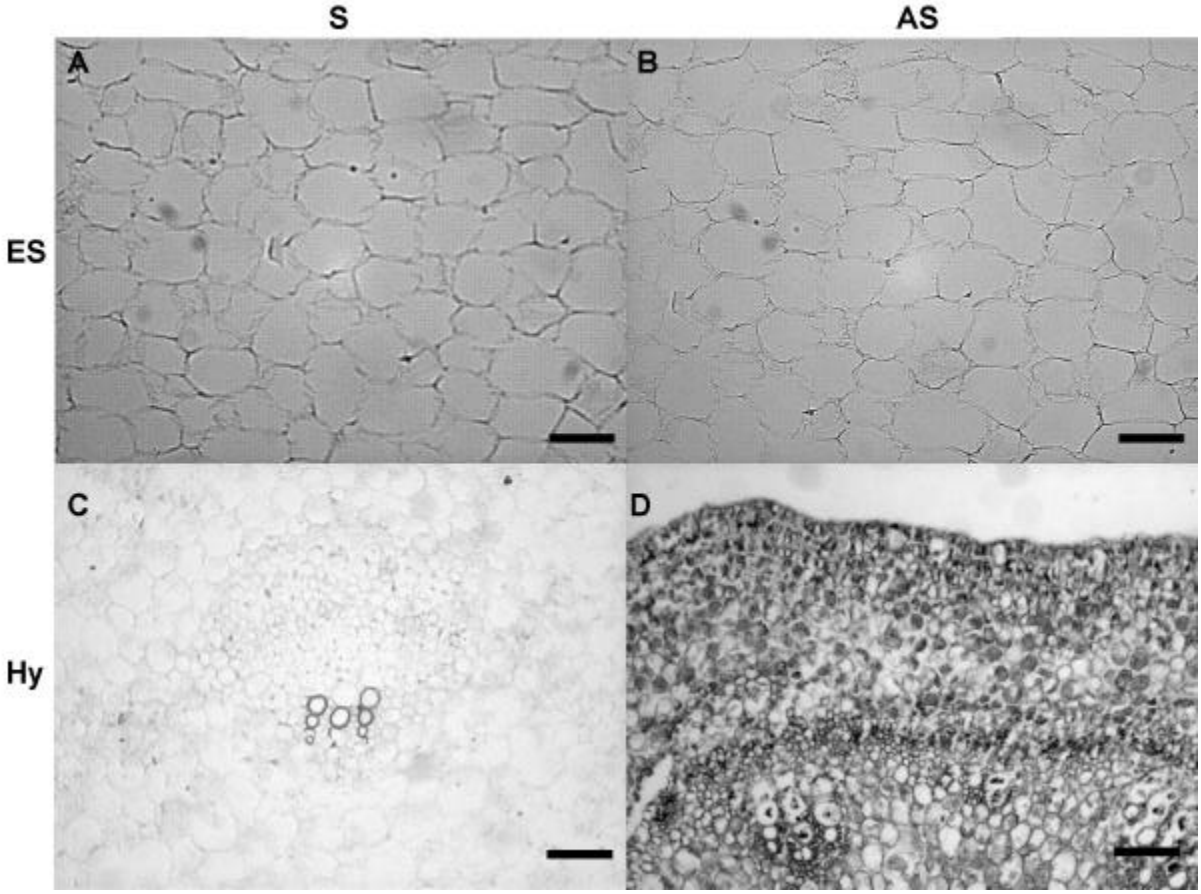


Figure 52 : *In situ* hybridization of *RcSCR2*. (A) and (B) are section of the endosperm; (C) and (D) are sections of hypocotyl. The sections of the endosperm and hypocotyl of *Ricinus communis* were hybridized with digoxigenin labelled full-length or hydrolyzed sense probes (A and C). No signal was found in the sections of the endosperm and hypocotyl. (B) and (D) are hybridized with digoxigenin labelled antisense probe. Hybridization signal was found in the section of hypocotyl (D). Bar in (A) and (B) = 25 µm. Bar in (C) and (D) = 100 µm.

C.3.2.4. *In situ* RT-PCR of *RcSCR2*

In order to find the expression site of *RcSCR2* in the endosperm and cotyledons, the *in situ* RT-PCR method was performed. The cDNA sequence of *RcSCR2* was compared with the *RcSCR1* sequence described before. The 3'-NTR of the *RcSCR2* is shorter than the one of *RcSCR1*. The *RcSCR2* gene specific primers pDEL4-For and pDEL4-Rev were used for *RcSCR2 in situ* RT-PCR. The PCR product is a 303 bp long DNA fragment.

Primer	Sequence
pDEL4-For	**5'-TATTGGCTACTTAGGTCATGATCTACCACC-3'**
pDEL4-Rev	**5'-ACAGTCATCAGCAAAAATAAGCACAAAGCA-3'**

To check the overall expression pattern of *RcSCR2* in the endosperm, the *in situ* RT-PCR was performed with 2 to 6 days old seedlings. Digoxigenin 11-dUTP was incorporated into the cDNA fragment directly. The positive and negative controls were performed as described in 'Material and Methods'.

The *RcSCR2* cDNA signal could be detected in the cotyledons and the endosperm of day 2 to day 5 samples (Figure 53). No significant hybridization signal was detected in the endosperm of the day 6 samples (Figure 53, M, N and O). A low amount of *RcSCR2* transcript was detectable in the cotyledons of day 6 samples (Figure 53, N).

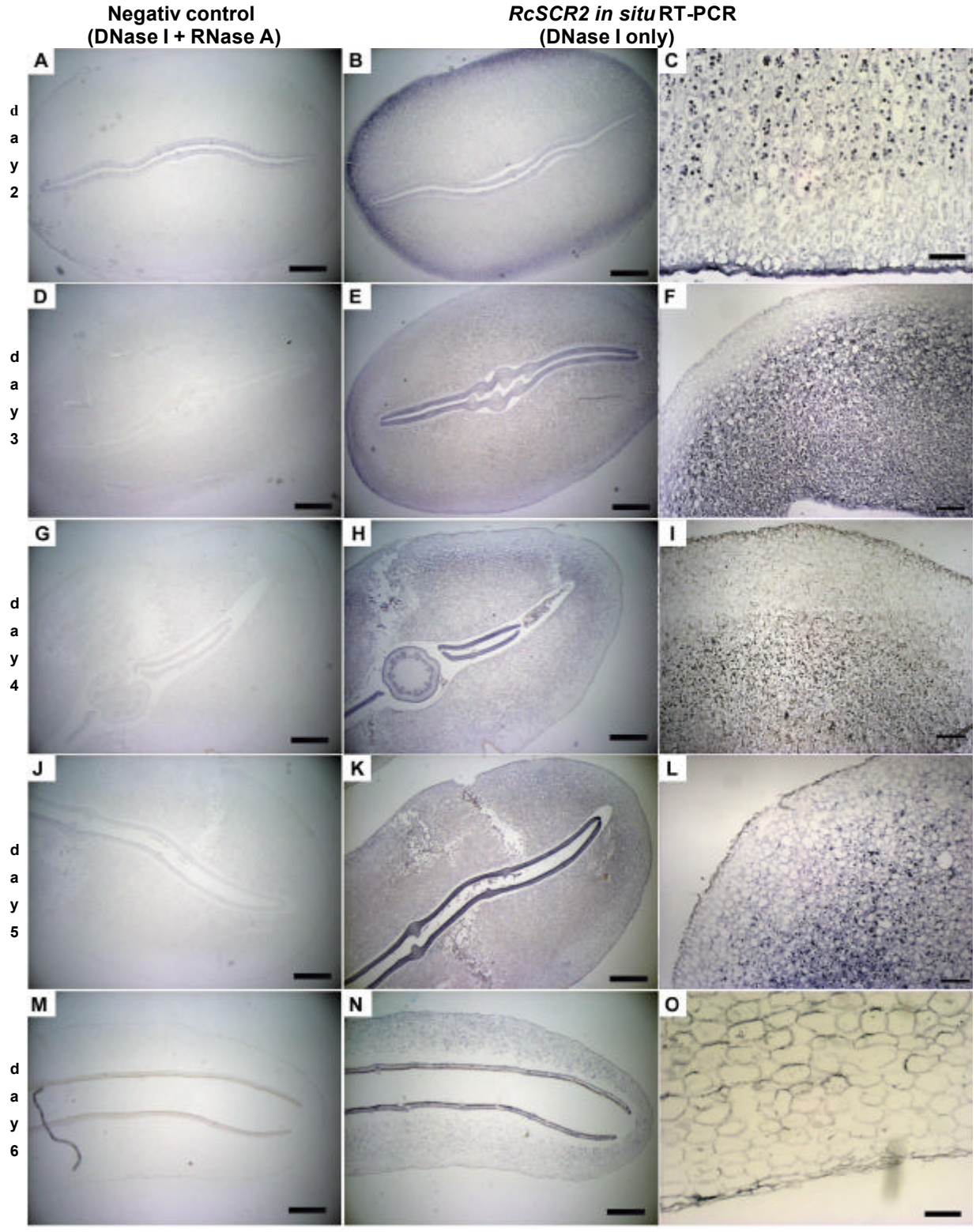
In day 2 sections, the *RcSCR2* RT-PCR signal could be detected in most of the cells of the endosperm (Figure 53, B and C), except of the 4 to 6 layers of cells near the cotyledons or seed coat. In day 3 sections, the *RcSCR2* expression was detected in most of the cells of the endosperm, *RcSCR2* expression also occurred in the cell layers near the cotyledons, but in the outer region (near the seed coat) of the endosperm, about 4 to 6 layers of cells, no signal of *RcSCR2* was visible (Figure 53, F, I and L).

In the day 4 and day 5 sections, the *RcSCR2* transcript could also be detected in the inner region of the endosperm. The *RcSCR2* expression also occurs in cotyledons (Figure 53, H and K). Only very weak expression could be detected in the outer layer of the endosperm (Figure 53, I and L). In the day 6 sections, the *RcSCR2* expression could be hardly

detected in the endosperm. But the *RcSCR2* transcript was detected in the cotyledon of day 6 samples. The signal of *RcSCR2* transcript could be observed mainly in the lower epidermis (Figure 53, N).

RcSCR2 expression was also found in other tissues. *RcSCR2* was found to be highly expressed in the young developing leaf (Figure 54 A). The localization is very similar to the expression of *RcSCR1*. The RcSCR2 was also found in the phloem of the hypocotyls (Figure 54 B).

Figure 53 : (next page) *In situ* RT-PCR of *RcSCR2*. All sections (10 µm thick) were treated with proteinase K. Negative controls (A), (D), (G), (J) and (M) were subsequently treated with DNase I. (B), (C), (E), (F), (H), (I), (K), (L), (N) and (O) were samples. The samples were treated with DNase I and RNase A and then RT-PCR was performed. (A), (B) and (C), cotyledons and the endosperm of day 2 (D), (E) and (F), cotyledons and the endosperm of day 3, (G), (H) and (I), cotyledons and the endosperm of day 4, (J), (K) and (L), cotyledons and the endosperm of day 5, (M), (N) and (O), cotyledons and the endosperm of day 6. Bar in (A), (B), (D), (E), (G), (H), (J), (K), (M) and (N) = 1 mm. Bar in (C) and (O) = 50 µm. Bar in (F), (I) and (L) = 200 µm.


Negativ control
(DNase I + RNase A)
RcSCR2 in situ RT-PCR
(DNase I only)
day 2
day 3
day 4
day 5
day 6
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

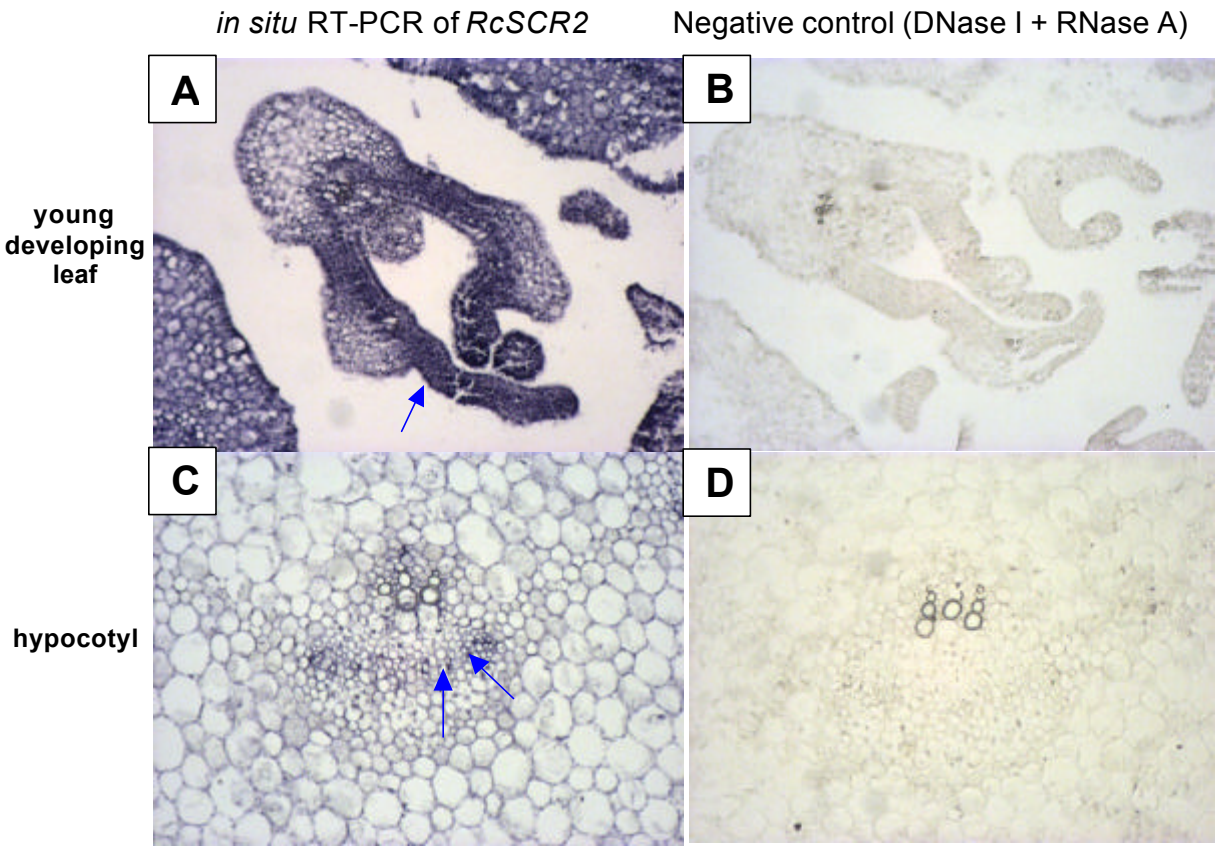


Figure 54 : *RcSCR2 in situ* RT-PCR in sections of young developing leaves and of hypocotyls. The *RcSCR2* was found in young leaves (A) and hypocotyls (C). The negative controls of (A) and (C) were (B) and (D). The arrows indicate the expression site of *RcSCR2*.

C.4. Sucrose phosphate phosphatase (SPP) in germinating *Ricinus communis* endosperm

C.4.1 Cloning of *RcSPP1*

A set of enzymes is responsible for sucrose biosynthesis in plants (Eastmond and Rawsthorne, 2000). Sucrose synthase under most physiological conditions is usually assigned a role in sucrose cleavage, rather than its synthesis (Wang *et al*., 1999; Hanggi and Fleming, 2001).

Sucrose phosphate synthase (SPS) catalyzes the synthesis of sucrose-6'-phosphate by a transglucosylation from UDP-glucose to fructose-6-phosphate. This enzymatic reaction produces sucrose-6'-phosphate and UDP. The sucrose-6'-phosphate is then hydrolyzed by sucrose phosphate phosphatase (SPP). This irreversible reaction by an enzyme that abundantly accompanies SPS in the cell prevents sucrose-6'-phosphate accumulation and provides an energetically favored and efficient production of sucrose even at low UDP-glucose and fructose-6-phosphate or at high sucrose concentrations (Lunn and MacRae, 2003).

In many plants, sucrose phosphate phosphatase is found at an ample level in the cytoplasm, together with and much higher than sucrose phosphate synthase. Until now, the biochemical and physiological study of sucrose phosphate phosphatase is limited by the difficulty in providing sufficient quantities of sucrose-6'-phosphate for use as the substrate. Recently, many sucrose phosphate phosphatase genes were cloned from different higher plants. These sucrose phosphate phosphatase genes share high homology. In order to study the sucrose synthesis in the endosperm of germinating Ricinus seedlings, the Ricinus sucrose phosphate phosphatase gene was cloned.

C.4.1.1. Degenerate PCR

In order to isolate a sucrose phosphate phosphatase from the endosperm of *Ricinus communis* by homology cloning, a degenerate PCR strategy was used. The first step of this process involved the design of degenerate primers based on the amino acid sequence

of previously cloned sucrose phosphate phosphatases from other plants. Different sucrose phosphate phosphatases from *Arabidopsis thaliana*, *Hordeum vulgare*, *Lycopersicon esculentum*, *Medicago trucatula*, *Pinus taeda*, and *Zea mays* were aligned as shown in Figure 55. The regions chosen are shown in Figure 55 and are detailed in Table 15.

