Doctoral Thesis

Transgene expression in rice controlled by nodulation related legume promoters

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Publication Date:
2000

Permanent Link:
https://doi.org/10.3929/ethz-a-003906844

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Transgene expression in rice controlled by nodulation related legume promoters

A dissertation submitted to the SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZÜRICH for the degree of Doctor of Natural Sciences

presented by
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March 2000
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Summary

Symbiotic nitrogen fixation by rhizobia in legumes contributes to approximately 50% of the total annually fixed nitrogen. The ability of biological nitrogen fixation (BNF) is restricted almost exclusively to leguminous plants. Due to the increasing need of N-fertiliser and the enormous input of energy during industrial N$_2$-fixation, considerable efforts are undertaken to transfer BNF to cereals. For our approach we assumed that most of the necessary genes exist in all plant species, where they have either other functions or are not active. The GUS marker gene offers a simple possibility to analyse expression patterns of promoters. In transgenic rice, we studied the promoter activity of the early nodulins MsEnod12A and MsEnod12B from alfalfa (Medicago sativa) and of the auxin inducible GH3 gene from soybean (Glycine max).

We showed activity of the MsEnod12 promoters in rice roots. The activity pattern is in agreement with the proposed function of ENOD12 proteins in the cell walls of legumes. The leguminous MsEnod12 promoters are probably recognised in a homologous signalling context in rice. One of the first steps of the interaction between rhizobia and legumes during nodulation is the induction of Enod genes and cell divisions by rhizobial Nod factors. In non-leguminous plants, Nod factors and chitin fragments (the backbone of Nod factors) are known to bind to defence related receptors. However, treatment of roots with a Nod factor or chitin fragments did not influence the activity pattern of the MsEnod12A and MsEnod12B promoters in rice.

Auxin is involved in nodulation of legumes. We transformed rice with the auxin inducible GH3 promoter fused to GUS, and showed that the GH3 promoter is also active in rice. Activity is restricted to single cells in front or edgewise of lateral root primordia, indicating a higher auxin concentration or higher auxin sensitivity in these cells. This suggests a function of auxin during the growth of lateral root primordia and confirms homology between nodulation and lateral root formation. External application of auxin increased the activity of the GH3 promoter. Auxin induced the GH3 activity largely in the cortical parenchyma of the lateral root formation zone.
During somatic embryogenesis, the $GH3$ promoter is active in somatic proembryos. Non-embryogenic cells of a callus show no $GH3$ activity. In contrast to non-embryogenic cells, somatic embryo cells express genes, which enable activation of the $GH3$ promoter, or the auxin concentration is higher in these cells. This higher auxin concentration could be due to de novo auxin synthesis in proembryos.
Zusammenfassung


In einem der ersten Schritte der Symbiose zwischen Rhizobien und ihrem Wirt induzieren bakterielle Nod Faktoren die Aktivierung von Enodgenen und Zellteilungen in Leguminosen. In Nichtleguminosen binden Nodfaktoren und Chitinfragmente (das Rückgrat der Nodfaktoren) an Rezeptoren des Abwehrsystems. In unseren Experimenten konnte allerdings die Behandlung der Reiswurzeln mit Nodfaktor oder Chitinfragmenten die Aktivität der beiden untersuchten Promotoren nicht verändern.

Auxin ist bei der Interaktion zwischen Rhizobien und Leguminosen involviert. Wir transformierten Reis mit dem Auxin induzierbaren Promotor GH3 von Soja und zeigten, das dieser Promotor auch in Reis aktiv ist. Die Aktivität war auf einzelne Zellen vor und seitlich von Seitenwurzelpromordien beschränkt und lässt auf eine erhöhte Auxinkonzentration oder erhöhte Sensitivität auf Auxin in diesen Zellen schliessen. Dies deutet darauf hin, dass Auxin eine Funktion während des Wachstums von Seitenwurzelpromordien hat und bestätigt

### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>2,4-D</td>
<td>2,4-dichlorphenoxy acetic acid</td>
</tr>
<tr>
<td>DIG</td>
<td>digoxigenin</td>
</tr>
<tr>
<td>GA&lt;sub&gt;3&lt;/sub&gt;</td>
<td>gibberellic acid</td>
</tr>
<tr>
<td>GH3</td>
<td>auxin inducible gene, isolated from soybean</td>
</tr>
<tr>
<td>GUS</td>
<td>β-glucuronidase</td>
</tr>
<tr>
<td>IAA</td>
<td>indole-3-acetic acid</td>
</tr>
<tr>
<td>kb</td>
<td>kilo bases</td>
</tr>
<tr>
<td>LRP</td>
<td>Lateral root primordium</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige-Skoog (medium)</td>
</tr>
<tr>
<td>MsEnod12</td>
<td>Enod12 gene from <em>Medicago sativa</em></td>
</tr>
<tr>
<td>NAA</td>
<td>α-naphthalene acetic acid</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
</tr>
<tr>
<td>X-Gluc</td>
<td>5-bromo-4-chloro-3-indolyl- β-glucuronide</td>
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1. Introduction

1.1. Rice production and use of fertiliser

Rice is the staple food for more than half of mankind. To keep pace with population growth, annual production must increase from present 520 million tons to about 760 million tons by 2020 (IRRI, 1993). Since future rice production has to come from the same or even reduced land area, due to urbanisation, desertification and changes in the use of arable land, productivity must be increased (Ladha and Reddy, 1995).

Nitrogen, after water, is the most critical factor for rice productivity (Ladha and Reddy, 1995). It takes 1 kg of N taken up by the plant to produce 15-20 kg of grain. In the tropics, lowland rice, which provides about 86 % of the world’s rice (Ladha and Reddy, 1995), yields 2-3.5 t/ha utilising naturally available N, derived from biological nitrogen fixation (BNF) by free-living and plant-associated diazotrophs (Watanabe and Roger, 1984; Ladha et al., 1993) and from mineralization of soil N (Bouldin, 1986; Kundu and Ladha, 1995). For higher yields, additional nitrogen must be applied (Ladha and Reddy, 1995). Reaching the higher rice yields needed by 2020 will require at least double of the 10 million tons of nitrogen fertiliser that is currently used each year for rice production (IFA-IFDC-FAO, 1992; IRRI, 1993).

Several reasons make this increasing amount of chemical N-fertiliser problematic. First, manufacturing fertiliser for today’s needs already requires about 544 x 10^9 MJ of fossil fuel energy (Mudahar, 1987a; Mudahar, 1987b). This energy is equivalent to about 13 million tons of oil – a non-renewable resource, the oxidised products of which pose hazards to the environment and to human health (Ladha and Reddy, 1995). Second, more than half of the applied fertiliser N is lost (through denitrification, ammonia volatilisation, leaching and runoff) because rice is grown in an environment conductive to N losses (George et al., 1992; Ladha et al., 1997). This may lead to considerable environmental pollution. Third, chemical N-fertiliser is not available for a large number of farmers, due to its high cost.
1.2. Conventional biological nitrogen fixation (BNF)

As already mentioned, diverse nitrogen-fixing microorganisms grow in wetland rice fields and contribute to the soil N pools. The major BNF systems known include cyanobacteria and photosynthetic bacteria that inhabit floodwaters and the soil surface, and heterotrophic bacteria in the root zone, or in the bulk soil (Ladha et al., 1997). Together with N derived from mineralisation of soil this supports a harvest of 2-3.5 t rice/ha (Ladha and Reddy, 1995), which is far lower than the yield of 15 t rice/ha which IRRI hopes to obtain under tropical conditions for the next 30 years (Bennett and Ladha, 1992).

The water fern Azolla and the semiaquatic legumes such as Sesbania or Astragalus are recommended green-manure plants for rice cultivation, since they fix N$_2$ symbiotically (Ladha et al., 1993). Azolla and Sesbania have the potential to support rice yields of 6-8 t/ha (Ladha et al., 1993). Farmers, however, usually have no economic advantage in choosing Azolla or Sesbania over fertiliser N, since additional costs such as labour, land opportunity, seed/inoculum, phosphorous, and pesticides make the use of Azolla and Sesbania uneconomical (Ladha and Reddy, 1995). But even if green manure had an advantage over chemical fertiliser – due to possibly higher production costs of chemical fertiliser in the future – the yield would still not reach the intended 15 t/ha.

Within this background, Bennett and Ladha (1992) suggest three unconventional approaches to BNF in rice that could give additional N inputs:

- a more efficient association between rice roots and a diazotroph
- symbiotic N$_2$ fixation through nodulation of rice with bacteria such as rhizobia or Frankia
- transfer of N$_2$ fixation capacity to the rice genome

Ladha and Reddy (1995) further suggested to search for stable and effective rice-endophytic diazotroph associations, which could contribute to solving the problem. The endophyte needs not be maintained intracellularly, like the rhizobial endosymbiont. It may be sufficient if it colonises rice intercellularly, as long as it fixes N$_2$ and excretes the fixed N for use by the plant (De Bruijn et al., 1995).
1.3. Unconventional approaches to BNF

All of the four above mentioned unconventional approaches to BNF would have the advantage that farmers could make use of them without the need of purchasing high-tech instruments. Further, farmers more easily adopt a genotype or variety with useful traits than they do crop and soil management practices that are often associated with additional costs (Ladha and Reddy, 1995).

Yields per hectare are critically dependent on the nature, amount, and timing of N supply (George et al., 1992). A system, which provides its own nitrogen, would probably not face these problems.

1.3.1. A more efficient association between rice roots and a diazotroph

Rice-diazotroph associations are well known, but their contribution to N supply is minor (Bennett and Ladha, 1992). In contrast, the association between sugarcane and diazotrophs is much more productive. Trials using $^{15}$N indicate that sugarcane obtains 20-55 % of plant N from associative fixation (Urquiaga et al., 1989; Urquiaga et al., 1992). An intimate association between rice and a free-living or loosely associated diazotroph might be forced by exploiting mechanisms operative in other plant-microbial interactions (Bennett and Ladha, 1992). Plant lectins can bind to specific chemical classes of carbohydrate including carbohydrates on bacterial surfaces (Etzler, 1985). Expression of an appropriate lectin gene in rice roots may promote an intimate association with a free-living diazotroph that displayed the corresponding carbohydrate moiety (Bennett and Ladha, 1992). The association might be enhanced further if exudates from the root could act as chemical attractants for the diazotroph or could be preferentially and specifically exploited by it as a C source (Bennett and Ladha, 1992). Such a system might be able to go into a tighter association between a diazotroph and rice, and therefore provide better transfer of fixed N between the partners than seen in the free-living associative bacteria. This might contribute to better N-supply for rice, but is probably not sufficient to fulfill the goal of 15 t rice yield/ha. But nevertheless, in combination with N fertiliser or green manure it could provide a useful tool in improving rice yield/ha.
1.3.2. Transfer of $N_2$ fixation capacity to the rice genome

To avoid the complex interactions between host and microsymbiont, it may be simpler to transfer the biochemistry of $N_2$ fixation to the host. In that case, one could concentrate on $nif$ and $fix$ genes and associated metabolic reactions (Vance, 1990; Rao et al., 1998). However, this approach raises several problems. First of all, how to 'stimulate' all the compounds to react in the plant in the wanted manner. Second, how to deal with the nitrogenase, which needs low O$_2$ tension? Bennett and Ladha (1992) suggested the chloroplasts of leaf cells as an ideal location, because they could supply ATP directly to the enzyme. However, how to deal with O$_2$, which accumulates as a by-product of chloroplast photosystem II? The authors suggest, that further insights into the detailed operation of (i) the rhizobia-leghaemoglobin model, (ii) the O$_2$ barrier of Frankia on Alnus, (iii) the heterocyst model of filamentous cyanobacteria such as Anabaena and Nostoc and (iv) the Gleocapsa model of the unicellular cyanobacterium Gleocapsa gallia (Gallon, 1980) may suggest a mechanism for the task of protection. Ladha and Reddy (1995) suggest alternatively that it may be possible to establish temporal separation of $N_2$-fixation and photosynthesis by regulating the $nif$ gene-expression in the dark.

Professor I. Potrykus follows a similar idea (pers. comm.). Assuming that plastid transformation in rice will be soon well established, his plans are to transfer all necessary $nif$ and $fix$ genes into chloroplasts of rice. This would have the advantage that most probably the chloroplast transcriptional/translational machinery could deal with the bacterial $nif$ and $fix$ operons. To handle the problem with oxygen, he suggests using promoters expressing these genes only in tissues with low oxygen tension, as an example in the stem vascular bundles.

1.3.3. Symbiotic $N_2$ fixation through nodulation of rice with bacteria such as Rhizobia or Frankia

The soil bacteria of the genus Rhizobium interact with leguminous plants, which leads to the formation of $N_2$-fixing nodules (see below). This symbiosis leads to
a high rate of biological N₂-fixation, which can then directly be used by the leguminous host.