Table 15. Degenerate primers used to amplify *RcSPP1*.

	Amino acid residues	Nucleotide sequence
Arabidopsis thaliana	KELRKEKP	AAAGAATTGAGAAAAGAGAAACC
Hordeum vulgare	KELRKEKP	AAGGAATTAAGGAAAGAGAAGCC
Lycopersicon esculentum	KQLRKEKP	AAGGAATTGAGGAAAGAAAAGCC
Medicago trucatula	KELRKEKP	AAACAACTGAGGAAAGAGAAGCC
Pinus taeda	KELRKEKP	AAAGAGCTGAGAAAGGAGAAACC
Zea mays	KELRKEKP	AAGGAATTAAGGAAAGAGAAGCC
SPP-D5F		AARSARYTRAGRAARGARAARCC

	Amino acid residues	Nucleotide sequence
Arabidopsis thaliana	GVMVSNSQ	GGTGTTATGGTGAGCAATTCCCAA
Hordeum vulgare	GVMVANAQ	GGTGTCATGGTTAGCAATGCCCAA
Lycopersicon esculentum	GVMVSNAQ	GGTGTAATGGTAGCTAATGCGCAG
Medicago trucatula	GVMVGNAQ	GGTGTCATGGTAAGCAATGCACAA
Pinus taeda	GVMVSNAQ	GGAGTCATGGTTGGTAATGCACAA
Zea mays	GVMVSNAQ	GGTGTCATGGTCAGCAATGCCCAA
SPP-D3R		GGWGTHATGGTNRSYAATKCVCAA

The thermocycling parameters for the PCR used were empirically determined, as an appropriate annealing temperature could not be calculated from the degenerate sequence of the primers. Although the homology of the primers to the *Ricinus communis* sequence was unknown, these PCRs suggested an annealing temperature of around 58° C. However, applying these parameters to a *Ricinus communis* endosperm cDNA template did not yield any specific amplification. In order to increase the potential for amplification from poorly annealing primers, the annealing temperature was decrease to 55° C. A successful amplification was eventually obtained using these PCR cycling parameters. The amplification product was used as a template to perform PCR again. After reamplification, three bands were seen and the 500 base pair amplification product was cloned into a pGEM-T vector and sequenced (Figure 56). The sequence data were used to search the

GenBank sequence databank, and the product was found to have high similarity to the sucrose phosphate phosphatase sequences.

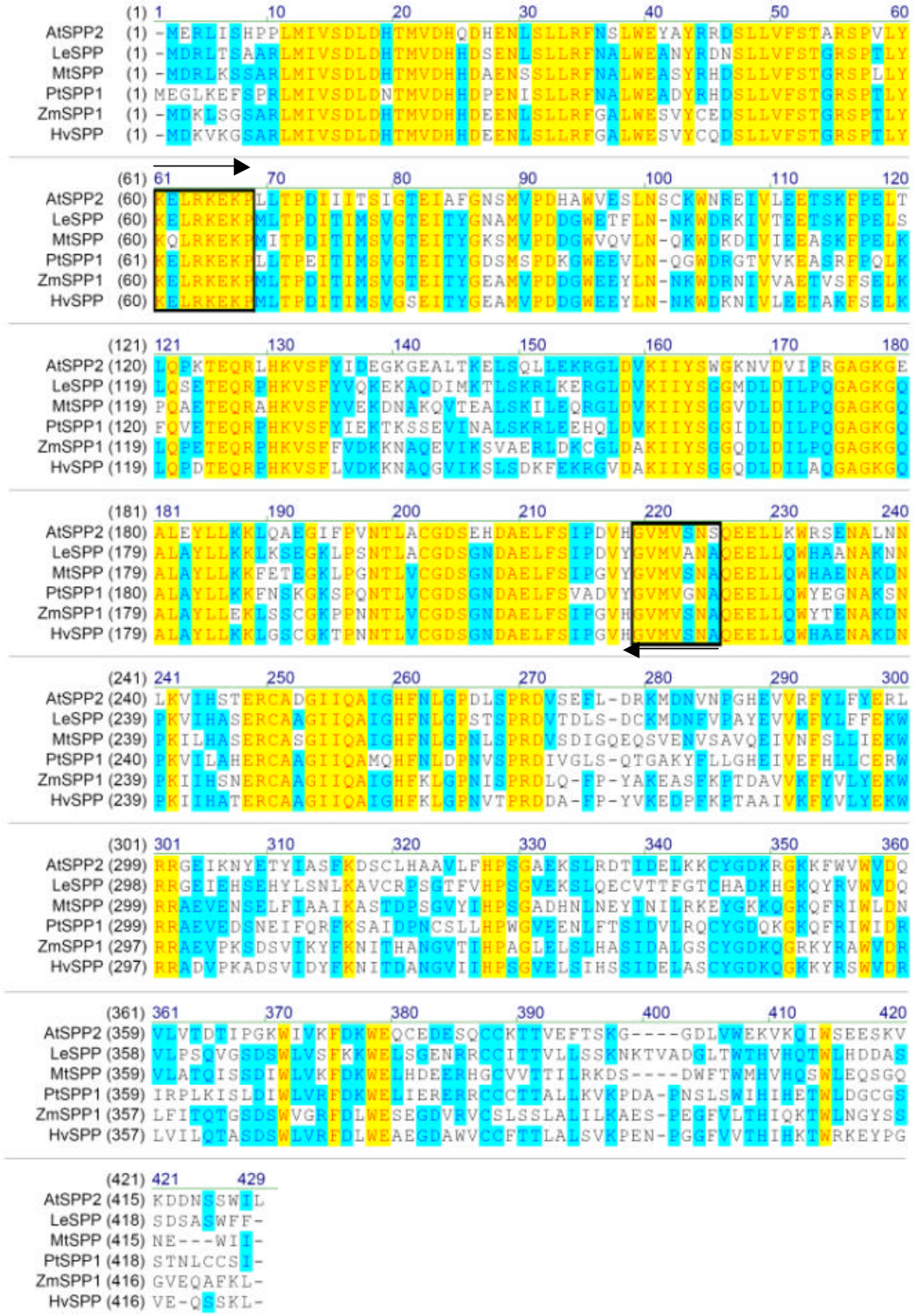


Figure 55: Degenerate primer design for sucrose phosphate phosphatase gene. An alignment of six sucrose phosphate phosphatases is shown. Amino acid residues shaded yellow are identical in all

species. The residues in the frames were used for degenerate primer design. Arrows designate primer directionality –SPP-D5F (forward primer (-->)) and SPP-D3R (reverse primer (<--)). The PCR product of RT-PCR, for which SPP-D5F and SPP-D3R were used, was approximately 500bp.

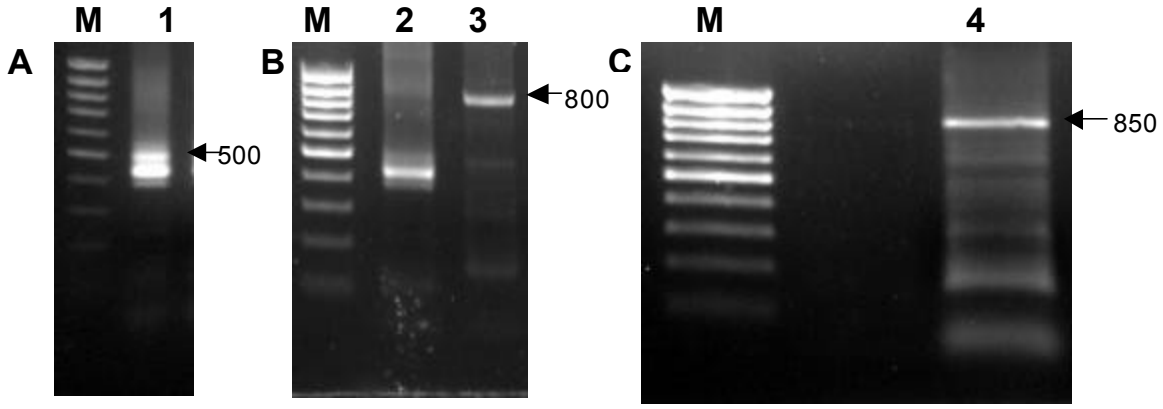


Figure 56: Gel electrophresis of RT-PCR and RACE products. Total RNA of the endosperm was used for reverse transcription. The product was used as template to carry out degenerate PCR and RACE. A. degenerate RT-PCR B. 3´-RACE C. 5´-RACE. M: 100 bp DNA marker. Lane 1: degenerate RT-PCR products. The 500 bp PCR product was cut from the gel and cloned. Lane 2: degenerate RT-PCR of RcSPP1. Lane 3: 3´-RACE of RcSPP1. An 800 bp PCR product was seen in the gel. The PCR product was cut from the gel and cloned. Lane 4: 5´-RACE of RcSPP1. An 850 bp PCR product was obtained. The band was cut from the gel and cloned to a pGEM-T vector.

C.4.1.2. 5'- and 3'- RACE

Using the sequence obtained from the initial degenerate PCR product, sequence-specific primers were designed with which 5´- and 3´-RACE reactions were carried out. The 3´-RACE reaction amplified a product of 800 bp, which was sequenced and also found to share high similarity with the clones listed above. The sequence contained the remainder of the 3´-coding sequence and the 3´-untranslated region (UTR), including a polyadenylation signal (Figure 56, B). With the 5´-RACE reaction, a product of around 850 bp was amplified (Figure 56, C). A multiple alignment of these related sucrose phosphate phosphatases strongly suggested that the assumed start codon obtained from 5´-RACE would be the favored site for transcription *in vivo* (Figure 58).

C.4.1.3. Sequence characteristics of *RcSPP1*

A full-length sequence was generated using the PCR products and was analyzed using the GCG Wisconsin Package. A Blast search of the GenBank database revealed the

deduced amino sequence to be 70 % identical to the *Medicago truncatula* sucrose phosphate phosphatase (*MtSPP*), and 69% identical to the functionally characterized *Lycopersicon esculentum* homologue. Thus, the coding sequence, named *RcSPP1*, represents the first reported cloning of the sucrose phosphate phosphatase homologue from *Ricinus communis*.

Based on the known sequence, specific primers were designed to amplify the entire known sequence, the full coding sequence as well as the 3´-UTR. The successfully amplified product was cloned into pGEM-T.

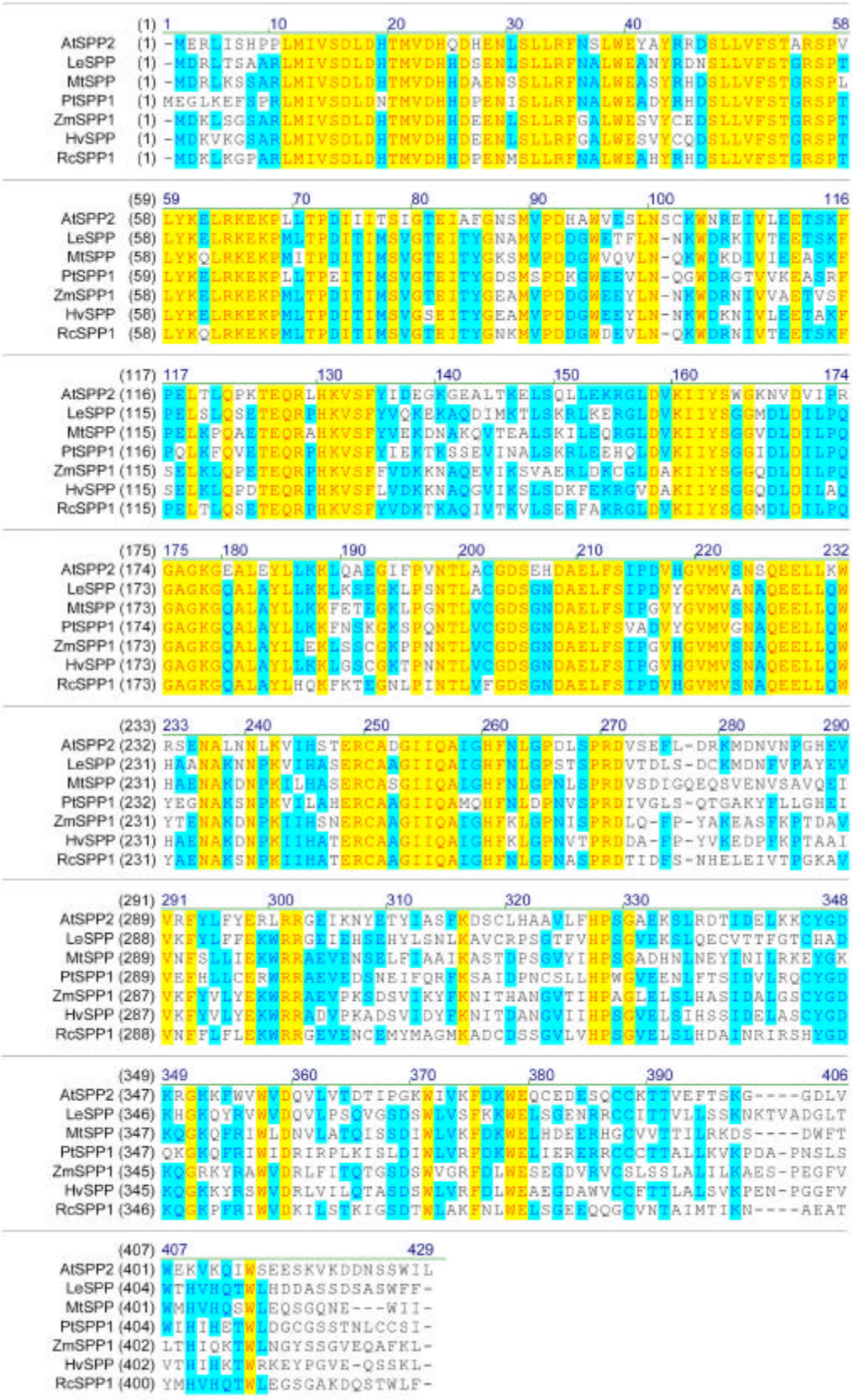
Further analysis of the sequence was performed to identify features of the primary amino acid sequence. In addition to the high similarity to the *Medicago truncatula* and *Lycopersicon esculentum* homologues (70 and 69 %, respectively), there was 41% identity - to the related sucrose phosphate phosphatases from *Nostoc sp.* PCC7120 and *Synechocystis sp.* PCC6803. RcSPP1 protein showed 27% identity to a sucrose phosphate synthase (SPS) from *Prochlorococcus marinus*, 23% identity to a SPS from *Oryza sativa* and 27% identity to a trehalose-6-phosphate synthase from *Azotobacter vinelandii* (data not shown). A sequence alignment is shown in Figure 57. The open reading frame of 421 amino acids predicts a calculated molecular mass of 47 kDa (Figure 58).

Table 16 : The similarity table of the deduced amino acid sequences of *RcSPP1* and other *SPP* genes. More information can be found in the description of Figure 57.

	RcSPP1	MtSPP	AtSPP2	LeSPP	HvSPP	ZmSPP1	PtSPP1
RcSPP1	100	70	60	69	65	64	61
MtSPP		100	57	66	62	60	62
AtSPP2			100	60	55	53	51
LeSPP				100	64	63	61
HvSPP					100	81	58
ZmSPP1						100	58
PtSPP1							100

Figure 57 : (Next page) Alignment of the deduced amino acid sequence of the maize SPP with SPP-like sequences and the carboxyl terminus of SPS from other species. The GenBank accession numbers of the sequences are as follows: ZmSPP1 (*Zea mays*), AF283564; AtSPP2 (*A. thaliana*), AF283563; LeSPP (*Lycopersicon esculentum*), AF493563; HvSPP (*Hordeum vulgare*), AF493562; MtSPP (*Medicago truncatula*), AF283566; PtSPP1 (*Pinus taeda*), AF460845; RcSPP1 (*Ricinus*

***communis* L.) . Sequences were aligned using the PILEUP program of the GCG Wisconsin sequence analysis package.**


(1) 1 10 20 30 40 58
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MtSPP (1) -MDRLKSSARLMIVSDLDHTMVDHHDAENSSLLRFNALWEASYRHDSLLVFSTGRSPL
PtSPP1 (1) MEGLKEFSPRLMIVSDLDNTMVDHHDPENISLLRFNALWEADYRHDSLLVFSTGRSPT
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HvSPP (1) -MDKVKGSARLMIVSDLDHTMVDHHDEENLSLLRFGALWESVYCQDSLLVFSTGRSPT
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(59) 59 70 80 90 100 116
AtSPP2 (58) LYKELRKEKPLLTPDIITSIGTEIAFGNSMVPDHAWVESLNSCKWNREIVLEETSKF
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(117) 117 130 140 150 160 174
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LeSPP (231) HAANAKNNPKVIHASERCAAGIIQAIGHFNLGPSTSPRDVTDLS-DCKMDNFVPAYEV
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PtSPP1 (232) YEGNAKSNPKVILAHERCAAGIIQAMQHFNLDPNVSPRDIVGLS-QTGAKYFLLGHEI
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PtSPP1 (347) QKGKQFRIWIDRIRPLKISLDIWLVRFDKWELIERERRCCCTTALLKVKPDA-PNSLS
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(407) 407 429
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MtSPP (401) WMHVHQSWLEQSGQNE---WII-
PtSPP1 (404) WIHIHETWLDGCGSSTNLCCSI-
ZmSPP1 (402) LTHIQKTWINGYSSGVEQAFKL-
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RcSPP1 (400) YMHVHQTWLEGSGAKDQSTWLF-

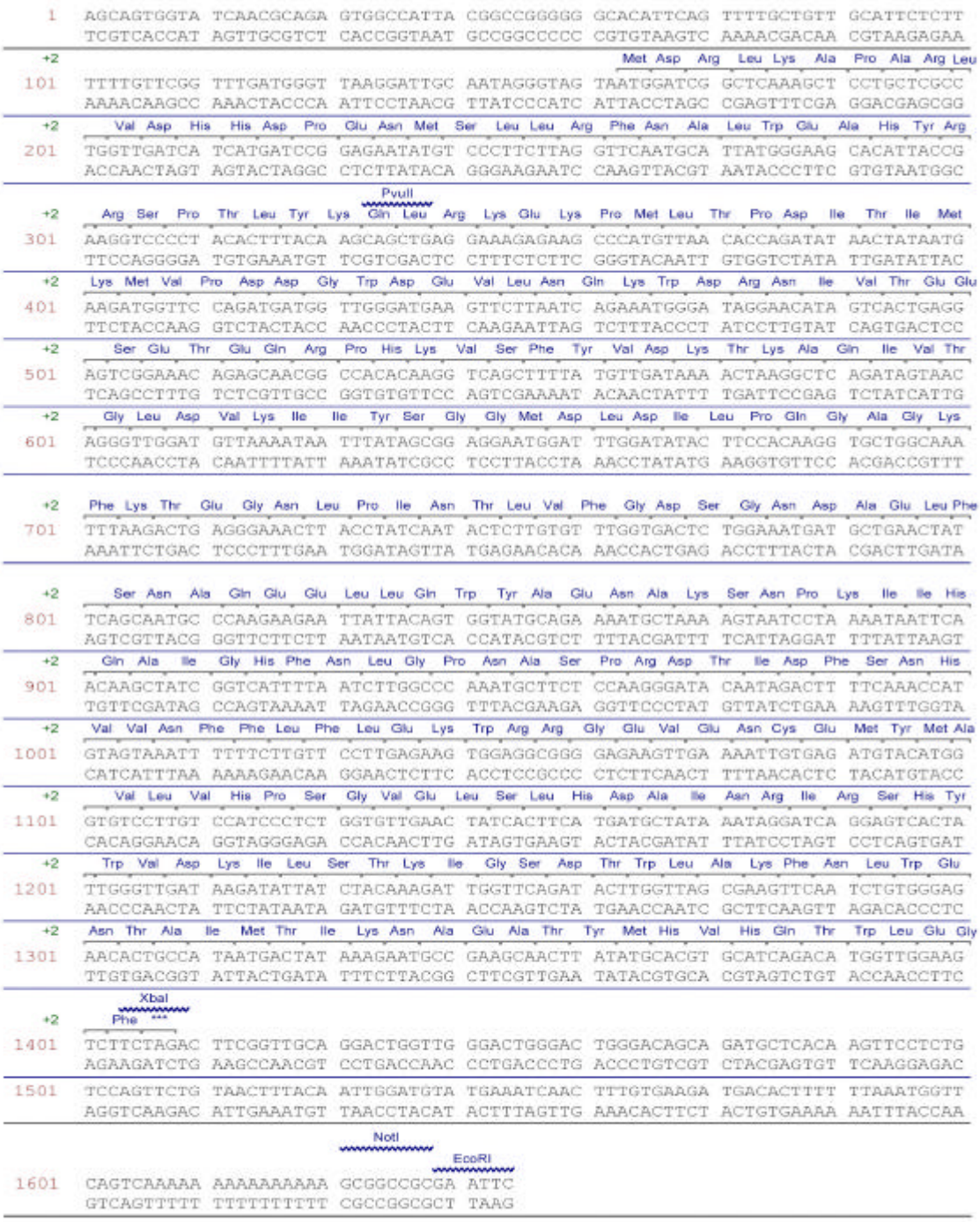

Figure 58 : *RcSPP1* cDNA and the derived amino acid sequence. In the coding region, *RcSPP1* cDNA has one *PvuII* and one *XbaI* restriction digestion site. *NotI* and *EcoRI* sites are designed in the oligo-dT primer.

C.4.2. Northern blot analysis of *RcSPP1*

In order to synthesize a digoxigenin labelled cRNA probe, the *RcSPP1* 3'-end cDNA fragment was subcloned into a pGEM-T vector. SP6 RNA polymerase was used to synthesize digoxigenin labelled cRNA probe. The cRNA probe was treated with DNase I and then formamide was added. Afterwards it was stored at –80°C.

All the procedures of Northern blot analysis were carried out as described in Materials and Methods. The results are shown in Figure 59. The intensity of the signals showed that *RcSPP1* expression level was very similar from day 2 to day 6 (Figure 59 B), only the expression of day 2 was weaker than other samples.

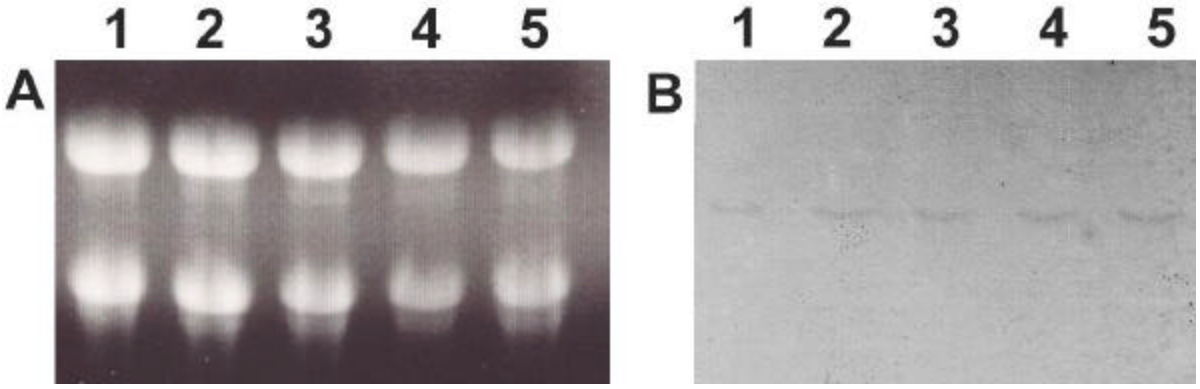

Figure 59 : Northern analysis of *RcSPP1* in the endosperm of 2-6 days old *Ricinus* seedlings. 5 µg of total RNA from the endosperm was loaded to each lane. A. RNA gel electrophoresis. B. Northern blot analysis. Lane 1: Day 2; Lane 2: Day 3; Lane 3: Day 4; Lane 4: Day 5; Lane 5: Day 6.

Part II

Starch accumulation and the expression of starch synthesis related genes in sugarcane plants

Introduction

Sugarcane (*Saccharum* spp. hybrid) ranks among the world's top 10 food crops and annually provides 60% to 70% of the sugar produced worldwide. Sugarcane yellow leaf syndrome is caused by the sugarcane yellow leaf virus (SCYLV)(Scagliusi and Lockhart, 2000; Rassaby *et al*., 2004). Sugarcane yellow leaf virus is a member of the luteovirus group of viruses that cause severe diseases in a number of other crops (Moonan *et al*., 2000; Moonan and Mirkov, 2002; Scagliusi and Lockhart, 1997, 2000; Smith *et al*., 2000). Yield reductions were shown to occur in sugarcane infected with SCYLV in several countries. Losses in tonnage of up to 40–60% were initially recorded in Brazil (Vega *et al*., 1997).

SCYLV is phloem limited, but its multiplication is only possible in tissues that have active nucleic acid and protein synthesis. SCYLV must be able to reach the sieve tubes after assembly. A massive accumulation of SCYLV particles could slow down assimilates transport drastically because carbohydrate movement depends on having intact phloem cells with control of plasmodesmal pore size. It was observed that sucrose concentration in leaves of virus-infected plants was permanently elevated, especially in the morning. Starch levels in infected leaves were also elevated. That is another sign of decreased carbohydrate export rate (Paul and Foyer, 2001). This indicates that sucrose translocation is slowed down by virus infection, but direct damage to the tissue caused by the virus could not be observed.

In order to find out the changes of the starch accumulation in virus-infected sugarcane plants, the starch synthesis was studied. In maize plants, using the PAS (Periodic acid and shift) procedure for staining polysaccharides heavy deposition of starch was illustrated in bundle sheath chloroplasts (Edwards *et al*., 2001), the site of Rubisco localization, as well as the appearance of starch in guard cells, while mesophyll cells were lacking starch. Starch granules in tissue sections can be stained also with KI. Using this method one can also find where starch is synthesized and accumulated. A set of enzymes is responsible for starch biosynthesis in plants (Haggi and Fleming, 2001; Hausler *et al*., 2000). The site of expression of starch synthesis related genes in the leaf of sugarcane is of interest. Starch staining in combination with *in situ* hybridization techniques can be siginificant value to provide spatial information on the site of starch synthesis.

In this work, the study is focused on starch accumulation and *in situ* hybridization of the genes related to starch synthesis in sugarcane leaves.

B. Material and Methods

B.1. Plant material

The plants were propagated from cuttings of varieties H73-6110, H78-4153 and H87-4094 obtained from the Hawaii Agriculture Research Center. The sugarcane was propagated by cuttings of the stalk containing two nodes with buds. The bud, a miniature stalk with its growing point and root and leaf primordia, forms the new shoot. In addition, a seed piece contains root primordia within its root band, which develop into set roots which function until the young shoot develops its own roots. The sugarcane plants were grown in the glasshouse at 24 °C with a 12-h photoperiod.

Plants of the sugarcane variety H87-4094 are sugarcane yellow leaf virus-free, but they are susceptible to SCYLV. H73-6110 plants are infected to 100% by SCYLV. H78-4153 plants are SCYLV tolerant plants. After virus inoculation, SCYLV can be detected in 4 % of the sugarcane plants.

B.2. Material and methods

All material and methods can be found in the ‘Material and methods’ of Part I.

C. Results

Starch is a very important substance in plants. The products of photosynthesis are assembled in plants to make glucose-phosphate. Glucose is converted into the polysaccharide starch, allowing the plant to store most of the energy collected in photosynthesis in the chemical bonds between the sugars. For plants, it is a compact form in which excess sugars produced during photosynthesis can be stored for later use.

The pathway of starch synthesis is known to involve the inter-conversion of sugars, sugarphosphates and nucleotide-sugars (Hajirezaei *et al*., 2003). ADP-glucose is synthesized by the enzyme ADP-glucose pyrophosphorylase, which is restricted to the plastid compartment of the cell. Amylopectin is synthesized by Starch synthases and branched by Branching Enzyme.

C.1. Starch staining of tissue sections

In order to study the starch accumulation in the leaves of virus-free and virus-infected sugarcane, tissue sections were stained with KI. Starch granules stained with KI are brown to dark brown in color. The starch area and cytoplasm area were then counted.

In the leaf of maize, starch granules were found in the bundle sheath cells (Edwards *et al*. 2001). In sugarcane leaves, starch granules were found only in the bundle sheath cells of H87-4094 (virus-free plants) and H78-4153 (virus tolerance) (Figure 60, A and B). The result was similar to that of maize. In virus-infected plants (H73-6110), starch granules were found not only in the bundle sheath cells, but also in mesophyll cells (Figure 60, C).

Comparing the ratio of starch area/ cytoplasm area in virus-free and virus-infected plants, the results indicate that virus-infected plants had a higher starch content in the leaf than virus-free plant (Table 17). This result was accordant with those of Lehrer (2001).

The results illustrate that the site of starch synthesis was affected by SCYLV. In virus-infected plants, starch synthesis was performed not only in bundle sheath cells, but also in mesophyll cells.

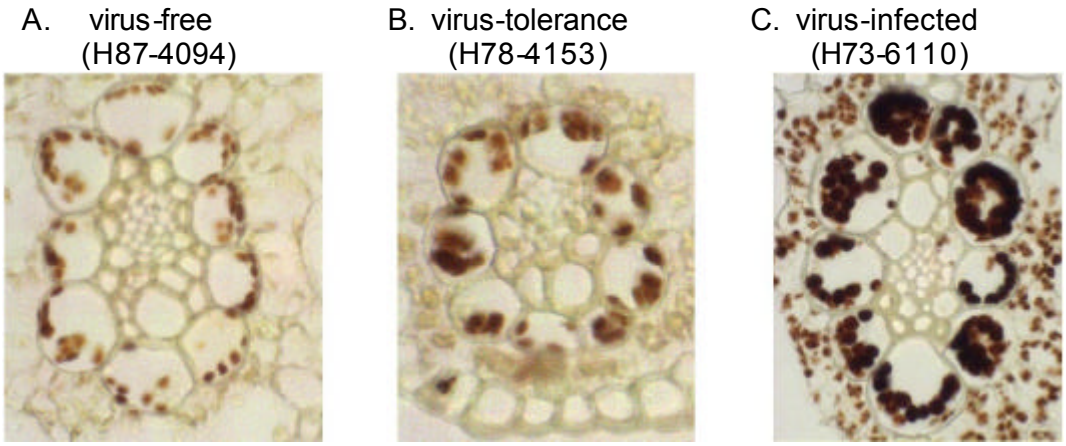

Figure 60 : Starch staining of sugarcane. The paraffin sections were dewaxed and then stained with KI. Starch could be detected in the bundle sheath cells of all plants (A, B and C). In virus-infected plants, starch could also be seen in mesophyll cells (C).

Table 17 : The starch area/ cytoplasmic area ratios of different sugarcane plants.

Samples	SCYLV infected	Number of analysed cells	Cytoplasmic area (μm^2)	Starch area (μm^2)	Starch area/ Cytoplasmic area
H87-4094	**No**	46	409,003	57,505	0,142
H78-4153	**Yes** (4 %)	51	285,395	73,426	0,254
H73-6110	**Yes** (100 %)	51	782.899	305,068	0,411

C.2. Partial cloning and *in situ* hybridization of the 18S ribosomal RNA gene

In order to obtain reliable results of *in situ* hybridization studies, positive and negative controls are always needed. A highly expressed and tissue/ organ specific gene is a good choice as a control. Total RNA from the leaf of sugarcane was extracted as described (see Material and Methods) and subjected to cDNA synthesis using random hexamer as the primer. Then 1 µl of cDNA was used for PCR amplification in the presence of a primer pair ("forward" primer: 5´-AGGGAGCCTGAGAAACGGCTACCACATC-3´ and "reverse" primer: 5´-TGGTTGAGACTAGGACGGTATCTGATC-3´). Amplification was achieved in a DNA thermal cycler (Mj Research.) with the following conditions: 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and elongation at 72°C for 1 min. Finally, the reaction mixture was kept at 72°C for 7 min. The PCR products were examined by electrophoresis in 1.2% agarose gels (Figure 61 A, lane 2). The fragments of the expected

size (around 600 bp) were retrieved with agarose DNA extraction kit (Roche) and cloned into pGEM-T Vector (Promega) according to the manufacturer's instructions. The plasmids (pGT-18S) generated were transformed into the *Escherichia coli* strain DH5a and then sequenced (Figure 62). The sequence data were used to search the GenBank sequence databank, and the product was found to have high similarity to the 18S ribosomal RNA gene.

In order to detect the 18S rRNA in the tissue sections, digoxigenin-labelled probes were used. The probes were synthesized by *in vitro* transcription. *In situ* hybridization was performed with digoxigenin-labelled sense and antisense RNA probes. The results are shown in Figure 63. It indicated that no hybridization signal was found in the sections, which were hybridized with sense probe (Figure 63, left). Strong signal could be seen in the sections, which were hybridized with the antisense probe (Figure 63, right). This result indicates that the hybridization was successful and 18S ribosomal RNA sense and antisense probes, which were made by *in vitro* transcription, could be used as positive and negative controls.