To induce a legume-rhizobia like symbiosis between rice and rhizobia or *Frankia* would have many advantages. First, nitrogen would be fixed within the plant and could directly be used, preventing possible losses due to denitrification and leaching, as happens with associative interactions between rice and microorganisms. Second, this system would be sustainable, preventing losses due to denitrification, ammonia volatilisation, leaching and runoff, which occur after N-fertilisation. Third, such a system would be of low expense regarding money and labour, compared with N-fertiliser or green manure. Fourth, farmers would not have to use sophisticated technology, just seeds of an improved genotype.

1.3.4. Development of rice-endophytic diazotroph associations

BNF systems comprised of diazotrophs endophytic to rice will have the advantage over free-living, associative and green manure systems in that fixed N₂ can directly be assimilated by the plant without loss (Ladha and Reddy, 1995). A N₂-fixing endophyte which is of considerable interest in this field is *Azoarcus*. *Azoarcus* inhabits the root of Kallar grass, which yields 20-40 t/ha/year of hay without the addition of any nitrogen fertiliser in soils having low fertility (Sandhu *et al.*, 1981). Reinhold Hurek and Hurek (1997) report that *Azoarcus* is able to colonise rice roots endophytically. However, bacteria do not appear to occur within a live plant cytoplast, thus can not be regarded as true endosymbiont (Reinhold Hurek and Hurek, 1997). Reported experiments showing a beneficial effect of rhizobia invading rice as endophytes are still lacking, and it is not clear whether such rhizobia can be regarded as real endophytes. Reddy *et al.* (1997) report a rhizobial invasion of rice roots through cracks in the epidermis and fissures created during emergence of lateral roots. However, endophytic colonisation was restricted to intercellular space or within host cells undergoing lysis (Reddy *et al.*, 1997).
1.4. The Legume - Rhizobia symbiosis

1.4.1. General introduction

Bacteria of the genera *Rhizobium*, *Azorhizobium*, *Bradyrhizobium* and *Sinorhizobium* (in the following collectively named rhizobia) enter into a symbiotic interaction with leguminous plants and with a single non-leguminous plant, *Parasponia*. Complex signal reactions between the plant partner and the microsymbiont partner lead finally to the formation of nodules on the roots of the host plant. These nodules provide an ideal environment for the rhizobia to fix nitrogen. The fixed nitrogen is afterwards transported to the plant. This makes the plant independent of combined nitrogen and therefore competitive in nitrogen-poor soils. The rhizobia profit in different aspects: first, the nodule provides the low O₂-concentration that is needed for the O₂-sensitive nitrogenase. Further, the plants deliver the energy for survival and N-fixation of the rhizobia. In addition, the rhizobia in the rhizosphere can profit also, since, due to nitrogen fixation, the plant becomes increasingly vigorous and secretes more secondary metabolites, including compounds that are specifically catabolised by those rhizobia (Goormachtig, 1996).

1.4.2. Signal exchange

Flavonoids in the root exudate of a host plant are the first signal, starting the complex interaction, leading finally to a functional nodule (Heidstra and Bisseling, 1996, see Fig. 1.1.). The flavonoids can bind to the constitutively expressed bacterial NodD protein (Göthals *et al.*, 1992), which turns this protein into a transcriptional activator of the other nod genes (Fisher and Long, 1992). The products of these nod genes are involved in the biosynthesis of the Nod factor. Rhizobia secrete the Nod factor, which is recognised by the plant, and as
a consequence, bacterial invasion and nodule formation will be initiated (for a more detailed description see Hirsch, 1992; Spaink, 1995).

The legume-rhizobia interaction shows certain specificity. Some rhizobia have a broad host range and can nodulate many different legumes, whereas others have a narrow host range and can nodulate only few species. Specificity is defined mainly in two steps. The first step is represented by the flavonoids. Only a flavonoid spectrum secreted by a certain legume will be recognised by the NodD protein of a given Rhizobium and subsequently activate the nod genes (Schultze et al., 1994). The second step are the Nod factors produced by
rhizobia. Legumes will only react to the Nod factors produced by their respective rhizobia.

1.4.3. Bacterial invasion

In most of the legumes studied, rhizobia enter the root via root hair infection and intracellular infection threads. Rhizobia attach to the tip of a young trichoblast (Smit et al., 1986). The root hair starts to curl and form the so-called shepherd’s crook (Kijne, 1992). Thus the rhizobia become entrapped and ingested at the tip of the root hair (Kijne, 1992). Degradation of the cell wall of the root hair and invagination of the host plasma membrane result in the formation of an infection thread (Kijne, 1992). The infection thread penetrates through the cell layers by a cell wall degradation and deposition mechanism, whilst growing and transporting the multiplying rhizobia.

Parallel to the infection process, cortical cells start to divide and form the nodule primordium (Dudley et al., 1987; Pawlowski and Bisseling, 1996). The infection thread grows towards this nodule primordium. Bacteria are released from the infection thread tips into cells of the nodule primordium by an endocytotic process during which bacteria stay outside the cytoplasm, enclosed by a plant cell membrane (Munoz et al., 1996). After the release into the plant cells, the bacteria differentiate into N₂-fixing bacteroids, while the primordium differentiates into a root nodule (Munoz et al., 1996).

1.4.4. Nodule formation

In the case of most temperate legumes, such as pea, alfalfa, vetch and clover, cell divisions are initiated in the inner cortex. These nodules have a persistent meristem and form a long-shaped indeterminate nodule. In a longitudinal section such an indeterminate nodule shows the following zones (Fig. 1.2.A): a meristematic zone (zone I), an infection zone (zone II), a fixation zone (zone III) where the bacteroids fix nitrogen, and in older nodules a senescent zone (zone IV). This zonation can be seen as long as the meristem provides new cells to the nodule. Subsequently, the nodule elongation ceases and the life of the nodule ends.
In case of most tropical legumes like soybean, bean and lotus, cell divisions are initiated in the outer cortex. The nodules do not have a persistent meristem and form therefore round-shaped, determinate nodules (Fig. 1.2.B). The nodules consist of a central tissue, containing large infected cells, interspersed by many smaller, uninfected cells. The central tissue is surrounded by a nodule parenchyma, where the vascular bundles of the nodule are situated. An outer cortical region containing sclerenchymatic cells surrounds the complete nodule (Goormachtig, 1996).

Fig. 1.2.: Indeterminate (A) and determinate (B) Nodules (derived from Pawlowski and Bisseling, 1996). For details see text, np: nodule parenchyma.

1.4.5. Nod factors

Nod factors are lipo-chitin oligosaccharides (LCOs), consisting of an oligosaccharide backbone of β-1,4-linked N-acetyl-D-glucosamine. The length of the backbone varies between 3 and 6 sugar units (Heidstra and Bisseling, 1996, see Fig. 1.3.). At the non-reducing end, the acetyl group is replaced by an acyl group. This basic structure is substituted by chemical groups attached at the reducing and non-reducing ends. Nod factors from different rhizobia differ in the length of their chitin backbone, properties of the acyl chain and in the substituents (Spaink and Lugtenberg, 1994; Mergaert et al., 1997). The structure of the Nod factor is involved in the host-specificity of nodulation, since
leguminous plants mainly select on the structure of the Nod factor (Heidstra and Bisseling, 1996; Long, 1996)

Fig. 1.3.: Nod factor structure, basic structure and possible modifications (from Long, 1996).

1.4.6. Nodulin genes

Nodule formation is accompanied by the expression of a set of organ specific genes, the nodulin genes (Van Kammen, 1984). Originally, by definition they are found only in root nodules, but not in non-infected roots nor in other parts of the host plant. However, some genes isolated first as nodulin genes, have later been shown to be also expressed in other plant tissues (Scheres et al., 1990; Kouchi and Hata, 1993; Bauer et al., 1997). Depending on the onset of expression, nodulin genes are divided into two groups (Van Kammen, 1984). Early nodulin genes (Enod genes) are expressed before the onset of N\textsubscript{2}-fixation. Their gene products are involved in infection and nodule formation. Late nodulin genes (Nod genes) are expressed once the N\textsubscript{2}-fixation starts and they are involved in nodule functioning. For an overview of the known Enod and Nod genes see Munoz et al. (1996).
1.5. **What supports the idea of nodulated rice?**

There are different arguments supporting the idea of nodulated rice:
- The symbiosis between *Parasponia* and Rhizobia
- Nodulin genes are not restricted to legumes
- The expression of nodulin genes is not restricted to nodule formation
- Non-leguminous plants can react to Nod factors

1.5.1. **The symbiosis between *Parasponia* and Rhizobia**

The non-leguminous plant *Parasponia* (Ulmaceae) forms N\textsubscript{2}-fixing nodules with rhizobia (Trinick, 1973), showing that nodulation is not restricted to legumes.

1.5.2. **Nodulin genes are not restricted to legumes**

There is more and more evidence that (probably most) nodulin genes are present in plant species other than legumes. Homologous genes of *Enod40* have been found in tobacco (Van De Sande et al., 1996) and rice (Kouchi et al., 1999). Reddy et al. (1998a) isolated two rice homologues of the soybean *GmEnod93*. A recent study of Reddy et al. (1999) showed further homologies of *Enod* genes in rice. Testing 80 rice accessions belonging to 23 *Oryza* species, they could show by Southern blot analysis homology to the tested early nodulins *PsEnod5, PsEnod12, PsEnod14, GmEnod2, GmEnod40, GmEnod55, GmEnod70* and *GmEnod93*. Not all tested lines showed the same strong cross-hybridisation signals, but at least for each tested *Enod* gene in some lines strong cross-hybridisation was found. This leads to the conclusion that these *Enod* genes are also present in rice, however with a different degree of conserved sequences. Further it has been demonstrated, that leghaemoglobin genes in legumes are diverged through gene duplication from pre-existing haemoglobin genes that are common in the plant kingdom (Andersson et al., 1996).
1.5.3. The expression of nodulin genes is not restricted to nodule formation

The original definition of nodulins was their exclusive expression during the nodulation. However, more and more nodulins, isolated during the last decades, are expressed also during root development and in further plant organs, although to a lower extent. The *Sesbania rostrata SrEnod40-1* is expressed in plant parts other than nodules (Corich et al., 1998). *SrEnod40-1* transcripts were associated in particular with leaf and stipula primordia and with young vascular bundles. Corich et al. (1998) conclude that *SrEnod40-1* expression were found to be associated with developing vascular bundles in all parts of the plant.

Kouchi and Hata (1993) report that a *GmN#36* (= *GmEnod40*, see Reddy et al., 1999) is not only active in nodules, but also in the cells adjacent to the secondary phloem in stems. They hypothesise that this nodulin is involved in morphogenesis and/or function of the nodule vascular system, which is supported by the observed expression in the stem (Kouchi and Hata, 1993).

Scheres et al. (1990) found *PsEnod12* not only expressed in nodules, but also in stem and flowers. Using the *in situ* technique, they localised *PsEnod12* mRNA in the stem internode section, in a zone of cortical cells, surrounding the central ring of vascular bundles and the interfascicular cambium cells (Scheres et al., 1990).

Bauer et al. (1997) showed that *MsEnod12* expression was associated with meristematic activity. Besides expression in nodules, they found expression additionally during root and lateral root development, behind the tips of developing principal or lateral roots.

1.5.4. Non-leguminous plants can react to Nod factors

Another point supporting the idea of approaching nodulated rice is the fact that non-leguminous plants have been reported to react to Nod factors. Reddy et al. (1996), Terada et al. (1996), and Reddy et al. (1998b) presented data from transgenic rice containing the *MtEnod12* or *MsEnod12B* promoter fused to the GUS marker gene. The reported expression pattern changed upon application
of Nod factors. Their results suggest that at least a portion of the signal transduction machinery, important for legume nodulation, is likely to exist in rice (Reddy et al., 1998b). In tomato cells, Nod factors are able to induce an alkalinization response at nanomolar concentrations (Staehelin et al., 1994).

The presence of Enod genes in non-leguminous plant species provides evidence that these homologous genes have arisen from a common ancestral plant. From the fact that Enod genes are not exclusively expressed during nodule formation, it can be surmised that they had a different function in such an ancestral plant. However, in legumes their function was increasingly specialised, ending as a fine tuned interaction between Rhizobia and the plant leading finally to nodulation of legumes.

And since some non-leguminous plants are able to perceive Nod factors it can be speculated that at least parts of the nodule formation program is present in non-leguminous plants as well.

1.6. MsEnod12::uidA in transgenic rice as a molecular tool to investigate effects of Nod factor and chitin fragment treatment of rice

1.6.1. Early nodulins12

The early nodulins12 are known from pea (Scheres et al., 1990), Medicago truncatula (Pichon et al., 1992), Medicago sativa (Allison et al., 1993), Vicia faba (Perlick and Pühler, 1993), and Vicia sativa (Vijn et al., 1995c). These proteins contain a putative membrane-translocation signal peptide and repetitive proline-rich motifs (Scheres et al., 1990; Pichon et al., 1992). It is assumed that Enod12 are cell wall proteins, involved in the infection process (Scheres et al., 1990; Schultze and Kondorosi, 1998).

1.6.2. MsEnod12A and MsEnod12B

In Medicago sativa, two differentially regulated Enod12 genes were found (Allison et al., 1993; Bauer et al., 1994; Bauer, 1995). MsEnod12A mRNA could be only detected in nodules, whereas low levels of MsEnod12B mRNA were found also in roots, flowers, stems, and leaves. MsEnod12B expression was enhanced early after inoculation with Rhizobium meliloti and expression
increased until nodules became visible. Bauer (1995) presumes that this early induction probably took place in the epidermis and in root hairs as demonstrated for the Enod12 genes in pea (Scheres et al., 1990) and M. truncatula (Pichon et al., 1992). Furthermore, an enhancement of MsEnod12B transcription in roots was detected after treatment with the Medicago specific Nod factor at a concentration of $1 \times 10^{-9}$ M (Bauer et al., 1994). This induction occurred in the root zones susceptible for root hair deformation.

After inoculation with *R. meliloti* or its purified Nod factors, MsEnod12A was induced much later than MsEnod12B (Bauer, 1995), and expression of MsEnod12A was only detectable when developing nodules were visible (Bauer et al., 1994). The authors assumed that transcription started in nodule primordia, where it increased rapidly to a high level during nodule development.

In summary, alfalfa possesses different mechanisms regulating MsEnod12A and MsEnod12B expression (Bauer et al., 1994). Whereas MsEnod12A expression is linked to meristematic activity during development of nodules and roots (Bauer et al., 1997), MsEnod12B induction is related to the action of Nod factors (Bauer et al., 1994; Bauer, 1995).

Since most of the Enod12 genes show a very fast, transient, Nod factor dependent activation, they are regarded as valuable molecular markers for studying early signal exchange between the two symbiotic organisms in legumes (Pichon et al., 1992; Journet et al., 1994). MsEnod12B may act as such a marker. On the other hand, MsEnod12A may serve as molecular tool for analysing meristem establishment during nodule and root development (Bauer, 1995; Bauer et al., 1997).

One scenario for the evolution of the Rhizobium-plant signalling system is that Nod factors, with their chitin backbone, could have co-opted for a plant defence response pathway originally responding to chitinaceous or other elicitors (Long, 1996). Stacey and Shibuya (1997) suppose that legumes possess two chitin receptors with differing chemical specificity. One of these receptors probably has a higher specificity for Nod factors and possibly plays a key role in determining nodulation specificity (Stacey and Shibuya, 1997). The other receptor shows lower specificity, can respond to chitin oligomers and may be a member of a hypothesised evolutionarily conserved family of chitin binding
proteins (Stacey and Shibuya, 1997). It has already been shown that oligochitin fragments generated from a cell wall polymer of various fungi are potent elicitors of defence responses in rice (Yamada et al., 1993). Therefore, at least parts of the signal transduction pathway which leads to the legume-rhizobia interaction probably also exist in monocots. Nod factors or chitin fragments may interfere with this existing signal transduction pathway. To monitor possible effects of such an interaction, MsEnod12A and MsEnod12B promoter:GUS fusions can be suitable tools.

Earlier experiments in our group showed that the MsEnod12A and MsEnod12B promoters are active in rice (Terada et al., 1996). After auxin pre-treatment, bombardment of Rhizobium meliloti Nod factor NodRm-IV(C16:2,Ac,S) reportedly changed the expression pattern of the MsEnod12B promoter in transgenic rice. This result supported the idea that at least parts of a signal transduction pathway leading to the activation of Enod genes upon application of Nod factors are available in rice. Unfortunately, all plants regenerated by Terada et al. (1996) proved to be sterile and the lines used in their experiments have been lost.

Additional support for the speculation that rice may react to Nod factors was given by Reddy and co-workers (Reddy et al., 1996; Reddy et al., 1998b). They showed, that MtEnod12::uidA, introduced into rice, is induced by Nod factor treatment. Their result provided additional support that rice (i) can react to Nod factors and (ii) that the Enod12 promoters may be a useful tool to monitor this reaction.

1.6.3. Nod factor and chitin fragment bombardment of rice roots

Previous experiments in our lab showed that Nod factor NodRm-IV(C16:2,Ac,S) only had an effect on MsEnod12B expression when delivered into the tissue by micro-projectile bombardment (Terada et al., pers. comm.). Terada and co-workers speculated that the bombardment helped to overcome a lack of access for the Nod factor to a putative receptor. In Vicia sativa the fatty acyl moiety of a Nod factor is essential for root nodule induction whereas the O-acetylated chitin oligosaccharide backbone alone is not active. However, when delivered into the
root tissue by microtargeting, O-acetylated chitin oligosaccharides were able to induce root cortical cell divisions (Schlaman et al., 1997). Therefore, in our experiments, besides incubation of rice roots in Nod factor solution, we additionally delivered the Nod factor mechanically, by bombardment, into the root tissue.

Several authors propose that legumes possess two chitin receptors with differing chemical specificity (Ardourel et al., 1994; Stacey and Shibuya, 1997). Ardourel et al. (1994) suggest that one of them, a signalling receptor, is able to interact with non-host-specific Nod factors and thereby triggers cell division. The other receptor, a stringent entry receptor, must interact with specifically tailored molecules (i.e. the host-specific Nod factors) before formation of infection threads and cell invasion can proceed. Stacey and Shibuya (1997) propose that one receptor probably may have a higher specificity for Nod factors and possibly plays a key role in determining nodulation specificity. The second receptor shows lower specificity, can respond to chitin oligomers and may be a member of a hypothesised evolutionarily conserved family of chitin binding proteins. Long (1996) argues that Nod factors, with their chitin backbone, could have co-opted a plant defence response pathway, originally responding to chitinaceous or other elicitors. Therefore one can assume that rice possesses receptors, which may recognise the chitin backbone of Nod factors. What kind of response these chitin fragments may trigger is unknown, but most probably they are connected to plant defence. The presence of chitinase in rice seeds and cultured cells, as well as the induction of the enzyme activity by chitin and related compounds has been reported (Hirano et al., 1990; Inui et al., 1991). Yamada et al. (1993) showed that purified chitin fragments (fragments larger than hexaose) induced defence responses in rice.

Besides the Nod factor NodRm-IV(C16:2,Ac,S) from Rhizobium meliloti we used therefore also chitotetraose and a chitopentaose to treat rice roots and to investigate, whether this treatment may influence the expression pattern of the MsEnod12A or MsEnod12B promoter.
1.7. *GH3::uidA* as molecular tool to monitor changes in the auxin concentration

Plant hormones are thought to play an important role during nodulation (Hirsch and Fang, 1994). For lateral root formation, the requirement for auxin has been shown (Wightman et al., 1980; Sussex et al., 1995), and, therefore, one can assume that auxin also plays a role during the nodulation process. Mathesius et al. (1998) made use of the auxin-inducible promoter GH3 to assess changes in the auxin balance during the earliest stages of root nodule formation. Spot-inoculation of the host-rhizobia on transgenic clover containing *GH3::uidA* showed that upon inoculation a rapid, transient and local downregulation of GH3 expression during nodule initiation, followed by an upregulation of the expression at the site of nodule initiation occurs. The respective Nod factor of the host-rhizobia and an O-acetylated chitin pentamer induced the same effects (Mathesius et al., 1998). Their results support the hypothesis that Nod factors and chitin oligosaccharides act by perturbing the auxin flow in the root during the earliest stages of nodule formation (Mathesius et al., 1998).

1.7.1. *GH3* – an auxin responsive promoter

The promoter of the auxin-responsive *GH3* mRNA of soybean (Hagen et al., 1984) was first described by Hagen and co-workers (Hagen et al., 1991). The gene encoding this *GH3* mRNA has been shown to be transcriptionally induced within five minutes after auxin application to plants (Ulmasov et al., 1995). *In situ* hybridisation showed that exogenous auxin induces the expression of *GH3* mRNA mainly in the vascular tissue in a wide variety of soybean organs (Gee et al., 1991). Hagen et al. (1991) fused 592 nucleotides of the *GH3* promoter and 157 nucleotides of the untranslated mRNA leader sequence to the *uidA* gene. In transgenic tobacco plants containing this construct, they observed strongest *GH3* expression in the absence of any auxin treatment in developing ovules and seeds, and in the vascular tissue of flower receptacles. Other organs showed no or only little staining. Weak staining was observed in young roots and trichomes of leaves, stems, petioles, and flower organs. After application of 10 μM NAA for 24 hours, a strong increase in *GH3* expression occurred.
Highest expression was detected in the root/shoot transition zone and the hypocotyl. Within root and hypocotyl tissue, GH3 is expressed most strongly in the vascular tissues, but expression was also detectable in the cortex and the epidermis. The fast reaction of the GH3 promoter to application of exogenous auxin probably provides a suitable tool to monitor also changes in the endogenous auxin concentration.

The same GH3::uidA construct as developed by Hagen et al. (1991) was used by Larkin and co-workers to study the involvement of auxin in root development of white clover (Larkin et al., 1996). In untreated tissue of transgenic white clover containing the GH3::uidA, GUS expression was significantly higher in stems than in roots and leaves. In shoot tissue, leaves and stems showed expression in the vascular traces and trichomes. In roots, the vascular tissue stained predominantly in the xylem parenchyma. In addition, intense blue islands were observed in the outer cortex, which, as shown by root sectioning, marked the location were lateral root primordia were initiated. Lateral roots grew towards and through these islands. After gravistimulation, a reaction could be seen within 60-90 minutes. GUS activity occurred on the non-elongating site of the root and appeared with the onset of curvature. Also in shoots responding to gravistimulation, a differential GUS staining was visible, but here GUS activity was located on the elongating side of the shoot. These expression patterns are in agreement with the Cholodny-Went hypothesis (Went and Thimann, 1937).

1.7.2. Lateral root development

Lateral root development is thought to be a two step process (Sussex et al., 1995). Initially, a large number of pericycle cells contribute to each lateral root as founder cells and form the primordium. Subsequently, a meristem is initiated in the primordium.

Celenza et al. (1995) suggest a model in which IAA is required for at least two steps in lateral root development. First to initiate cell division in the pericycle and second to promote cell divisions and maintain cell viability in the developing lateral root. IAA is thought to be the signal for lateral root initiation in planta. Two lines of evidence support this statement: (i) exogenous application of IAA induces the formation of lateral roots (Torrey, 1950; Blakely et al., 1982), (ii)
transgenic plants overexpressing the bacterial IAA biosynthetic genes have increased lateral root production (Klee et al., 1987; Kares et al., 1990). The IAA to initiate cell divisions in the pericycle is presumed to be transported from other parts of the plant (Charlton, 1996), which is presumably the shoot apical meristem (Zhang and Hasenstein, 1999).

As mentioned above, Larkin et al. (1996) showed a distinct expression of the GH3 promoter in white clover roots during lateral root formation. These intense blue islands in the outer cortex marked the location were lateral root primordia were initiated. Therefore the GH3 promoter might be a useful tool to investigate the role of auxin in lateral root initiation and development in rice.

1.7.3. Somatic embryogenesis

Embryogenesis from cultured somatic tissues was reported as early as 1958 (Reinert, 1958; Steward et al., 1958). Most of the work published so far was relates to carrot somatic embryogenesis. This work showed that auxin appears to play important roles both in the induction and continued development of embryos in culture (Zimmerman, 1993). In carrot somatic embryogenesis, cells require a minimum exposure to the exogenously applied auxin 2,4-D, in order to acquire the competence to eventually undergo somatic embryo development (Michalczuk et al., 1992a). In the further process, carrot proembryogenic masses must be transferred to 2,4-D free medium, otherwise the carrot embryos revert to proliferating calli (Schiavone and Cooke, 1987). However, endogenous auxin is, in contrast, required for the successful completion of embryo development (Michalczuk et al., 1992a). Therefore, it seems that once the embryogenesis has been induced by exogenously applied 2,4-D, the role of auxin changes and the embryos begin to synthesise their own auxin (Michalczuk et al., 1992a; Michalczuk et al., 1992b). Little is known about synthesis and transport of auxin in the plant embryo (Liu et al., 1993). However, a proper polar transport of auxin within the embryo, seems to be a prerequisite for normal morphogenesis (Schiavone and Cooke, 1987; Liu et al., 1993; Fischer and Neuhaus, 1996; Fischer et al., 1997).

During rice embryogenesis, callus induction and proliferation occurs under high 2,4-D concentrations (1 or 2 mg/l medium), whereas plants regenerate on
medium containing 1 mg IAA per litre. However, somatic embryos form and mature already in the presence of the high 2,4-D concentration (Jones and Rost, 1989). Therefore, it is not clear whether the data from carrot embryogenesis can be transferred to rice somatic embryogenesis. We made use of the GH3::uidA construct to monitor changes in the auxin concentration during somatic embryogenesis in rice. Callus cultures induced from mature embryos of a transgenic line, or callus cultures obtained during the rice transformation process were used to make sections through calli and to investigate the GH3 expression.

1.8. Aim of the work

In Medicago sativa, MsEnod12B was reported to be a marker to study early steps of nodulation, whereas MsEnod12A may serve as molecular tool to analyse meristem establishment during nodule and root development. In order to study putative nodulation related compounds in rice, these two promoters appeared to be useful tools. In a first step, the activity of the MsEnod12A and 12B promoters, fused to the uidA gene, had to be investigated in rice. In a second step, a putative effect of Nod factor and chitin fragment application on MsEnod12A and 12B activity in rice was to be analysed.