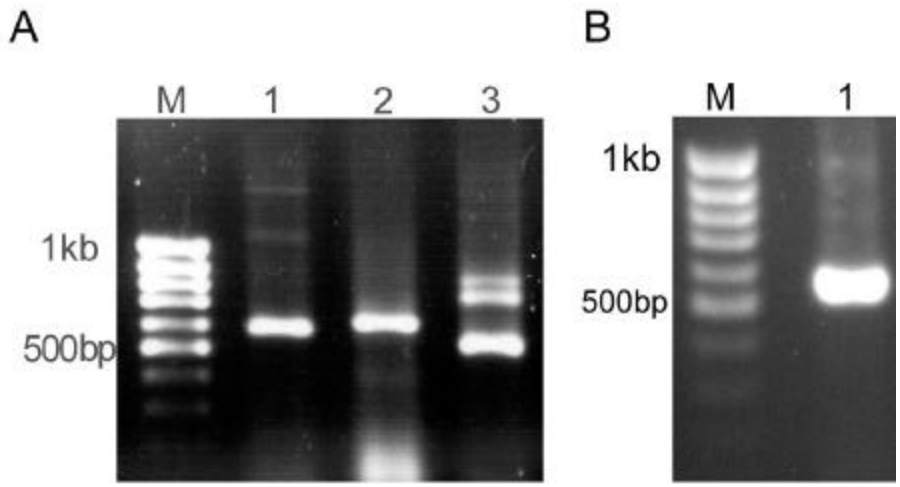

Figure 61 : Gel electrophoresis of RT-PCR products of ADP-glucose pyrophosphorylse, 18S rRNA, starch branching enzyme and SCYLV. (A) RT-PCR products of ADP-glucose pyrophosphorylase, 18S rRNA and starch branching enzyme were checked in a 1.2% agarose gel. M: 100bp marker; Lane 1: ADP-glucose pyrophosphorylase. The size of the RT-PCR fragment is around 600bp; Lane 2: 18S rRNA. The amplified DNA band (around 600 bp) was of the appropriate length; Lane 3: starch branching enzyme. The amplified DNA band (around 500 bp) had the appropriate length. (B) The RT-PCR product of the SCYLV fragment was checked in a 1.2% agarose gel. The size of the RT-PCR fragment is around 550bp as expected.

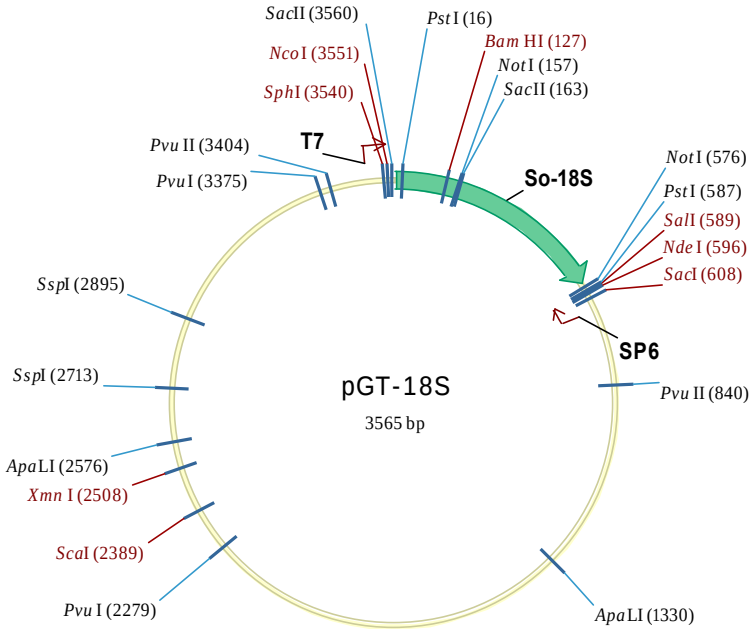

Figure 62 : Restriction map of pGT-18S. The partially cloned 18S rRNA gene of sugarcane was ligated into a pGEM-T vector. This plasmid was used as the template to synthesize digoxigenin-labelled sense and anti-sense probes for *in situ* hybridization.

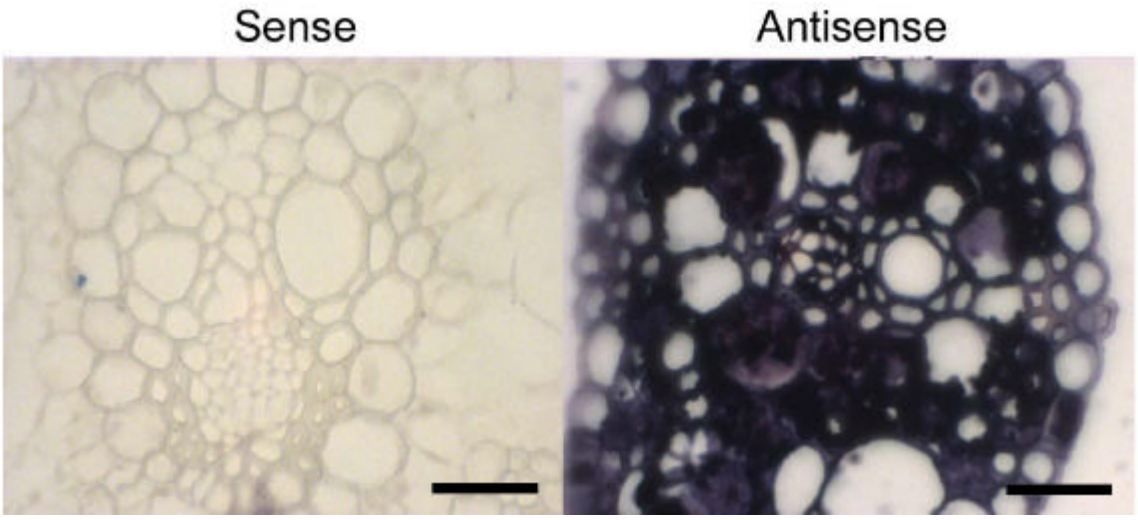

Figure 63: *In situ* hybridization of 18S ribosomal RNA. The paraffin sections of sugarcane leaves were dewaxed and then hybridized with digoxigenin-labelled probes. No signal could be detected in the tissue sections, which were hybridized with the sense probe. The sections, which were hybridized with the antisense probe, showed very strong hybridization signal. Bar = 40 µm.

C.3. Partial cloning and *in situ* hybridization of sugarcane yellow leaf virus

Total RNA for RT-PCR was isolated from the leaves of sugarcane. Genomic DNA contamination was then digested by DNase treatment. First-strand cDNA synthesis was primed with random hexamers and catalysed by M-MuLV reverse transcriptase. PCR amplification of 550 bp of the SCYLV fragment was performed using the forward primer 5´-CACACATCCGAGCGATAGTGAATGAAT -3´ and the reverse primer 5´-GTCTCCATTCCCTTTGTACAGCAACCA -3´. Amplification was achieved in a DNA thermal cycler (Mj Research.) with the following conditions: 30 cycles of denaturation at 94° C for 1 min, annealing at 56° C for 1 min, and elongation at 72° C for 1 min. Finally, the reaction mixture was kept at 72° C for 7 min. Amplified DNA bands of the appropriate length (around 550 bp) were cloned into a pGEM-T vector and analyzed by gel electrophoresis (Figure 61, B). The plasmids generated were transformed into the *Escherichia coli* strain DH5a and then sequenced. The sequence data were used to search the GenBank sequence databank, and the product was found to have high similarity to the sugarcane yellow virus.

In order to detect the SCYLV RNA in the leaf of sugarcane, digoxigenin-labelled RNA probes were used. RNA probes were synthesized by *in vitro* transcription. The plasmid, which contains SCYLV genome fragment, was used as template to perform *in vitro* transcription. *In situ* hybridization was performed with digoxigenin-labelled sense and antisense RNA probes.

The results of *in situ* hybridization are shown in Figure 64. They indicate that no hybridization signal was found in the sections, which were hybridized with the sense probe (Figure 64, A and B). Strong signals could be seen in the phloem tissue of the sections of the virus-infected plants, which were hybridized with the antisense probe. No signal was found in the sections of the virus-free plant, which were treated with the antisense probe (Figure 64, C). The results are accordance with the previous results of tissue blot immuno analysis (Lehrer, PhD thesis, Bayreuth 2001). This indicates that the hybridization was successful and SCYLV sense and antisense probes, which were made by *in vitro* transcription, could also be used as positive and negative controls.

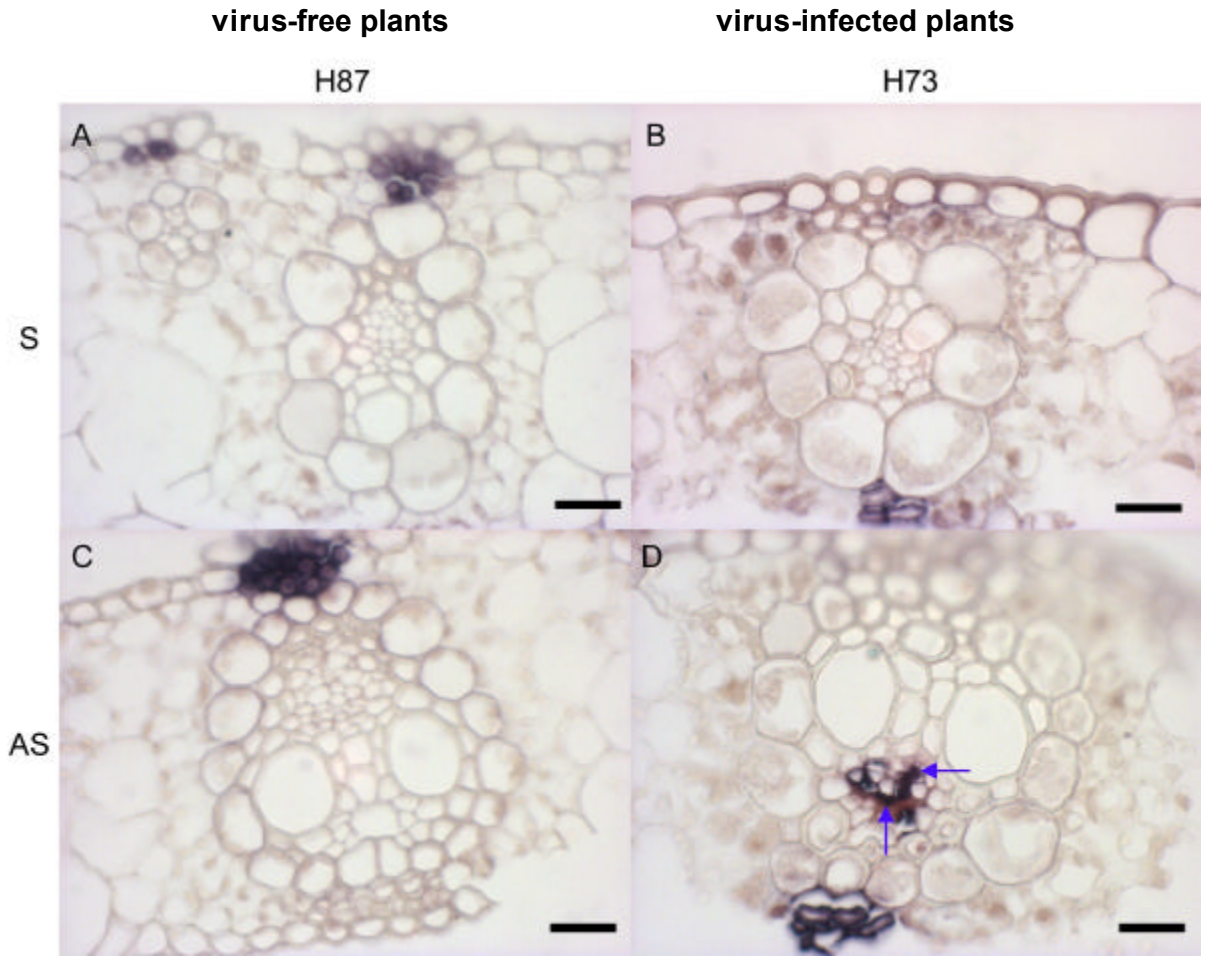

Figure 64 : *In situ* hybridization of SCYLV. Tissue sections of sugarcane leaves were dewaxed and then hybrid ized with digoxigenin -labelled probes. The blue arrows indicate the hybridization signal. (A) and (C) are sections of virus-free plants. (B) and (D) are sections of virus-infected plants. No hybridization signals could be detected the sections from virus-free and virus-infected plants, which were hybridized with the sense probe (A and B). No signal was found in the sections from virus-free plants, which were treated with the antisense probe (C). The hybridization signal could only be detected in sections from the virus-infected plant (D), which were hybridized with the antisense probe. The signal could be found in the phloem tissue (D). Bar = 25 µm.

C.4. Partial cloning and *in situ* hybridization of starch synthesis related genes

In order to study the site o f starch synthesis in the sugarcane leaf, starch synthesis related genes, ADP-glucose pyrophosphorylase and starch branching enzyme (SBE), were partially cloned. The cloned cDNA fragments were used as templates for synthesizing digoxigenin-labelled RNA probes.

C.4.1. Partial cloning and *in situ* hybridization of the ADG-glucose pyrophosphorylase gene

In order to clone the ADP-glucose pyrophosphorylase gene in source leaves of sugarcane, an RT-PCR approach was applied. First-strand cDNA was obtained by reverse transcription of poly-A RNA with an oligodT(30) primer. From a comparison of the amino acid sequences of several cDNA clones for plant ADP-glucose pyrophosphorylase, two highly conserved regions, IASMGIYV and RETDGYFI, were selected. Using degenerate primers (ADP-Glc-PP-5d-for primer: 5´- ATHGCBDSHATGGAAGTNTAYRTH -3´ and ADP-Glc-GPP-3d-rev primer: 5´- ATHGCBDSHATGGAAGTNTAYRTH -3´) from these peptide sequences, cDNA fragments of putative ADP-glucose pyrophosphorylase from source-leaf RNA of sugarcane were amplified. Amplification was achieved in a DNA thermal cycler (Mj Research.) with the following conditions: 30 cycles of denaturation at 94° C for 1 min, annealing at 56° C for 1 min, and elongation at 72° C for 1 min. Finally, the reaction mixture was kept at 72° C for 7 min. The fragment had the predicted size of 600 bp (Figure 61 A, line 1). Amplified DNA bands of the appropriate length were cloned into a pGEM-T vector. The plasmids generated were transformed into the *Escherichia coli* strain DH5a and then sequenced. The sequence data were used to search the GenBank sequence databank, and the product was found to have high similarity to the ADP-glucose pyrophosphorylase sequences (Figure 65).

In order to detect the mRNA of ADP-glucose pyrophosphorylase in the leaf of sugarcane, digoxigenin-labelled RNA probes were synthesized. RNA probes were synthesized by *in vitro* transcription. The plasmid, which contains the fragment of the ADP-glucose pyrophosphorylase gene, was used as template to perform *in vitro* transcription (Figure 66). *In situ* hybridization was performed with digoxigenin-labelled sense and antisense RNA probes.

Figure 65 : Alignment of different ADP-glucose pyrophosphorylases. A partial sequence of the ADP-glucose pyrophosphorylase gene of sugarcane was aligned to ADP-glucose pyrophosphorylase sequences from Arabidopsis (AtAGPP, accession number: NM121927), barley (HvAGPP, accession number: HVU68876; HV-AGPP-1, accession number: X62243), *Cicer arietinum* (CsAGPP, accession number: AF356002), maize (ZmAGPP, accession number: AY032604), wheat (TAAGPP, accession number: X14348).

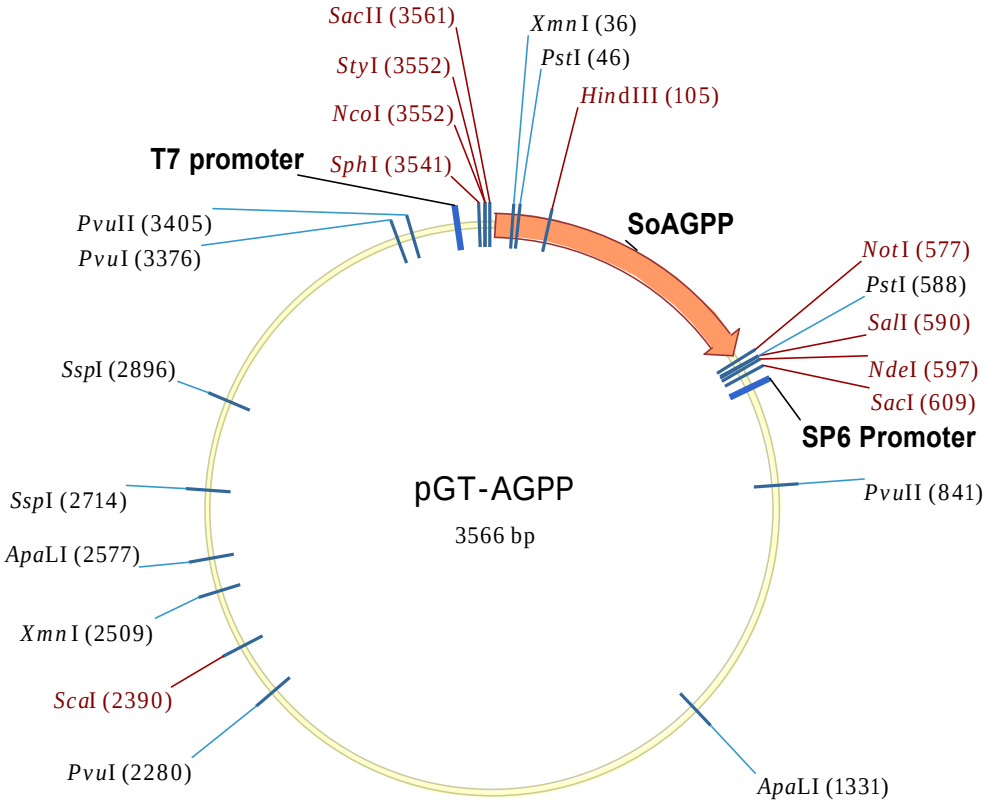

Figure 66 : Restriction map of pGT-AGPP. ADP-glucose pyrophosphorylase of sugarcane was partially cloned to a pGEM-T vector. This plasmid was used as template to carry out *in vitro* transcription to synthesize digoxigenin-labelled sense and antisense probes for *in situ* hybridization.