Auxin plays an important role during lateral root formation, nodulation, and also during somatic embryogenesis. The auxin responsive soybean promoter GH3 was reported to be a useful tool in investigating changes in the endogenous auxin concentration. In order to study the role of auxin, we monitored the activity of the GH3 promoter, fused to the uidA and gfp genes, during lateral root formation and somatic embryogenesis in rice.
2. Material and Methods

2.1. Tissue culture and transformation

2.1.1. Cultures

2.1.1.1. Plants

Rice plants (*Oryza sativa* L.), japonica cultivar Taipei 309, were grown in the greenhouse at 28 °C day and 21 °C night temperatures. The light regime was 12 h light, supplemented with light from 400 W fluorescent lamps (MT 400 DL/BH, 400 W mercury lamp, Iwasaki, Tokyo) if a minimum of 100,000 lx, measured outside the greenhouse, was not reached during the day. Humidity was set to 80% during the day and 60% during the night. Seedlings were transplanted to pots around 20 days after germination. The soil was paddled and fertilised with 1 g/l Nutricote (NPK 16-10-10; Maag, Dielsdorf, CH), 1 g/l Plantomaag 4D (NPKMg 20-10-15-3.6; Maag) and 0.25 g/l FeEDTA (Fluka, Buchs, CH). Three plants were grown in one pot, containing 2 l soil.

2.1.1.2. Germination and selection of progeny of transgenic rice lines

Rice seeds from primary transformants or of further generations were dehusked and surface sterilised for 1 hour in 6-7% sodium hypochlorite solution supplemented with 0.01% Tween 20, followed by three washes in sterile water. Seeds were germinated on MS-medium (Murashige and Skoog, 1962; Duchefa, Haarlem, NL), supplemented with 30 g/l sucrose and solidified with 3 g/l gelrite (Duchefa).

To exclude those progeny, which had lost their gene of interest due to mendelian segregation, from further cultivation, two screening methods were used. The first one was a histochemical GUS assay. Roots of the plantlets were stained for GUS and only plants having roots staining blue were used for further cultivation. The second test was an indirect test, assessing the plants for the presence of the hygromycin resistance gene. Seedlings were transferred 4-6 days after germination onto a metal grid within a glass jar, containing liquid MS-medium, supplemented with 15 g/l sucrose and $2 \times 10^4$ U/l hygromycin B.
(Duchefa), to the level of the metal grid. Growing exposed to the selective agent, transgenic plantlets developed healthy roots demonstrating normal lateral root formation, whereas non-transgenic plantlets showed very weak root growth without lateral root formation and finally they died. This method was used to efficiently screen a large number of progeny.

2.1.1.3. Isolation and culture of immature embryos

Immature caryopses, 10 to 15 days after pollination (with liquid endosperm, Fig. 2.1.a) were collected from the plants in the greenhouse. They were surface sterilised by incubation in 6-7 % sodium hypochlorite solution (Fluka) containing 0.01 % Tween 20 for 20 minutes, and subsequently washed three times with sterile water. Immature embryos (Fig. 2.1.b) were excised and placed, scutellum side up, on callus induction medium (Medium1; MS salts and vitamins (Duchefa), 2 mg/l 2,4-D, 30 g/l sucrose and solidified with 3.5 g/l agarose type I (Sigma, Buchs, CH) (Fig. 2.1.c). Isolated embryos were cultured at 30 °C in the dark. Between 3 and 4 days after isolation, the coleoptiles, emerging from the embryos, were removed. Another 3 to 4 days later, embryos were ready for bombardment (in total 6 to 8 days after isolation).

2.1.1.4. Establishment of rice embryogenic callus suspensions

Immature or mature embryos were cultured for 2-3 weeks on medium 1 (isolation and cutting of coleoptiles as described above). About 5-10 embryos were transferred to 20 ml of culture medium R2 (Medium R2; R2 salts (Duchefa), 30 g/l sucrose, 1 mg/l 2,4-D and 1 mg/l thiamine). Cultures were maintained at 30 °C in light on a rotary shaker at 85-95 rpm. The medium was changed every 5-7 days. Calli of different sizes arose from the immature embryos. After 2-3 weeks, immature embryos and the large calli were discarded. About 1.5 to 3 months after the embryos had been isolated, callus suspensions reached a critical mass suitable for bombardment.
2.1.2. Particle Bombardment

2.1.2.1. Coating of gold particles

For bombardment of immature embryos or callus suspensions, a modified (Iglesias, 1994) particle inflow gun (PIG) according to Finer et al. (1992) was used. Bombardments were carried out following the protocol of Iglesias (1994). Spherical gold particles with a diameter in the range of 1.5-3 μm (Aldrich, Buchs, CH) were suspended in 50% glycerol at a concentration of 50 mg/ml. For bombardment, about 1 μg DNA of each plasmid was used per shot. An equimolar amount of each plasmid was utilised. For 5 shots, the following protocol was used: 50 μl of gold stock suspension were added to the plasmid DNA. Fifty microlitre of ice cold CaCl₂ (2.5 M) was added, and briefly vortexed. After addition of 20 μl cold spermidine (0.1 M), the suspension was vigorously vortexed for 1-2 minutes. DNA was precipitated with 300 μl cold EtOH (100%) and the mixture stored at -20 °C for 20 min to 2 hours. During this time, the embryos or suspensions were prepared for bombardment. Directly before bombardment, samples were spun down for 5 seconds at low speed and the coated gold was resuspended in 36 μl sterile water. Per shot, 7 μl of gold particle suspension was pipetted to the particle holder.

2.1.2.2. Preparation of immature embryos

About 15 to 30 minutes before bombardment, precultured rice embryos were placed in the centre of a 5 cm petri dish (similar to Fig. 2.1.d) containing high plasmolysis medium (Medium 6; MS salts and vitamins, 100 g/l sucrose, 2 mg/l 2,4-D, solidified with 4 g/l agarose type I).

2.1.2.3. Preparation of rice embryogenic callus suspensions

About 2 days after subculturing, suspension calli were used for bombardment. Suspension calli were incubated in liquid plasmolysis medium (R2 salts, 100 g/l sucrose, 1 mg/l 2,4-D, 1 mg/l thiamine) for 30 minutes. Afterwards, calli were spread in the center of a 5 cm petri dish, containing 8 ml of liquid plasmolysis medium, solidified with 4 g/l agarose type I (Fig. 2.1.d). As much remaining
liquid medium as possible was removed by pipetting. Directly before bombardment, removal of liquid was repeated.

2.1.2.4. Bombardment

For the bombardment of precultured embryos and callus suspensions, the target plates were placed 14 cm from the particle holder. A nylon baffle grid was placed between the particle holder and the plates at a distance of 9 cm from the holder, in order to reduce noise impact on the tissue. The chamber was evacuated to minus 90 mbar and the particles were accelerated by a helium jet of 6 bar pressure for 50 milliseconds. After bombardment, embryos and suspensions were left overnight on the high osmotic medium.

2.1.3. Selection of resistant calli after bombardment

2.1.3.1. Bombarded immature embryos

After the overnight culture on high osmotic medium, the embryos were transferred to solid selection medium (Medium 3; MS salts and vitamins, 30 g/l sucrose, 2 mg/l 2,4-D, 3.5 g/l agarose type I and 3 x 10^4 U/l hygromycin B). After one week, embryos were transferred to a 6 or 24 well plate, containing R2 selection medium (Medium R2, containing 3 x 10^4 U/l hygromycin B). The medium was refreshed weekly. After 3 weeks, embryos were screened for resistant calli (Fig. 2.1.f).

2.1.3.2. Bombarded callus suspensions

After overnight culture on high osmotic medium, calli were subcultered to a 6 well plate, containing culture medium R2 (see above). After a week, medium was changed to R2 selection medium (see above). The medium was refreshed weekly, and after 3 weeks, the suspensions were screened for resistant calli. Several resistant calli (Fig. 2.1.e) were chosen per well, but eventually only one line per well was regenerated, in order to prevent the regeneration of several dependent lines.
2.1.4. Regeneration of rice plants from resistant calli

Resistant calli picked from liquid or solid selection medium were placed on callus increasing medium (Medium R2I; R2 salts, 60 g/l sucrose, 2 mg/l 2,4-D, 3 x 10^4 U/l hygromycin B, 100 mg/l inositol, 10 ml/l of a 100 x MS-vitamins stock solution (Murashige and Skoog, 1962), solidified with 5 g/l agarose type I). Calli were transferred to fresh medium weekly. Only the most embryogenic parts of the calli were considered for further cultivation. From 2 weeks on, calli having cauliflower-like embryogenic structures were transferred to regeneration medium (Medium Rzs; R2 salts, 20 g/l sucrose, 30 g/l sorbitol, 1 mg/l zeatin (Duchefa), 0.5 mg/l IAA (Sigma), 10 ml/l MS-vitamins 100 x stock solution, solidified with 6.5 g/l agarose type I). Subsequently, the calli were air dried for 5-10 minutes to enhance desiccation of the calli (Rancé et al., 1994). Calli were cultivated in culture chambers (Weiss, Reiskirchen, FRG) at 30°C, 16 hours in light and 8 hours in dark. Calli were subcultured every 1-2 weeks. Calli with shoots longer than 0.5 cm and small roots (Fig. 2.1.g, h) were transferred to MS-medium (MS salts and vitamins, 30 g/l sucrose, 3 g/l gelrite). When plantlets reached a size of about 15-20 cm (Fig. 2.1.i), they were transferred to soil and grown to maturity (Fig. 2.1.j).
Material and Methods

Fig. 2.1.: Transformation of rice. a: immature caryopses, b: isolated immature embryo, c: immature embryos on callus inducing M1 medium, d: calli derived from embryogenic cell suspension on high osmotic medium, ready for bombardment, e: hygromycin-resistant calli after suspension bombardment (arrows), f: resistant callus after bombardment of immature embryos (arrow), g, h: young plantlets on regeneration medium, i: young plantlets on MS medium, before transfer to the greenhouse, j: rice plants in the greenhouse.
2.2. Plasmids

2.2.1. MsEnod12A and MsEnod12B

The two plasmids pPR92 and pPB12B were kindly provided by Petra Bauer (Bauer, 1995). pPR92 contains the *Medicago sativa* *Enod12A* promoter (Allison *et al.*, 1993), driving expression of the *uidA* gene (Jefferson, 1987). pPB12B contains the *Medicago sativa* *Enod12B* promoter (Bauer, 1992), driving expression of the *uidA* gene (Fig. 2.2.). To avoid the problem of resistant plants containing only the selectable marker but not the gene of interest, constructs containing the gene of interest and the marker gene were generated. pPR92 was digested with *EcoR* I and *Bgl* II to release a 7.8 kb fragment, containing the complete gene of interest. This fragment was blunt end inserted into *Asp* 718 cleaved pAcH1 (Bilang *et al.*, 1991), resulting in pRIA21 (Fig. 2.2.a).

pPB12B was digested with *Xba* I and *Bgl* II, releasing a fragment of 6.3 kb, containing the complete gene of interest. This fragment was blunt end inserted into *Asp* 718 cleaved pAcH1, resulting in pRIB9 (Fig. 2.2.b). These cloning steps were performed by Rie Terada.

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**Fig. 2.2.:** Construction of the plasmids pRIA21 and pRIB9.
Testing of the original plasmids pR92 and pPB12B did not result in the expected restriction patterns. All parts related to the gene of interest were proven to be correct, however the restriction pattern of the backbone stayed unclear (Petra Bauer, pers. comm.). In addition, in the two plasmids pRIA21 and pRIB9, we cannot exclude an effect to the gene of interest by the strong Actin promoter lying upstream. Therefore we cloned for our last experiment and further transformation steps a new set of plasmids (Fig. 2.3.). To construct pNAN26, a 3550 bp SstI-PstI fragment from pRIA21, containing 1450 bp 3' end of the MsEnod12A promoter and the uidA gene, was ligated between the SstI and PstI sites of the vector pNAN7 to produce pNAN9 (Fig. 2.3.a). About 3800 bp SalI (blunt) and NcoI fragment from pRIA21 were introduced between HindIII (blunt) and NcoI of pNAN9 to produce pNAN26.

To construct pNAN10, two fragments of pRIB9, a NcoI-PstI fragment of about 2500 bp (containing the MsEnod12B promoter) and a SstI-NcoI fragment (containing the uidA gene), were assembled between the SstI and PstI site of the vector pNAN7 (Fig. 2.3.b). These cloning operations were performed by Yakandawala Nandadeva.

Fig. 2.3.: Construction of the plasmids pNAN10 and pNAN26.
2.2.2. VsEnbpl

The vetch Enbp1 cDNA was kindly provided by Erik Jensen (Christiansen et al., 1996). Starting from VsEnbp1 cDNA in pBluescript, partial digestion with Sac I released a 5.5 kb fragment, containing the complete cDNA. Plasmid PLS1 GUS (kindly provided by J. Fütterer, ETH Zürich) was cleaved with Xho I and Pst I to get a 3.5 kb fragment, containing the pBluescript backbone together with the 35S promoter combined with a S1-translational enhancer sequence (Fütterer et al., 1990). Further, PLS1 GUS was digested with Pst I and Sac I to release a 300 bp fragment, containing the nopaline synthase termination signal Tnos. Combining these 3 fragments resulted in the plasmid 35SE1, containing the Vsenbp1 cDNA, driven by the 35S promoter (Fig. 2.4.).

![Fig. 2.4.: Construction of the plasmid 35SE1.](image)

The two constructs 35S-E-EG and 35S-EG were kindly provided by Erik Jensen (Hansen et al., 1999; Fig. 2.5.). The construct 35S-E-EG contains the PsEnod12B promoter, controlling the uidA gene, and in addition the 35S promoter driving the Vsenbp1 gene. The construct 35S-EG serves as a negative control. In contrast to 35S-E-EG, 35S-EG does not contain the Vsenbp1 gene, whereas the rest of the plasmid is the same.

![Fig. 2.5.: Plasmids 35S-E-EG and 35S-EG.](image)
2.2.3. GH3

The auxin-inducible promoter GH3 (Hagen et al., 1991) was kindly provided by Michael Djordjevic (University of Canberra). Hagen et al. (1991) fused a 749 bp sequence of the soybean GH3 gene, containing 592 bp of the promoter and 157 bp 5' untranslated leader sequence, to the GUS coding sequence. Therefore, when referring to the GH3 promoter, the entire 749 bp sequence is meant, including the GH3 promoter and the leader sequence. For our purposes we used a modified construct, kindly provided by Herman Spaink (Leiden University), who fused the gene for the green fluorescence protein (gfp) to the 3' end of the original GH3::uidA construct (Fig. 2.6.).

![Plasmid pMP3631, containing the GH3 promoter driving the uidA and gfp genes.](image)

2.3. Analysis of regenerated plants

2.3.1. Molecular analysis

2.3.1.1. Digoxigenin-labelled probes for Southern blot

Southern analysis was performed using the Digoxigenin system from Boehringer (Boehringer Mannheim, 1993). The plasmids pRIA21, pRIB9, and pMP3631 (Fig. 2.2., 2.6.) were used as templates. The digoxigenin-linked dUTP nucleotides were incorporated into the probes by PCR using specific primers (Table 2.1.), following the protocol of Boehringer.
Material and methods

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>$T_{An}$</th>
<th>Fragment</th>
<th>probe</th>
</tr>
</thead>
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<tr>
<td>GUS5'</td>
<td>5'GCATTAATGGACTGGATTTGGG3'</td>
<td>56 °C</td>
<td>530 bp</td>
<td>uidA,</td>
</tr>
<tr>
<td>GUS3'</td>
<td>5'CAGGGGAGGATTTGGG3'</td>
<td></td>
<td></td>
<td>probe 1</td>
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<tr>
<td>ProEnod12</td>
<td>5'CGGTCCAGACATTGCAC3'</td>
<td>54 °C</td>
<td>390 bp</td>
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</tr>
<tr>
<td>5'GUS</td>
<td>5'GATGCCCACAGGCGGTCCGAG3'</td>
<td></td>
<td>450 bp</td>
<td>probe 3</td>
</tr>
<tr>
<td>GH3Prom1-5'</td>
<td>5'CCACAGGGATTTGGATCCGTG3'</td>
<td>52 °C</td>
<td>401 bp</td>
<td>GH3-P</td>
</tr>
<tr>
<td>GH3Prom2-3'</td>
<td>5'GGGAGCTGAAACATGTG3'</td>
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<td></td>
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<td>GH3Prom3-5'</td>
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<td>52 °C</td>
<td>109 bp</td>
<td>GH3-S</td>
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<td>GH3Prom4-3'</td>
<td>5'GCTAATGGTACGCGCGTAG3'</td>
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</tr>
</tbody>
</table>

Table 2.1.: Primers used to generate the specific probes. $T_{An}$ gives the annealing temperature used for the PCR

We generated a specific probe for the uidA gene with the primers GUS5' and GUS3' (probe 1). Primer ProEnod12 anneals to the 3' of the MsEnod12A and MsEnod12B promoters. This results, together with the primer 5'GUS which anneals at the 5' end of the uidA gene, in a fragment of 390 bp for the MsEnod12A (probe 2) and 450 bp for the MsEnod12B (probe 3). Primers GH3Prom1-5' and GH3Prom2-3' generated a probe at the 5' end of the GH3 promoter (GH3-P), whereas primers GH3Prom3-5' and GH3Prom4-3' generated a probe at the 3' end of the GH3 promoter, within the leader sequence (GH3-S). The PCR was run with denaturation at 94 °C for 5 min, 45 amplification cycles (denaturation at 94 °C for 1 min, annealing at $T_{An}$ (Table 2.1.) for 30 sec and extension at 72 °C for 1 min), followed by a final extension at 72 °C for 7 min. The produced probes were purified with a PCR product purification kit (QIAGEN, Hilden, D). For one Southern blot half of the purified PCR product was used.

2.3.1.2. Isolation of plant DNA

Material of young leaves was ground under liquid nitrogen and DNA was extracted using the Nucleon Phytopure Plant DNA extraction kit (Amersham Pharmacia Biotech, Uppsala, SW).
2.3.1.3. Southern blot analysis

About 5 \( \mu g \) of total genomic DNA was digested with the appropriate enzymes. Fragments were electrophoretically separated on a 0.8 % agarose (Sigma, type I) gel. After running for 5-8 hours at 120 V or overnight at 30 V, the gel was incubated for 10 min in 0.25 M HCl, denatured for 30 min in denaturation solution (0.5 M NaOH, 1.5 M NaCl) and washed 2 times for 15 min in neutralisation buffer (0.5 M Tris-HCl pH 7.4; 1.5 M NaCl; 1 mM EDTA). DNA was transferred overnight to a positively charged nylon membrane (Boehringer) and UV cross-linked (UV Stratalinker, Stratagene, USA). The membranes were hybridised with the DIG-labelled probes and washed and detected following the protocol of Boehringer. Signals were visualised on Kodak Bio Max MR films (Kodak, Rochester, USA).

2.3.2. Histochemical analysis

2.3.2.1. Histochemical GUS assay

Expression of GUS was detected histochemically by incubating the plant material in GUS staining solution (0.1 M K/Na-phosphate pH 7.0, 0.1 % Triton X-100, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.1 % X-Gluc (5-Bromo-4-chloro-3-indolyl-beta-D-glucuronide acid cyclohexylammoniumsalt; Biosynth, CH; Jefferson, 1987). Samples were kept under low pressure for 10 min to remove air and improve penetration of the solution, then incubated at 37 °C for 1 to 24 hours. Samples were washed 3 times with 0.1 M K/Na-phosphate buffer (pH 7.0) and analysed under a stereomicroscope without sectioning. Hand or microtome sections were performed to analyse the staining pattern with a microscope.

2.3.2.2. Hand sections

For hand sections, material was fixed with 70 % EtOH for at least 3 days. Material was embedded in a piece of pith, and sectioned with a razor blade.
2.3.2.3. Microtome sections

For microtome sections, plant material was embedded in hydroxyethyl methacrylate Technovit 7100 (Heraeus Kulzer, D) following the manufacturer's protocol. After staining, plant material was fixed overnight with 2.5% glutaraldehyde (Sigma). After dehydration through alcohol solutions (30 – 50 – 70 – 90 – 100%), samples were incubated in preparation solution overnight. After adding hardener II, samples were kept for 1 hour at 37 °C and further at RT until the Technovit 7100 solidified. Technovit blocks were trimmed and sectioned with a microtome (Leica RM2145, Glattbrugg, CH). Sections were either directly embedded in Euparal (Schmid + Co., Köngen/N, D) or stained first for 5 min with 0.05 % toluidine blue, washed twice with ddH2O and then embedded in Euparal.

2.3.2.4. In situ hybridisation

Plasmid pHCntG (J. Fütterer, ETH Zürich, see Burkhardt, 1996), containing the uidA gene driven by a 35S promoter, was digested with EcoR V and PstI to release a fragment of 1060 bp at the 3' end of the uidA gene. This fragment was cloned into plasmid pBSK (-) (Stratagene, USA). The obtained plasmid was digested with EcoR V or Xba I to synthesise the antisense probe (with the T3 polymerase) or the sense probe (with the T7 polymerase), respectively. To prepare the non-radioactive probe, a protocol based on Coen et al. (1990) was followed. To prepare the radioactive probe a modified protocol of Sambrook et al. (1989) was followed. Plant material was fixed overnight at 4 °C in 4 % paraformaldehyde and 0.25 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), and embedded in paraffin according to Cox et al. (1984). Ten-micrometer thick sections were cut on the microtome and mounted on polysine coated microscope slides (BDH, Chemie Brunschwig AG, Basel, CH). In situ hybridisation was performed according to Goormachtig et al. (1997) for the radioactive probe and based on Coen et al. (1990) for the non-radioactive hybridisation.
2.4. Treatment of transgenic rice with Nod factor and chitin fragments

Transgenic plants containing the MsEnod12A or 12B promoter-uidA fusion were treated with either a Nod factor or with Chitin fragments, to study putative effects of these substances on the GUS expression pattern.

2.4.1. Nod factor bombardment

Seeds were sterilised and germinated on R2 medium (see above), with either low concentration of combined nitrogen (medium R210N; similar to medium R2, but with only 10% of KNO3 and 10% of (NH4)2SO4), or R2 medium without any combined nitrogen (medium R2/N; similar to medium R2 but without KNO3 and without (NH4)2SO4, instead with 2.5 mM K2SO4 to maintain the SO42- concentration). For seed germination, these media were solidified with 3 g/l gelrite. Three to five days after germination, plantlets were transferred to metal grids in glass jars, which were filled with the respective liquid medium. Another 3-5 days later, different concentrations of 2,4-D were added. Plants were pre-treated like this for different time. For bombardment, plantlets or roots were treated with osmoticum (100 g/l sucrose added to the respective medium) for 15-30 min. For the preparation of the gold particles, a simplified protocol was used. Three hundred microlitres of EtOH (100%) was added to 50 µl of gold stock suspension (see above), shortly spinned down and the supernatant discarded. Nod factor solution (36 µl) of the desired concentration was added to the gold particles. Per shot 7 µl gold suspension were used. For bombardment the same parameters as for rice transformation were used (see above). Before and after bombardment, 5-10 µl of the respective Nod factor solution was pipetted on every root. Bombarded roots were kept for 24 hours on the high osmotic medium. Subsequently, they were either directly stained or subcultivated onto solid R2, R210N, or R2/N medium containing the appropriate 2,4-D concentration and cultivated for another 2 days. The Nod factor used was NodRm-IV(C16:2, Ac, S; M. Schultze, CNRS Gif sur Yvette, F) (Fig. 2.7.a). This is the biologically most active Nod factor of Rhizobium meliloti on Medicago sativa (Lerouge et al., 1990, Schultze et al., 1992). NodRm-IV(C16:2, Ac, S) is based on a tetrameric chitin backbone. At the non-reducing end, this backbone
contains an unsaturated C16:2 acyl chain and an O-acetyl group, and at the reducing end a sulfate group.

![Diagram](image)

**Fig. 2.7.** a: Structure of NodRm-IV(C16:2, Ac, S) (Lerouge et al., 1990, Schultz et al., 1992), b: General structure of a chitin fragment. In the case of the Chitotetraose and Chitopentaose, n is 2 and 3, respectively.

### 2.4.2. Incubation with Nod factor

Germination, cultivation in glass jars and the 2,4-D pre-treatment were performed as described above. After pre-treatment, roots were dissected into pieces of 0.5-1 cm and incubated in the appropriate medium containing Nod factor, 2,4-D, both or none of them for 1, 3, or 5 days.