The results are shown in Figure 67. No hybridization signal was found in the sections, which were hybridized with the sense probe (Figure 67, C and D). Strong signals could be seen in the tissue sections of leaf blades, which were hybridized with the antisense probe. Hybridization signals could be found in bundle sheath cells and mesophyll cells of virus-free and virus-infected plants (Figure 67, A and B). In the sections of virus-infected plants, however, the mesophyll cells exhibited the most intense hybridization signal (Figure 67, B).

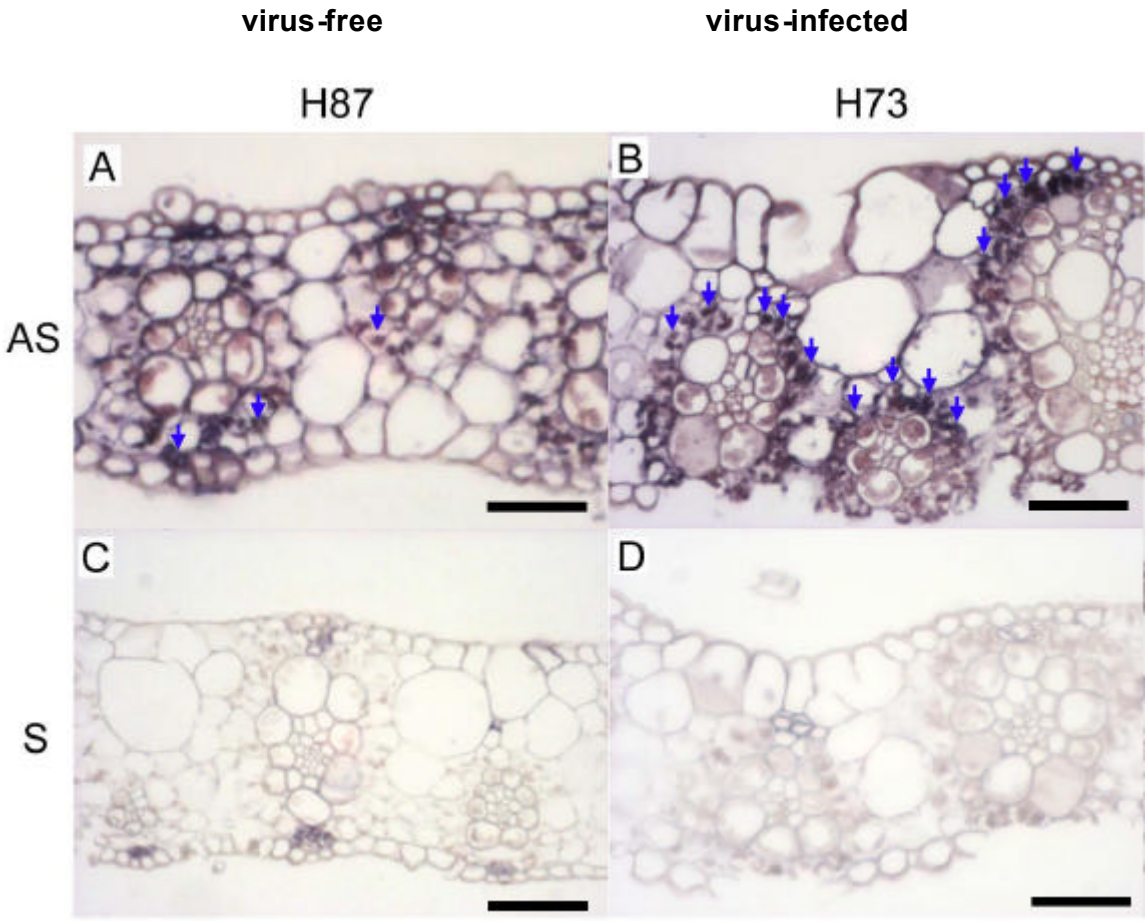

Figure 67 : *In situ* hybridization of ADP-glucose pyrophosphorylase in sugarcane leaves. Tissue sections of sugarcane leaves were dewaxed and then hybridized with digoxigenin-labelled probes. (A) and (C) are sections of virus-free plants. (B) and (D) are sections of virus-infected plants. The hybridization signals could be detected in the sections, which were hybridized with the antisense probe (A and B). The signals were found in the bundle sheath cells and mesophyll cells. No hybridization signal could be detected in the sections from virus-free and virus-infected plants, which were hybridized with the sense probe (C and D). Bar = 40 µm.

C.4.2. Partial cloning and *in situ* hybridization of the gene of the starch branching enzyme (SBE)

For the isolation of starch branching enzyme cDNA in source leaves of sugarcane, a RT-PCR was carried out. First-strand cDNA was obtained by reverse transcription of poly-A RNA with an oligodT(30) primer. A DNA fragment corresponding to a 500-bp-long domain of plant starch branching enzyme was amplified by PCR with the degenerated primers (SBE-5d-for: 5´- AAAAGATGA TTC ACT TCA TYA CRA TG -3´ and SBE-3d-rev: 5´-

TTGCCGGTMGTTGAASTTTGTTTCWGGCAC-3´). Amplification was achieved in a DNA thermal cycler (Mj Research.) with the following conditions: 30 cycles of denaturation at 94° C for 1 min, annealing at 56° C for 1 min, and elongation at 72° C for 1 min. Finally, the reaction mixture was kept at 72° C for 7 min. Reverse-transcription PCR products were analyzed by agarose-gel electrophoresis. Amplified DNA bands (around 500 bp) of the appropriate length were cloned into a pGEM-T vector (Figure 61 A, Lane 3). The plasmids generated were transformed into the *Escherichia coli* strain DH5a and then sequenced. The sequence data were used to search the GenBank sequence databank, and the product was found to have high similarity to the sequences of starch branching enzymes.

In order to study the site of expression of the starch branching enzyme gene in the sugarcane leaf, digoxigenin-labelled RNA probes were synthesized. RNA probes were synthesized by *in vitro* transcription. The plasmid, which contains the fragment of the starch branching enzyme gene, was used as template to perform *in vitro* transcription (Figure 68). *In situ* hybridization was performed with digoxigenin-labelled sense and antisense RNA probes.

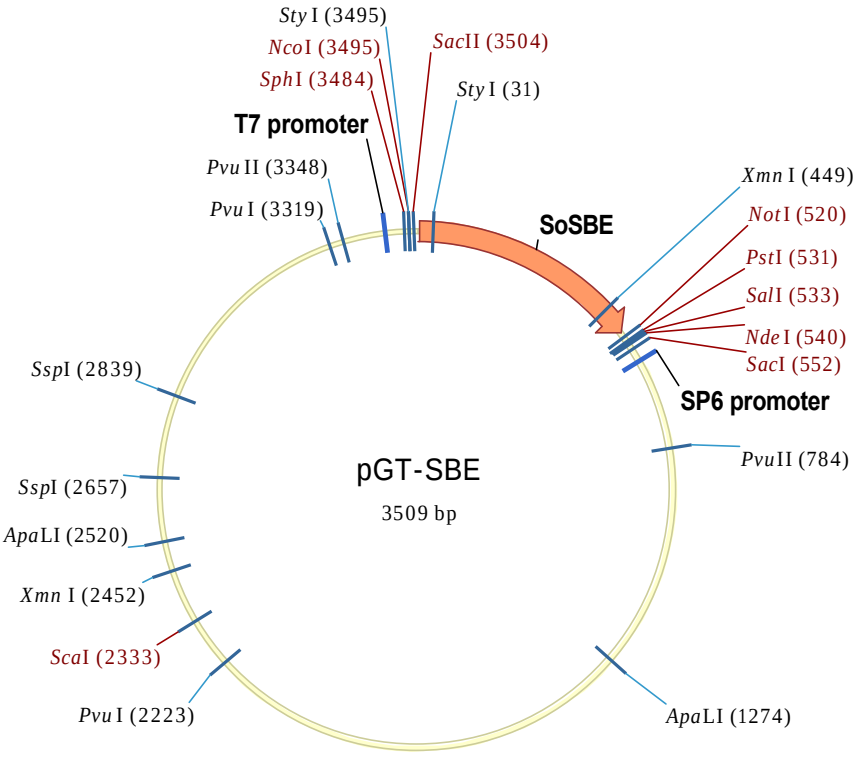

Figure 68 : Restriction map of pGT-SBE. The gene of starch branching enzyme of sugarcane was partially cloned to a pGEM-T vector. This plasmid was used as template to carry out *in vitro* transcription to synthesize digoxigenin-labelled sense and antisense probes for *in situ* hybridization.

The results of the *in situ* hybridization are shown in Figure 69. No hybridization signal could be detected in the sections, which were hybridized with the sense probe (Figure 69, A and C). Strong signal could be seen in the tissue sections, which were hybridized with the antisense probe (Figure 69, B and D). Hybridization signals were found mainly in bundle sheath cells. There was no significant difference between the virus-free and virus-infected plants. Compared to the signals in bundle sheath cells in virus-free and virus-infected plants, they had similar expression in bundle sheath and mesophyll cells.

The result indicated that the expression level of starch branching enzyme gene in the mesophyll cells might be elevated in virus-infected-plants.

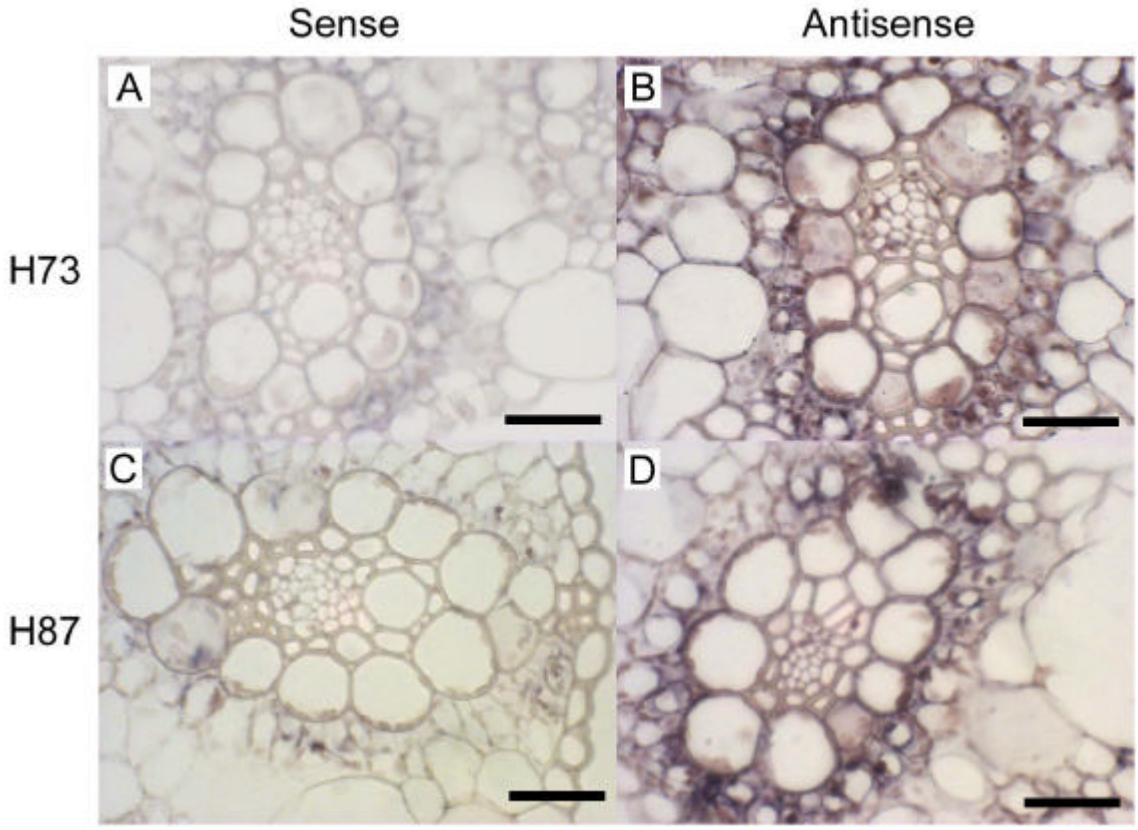

Figure 69: *In situ* hybridization of starch branching enzyme (SBE). (A) and (B) are sections of virus-infect plants. (C) and (D) are sections of virus-free plants. The sections were dewaxed and hybridized with sense and antisense digoxigenin-labelled probes. No signal could be found in the sections, which were hybridized with the sense probe (A and C). Hybridization signals were found in sections, which were hybridized with the antisense probe (B and D). Bar = 40 µm.

Discussion

1. The function of *RcSCR1*

Sucrose is the major mobile carbohydrate in the majority of higher plants. Sucrose is loaded into the phloem against a large concentration difference and is transported to heterotrophic tissues where it is used for metabolism or storage. Our knowledge of sucrose translocation has increase to a large extent by the biochemical and molecular characterization of the sucrose transporter (SUT) family of low- and high-affinity sucrose transporters in the last decade.

Phloem loading is catalyzed by specific sucrose transporters, which couple the uptake of sucrose to the electrochemical potential difference of protons generated by the H+/ATPase (Buckhout, 1989, 1994; Bush, 1989, 1990, 1993a; Williams *et al.*, 1990). In higher plants, three major members of the sucrose transporter subfamilies with different properties were identified: SUT1, a high-affinity sucrose/H+ cotransporter; SUT4 (Weise *et al.*, 2000), a low-affinity sucrose/H+ cotransporter; and SUT2 (Lalonde *et al.*, 1999; Barker *et al.*, 2000), which -when expressed- in yeast is only weakly active and may have similar functions as the yeast sugar sensors RGT2 and SNF3 (Lalonde *et al.*,1999).

The proton-coupled sucrose uptake transporter (SUT) subfamilies play essential roles in long-distance transport of sucrose within the vascular tissue of plants. The SUT1 subfamily is a high-affinity low-capacity subfamily and has been identified in many plant species. In most higher plants, high-affinity sucrose transporter SUT1 is essential for phloem loading. Using antisense inhibition or T-DNA insertion to reduce SUT1 transcript levels shows the importance of the sucrose transporter SUT1 in plants. These transgenic tobacco or potato plants have severe physiological problems, such as reduced growth, curled leaves with partial chlorosis and necrosis, and accumulation of carbohydrates such as glucose, fructose and sucrose in leaves. (Riesmeier *et al.*, 1994; Kühn *et al.*, 1996; Bürkle *et al.*, 1998). Arabidopsis plants, with T-DNA insertion, exhibit the similar syndromes as tobacco plants with antisense inhibition (Gottwald *et al.*, 2000). RcSCR1, which belongs to the SUT1 subfamily, was found in the phloem of the hypocotyl and in the petioles of source leaves. This suggests that RcSCR1 may have the same function in *Ricinus* plants, it retrieves the escaped sucrose from sieve tubes.

Certain seeds accumulate storage reserves in the endosperm during development. Upon germination, these are hydrolyzed and the product, including sucrose, is released into the apoplastic space. *RcSCR1* transcript was also found in the lower epidermis of the cotyledons. It suggests that RcSCR1 protein may play a role in the uptake of sucrose into the cotyledons. But *RcSCR1* transcripts and RcSCR1 protein were not found in the phloem tissue of cotyledons. Thus, the question is: if RcSCR1 protein does not exist in the phloem of cotyledons, how is sucrose transported into the phloem in cotyledons of the *Ricinus* seedling? Is it possible that there are two or more types of sucrose transporters in *Ricinus* seedling? Are other SUT1 used for transporting sucrose into the phloem of cotyledons?

A partial-length cDNA clone, *RcSUT1*, was isolated by RT-PCR from *Ricinus* seedling RNA (Bick *et al.* 1998). The *RcSUT1* cDNA clone differs from *RcSCR1* cDNA clone by only two bases. *RcSUT1* is almost identical to the cDNA of sucrose transporter *RcSCR1*, which was previously isolated by library screening (Weig and Komor, 1996). However, this difference does not alter the overall deduced amino acid sequence composition. By *in situ* hybridization, *RcSUT1* signals were observed in the lower epidermal layer of the cotyledons and phloem tissue, consistent with a role for active sucrose uptake of these cells (Bick *et al.* 1998). But in this work, the 3'-UTR sequence of *RcSCR1*, a relative gene specific sequence, was used as template to synthesize anti-sense probe to perform *in situ* hybridization, rather than the conserved region of all SUT1. We have found that the expression site of *RcSCR1* could be found in lower epidermis or palisade parenchyma cells of cotyledons, but not in the phloem tissue. The results were different to it of *RcSUT1*. It suggests that ether *RcSUT1* and *RcSCR1* are different sucrose transporter genes of *Ricinus* or they are same gene, but it exists another sucrose transporter gene of *Ricinus communis*.