### 2.4.3. Chitin fragment bombardment

Plants were germinated and prepared in the same way as for Nod factor bombardment. The chosen media were either R210N or R2/N. Plants were pre-treated with or without 2,4-D (4.5 × 10^{-6} M) for 1 or 3 days. Of each plant, half of the roots were bombarded with gold suspended in water (negative control), or gold suspended in 10^{-6} M chitin fragment solution. The chitin fragments used were Chitotetraose and Chitopentaose (Fig. 2.7.b, Seikagaku, Tokyo, J). Before and after bombardment, 5-10 μl of water or chitin fragment solution were added to each root. After bombardment, roots were left on the high osmotic medium for 1 day. Then roots were either stained or replaced on the same medium as before bombardment for another 2 days.
2.5. Rice cell protoplast preparation and transfection

Protoplasts were isolated from cell suspension culture of *Oryza sativa* L. (line Oc) and transfected according to Chen *et al.* (1994) with modifications. Suspensions were cultured in media containing MS salts and vitamins, 2 mg/l 2,4-D, 6 % sucrose and 2.5 mM 2-morpholinoethanesulfonic acid (MES). Digestion was performed using 3 % cellulase Onozuka R-10, 1 % macerozyme R-10 (both Yakult Pharmaceutical Ind. Co., Ltd., Tokyo, Japan), 8 % mannitol and 1 mM CaCl₂ for 16 h with slow rotation at 30 °C. Protoplasts were transformed with the PEG method with 5 or 10 μg DNA of each plasmid, cultured in K3 medium (K3 macro elements, B5 micro elements, 0.4 M sucrose, 0.25 g/l D(+) -Xylose, 100 mg/l inositol, 1 mg/l 1-naphthylacetic acid (α), 0.2 mg/l N⁶-benzyladenine, pH 5.6), and harvested 16 h later (Chen *et al.*, 1998). The protoplasts were analysed for activity of β-glucuronidase according to Jefferson (1987).
3. Results

3.1. Morphology of rice roots and the effect of auxin-treatment

3.1.1. Rice root anatomy

The anatomy of rice roots has been described (Clark and Harris, 1981; Harada and Yamazaki, 1993). However, in these and in further publications, the nomenclature for cross-sections through rice roots was not handled consistently. To keep the nomenclature consistent throughout this thesis, and enable comparison of our data with published work, in the following we describe the observed structures in a rice root cross-section and define a nomenclature in accordance with the tradition in plant anatomy.

The rice root roughly consists of a central cylinder and a cortex. On its inner side, the cortex is bounded by the endodermis, on its outer side by three layers: solumenchyma, exodermis, and epidermis (Fig. 3.3.). Various publications differ in the designation of the endodermis.

Harada and Yamazaki (1993) showed some figures, where it was obvious that the endodermis consisted of uniform cells and the pericycle was the first cell layer which contained cells of a various size (Fig. 2-14b, c on page 146; Fig. 2-29b, d on page 158; Fig. 3.1.a in this thesis). In this case, the pericycle contained, in the young root, protoxylem vessels. There were no intercellular spaces observable between the pericycle and the endodermis. In other figures of Harada and Yamazaki (1993), the pericycle was the cell layer with uniform cells and was adjacent to the cell layer that contained the protoxylem vessels (Fig. 1-43 on page 125; Fig. 3.1.b in this thesis). The endodermis was adjacent to the pericycle and intercellular spaces were visible between pericycle and endodermis. Compared with the description of root sections on pages 146 and 158 in Harada and Yamazaki (1993), the pericycle and endodermis were located one cell layer closer to the epidermis in the description of page 125.

In another publication where cell layers were described (Reddy et al., 1997), the authors used the nomenclature as described on page 125 of Harada and Yamazaki (1993).
A comprehensive overview was given by Clark and Harris (1981). According to their study, the pericycle contained protoxylem vessels, and the endodermis was the first layer with uniform cells (Fig. 3.2.a). Clark and Harris (1981) described cells of the endodermis near the root tip as more rectangular than the other cortical cells. The endodermis was in close contact with the pericycle, without intercellular spaces.

In order to clarify the situation, we studied the anatomy of the young rice root. The Casparian strip, which defines the endodermis, was not clearly visible in sections, made within the elongation and differentiation zone of 4 to 14 days old rice roots. However, in 4 μm thick microtome sections, wall thickenings were observed in the radial walls of a single distinct cell layer. In toluidine stained sections, these thickenings stained slightly darker than the adjacent cell walls. (Fig. 3.2.b-d, arrows). We considered these wall thickenings as the early stage of a Casparian strip. This cell layer with the cell wall thickenings consisted of rectangular cells and was in close contact to the inner adjacent cell layer, which was in accordance to Clark and Harris (1981). The presence of the Casparian strip defined this cell layer as endodermis. Therefore, according to our observation, the endodermis was closely connected to the pericycle without any intercellular spaces between these two layers. On the side of the cortical parenchyma, intercellular spaces occurred between the endodermis and the adjacent cell layer.
Conclusion: In the subsequent parts of this thesis, we use the term pericycle always for the last (from the inside to the outside) cell layer with cells of various sizes and the term endodermis for the next (from the inside to the outside) cell layer, which is in close contact to the pericycle and exposes the first intercellular spaces towards the outer layers of the cortex.

Figure 3.3 summarises the use of anatomical terms for the present thesis. The central cylinder contains the phloem and xylem vessels. The pericycle is the outer layer of the central cylinder. The cortex consists of four distinguishable layers. Adjacent to the pericycle, the endodermis, containing the Casparian strip, is the innermost cell layer of the cortex. The second layer is the cortical parenchyma, showing large intercellular spaces in the older root. The cortex is bordered at the periphery by the solorenchyma and the exodermis. The
solorenhyma consists of short fibres with thick cell walls (Clark and Harris, 1981). In older roots, these two cell layers take the function of the epidermis, which borders the young root and is shed from the mature root (Juliano and Aldama, 1937; Clark and Harris, 1981).

**Fig. 3.3.:** Scheme of the nomenclature used in the present thesis.
3.1.2. Comparison of lateral roots and auxin-induced structures

Auxin induced, nodule-like structures have been described in a number of monocotyledonous plant species (Ridge et al., 1992a; Ridge et al., 1992b; Christansen Weniger and Vanderleyden, 1994b; Gantar and Elhai, 1999; El Khawas and Adachi, 1999). Incubation of rice roots in 2,4-D induced the formation of nodule-like structures (Ridge et al., 1992b). These structures appeared to result from the fusion of multiple meristems induced in very close proximity to each other. The internal tissue differentiation of some nodule-like structures was found to be similar to that of both Parasponia nodules and some kinds of determinate legume nodules (Ridge et al., 1992b).

The term 'nodule-like' implies a similarity in structure or function to the known nodules of legumes, Sesbania, Parasponia or actinorhizal plants. We studied the effect of auxin on rice roots to determine whether the term 'nodule-like' is accurate to describe these induced structures.

Seeds of TP309 were germinated on MS-medium. Four to six days after germination, seedlings were placed into glass jars containing liquid MS-medium. After two to three days, 2,4-D was added to a final concentration of $5 \times 10^{-8}$ M or $1 \times 10^{-6}$ M, IAA to a final concentration of $1 \times 10^{-5}$ M. Root samples were taken after one to ten days of incubation.

The artificial auxin 2,4-D in both concentrations induced more and larger abnormal lateral roots than IAA, and $1 \times 10^{-6}$ M 2,4-D produced a stronger effect than $5 \times 10^{-8}$ M. Exposure to IAA at a concentration of $1 \times 10^{-5}$ M for three days did not induce the clearly recognisable abnormal lateral roots, but somewhat stunted roots. Three or more days incubation in 2,4-D ($5 \times 10^{-8}$ M or $1 \times 10^{-6}$ M) inhibited root growth and induced numerous outgrowths. These outgrowths deformed the root morphology completely. Therefore, we incubated rice in medium containing $1 \times 10^{-6}$ M or $5 \times 10^{-8}$ M 2,4-D for one or two days. These treatments clearly induced new structures, however, apart from the new structures, root morphology still remained intact.

In general, external auxin induced different responses in different areas of the root. Older parts of the roots hardly reacted to the auxin treatment, whereas younger parts showed a strong reaction. Young lateral roots stopped growth,
and thickening and stunting occurred on the previously formed lateral roots and lateral root primordia. Occasionally, de novo formed abnormal lateral roots were visible in the area of lateral root formation. The root hair zone and the early mature zone formed many abnormal lateral roots. Even after one day, abnormal lateral roots were clearly visible, however, still within the root cortex. After three days of 2,4-D incubation, these abnormal lateral roots penetrated and destroyed the root epidermis (Fig. 3.4.a).

To study the development of lateral roots and the auxin-induced structures, 4 μm thick microtome sections were cut. Figure 3.4. gives an overview of the sections of 2,4-D treated roots, Figure 3.5. an overview of the sections of untreated control roots. Figures 3.4.a and 3.5.a show an unsectioned root. Two differences to normal lateral root development were obvious in abnormal lateral root development: (i) induced structures were broader as compared to normal lateral roots. (ii) many abnormal lateral roots started to develop and the average distance between the abnormal lateral roots was smaller as compared to normal lateral root development. Cross sections showed that the new structures were induced in the central cylinder, similar to the lateral root primordia (LRP) (Fig. 3.4.b, c). However, in contrast to the LRP, the induction of the new structures was not restricted to the pericycle. Their development included additional cells between the xylem rays, and thus, during further development they prised the originally adjacent xylem rays apart (Fig. 3.4.e-h, arrowheads).

During LRP development, the position of the xylem rays stayed almost constant (Fig. 3.5.c-e). The 2,4-D induced structures were more round shaped and lacked the distinct longitudinal polarity of normal lateral roots (Fig. 3.4.a, d-g).

The anatomy of auxin induced structures was simple. Various tissues did not differentiate. Several cell layers at the periphery were apparently different from the rest of the tissue (Fig. 3.4.f, h: asterisks). Even older induced structures were devoid of any kind of vessels or a central cylinder with connection to the main root (Fig. 3.4.g-i). In contrast, normal lateral roots showed very early a clear tissue differentiation (Fig. 3.5.c-e).
Fig. 3.4.: Rice roots, treated with 2,4-D. a: low magnification overview, arrowheads indicate abnormal lateral roots, b-l: 4 μm microtome cross-sections. a, c, d, e, f: 1 day treatment with \(1 \times 10^{-6}\) M 2,4-D. b, g, h: 1 day treatment with \(9 \times 10^{-6}\) M 2,4-D, i: 36 days treatment with \(4.5 \times 10^{-5}\) M 2,4-D. d-f, i: stained with toluidine blue, e-h: arrowheads mark xylem vessels, f,h: asterisks mark cell layers at the periphery.
Fig. 3.5.: Lateral root development in rice. a: low magnification overview, arrowheads indicate lateral roots b-e: 4 µm microtome cross-sections, d: stained with toluidine blue.
3.2. MsEnod12A and MsEnod12B

3.2.1. Transformation

3.2.1.1. Transformation with _MsEnod12A::uidA_

In an initial experiment, 279 embryos were co-bombarded with pPR92 and pAcH1. Out of twelve regenerated lines, seven were shown to contain the complete gene insert by Southern blot analysis of genomic DNA. Only three of these seven lines were fertile and none of these expressed _MsEnod12A_ (determined by GUS histochemistry), not even after pre-treatment with 2,4-D and bombardment of Nod factor.

In a second experiment, cell suspensions were bombarded with the plasmid pRIA21. Out of this experiment, 52 independent lines were regenerated, 26 of these were shown to contain the complete insert, proven by Southern blot analysis. Only four out of these 26 lines were fertile. The four lines were tested for GUS staining with and without 2,4-D treatment and after 2,4-D pre-treatment and Nod factor bombardment. Only one line (line 58.1 26/3) showed blue staining.

In a third experiment, twelve plates were co-bombarded with the plasmids pPR92 and pAcH1. Out of this experiment, 44 independent lines were regenerated, of which 21 lines were positive according to Southern blot analysis. Of the 21 transgenic lines with the correct insert, twelve were fertile. They were all tested for basic expression by GUS staining. They were also tested for _uidA_ expression after 2,4-D treatment. In addition, six of these lines were pre-treated with 2,4-D followed by Nod factor bombardment. One single line (70.1 6) showed GUS activity.

In order to get more transgenic and _MsEnod12A_ expressing lines, an additional experiment was performed. Two cell suspension plates were bombarded with the plasmid pRIA21. After selection on hygromycin, one half of each resistant callus was tested for GUS activity. Only calli staining blue were considered for further cultivation. With this procedure, five independent, blue staining lines were regenerated. Unfortunately, all plants of these five lines grew very poorly,
and finally none of them was fertile. However, these lines were useful for experiments during tissue culture.

In summary, 279 embryos and 24 suspension plates were either bombarded with pRIA21 or co-bombarded with pPR92 and pAcH1. Out of these experiments, in total 113 independent transgenic lines were regenerated (Table 3.1.). Fifty-nine lines were shown to contain the expected insert, but only 19 of these 59 lines were fertile. Moreover, most of the fertile lines had only very weak seed set. Out of these 19 positive and fertile lines, only two lines showed blue staining. In addition, the five lines of the last experiment were used for further experiments.

<table>
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<tr>
<th>Total of regenerated, independent lines</th>
<th>Lines containing the correct insert</th>
<th>Fertile lines out of the 59 lines containing the correct insert</th>
<th>GUS positive, fertile lines</th>
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<tr>
<td>113</td>
<td>59</td>
<td>19</td>
<td>2</td>
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</table>

Table 3.1.: Summary of the regenerated lines from bombardments with pRIA21 and pPR92.

3.2.1.2. Transformation with MsEnod12B:uidA

In a first experiment, seven cell suspension plates were co-bombarded with pPB12B and pAcH1. Out of 30 regenerated lines, 21 were shown to contain the complete insert by Southern blot analysis. Eight of the 21 lines were fertile. They were tested for basic expression by GUS staining. In addition, they were tested for MsEnod12B expression after 2,4-D treatment or 2,4-D pre-treatment and Nod factor bombardment. Only one line expressed MsEnod12B to some extent.