The developing embryo is symplastically isolated from maternal tissues and is dependent on the import of nutrients from the surrounding apoplastic space. Sugars are essential for embryo development and for the deposition of storage compounds necessary for germination. During later stages of *Vicia faba* embryo development, *VfSTP1* (hexose transporter) is replaced by the sucrose transporter *VfSUT1* (sucrose transporter) (Weber *et al.* 1997). *VfSUT1* expression is highest in epidermal cells with transfer-cell morphology

and with storage activity in the underlying parenchyma, indicating that the encoded transporter is responsible for providing substrate for the synthesis of storage compounds. In fava bean cotyledons, carrier-specific inhibitors as well as the removal of the outer cell layers of the cotyledons reduced sucrose uptake considerably (McDonald *et al*., 1995), indicating that sucrose is taken up predominantly by the outer epidermis. The RcSCR1 protein was also found in the developing seed of Ricinus, it is supposed that RcSCR1 protein might uptake sucrose into endosperm storage cells.

RcSCR1 transcript is also found in the endosperm of germinating *Ricinus* seeds. The *RcSCR1* expression level in the germinating endosperm is relative lower than that in other tissues. Northern blotting with *RcSCR1* as the probe was used to characterize the expression of the sucrose transporter in *Ricinus* endosperm. The *in situ* RT-PCR result revealed that the *RcSCR1* expression is not equally in endosperm. The expression level is higher in the endosperm middle layer than in the innermost layer and in the outermost layer. It is supposed that RcSCR1 protein in the endosperm might retrieve escaped sucrose. Sucrose can diffuse either to cotyledons or seed coat. To avoid sucrose efflux, RcSCR1 protein retrieves sucrose into the cells. However, the function of *RcSCR1* in the endosperm is still unclear.

SUT1 proteins were found not only in source tissues but also in sink tissues (Riesmeier *et al*., 1994; Kühn *et al*., 1997). However, the role of SUT1 in sink tissues is still not understood. The expression of sucrose transporter all over this tissue might accelerate the uptake of sucrose into it.

2. The function of *RcSCR2*

Physiological studies indicate that plants contain multiple sucrose transport systems. A saturable, low-affinity high-capacity (LAHC) sucrose uptake system has been studied in leaves (Delrot and Bonnemain, 1981), and up to now only few genes have been assigned to this function. The new SUT4 subfamily of sucrose transporters has known members in Arabidopsis (*AtSUT4*, Weise *et al*., 2000), potato (*StSUT4,* Weise *et al*., 2000), tomato (*LeSUT4,* Weise *et al*., 2000) and Lotus japonicus (*LjSUT4*, Flemetakis *et al*., 2003), grape berry (VvSUC12, Manning *et al*., 2001). Since expression of *AtSUT4* and *StSUT4* in yeast conferred low-affinity sucrose uptake activity, *SUT4* appears to represent a LAHC

sucrose transport system. The *Km* values of these SUT4 sucrose transporters are higher than those of SUT1 sucrose transporters. The *Km* values of SUT1 are between 0.2 and 2.0 mM and the *Km* values of SUT4 are in the range of about 6 mM at optimal pH. However, the transport rate of SUT4 relative to SUT1 is not known.

RcSCR2, encoding a putative sucrose transporter, was identified in a *Ricinus communis* seedling cDNA library. The RcSCR2 protein contains 509 amino acids and the deduced amino acid sequence showed that RcSCR2 protein might also have 12 transmembrane domains typical for this transporter family. The multiple amino acid sequence alignment of RcSCR2 protein with other known plant sucrose transporters expressed in sink tissues, revealed that the RcSCR2 protein exhibits similarity to LeSUT4 (72%), StSUT4 (71%), LjSUT4 (70%), AtSUT4 (69%) and RcSCR1 (50%) (Table 18). The result of multiple alignments of these sucrose transporters suggests that AtSUT4, LeSUT4, LjSUT4, RcSCR2 and StSUT4 belong to the same sucrose subfamily, SUT4. There is only 50% similarity between the deduced amino acid sequences of RcSCR1 and other SUT1 proteins. Most similar regions locate in the 12 transmembrane domains. The SUT4 proteins are phylogenetically distinct from the SUT1 group, however, both show a similar expression pattern in phloem cells of *Arabidopsis*, tomato and potato (Weise *et al*., 2000).

Table 18 : The similarity table of different SUTs. RcSCR2 is compared to different sucrose transporters. AtSUT4 (accession no. AF175321); RcSCR1 (accession no. Y16766); LeSUT4 (accession no. AF176950); StSUT4 (accession no. AF237780); LjSUT4 (accession no. AJ538041). DcSUT1 belongs to SUT1 subfamily.

	RcSCR2	AtSUT4	LjSUT4	LeSUT4	StSUT4	RcSCR1
RcSCR2	100	69	70	72	71	50
AtSUT4		100	67	68	67	50
LjSUT4			100	50	50	49
LeSUT4				100	96	50
StSUT4					100	50
RcSCR1						100

The SUT4 sucrose transporters are expressed in source and sink tissues. AtSUT4, LeSUT4 and StSUT4 are expressed differently: An *AtSUT4* promoter-GUS fusion transgenic plant showed to be promoter active in minor veins in source leaves and *AtSUT4* is also expressed in sink leaves, flowers, and fruits. In sink tissue, *AtSUT4* is supposed to have a function in sucrose uptake into sink cells (Weise *et al*., 2000). The cell-specific localization of AtSUT4 is still unknown (Weise *et al*., 2000). LeSUT4 and StSUT4

transcripts were detected using the RNase protection assay (RPA) in source and sink leaves, in green tomato fruits and in the ovaries of flowers. The proteins were localized to the sieve elements (SE) of major veins of source and sink leaves and in SE of the midrib, the petiole and the stem (Weise *et al.*, 2000). It is reasonable that high rates of sucrose uptake into sink cells would require a LAHC transport system. The phloem loading is thought to occur in minor veins. Thus in Arabidopsis, expression of AtSUT4 in source leaf minor veins suggests a function in phloem loading.

Expression of *AtSUT4* and *StSUT4* in SUSY7 yeast strain allowed yeast growth on sucrose, providing the indication that *AtSUT4* and *StSUT4* encode functional sucrose transporters. The LeSUT4 did not take up sucrose in the yeast sucrose uptake experiment (Weise *et al*., 2000). Functional analysis of *RcSCR2* was performed in a yeast mutant SEY2102. Although for the RcSCR2 protein, no sucrose transporter activity could be detected in a transformed yeast mutant SEY2102 (Eisenbarth, Diploma thesis, Bayreuth 1999), the deduced amino acid sequence suggests that RcSCR2 protein has a similar function as other SUT4 proteins.

It is supposed that SUT4 may have the following functions in sink tissues: if *SUT4* is expressed outside of the phloem, it may function directly in sucrose uptake into sink cells and have a role in determining sink strength; or if *SUT4* is expressed in sieve elements in sink tissue, then it could function in regulating the concentration of extracellular sucrose by way of reuptake. The *in situ* hybridization result indicated that *RcSCR2* was also expressed in young developing leaves where no phloem tissue was found. The result suggests that RcSCR2 protein may take the role of high capacity sucrose transporter to transport sucrose into sink tissues rather than to regulate the sucrose concentration in the extracellular space.

In the phloem, sucrose transporters, which have different affinities, may be expressed to optimize the capacity and affinity of sucrose uptake into the sieve elements. HALC and LAHC sucrose transporters are expressed in the loading zone and along the path (Lalonde *et al.*, 1999). In potato plants, the result of immunolocalization indicated the co-localization of three different sucrose transporters (SUT1, SUT2 and SUT4) at the plasma membrane of the same enucleate sieve element. It is suggested that the sucrose transporters also exist as oligomer (homo- or hetero-) in planta (Reinder *et al*., 2002). The function of

sucrose transporter hetero-oligomers in plant cells is still unclear. It is suggested that they may regulate the uptake of sucrose. In spatial studies, the results indicate that *RcSCR1* and *RcSCR2* were both expressed in the young developing leaves, in the phloem of the hypocotyl and in the endosperm. But whether RcSCR1 and RcSCR2 proteins formed hetero-oligomers in the same cell could not be distinguished.

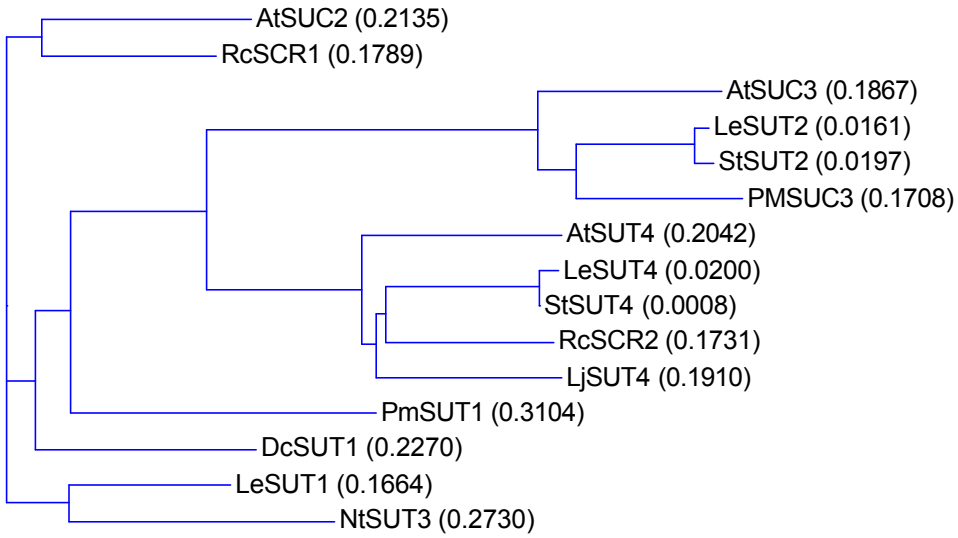

Figure 70 : Phylogenic tree of sucrose transporters. The tree is based on the alignment of amino acid sequences of sucrose transporters from Arabidopsis thaliana (AtSUC2: accession X75382, AtSUC3: accession AJ289165, AtSUT4: accession AJ289166), Nicotiana tabacum (NtSUT3: AF149981), Lycopersicon esculentum (LeSUT1: accession X82275, LeSUT2: accession AF166498, LeSUT4: accession AF176950), Solanum tuberosum (StSUT2: accession AY291289, StSUT4: accession AF23778), Daucus carota (DcSUT1: accession Y16766), Ricinus communis (RcSCR1: accession Z31561), Plantago major (PmSUT1: accession X84379, PmSUT2: accession X75764).

The *in situ* RT-PCR results indicate that the *RcSCR2* transcript is present in the middle layer of the germinating endosperm. Thus, the RcSCR2 protein may also be located in the middle layer of the endosperm. In order to know the real localization of the RcSCR2 protein in *Ricinus* plants, immunolocalization is needed. For these studies an antibody against RcSCR2 would be required.

3. Can RcSCR1 and/or RcSCR2 serve as an efflux transporter in the endosperm?

To date, the knowledge of sucrose transporters is limited to transporters which function in sucrose uptake. The characteristics of efflux transporters on the molecular level are still unknown. Efflux of sucrose into the leaf apoplasm from bundle sheath/vascular parenchyma cells has to occur, however, to supply sucrose for loading into the SE-CCC of minor veins. It is supposed that sucrose efflux is achieved by facilitated membrane transport (Giaquinta 1983). In cereals facilitated diffusion of sucrose is found (Porter *et al*., 1987; Wang & Fisher 1995). Facilitated diffusion may be mediated by sucrose/H+ symporters in the absence of a proton difference (Lemoine *et al*., 1996). This conclusion is supported by observations of sucrose efflux from leaf discs, protoplasts and plasma membrane vesicles (Laloi *et al*., 1993).

It is suggested that phloem loading of sucrose is carried out in the minor veins and the SUC3/SUT2 subfamily is mainly expressed in the minor veins of source leaves. In this background, AtSUC3 protein was immunolocalized in phloem parenchyma cells of minor veins where it may function as a facilitator for sucrose release (Barker *et al*., 2000; Meyer *et al*., 2000). It is also postulated that AtSUC3 serves a role as a sucrose sensor because of its structural characteristics and high *Km* value. The function of SUC3/SUT2 type sucrose transporter is still unclear.

Whether efflux of sucrose is energy coupled is ambiguous. It is supposed that there is a sucrose/H+ antiporter or a facilitating system for sucrose efflux (Patrick and Offler, 1995; Wang *et al*., 1995; Patrick, 1997). The model for sucrose/proton antiport located on plasma membranes has been derived from experiments with coats of french and broad bean (Borisjuk *et al*., 2003; Laloi *et al*., 1993; Fieuw and Patrick 1993; Walker *et al*., 1995 and 2000). In isolated plasma membrane vesicles from sugar beet leaves sucrose export was also detected (Laloi *et al*., 1993). Energy-coupled sucrose release in seed coats of French and broad bean exhibits properties consistent with sucrose/H+ antiport (Fieuw and Patrick, 1993; Walker *et al*., 1995, 2000). The sucrose efflux rates of developing bean seeds were turgor dependent. The efflux increased with increasing turgor. In addition, the sucrose efflux is inhibited by PCMBS. This suggests that the sucrose efflux is transporter-mediated (Patrick, 1994; Walker *et al*., 2000). However, none of these sucrose efflux

transporters has been described at the molecular level.

A requirement for energized transport to drive sucrose efflux is not immediately self-evident for phloem unloading. Energy-coupled sucrose release from French and broad bean seed coats accounts for 50% of their total sucrose flux (Fieuw & Patrick 1993; Walker *et al*., 1995, 2000). The remaining passive flux might occur through non-selective channels reported to support sucrose and amino acid efflux from seed coats of pea (De Jong *et al*., 1996, 1997). Interestingly, a non-selective channel has been detected in release cells of *Phaseolus* seed coat that is permeable to a wide range of electrolytes including large organic ions such as glutamate (Zhang *et al*., 2002).

Antisense inhibition of the sucrose transporter SUT1 in potato tubers impairs early tuber development. In transgenic potato SUT1 antisense plants using the tuber-specific patatin promoter B33, the development of tubers was retarded (Kühn *et al*., 2003). A remarkable reduction in tuber size was observed in antisense plants at early stages of tuber development. In addition, starch and amino acid content were altered in antisense tubers at these early developmental stages. Later tuber development did not appear to be disturbed, and antisense plants were able to produce the same amount of tuber fresh weight compared with wild type. This is in agreement with observations in transgenic potato plants expressing yeast-derived invertase either in the apoplasm or in the cytosol of storage parenchyma cells (Sonnewald *et al*., 1997; Tauberger *et al*., 1999). In both sets of plants, the tuber yield is reduced, indicating that sucrose passes through the apoplasm.

Transcripts of *RcSCR1* and *RcSCR2* were found in the germinating *Ricinus* endosperm. These sucrose uptake transporters can retrieve sucrose from the apoplastic space. The question is whether *RcSCR1* and *RcSCR2* could also serve as efflux transporters in germinating endosperm. So far there has been no indication that RcSCR2 protein could release sucrose in germinating endosperm. It is suggested that a sucrose uptake transporter can serve as an efflux transporter when there is no energy supply. If this postulate were true, could it be that RcSCR1 and RcSCR2 proteins are dual functional transporters? If RcSCR1 and RcSCR2 proteins were dual functional transporters, how do RcSCR1 and RcSCR2 control the uptake and efflux of sucrose? New methods need to be developed and used to study this question.

4. How do *Ricinus communis* endosperm cells synthesize and release sucrose?

4.1. Sucrose synthesis in germinating endosperm

Sucrose is universal in plants and plays many roles: compatible solute, storage reserve, transport sugar. Sucrose can also affect the expression of genes that are involved in cell division and differentiation (Blazquez *et al*., 1998; Gaudin *et al*., 2000; Ohto *et al*., 2001), and has been implicated in the control of many developmental processes, including the induction of flowering (Ohto *et al*., 2001; King and Ben-Tal, 2001), the differentiation of vascular tissue (Uggla *et al*., 2001), seed development (Iraqi *et al*., 2001) and the accumulation of storage products (Rook *et al*., 2001; Davoren *et al.*, 2002). Thus, sucrose synthesis must be well regulated.

The sucrose synthase catalyses the reversible conversion of sucrose and UDP to UDP-glucose and fructose. Its activity may correlate with starch synthesis in maize (Chourey and Nelson, 1976) and developing pea seeds (De´jardin *et al*., 1997). The possible function of sucrose synthase in plant cells may be continued rapid degradation of sucrose in the cytosol rather than sucrose synthesis (Nguyen-Quoc and Foyer, 2001).

The main pathway to synthesize sucrose is via sucrose phosphate synthase and sucrose phosphate phosphatase. The first step in the pathway is catalyzed by sucrose-phosphate synthase (SPS). The SPS activity is under transcriptional, posttranslational and allosteric control. The gene encoding the last enzyme in the pathway, sucrose-phosphate phosphatase (SPP), has been cloned. The sequences revealed that SPP has an SPS-like domain at the N-terminus, to which SPS might bind.

In photosynthetically active cells, sucrose is synthesized from UDP-glucose and fructose-6-phosphate in a sequence of two reactions catalysed by SPS and SPP. SPS and SPP activities are localized in the cytosol. *RcSPP1* transcripts were found in the endosperm of *Ricinus* when seeds germinate. The *RcSPP1* is expressed at very early stage (day 2 or earlier) of germination. Thus, in the endosperm cells, the synthesis of sucrose may start at day 2 or earlier.

The presence of sucrose in vacuoles is well known in some plants. That it is actually accumulated in the vacuole is proven by the fact that in sugar beet taproot tissue, the cytosolic sugar concentration is only 76mM compared to a vacuolar concentration of 514mM (Saftner *et al.*, 1983). It was found that sucrose could be transported in the vacuoles of leaf tissue of barley plants (Kaiser and Heber, 1984). Sucrose-6-phosphate is not transported across the tonoplast (Echeverria and Salvucci, 1991). Thus, the accumulated sucrose in vacuoles must be synthesized in the cytosol and then taken up into the vacuole.

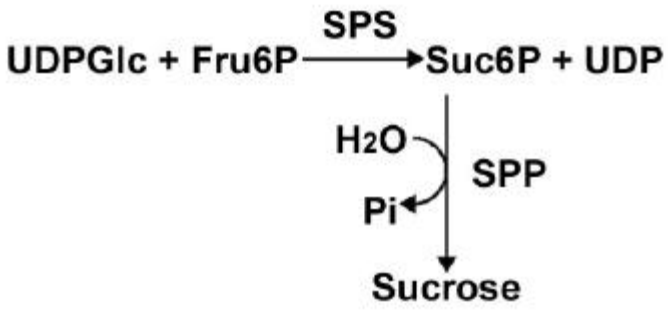

Figure 71 : The possible sucrose synthesis pathway in germinating endosperm of *Ricinus*. Sucrose phosphate synthase (SPS) catalyses the synthesis of sucrose-6F-phosphate (Suc6P). Suc6P is then hydrolyzed to sucrose and phosphate by sucrose-phosphatase (SPP).

Experimental evidence for sucrose uptake into the vacuole came from studies with isolated tonoplast vesicles of red beet (Voss and Weidners, 1988). Later, evidence indicated was presented that a sucrose/H+ antiporter exists in the tonoplast (Getz and Klein, 1995; Greutert and Keller, 1993). In intact red beet vacuoles sucrose was taken up against a 200-fold concentration difference in the presence of Mg-ATP (Getz, 1991). On the other hand, experiments with sugarcane suspension cultures led to different results (Preisser and Komor, 1991). The equal distribution of sucrose between cytosol and vacuole in all phases of the growth cycle of the suspension culture cells of sugarcane indicated that sucrose uptake into sugarcane vacuoles is a passive process. A postulated sucrose/H+ antiporter in the tonoplast of sugar beet has been cloned (Chiou and Bush, 1996). This protein mediates sucrose transport between cytosol and vacuole. This protein has an estimated molecular mass of 54kD (Chiou and Bush, 1996). This is very close to the mass of the sucrose transport protein in red beet tonoplast identified by Getz *et al.* (1993).

As indicated before, transporters of the tonoplast are supposed to work as H+/sucrose antiporters (Briskin *et al.*, 1985). An immunological approach by Getz *et al*. gave some

indications that the sucrose transport activity from red beet tonoplast was associated with polypeptides in the range of 55~60 kDa when reconstituted in proteoliposomes (Getz *et al*., 1993). However, no further characterisation was reported. Only in one case a protein was shown to be associated with the tonoplast (Chiou and Bush, 1996). However, the corresponding cDNA is not closely related to any of the other sucrose transporters of plants (but there is no indication that a sucrose/proton antiporter and a sucrose/proton symporter should share extensive sequence homologies) and no function could be attributed to this transporter after expression in yeast.

Although sucrose can be found in vacuoles of red beet cells, it is unknown for endosperm cells of *Ricinus* whether newly synthesized sucrose is taken up and accumulated in the vacuole or whether it is released to the apoplasm immediately after synthesis. It is supposed that sucrose is accumulated in the vacuoles of endosperm cells. The accumulated sucrose in the vacuole can reduce the water potential. Thus, water must be transported into the vacuole leading to a volume increase. This could explain the increase in size of endosperm cells during germination.

4.2. How is sucrose released from the endosperm of *Ricinus*?

When *Ricinus* seeds germinate, the endosperm provides the energy and nutrients for the other parts of the seedling. The most important carbohydrate is sucrose. The cotyledons are symplastically isolated from the endosperm. Thus, sucrose must be released from the endosperm first and then taken up into the cotyledons. The mechanism of sucrose efflux is still unknown. It is supposed that sucrose is released by: 1. an efflux transporter, 2. a transport vesicle system and 3. programmed cell death.

1. Sucrose efflux transporter

In source leaves during the process of apoplastic phloem loading, sucrose has to be released from the mesophyll cells, where it is synthesized, into the apoplasm. Sucrose efflux was measured with isolated plasma membrane vesicles from sugar beet leaves (Laloi *et al.* 1993). A mechanism for a sucrose proton antiport or a facilitating system was postulated for the efflux of sucrose (Patrick and Offler, 1995; Wang and Fisher, 1995; Patrick, 1997). Sucrose efflux must also occur before the uptake of sucrose from the apoplasm by sink organs, which are symplastically isolated. *Ricinus* cotyledons are

symplastically isolated from the endosperm. Sucrose must be released from endosperm and then taken up by the cotyledons. However, no sucrose efflux carrier has been cloned yet. New methods and strategies must be developed for the finding of sucrose efflux transporters.

2. Transport vesicle

The traditional view of the plant cell vacuole as a storage compartment has been replaced in recent years. The plant vacuole is thought to be a multipurpose organelle since the vacuole carries out numerous metabolic functions (Wink, 1993). The vacuole participates in the efflux of a variety of solutes ranging from organic acids to amino acids and mineral ions.

The sugar solutions, which are secreted from plant nectaries, often contain different amounts of other hydrophilic substances such as oligosaccharides, amino acids, organic acids, ions, vitamins, etc. (Fahn, 1979; Caldwell and Gerhardt, 1986). The sugar concentration of the nectar is very high. The differences in concentration between the nectar and the phloem sap indicate that at some point selective transport must have occurred against a concentration difference (Pate *et al*., 1985). The ultrastructural observation revealed that during nectar secretion, the cellular architecture of the nectar-secreting cells is extremely dominated by an extensive network of ER. Similar phenomena have been reported in the species including *Tropaeolum majus* (Rachmilevitz and Fahn, 1975), *Achillea millefolium* (Figuereido and Pais, 1994) and *Cucurbita pepo* (Nepi *et al*., 1996). Some studies of chickpeas (*Cicer arietinu*) and red beet (*Beta vulgaris*) hypocotyl offer additional evidence in favor of a direct vesicular transport of solutes from the vacuole to the plasmalemma (Lazzaro and Thomson, 1992a, 1992b, 1996; Echeverria and Achor, 1999). Movement of vacuolar solutes to the plasma membrane within vescles is advantageous in that large amounts of metabolites can be protected from enzymatic attack by cytosolic enzymes when they are transported through the cytosol.

It was shown that stimulation of the action potential in motor cells also involves release of sucrose (Fromm and Eschrich, 1993). Sucrose release seems to be involved with turgor loss from the stomatal guard cells as well. The simultaneous release of ions and sucrose strongly suggest a common transport mechanism difficult to explain by movement through membrane channels alone. In all cases, motor cells cycle between a turgid state with one

single vacuole (open stomata and open leaf blades) and a shrunken state of much reduced vacuolar volume with many small vacuoles (during stomatal closure and folded leaf blades). It is supposed that plant cells may release sucrose by vesicle transport. Although sucrose may be released by vesicle transport in guard cells and nectaries, up to now there is no evidence for this mechanism in the endosperm of *Ricinus*. Most of the evidences, which support the vesicle transport, are ultrastructural observations. To prove the vesicle transport model needs more biochemical and molecular studies.

3. Programmed cell death

Programmed cell death (PCD) is a process in many organisms by which cells die. The basic morphological and biochemical features of PCD are conserved between the animal and plant kingdoms. Plants process PCD during certain situations that require substantial nitrogen and carbon mobilization, such as leaf senescence. Cell death is a basic biological process that functions in many aspects of animal and plant development and in their responses to stress (Greenberg, 1994; Wang *et al*., 1996; Martins and Earnshaw, 1997). The PCD process is well regulated. It has also been shown that a long period of sucrose starvation induced PCD in suspension cultures of *Acer* spp. cells (Aubert *et al*., 1996). In this case, a massive breakdown of membrane lipids was found. When *Ricinus* seeds germinate, a high level of *nsLTPc1* expression can be found in the lower epidermis of the cotyledons. It supposed that nsLTPc1 protein could retrieve the breakdown products of membrane lipids that are released from dead endosperm cells.

Endosperm provides nutrients and energy during seed germination. Finally, endosperm cells undergo the last step of senescence by programmed cell death (PCD; Schmid *et al*., 1999). The PCD of endosperm cells was found during seed germination (Schmid *et al*., 1999; Eklund and Edqvist, 2003). Another characteristics of PCD were proteinase and RNase accumulation. Nuclear DNA fragmentation was also observed in *Ricinus* endosperm during germination. After PCD, cells release all their contents, and the cotyledons take them up as nutrients and energy resource.

The observation of the endosperm revealed that cell death begins in the cell layers next to the cotyledons. At the early stage of germination, oleosomes and storage protein grains were found in endosperm cells. Only a few cells next to the cotyledons exhibited the nuclear DNA fragmentation. At a later stage, the nuclear DNA fragmentation was found in

all cells of the endosperm. Starch accumulation in cotyledons coincides with the cell death of the endosperm. The starch accumulation was found in cotyledons at day 6. The PCD is well controlled during germination to avoid the loss of nutrients and energy.

Can endosperm cells release sucrose continuously or do they release sucrose when they collapse? If endosperm cells release sucrose continuously, endosperm cells must use efflux transporters or other mechanisms to release sucrose before cells die. On the other hand, if endosperm cells release sucrose after cell death, the sucrose must be accumulated in a high concentration in endosperm cells. In senescence leaves, mesophyll cells release all nutrients before PCD. No evidence can prove that *Ricinus* endosperm releases sucrose only after cell death. More studies are needed. *In vivo* detection of sucrose concentration (Borisjuk *et al*., 2002) needs to be developed.

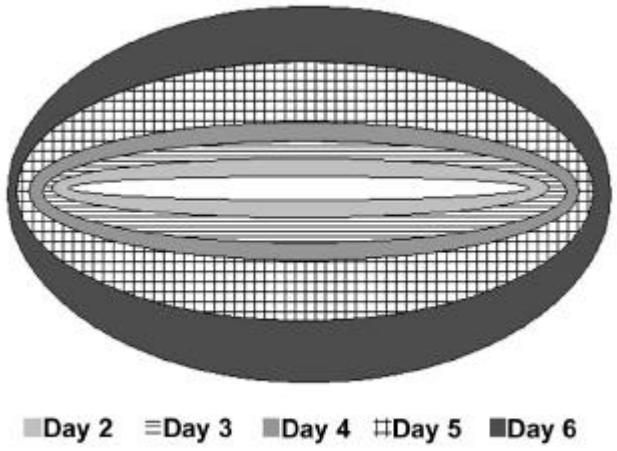

Figure 72 : PCD in Ricinus endosperm. Programmed cell death (PCD) is found in *Ricinus communis* L. endosperm during germination. The process begins at the innermost layers next to the cotyledons. At the beginning, about 5 layers of cells are degraded per day, but more than 20 layers of cells collapsed were found during the 5th-6th days after germination. After 6 days, the nutrients stored in the endosperm were depleted, and then the endosperm is separated from the cotyledons.

5. The Yeast screening system

In the yeast screening system applied, RcSCR1 protein was used to transport sucrose into the invertase deficient yeast mutant SEY2102. The accumulated sucrose in the yeast cell

reduces the water potential and then water flows into the cell producing a high turgor pressure. Under this condition, the yeast suffers from osmotic stress and grows slowly. If sucrose could be transferred out of cell, growth of the yeast transformants might partially recover. The result of the screening revealed that most of the original cDNA library::nSC4+ double transformants could grow more quickly than negative controls after a week of selection. But after retransformation it was shown that growth was as slow as the control.

All transformants were checked by PCR. An amplified fragment (URA3-PMA1 promoter-*RcSCR1*-PMA terminator) of nSC4+ from all transformants should have been 5.3 kb. But PCR products of all transformants were much smaller than 5.3 kb.

High-level expression of a foreign gene may place a metabolic problem on yeast cells, reducing its growth rate and the efficiency of gene expression. Expression of some genes causes a more serious effect, either through a severe effect on metabolism or by direct toxicity. A consequence of this is the not deliberate selection of variants expressing lower levels of protein, particularly when constitutive expression systems are used. Even with regulated promoters, slow accumulation of a toxic protein through leaky expression may have the same effect. In order to reduce the stress, which is induced by producing toxic or heterologous proteins, mutation or recombination of the plasmid may take place. The foreign gene may be eliminated by recombination with native 2µ-plasmid. Low yield of RcSCR1 may be the reason of the failure of screening. Reduced copy number, rearrangement, or mutation of the vector can reduce the yield of foreign protein (Romanos *et al.* 1992). Intergrating vector or ARS/CEN vector may be used to substitute 2µ-derived vector for producing RcSCR1 protein (Table 19).

Table 19 : *S.cerevisiae* vector systems

Vector	Yeast Sequence	Copy number per cell	Stability
Integrating	Homologous DNA	= 1	Stable
Centromere	ARS/CEN	1-2	Stable
2µ-derived	2µ replication origin	50-200	-

6. *In situ* hybridization of SCYLV in sugarcane leaves

The results of the *in situ* studiy hybridization indicated that the SCYLV was limited in the phloem tissue. The result is accordance with previous studies with tissue blot immuno

assay (Lehrer, Ph D thesis, 2001). *In situ* hybridization can thus be used as another choice to detect the virus.

The tissue blot immunoassay (TBIA) that was developed using a polyclonal rabbit anti-SCYLV antiserum is now the standard test method in Hawaii and most parts of the world. Since the supply of the antiserum is nearly depleted, efforts were made to produce a new polyclonal antiserum with an equal or greater specificity. *In situ* hybridization provides another possibility to detect SCYLV. Although virus detection with the *in situ* hybridization technique is too laborious for the large-scale applications, *in situ* hybridization has some advantages. Compared to tissue blot immunoassay, *in situ* hybridization does not require anti-SCYLV serum and by *in situ* hybridization the morphology is preserved and the site of the virus can be localized.

7. Starch accumulation in SCYLV infected and SCYLV free sugarcane plants

It is already known that the starch content in virus-infected sugarcane plants is higher than in virus-free plants (Lehrer, PhD thesis, Bayreuth 2001). In that study, enzymatic methods were used to measure the starch content in tissue extracts and no information was gained about the cell types in which starch was accumulated.

By staining of starch in the tissue sections of sugarcane leaves, we found that starch accumulation in virus-infected plants is different from that in virus-free plants. In virus-free plants, starch granules can be found only in bundle sheath cells, whereas in virus-infected plants, starch granules can be found not only in bundle sheath cells, but also in mesophyll cells. The site of starch synthesis is obviously changed in virus-infected plants.

8. *In situ* hybridization of starch related genes in sugarcane plants

Starch synthesis is a very complicated process and many different enzymes are involved. There are three major enzymes related to starch synthesis, ADP-glucose pyrophosphorylase, starch synthase and starch branching enzyme.

The genes for the ADP-glucose pyrophosporylase and the starch branching enzyme were partially cloned and the *in situ* hybridization of these two genes was done also. The results of starch staining indicated that SCYLV could affect the expression of starch metabolism related genes. The site of expression of ADP-glucose pyrophosphorylase was found in bundle sheath cells and mesophylls. In virus-free plants, the expression of ADP-glucose pyrophosphorylase gene in mesophyll cells is weaker than it in virus-infected plants. This result is similar to it of starch staining. The *in situ* hybridization results of starch branching enzymes were not so clearly. Maybe the expression level of starch branching enzyme was not changed too much. It is possible that the digoxigenin-labelled probes hybridized to similar sequence, for example, the mRNA of the isozymes. Maybe using the 5'-UTR or 3'-UTR to synthesize probe is better than using the conserved sequence.

It is possible that the expression level of starch degradation related enzymes in mesophyll cells of virus-infected plants were reduced. The metabolism of carbohydrates is very complicated. The mechanism of starch accumulation in mesophyll cells is still unknown. The expression of the gene for starch synthesis and degradation, sugar synthesis and degradation and sucrose transporters needs to be studied to ravel how starch is accumulated in mesophyll cells.

9. Future aspect

9.1. Suggestions to improve the yeast screening system

There are some problems of the screening system. (1) The nSC4+ plasmid is not stable during the screening process. (2) The screening time is too long and not efficient. New plasmids and a new strategy must be developed.

A strong constitutive promoter-PMA1 was used to express *RcSCR1*. It is necessary to avoid overexpression of RcSCR1 and DNA rearrangement and reduce the expression of RcSCR1 to a moderate level. The RcSCR1 should be subcloned to non-2μ derived vector and/or the strong promoter *PMA1* may be changed. In addition, nSC4+ plasmid contains a 2μ-replication origin. The nSC4+ plasmid may recombine with native 2μ plasmid and eliminate the RcSCR1 sequence. For yeast, 2μ-replication origin derived plasmids are high copy number plasmids and the *ARS/CEN* derived plasmid can only have one or two

copies in a yeast host cell. The integrating plasmid can also be used for reducing the expression. PMA1 promoter-RcSCR1-PMA terminator fragment can be cloned to an integrating vector or an *ARS/CEN* derived vector to reduce the copy number. Using a weak promoter such as engineered *cyc1* promoter can reduce the expression level (Mumberg *et al.* 1995). The PMA1 promoter can be replaced with a weaker promoter to reduce the expression level.