In a second experiment, seven plates were bombarded with the plasmid pRIB9. From this experiment, 21 lines were regenerated. Twelve of them contained the complete insert, according to Southern blot analysis. Only four out of these twelve positive lines were fertile. They were tested for expression with or without 2,4-D treatment or after 2,4-D pre-treatment and bombardment of Nod factor. None of the four lines showed any MsEnod12B expression at all.

In order to get more transgenic MsEnod12B expressing lines, an additional experiment was performed. Two cell suspension plates were bombarded with pRIB9. After selection on hygromycin, resistant calli were divided and one half
of each callus was stained. Only blue staining calli were considered for further cultivation. With this procedure, four independent lines were regenerated, of which three showed MsEnod12B expression. Only one of these lines grew very well, and eventually none of them was fertile. However, these lines were useful for experiments during tissue culture.

In summary, 14 cell suspension plates were bombarded. In total 55 lines were regenerated, 36 were shown to have the complete insert (Table 3.2.). Out of these 36 lines, 12 were fertile, but only one line showed uidA expression. In addition, the two blue staining lines of the last experiment were used for experiments.

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<thead>
<tr>
<th>Total of regenerated, independent lines</th>
<th>Lines containing the correct insert</th>
<th>Fertile lines out of the 36 lines containing the correct insert</th>
<th>GUS positive, fertile lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>55</td>
<td>36</td>
<td>12</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 3.2.: Summary of the regenerated lines from bombardments with pRIB9 and pPB12B.

### 3.2.2. Southern blot analysis

#### 3.2.2.1. MsEnod12A::uidA

In order to define lines that contain the complete gene of interest, Southern blot analysis of genomic DNA was performed. Leaf material from regenerated plants was collected and DNA isolated. In a first approach, plant genomic DNA was digested with Sal I and EcoR I (Fig. 3.6.). This digestion released a fragment of 7.8 kb (Fig. 3.6.a), which was detected by a probe hybridising to the uidA gene (probe 1, Fig. 3.6.a). The Southern blot shows a sample of 15 independent lines. Ten of these lines had the correct insert, only one of them was fertile. None of these lines showed blue staining.

This approach of releasing the complete gene of interest had the disadvantage of a low resolution, since the excised fragment had a length of 7.8 kb. In this size range, the resolution of a Southern blot is quite low, and it is difficult to define the correct band size. Therefore, in further experiments, the DNA was digested with enzymes releasing two fragments, which were both detected by the probe 2 (Fig. 3.7.a, Fig. 3.8.a). Probe 2 extends to the left and right of the restriction site of Asp 718, which cuts the entire gene of interest into two
fragments of 3.6 kb and 4.1 kb, respectively. Therefore, this probe detects both fragments. The digestion of genomic DNA of Line 70.1 6 with Asp 718 and Bgl II proved that this line has the correct insert (Fig. 3.7.b, lane 2). Besides the expected two bands several additional bands were also detected. Therefore this line contains besides the completely inserted gene of interest several truncated copies. Lane 1 shows the undigested genomic DNA. The high molecular weight signal proves that the gene of interest is inserted into the plant genome.

**Fig. 3.6.** Southern blot analysis of plants transformed with pRIA21. a: Plasmid pRIA21, shown are the restriction sites and the position of the probe 1, b: Southern blot of 15 independent lines. wt: negative control, pl: positive control, M3: Marker M3, Boehringer, *: positive, sterile line; +: positive, fertile line.
Fig. 3.7.: Southern blot analysis of line 70.1 6. a: Plasmid pPR92, used for transformation. Shown are the restriction sites and the position of the probe 2, b: Southern blot of line 70.1 6. 
pl: positive control, A: Asp 718, B: BglII, -: undigested DNA.

Fig. 3.8.: Southern blot analysis of plants, transformed with pRIA21. a: Plasmid pRIA21, shown are the restriction sites and the position of the probe 2, b: Southern blot of five independent transgenic lines. wt: negative control, pl: positive control, M3: Marker M3, Boehringer, *: positive, sterile line; +: positive, fertile line; bold: expressing line. -: undigested DNA, A: Asp 718, E: EcoR I, H: Hind III.
The third Southern blot (Fig. 3.8.b) shows the result of the analysis of five blue staining lines. For each plant line, the first lane shows undigested DNA, the second lane shows a single cut with Hind III, in order to determine the copy number, and in the third lane the double digestion with Asp 718 and EcoRI is shown, which releases the two fragments of 3.6 kb and 4.1 kb. All five lines show the two correct inserts. Lines A5, A7, and A18 have more than ten copies, most of which are truncated. Line 58.26/3 has two copies, one of the copies is truncated. Line A13 has four copies, whereof at least one of the copies is truncated (lines A13/1 and A13/3 are dependent).

3.2.2.2. MsEnod12B::uidA

In a first screening, genomic plant DNA was digested with EcoRI to release a fragment of 5.5 kb, which was detected by probe 1 (Fig. 3.9.a). This fragment contains most of the pMsEnod12B promoter and the uidA gene. The Southern blot (Fig. 3.9.b) shows a sample of 14 lines. Out of these 14 lines, 11 had the correct insert. Three of them were fertile, but none showed any MsEnod12B expression. The lines 52/12, 52/12 1.1 and 52/12 1.2 are dependent. A more detailed analysis was performed with the MsEnod12B expressing line used for experiments (Fig. 3.10.b). The first lane shows undigested DNA, the second lane a Hind III digestion in the inserted construct, which gives the information about the copy number. In the third lane, the digestion with Asp 718, Nsi I and Pst I releases two fragments of about 2.4 kb and 2 kb, which are both detected by the probe 3, which extends left and right of the restriction site of Asp 718 (Fig. 3.10.a). Line B9 contains three copies, two of them are truncated.
Fig. 3.9.: Southern blot analysis of plants, transformed with pRIB9. a: Plasmid pRIB9, shown are the restriction sites and the position of probe 1, b: Southern blot of 14 lines, plant DNA digested with EcoR I.
wt: negative control, pl: positive control, M3: Marker M3, Boehringer, *: positive, sterile line; +: positive, fertile line.

Fig. 3.10.: Southern blot analysis of line B9. a: Plasmid pRIB9, used for transformation. Shown are the restriction sites and the position of probe 3, b: Southern blot of line B9. pl: positive control, M3: Marker M3, Boehringer, -: undigested genomic DNA, A: digested with Hind III, B: digested with Asp 718, Nsi I, and Pst I.
3.2.3. Basic activity of MsEnod12A and MsEnod12B in transgenic rice

3.2.3.1. Basic activity of MsEnod12A::uidA in transgenic rice

The expression pattern and the activity of MsEnod12A::uidA were different in the various transgenic lines. Figure 3.11. shows low magnification micrographs, Figure 3.12. 4 μm thick microtome cross-sections, and Figure 3.13. hand-sections. The cells of the root cap showed blue staining (Fig. 3.11.b). The root tip, including the meristem and part of the growing zone, expressed little GUS activity (Fig. 3.11.a, Fig. 3.12.a). The blue staining was distributed over the entire cross section, with slightly stronger staining in the epidermal cells. Further basal, the expression in the epidermal cells increased, forming a blue band (Fig. 3.11.a, Fig. 3.12.b). Staining in the cortex remained weak. In the root hair zone, uidA expression was mainly visible in the stele, the endodermis and the epidermis (Fig. 3.11.c, Fig. 3.12.c). In the epidermis, only trichoplasts stained blue (Fig. 3.11.b,d, Fig. 3.12.c). Atchoblasts did not stain at all. The blue staining in trichoblasts was visible very early in their differentiation. In the young lateral root primordia zone, there was little or no uidA expression in the cortex (Fig. 3.12.c, Fig. 3.13.b). Young lateral root primordia showed only very weak or even no MsEnod12A activity (Fig. 3.11.d, Fig. 3.12.e, and Fig. 3.13.f). In the vicinity and mainly in front of the lateral root primordium strong blue staining was detectable (Fig. 3.11.d, Fig. 3.12.d,e). The staining in front of a lateral root primordium was detectable already in the early stage of a primordium formation (Fig. 3.12.d). Later in their development, lateral roots showed the same expression pattern as the main root (Fig. 3.11.e, f, Fig. 3.12.f). Vascular bundle staining differed in the various lines from very weak to very strong (Fig. 3.12.c, f, Fig. 3.13.a-f). The blue staining of the endodermis was also quite heterogeneous. Within the same line it varied from no staining (Fig. 3.12.c-f) to clear blue staining (Fig. 3.13.a). Only one single transgenic line differed in its expression pattern. This line showed, in general, very intense staining. Furthermore, in this line lateral root primordia showed very strong blue staining, similar to the observed expression of Enod12B.
Fig. 3.11.: GUS-staining in rice roots containing MsEnod12A::uidA. a-d: line 70.1 6, e: line A9, f, g: line A7. Arrow: root cap, arrowhead: root hair, asterisk: blue staining around a lateral root primordium, cross: indicates the blue band behind the root tip.
Fig. 3.12.: GUS expression in roots of line 70.1 6, containing the MsEnod12A::uidA construct. Cross sections near the root tip (a,b), the elongation zone (c), the lateral root primordium zone (d,e) and the lateral root zone (f) are shown. Arrows: blue staining in the stele, arrowheads: root hairs, +: blue staining epidermal cells in the root tip region, *: indicates the blue staining region around lateral root primordia.
Fig. 3.13.: Blue staining in the elongation and young lateral root primordium zone of five different transgenic rice lines, containing the $MsEnod12A::uidA$ construct, a: line 7016, b,c: line A7, d: line A18, e: line A13, f: line A9. Note the different intensity of $MsEnod12A$ expression in the stele.
3.2.3.2. Basic activity of \textit{MsEnod12B::uidA} in transgenic rice

The expression pattern of \textit{MsEnod12B::uidA} was in general similar to that of \textit{MsEnod12A::uidA}. Figures 3.14. and 3.15. show low magnification micrographs and microtome sections. In contrast to the \textit{MsEnod12A} promoter, the \textit{MsEnod12B} promoter was hardly active in the root cap, but strongly in the root tip, including the meristem and the mature zone (Fig. 3.14.a, b, e, Fig. 3.15.c). Similar to \textit{MsEnod12A}, the \textit{MsEnod12B} promoter was active in root hairs (Fig. 3.14.a, Fig. 3.15.a). However in contrast to \textit{MsEnod12A} activity, occasionally \textit{12B} was active as well in epidermal cells others than root hairs (Fig. 3.15.a). The central cylinder stained very strongly (Fig. 3.15.a, e), sometimes a weak staining in the endodermis was visible (Fig. 3.15.b). The most significant difference between the \textit{MsEnod12A} and the \textit{MsEnod12B} activity pattern was obvious in lateral root primordia. \textit{MsEnod12B} was highly active in the cells of young lateral root primordia (Fig. 3.14.c, Fig. 3.15.a, b, f), in contrast to the low or even absent activity of \textit{MsEnod12A} in young lateral root primordia (Fig. 3.11.e, Fig.3.12.e, Fig. 3.13.f). In addition to the \textit{uidA} expression inside lateral root primordia, \textit{12B} directed \textit{uidA} expression also outside the primordia in the cortex. Thus \textit{12B} activity was very similar to the activity of \textit{12A} in the cortex around lateral root primordia, however, the apparent GUS staining was lower in \textit{12B}.

Hand cut sections of older parts of roots showed that the expression pattern was different to that observed in younger root parts. In younger root parts, the central cylinder showed strong \textit{uidA} expression and the cortex was, with the exception of the vicinity of lateral root primordia, nearly free of any blue staining (Fig. 3.16.a, Fig. 3.14.g, h). In older root parts, the central cylinder showed no \textit{uidA} expression and the cortex showed weak blue staining, mainly in the first cell layer of the cortical parenchyma (Fig. 3.16.b).
Fig. 3.14.: GUS staining of line B9, containing the MsEnod12B::uidA construct. a-d: Low magnification micrographs, e-f microtome sections from the root tip (e,f) and the lateral root primordium zone (g,h). Arrows indicate the strong MsEnod12B expression in the root tip. Lateral root primordia stain completely blue (c,g,h). Arrowheads indicate root hairs, crosses indicate the blue staining vascular tissue and asterisks mark the blue staining cortex around a lateral root primordium.
Fig. 3.15: *MsEnod12B* expression in line B16. Microtome sections are shown from the root tip (a,b) and the lateral root primordia zone (c,d). Asterisk: blue staining in a lateral root primordium, cross: blue staining in the stele.