Using *ARS/CEN* plasmid or integrating plasmid cannot only reduce the copy number of the plasmid can be reduced and therefore the expression of RcSCR1 protein, but also recombination with the native 2µ-plasmid can be avoided. Both 181A1NE and nSC4+ plasmids are 2µ-derived plasmids and they all use *Amp*^{r} as the antibiotic selection gene in *E.coli*. The nSC4+ may recombine with cDNA-181A1NE plasmid because they all have the same 2µ-replication origin. Then the *URA3* selection marker gene may be retained in the yeast cell but partial or full length RcSCR1 fragment may be eliminated. To reduce the screening work, *PMA1* promoter-RcSCR1-*PMA1* terminator should be subcloned to an *ARS/CEN* derived plasmid, which uses *Kan*^{r} or another gene, rather than *Amp*^{r}, for antibiotic selection. Certainly, the *PMA1* promoter-RcSCR1-PMA terminator can be subcloned to an integrating vector. This would increase the possibility to extract plasmids from yeast transformants containing a putative sucrose efflux transporter cDNA.

The screening may be modified in some steps. It may save time and also reduce the possibilities of rearrangement of promoter-*RcSCR1* during the screening. After cDNA library transformation, the transformants can be plated on a sucrose-free selection media plate. After several days, when colonies should be seen, single colonies are picked and cultured overnight. The growth curves of these cultures should be compared to the negative control. The transformants, which can grow more quickly than the negative control in a sucrose-containing medium, may have the sucrose efflux transporter cDNA. With this direct screening method 1 week selection time can be saved (Figure 73).

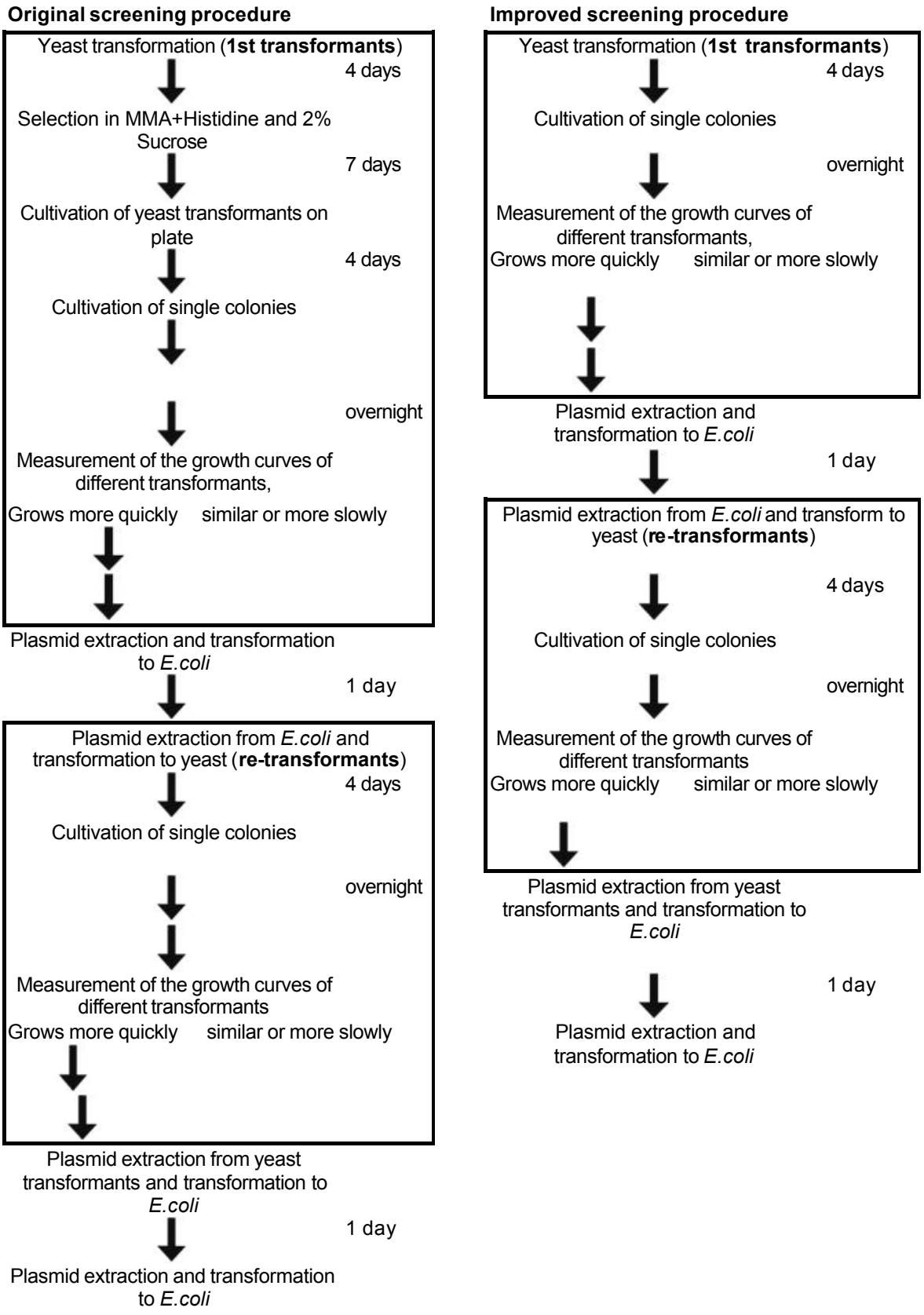

Figure 73 : Flowchart of the original screening procedure and the improved procedure.

9.2. Starch synthesis and SCYLV in sugarcane plants

In grains of winter wheat diurnal changes in ATP content and activities of enzymes catalyzing starch synthesis have been reported (Jiang *et al*. 2004). The enzymes included sucrose synthase (SuSy; EC 2.4.1.13), ADP-glucose pyrophosphorylase (ADPGPPase; EC 2.7.7.27), soluble starch synthase (SSS), and starch granule-bound synthase (GBSS; EC 2.4.1.21). The enzyme activities were generally higher during the nighttime than the daytime, but ATP content showed an opposite pattern (Jiang *et al*. 2004). The presence of starch and sucrose synthesis related genes in different tissues, specific biochemical properties and various regulations could lead to optimization of the control of starch and sucrose metabolism, partitioning and storage in the sugarcane plant. The expression of these genes may be affected by SCYLV and lead to starch accumulation in the mesophyll cells of leaves. Further studies using Northern hybridization, quantitative Real time RTPCR, *in situ* RT-PCR, *in situ* hybridization, immunolocalization, cDNA isolation and promoter analysis are needed to fully understand the regulation of the starch and sucrose synthesis related genes and sucrose transporter genes.

Summary

In order to find the sucrose efflux transporter of the endosperm of *Ricinus communis* L., the yeast complementation selection method was used, but it was unsuccessful. Mutation was occurred on the nSC4+ plasmid during the selection. Maybe the stress of the yeast cells was too strong and then induced the mutation in the yeast cells. Using a weak promoter and reducing the copy number of the plasmid may avoid the mutation occur during the selection.

The endosperm of *Ricinus communis* L. stores lipid and converts it to sucrose for the growth of seedlings. Sucrose phosphate phosphatase gene, *RcSPP1*, was cloned from the endosperm of the germinating seedling of *Ricinus communis* L. The endosperm cells synthesize sucrose by using SPS and SPP rather than sucrose synthase. Northern blot analysis indicated that the *RcSPP1* expression level of the germinating endosperm was very similar from day 2 to day 6.

The expression of *nsLTPc1* is cotyledon-specific. It is also confirmed by *in situ* hybridization. The results of *nsLTPc1 in situ* hybridization indicate that the expression of *nsLTPc1* was a cell-specific. The expression of *nsLTPc1* was found only in the lower side of the cotyledons of *Ricinus communis* L.

The expression of *RcSCR1* is found in the endosperm, hypocotyl and cotyledons of the *Ricinus communis* L. geminating seeds. By northern blot analysis of the RNA from different days old endosperm, it indicates that the *RcSCR1* has a highest expression level at day 5. By *in situ* hybridization and immunolocalization, the results illustrate that the mRNA and protein can be found in the lower epidermis of cotyledons from day 2 to day 5. In the 6-day-old cotyledons, the mRNA and protein of *RcSCR1* are predominantly found in palisade parenchyma cells, but they are also found in the lower epidermis of cotyledons. The results of *in situ* hybridization indicate that the transcript of *RcSCR1* can be found in most of the endosperm cells. *RcSCR1* can be found in the middle layer of the endosperm from day 2 to day 5, no transcript of *RcSCR1* is found in the cell layers near the seed coat. On the day 6, no *RcSCR1* transcript can be detected in the endosperm cells. It is suggest that the function of RcSCR1 protein is to retrieve the sucrose from apoplastic space to avoid sucrose escape.

Compared to the amino acid sequence of known sucrose transporters, the putative sucrose transporter of *Ricinus communis*, RcSCR2, belongs to SUT4 subfamily. The transcript of *RcSCR2* is found in the endosperm, hypocotyl and cotyledons of the *Ricinus communis* L. germinating seeds. The expression of RcSCR2 is very weak. The expression level of RcSCR2 cannot be detected by northern analysis. By quantitative real time RT-PCR, it indicates that the *RcSCR2* has a highest expression level at 3 day. By *in situ* hybridization, the results illustrate that the mRNA cannot be found in the endosperm, cotyledons and hypocotyl. The results of *in situ* RTPCR indicate that the transcript of *RcSCR2* can be found in most of the endosperm cells. *RcSCR2* can be found in the middle layer of the endosperm from day 2 to day 5, no transcript of *RcSCR2* is found in the cell layers near the seed coat. On the day 6, no *RcSCR2* transcript can be detected in the endosperm cells. Although RcSCR2 in yeast does not function properly, but it shares high homology to other SUT4 type transporters, so they may have the same function to take up sucrose into cells. It is suggest that the function of RcSCR2 protein is to retrieve the sucrose from the apoplastic space to avoid sucrose escape. How the expression of RcSCR1 and RcSCR2 is regulated in the endosperm is still unknown.

Sugarcane is a very important food crop. Sugarcane yellow leaf virus leads to sugarcane yellow syndrome and reduces the sugar production. Starch accumulation was found in the virus-infected plants. Within the starch staining, the results indicate that starch is accumulated in bundle sheath cells and mesophyll cells of virus-infected plants, however, starch can be found only in the bundle-sheath cells of virus-free plants. The *in situ* hybridization study indicates that the expression of ADP-glucose pyrophosphorylase in the mesophyll cells of virus-infected plant is stronger than it in virus-free plants. The results of *in situ* hybridization of starch branching enzyme indicates that no significant difference between the virus-free-plants and virus-infected. The results are different to it of starch staining. The mechanisms are still unclear, more carbohydrate metabolism related genes must be studied.

Zusammenfassung

Um den Saccharose-Efflux-Transporter des Endosperms von *Ricinus communis L.* zu finden, wurde die Methode der Hefekomplementierung. Da während der Selektion eine Mutation auf dem nSC4+ Plasmid auftrat, war dieser Ansatz zunächst nicht erfolgreich. Möglicherweise war die Mutation durch zu hohen Stress in den Hefezellen induziert worden. Folgerichtig konnte die Verwendung eines schwachen Promotors und die Reduktion der Kopienzahl des Plasmids verhindern, dass die Mutation während der Selektion auftrat.

Das Endosperm von *Ricinus communis L*. dient als Speicher für Lipide, die während des Wachstums des Keimlings in Saccharose umgewandelt werden. Das Gen der Saccharose-phosphat-phosphatase RcSPP1 wurde aus dem Endosperm des Keimlings von *Ricinus communis L.* kloniert. Die Endospermzellen synthetisieren Saccharose bevorzugt durch SPS und SPP, weniger durch die Saccharose Synthase. Eine Northern Blot Analyse zeigte, dass RcSPP1 von Tag 2 bis 6 gleich stark exprimiert wurde.

Die Expression von nsLTPc1 ist keimblattspezfisch, was durch eine *in situ* Hybridisierung bestätigt wurde. Des weiteren zeigte die nsLTPc1 *in situ* Hybridisierung, dass nsLTPc1 zellspezifisch exprimiert wurde, nämlich nur auf der Unters eite der Kotyledonen von *Ricinus communis L*.

RcSCR1 wird im Endosperm, Hypocotyl und den Kotyledonen der Keimlinge von *Ricinus communis L.* exprimiert. Eine *Northern Blot* Analyse von verschiedenen Tage altem Endosperm zeigte, dass RcSCR1 die höchste Expressionsstärke an Tag 5 hatte. Die Ergebnisse der *in situ* Hybridisierung und Immunolokalisierung zeigten, dass sowohl mRNA als auch das Protein von Tag 2 bis 5 in der unteren Epidermis der Keimblatter gefunden werden konnten. In 6-Tage-alten Keimblättern, fanden sich mRNA und Protein von RcSCR1 vorwiegend in den Palisaden-Parenchym-Zellen, jedoch auch in der unteren Epidermis der Kotyledonen. Die *in situ* Hybridisierung zeigte weiterhin, dass das Transkript von RcSCR1 in den meisten Endosperm-Zellen gefunden werden konnte. RcSCR1 konnte von Tag 2 bis 5 in der mittleren Schicht des Endosperms gefunden werden; kein Transkript von RcSCR1 wurde in den Zellschichten nahe des Samenmantels gefunden.

An Tag 6 konnte kein Transkript von RcSCR1 in den Endosperm-Zellen nachgewiesen werden.

Aufgrund des Vergleichs der Aminosäuresequenz mit bekannten Saccharose-Transportern wurde der mutmaßliche Saccharose-Transporter von *Ricinus communis L.*, RcSCR2, der SUT4 Subfamilie zugeordnet. Das Transkript von RcSCR2 wurde liche Saccharose-Transporter von *Ricinus communis L.*, RcSCR2, der SUT4 Subfamilie zugeordnet. Das Transkript von RcSCR2 wuttern von keimenden Sämlingen von *Ricinus communis L.* gefunden. Die Expression von RcSCR2 warsehr schwach, so dass eine Änderung der Expressionsstärke nicht durch eine Northern Blot Analyse nachgewiesen werden konnte. Durch eine quantitative *real time* RT-PCR wurde gezeigt, dass RcSCR2 an Tag 3 am stärksten exprimiert wu rde. Die Ergebnisse der *in situ* Hybridisierung zeigten, dass die mRNA nicht im Endosperm, Keimblättern und Hypocotyl gefunden werden konnte. Die Ergebnisse der*in situ* RT-PCR zeigten, dass das Transkript von RcSCR2 in den meisten Endosperm-Zellen gefunden werden konnte. RcSCR2 wurde in der mittleren Schicht des Endosperms von Tag 2 bis Tag 5 transkribiert, nicht jedoch in den Zellschichten in der Nähe des Samenmantels. An Tag 6 ko nnte kein RcSCR2-Transkript nachgewiesen werden.

Zuckerrohr ist eine sehr wichtige Kulturpflanze. Der Zuckerrohr-Gelb-Blatt-Virus führt zum Zuckerrohr-Gelb-Syndrom und reduziert die Zuckerproduktion. In virus-infizierten Pflanzen wurde eine Anreicherung von Stärke gefunden. Die Ergebnisse der Stärkeanfärbung zeigten, dass in virus-infizierten Pflanzen die Stärke in den Bündelscheidezellen und den Zellen des Mesophylls akkumulierte; dagegen konnte in virus-freien Pflanzen Stärke nur in den Bü ndelscheidezellen gefunden werden. Eine *in situ* Hybridisierung zeigte, dass die Expression von ADP-Glucose Phyrophosphorylase in den Mesophyll Zellen von virus-infizierten Pfalnzen stärker war als in virus-freien Pflanzen. Die Ergebnisse der *in situ* Hybridisierung des *starch branching enzyme* zeigten keine signifikanten Unterschiede zwischen virus-freien und virus-infizierten Pflanzen und unterschieden sich damit von den Ergebnissen der Stärkefärbung. Um den Mechanismus des Kohlenhydratstoffwechsels weiter aufzuklären, müssen weitere damit verbundene Gene untersucht werden.

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Erklärung

Hiermit erkläre ich, daß ich die Arbeit selbständig verfaßt und keine anderen als die von mir angegebenen Quellen und Hilftsmittel benutzt habe.

Ferner erkläre ich, daß ich nicht anderweitig mit oder ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen.

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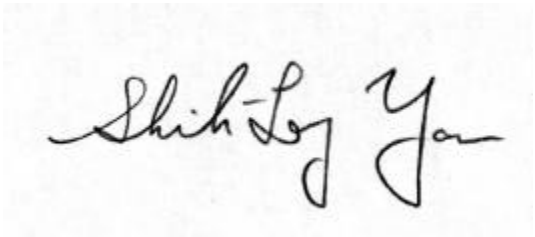

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