Fig. 3.16.: Expression of *MsEnod12B:uidA* in rice. a: in the lateral root primordia zone, b: in the end of the young lateral root zone. Note the change of the expression from the stele (+) to the cortex (*).
3.2.4. Effects of plant hormones on *MsEnod12A::uidA* and *MsEnod12B::uidA* activity

3.2.4.1. Auxin

Auxin application to rice inhibits root growth and induces the formation of abnormal lateral roots. Addition of auxin to transgenic plants containing the *MsEnod12A::uidA* construct increased the expression driven by the *MsEnod12A* promoter strongly (Fig. 3.17.). Binocular and microscopic observation showed however, that this effect was only an indirect increase due to the change in the morphology of the treated roots. Treatment with $5 \times 10^{-8}$ M 2,4-D for three days caused symptoms shown in Fig. 3.17. There was an enormous amount of root hairs, and of the auxin-induced abnormal lateral roots (Fig. 3.17.a,c). Root hairs stained blue as already seen in the basic expression, but since the number of root hairs was increased, it gave the impression of a stronger GUS staining. The abnormal lateral roots did not stain at all. This was in contrast to the lateral root primordia, in which a weak *uidA* expression could be observed. The vicinity of the abnormal lateral roots stained strongly blue, as was already observed in the vicinity of lateral root primordia. Due to the high amount of abnormal lateral roots, this gave the impression of an increased *uidA* expression. However, the expression pattern remained the same. The root cap as well as the root tip including the meristem and part of the growing zone did not stain in auxin-treated roots (Fig. 3.17.a). This was in contrast to the basic expression, where the root cap was staining strongly blue, whereas the root tip showed a weak *uidA* expression.

Due to the addition of auxin, already emerged lateral roots became stunted (Fig. 3.17.d), showing also a lot of root hairs. The expression pattern of these stunted lateral roots was the same as for the main root.

Treatment with $1 \times 10^{-6}$ M or $4.5 \times 10^{-6}$ M 2,4-D for one or three days produced similar effects on *uidA* expression. With this high concentration of 2,4-D, root hair growth was suppressed. Since in the root hair zone the main staining was in root hairs, such treated roots showed less blue staining than roots treated with the lower 2,4-D concentration. The large auxin-induced structures did not stain, whereas the surrounding cortex tissue did (Fig. 3.17.f).
Fig. 3.17.: MsEnod12A expression after auxin-treatment. a-d: treatment for 3 days with $5 \times 10^{-8}$ M 2,4-D. e, f: treatment for 3 days with $4.5 \times 10^{-6}$ M 2,4-D. Arrowhead, asterisk: indicate the auxin-induced structures. Arrows: indicate the non-staining root tip.
Upon auxin induction, the line B9, containing the \textit{MsEnod12B::uidA} construct, showed the same staining pattern as the \textit{MsEnod12A} lines. Staining was visible in the cortex in the vicinity of abnormal lateral roots, and in the trichoblasts. Also in this case, the induced abnormal lateral roots did not stain blue at all. This, however, is in contrast to the basic expression pattern, where lateral root primordia showed a strong blue staining.

3.2.4.2. Kinetin, GA$_3$ and the auxin transport inhibitor TIBA

Plants containing the \textit{MsEnod12A::uidA} construct were treated with $1 \times 10^{-5}$ M kinetin, $1 \times 10^{-6}$ M GA$_3$, and $1 \times 10^{-6}$ M TIBA for one and three days. All these substances affected root growth as expected from descriptions in the literature. Kinetin and TIBA both inhibited lateral root primordium initiation. GA$_3$ had little effect at all. However, in all cases the expression pattern of the \textit{MsEnod12A} promoter did not change.

3.2.5. Bombardment of Nod factor

For bombardment of Nod factors, plants were cultured on liquid R2 medium. Either normal R2 medium, R2 medium with only 10% of combined nitrogen (R210N) or R2 medium without any nitrogen (R2/N) was used. Before bombardment, some plants were pre-treated with 2,4-D ($4.5 \times 10^{-6}$ M or $5 \times 10^{-8}$ M). For bombardment, either intact plants or dissected roots were incubated on high osmoticum (10% sucrose) for 30 min in the respective pre-treatment medium. Before and after bombardment, 5-10 µl of Nod factor solution (same concentration as used for bombardment) were applied to each root. After bombardment, roots and plants were kept on the high osmotic medium for 24 hours. Afterwards, roots were either directly stained in X-Gluc solution overnight, or the explants were transferred to the regular pre-treatment medium and stained three days after bombardment. An overview of all experiments is given in Table 3.3. Experiments were carried out with two to five lines containing the \textit{MsEnod12A::uidA} and with one line containing the \textit{MsEnod12B::uidA}. Under these experimental conditions we never observed any change in \textit{uidA} expression due to bombardment with Nod factor, as compared to bombardment with water alone. The basic expression was also not changed.
Results

by the bombardment with water. These results are in contrast to the results of Terada et al. (1996) who showed a change in uidA expression after the bombardment of 2,4-D pre-treated rice roots with the same Nod factor we used.

<table>
<thead>
<tr>
<th>Nod factor concentr.</th>
<th>Medium</th>
<th>2.4-D pre-treatment</th>
<th>explant</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1 \times 10^{-9}$ M</td>
<td>R2</td>
<td>none</td>
<td>root cut</td>
<td>3 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$5 \times 10^{-8}$ M</td>
<td>root cut</td>
<td>3 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$4.5 \times 10^{-8}$ M</td>
<td>root cut</td>
<td>3 days</td>
</tr>
<tr>
<td></td>
<td>R210N</td>
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<td>root cut</td>
<td>3 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$5 \times 10^{-8}$ M</td>
<td>root cut</td>
<td>3 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$4.5 \times 10^{-8}$ M</td>
<td>root cut</td>
<td>3 days</td>
</tr>
<tr>
<td></td>
<td>R2/N</td>
<td>none</td>
<td>root cut</td>
<td>3 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$4.5 \times 10^{-8}$ M</td>
<td>root cut</td>
<td>3 days</td>
</tr>
<tr>
<td>$1 \times 10^{-8}$ M</td>
<td>R2/N</td>
<td>none</td>
<td>root cut</td>
<td>1 day</td>
</tr>
<tr>
<td></td>
<td></td>
<td>whole plant</td>
<td>1 day</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>whole plant</td>
<td>3 days</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$4.5 \times 10^{-8}$ M</td>
<td>root cut</td>
<td>1 day</td>
</tr>
<tr>
<td></td>
<td></td>
<td>root cut</td>
<td>3 days</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>whole plant</td>
<td>1 day</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>whole plant</td>
<td>3 days</td>
<td></td>
</tr>
<tr>
<td>$1 \times 10^{-6}$ M</td>
<td>R2</td>
<td>none</td>
<td>root cut</td>
<td>1 day</td>
</tr>
<tr>
<td></td>
<td></td>
<td>root cut</td>
<td>3 days</td>
<td></td>
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<tr>
<td></td>
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<td>whole plant</td>
<td>1 day</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>whole plant</td>
<td>3 days</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R210N</td>
<td>none</td>
<td>root cut</td>
<td>3 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$4.5 \times 10^{-8}$ M</td>
<td>root cut</td>
<td>3 days</td>
</tr>
<tr>
<td></td>
<td>R2/N</td>
<td>none</td>
<td>root cut</td>
<td>1 day</td>
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<td>whole plant</td>
<td>3 days</td>
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<tr>
<td></td>
<td></td>
<td>$4.5 \times 10^{-8}$ M</td>
<td>root cut</td>
<td>1 day</td>
</tr>
</tbody>
</table>

Table 3.3.: Overview of all bombardments on roots of transgenic rice containing MsEnod12A::uidA and MsEnod12B::uidA respectively. Pre-treatment indicates, whether the plants were first treated with 2,4-D for 2-3 days or not, and in which concentration. Explant indicates, whether the roots were dissected from the plant prior to shooting (root cut), or whether whole plants were placed on the plates for bombardment (whole plant). Finally, incubation time means whether roots were stained one day or three days after bombardment.
3.2.6. Incubation in Nod factor

The simplest way to apply Nod factors to roots is incubation of roots in Nod factor solution. Reddy et al. (1998b) reported a more significant effect of Nod factor on excised root sections as compared to its application to the roots of intact plants. From MsEnod12A::uidA transgenic lines excised root pieces of about 0.5 cm length were incubated in Nod factor solution. The experiment was performed according to Reddy et al. (1998b). Plants were grown in liquid MS medium, without any pre-treatment. Excised roots were incubated in liquid MS medium without any addition, with $1 \times 10^{-9}$ M Nod factor, with $4.5 \times 10^{-6}$ M 2,4-D or with both together (Table 3.4.). In a second experiment, three MsEnod12A lines were grown on R2/N with or without pre-treatment of 2,4-D ($4.5 \times 10^{-6}$ M) for 1 day. Roots were cut into sections of about 0.5 cm length and incubated in R2/N medium, with or without 2,4-D (depending on the pre-treatment) and 0, $1 \times 10^{-8}$ M or $1 \times 10^{-7}$ M Nod factor for 1, 3 or 5 days in the dark (Table 3.4.). Subsequently, roots were stained for GUS activity for 24 hours.

<table>
<thead>
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<th>Medium</th>
<th>Pre-treatment</th>
<th>Treatment</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
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<td>none</td>
<td>1 day</td>
</tr>
<tr>
<td>MS</td>
<td>none</td>
<td>$10^{-9}$ M Nod factor</td>
<td>1 day</td>
</tr>
<tr>
<td>MS</td>
<td>none</td>
<td>$10^{-9}$ M N, $4.5 \times 10^{-6}$ M 2,4-D</td>
<td>1 day</td>
</tr>
<tr>
<td>MS</td>
<td>none</td>
<td>$4.5 \times 10^{-6}$ M 2,4-D</td>
<td>1 day</td>
</tr>
<tr>
<td>R2/N</td>
<td>none</td>
<td>Nod factor: $0$, $10^{-8}$ M, $10^{-7}$ M</td>
<td>1, 3, 5 days</td>
</tr>
<tr>
<td>R2/N</td>
<td>$4.5 \times 10^{-6}$ M 2,4-D</td>
<td>Nod factor: $0$, $10^{-8}$ M, $10^{-7}$ M</td>
<td>1, 3, 5 days</td>
</tr>
</tbody>
</table>

Table 3.4.: Overview of the different incubation treatments of excised root segments.

The results of these experiments confirmed the results obtained with Nod factor bombardment, i.e. 2,4-D, but not Nod factors, affect uidA expression in the transgenic roots.

3.2.7. Bombardment with Chitin fragment

The Nod factor we used for bombardment was a specific Nod factor of Rhizobium meliloti, which triggers nodulation in Medicago sativa. There is no information available, whether such a Nod factor can be recognised by monocotyledons at all. Chitin fragments, the backbone of Nod factors, are more general elicitors, inducing defence reactions in a wide variety of plants (see
Stacey and Shibuya, 1997). We bombarded two different chitin fragments, a chitotetraose and a chitopentaose, onto roots of transgenic plants, containing the MsEnod12A::uidA or MsEnod12B::uidA construct. Plants were prepared in a way similar to Nod factor bombardment. We chose either R210N or R2/N medium. Plants growing on R210N were not pre-treated, while plants on R2/N were pre-treated with 2,4-D (4.5 x 10⁻⁶ M) for one day. To avoid different expression levels resulting solely from the different viability of the plants, we dissected roots from each plant for the bombardment and half of the roots were either bombarded with chitin fragments or with empty gold particles for control. Roots were incubated for 30 min on osmoticum (10 % sucrose) in the pre-treatment medium. Before and after bombardment, 5-10 μl of the respective chitin fragment solution was applied to the roots, or water for the negative control. After bombardment, roots were left on the high osmotic medium for 24 hours. Then roots were either stained directly or returned to the pre-treatment medium for another two days. Subsequently, these explants were stained three days after bombardment. An overview of the experimental parameters is given in Table 3.5. In all experiments, the bombardment with chitin fragments did not change the expression pattern of MsEnod12A and MsEnod12B.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Pre-treatment</th>
<th>Bombardment of</th>
<th>Cultivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>R210N</td>
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<td>Chitotetraose, Chitopentaose</td>
<td>1, 3 days</td>
</tr>
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<td>1, 3 days</td>
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<tr>
<td>R2/N</td>
<td>4.5 x 10⁻⁶ M 2,4-D</td>
<td>Chitotetraose, Chitopentaose</td>
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</tr>
<tr>
<td>R2/N</td>
<td>4.5 x 10⁻⁶ M 2,4-D</td>
<td>Chitotetraose, Chitopentaose</td>
<td>1, 3 days</td>
</tr>
</tbody>
</table>

Table 3.5.: Different approaches used for the bombardment with chitin fragment.

3.2.8. Effect of VsENBP1 on early nodulins in rice

The protein ENBP1 was isolated from Vicia sativa (Christiansen et al., 1996). ENBP1 is a DNA-binding protein that interacts with a conserved sequence in the Enod12 promoter of pea. The authors suggest that ENBP1 is a potential regulator of Enod12 expression (Christiansen et al., 1996).
3.2.8.1. Bombardment of VsEnbp1 to rice, containing either MsEnod12A::uidA or MsEnod12B::uidA

Rice suspensions, derived from transgenic rice containing either MsEnod12A::uidA or MsEnod12B::uidA were produced. For the experiment, the suspensions were incubated for four days in nitrogen-free medium (R2/N). They were bombarded according to the rice transformation protocol. Per shot, 0.5 µg DNA of plasmid 35S1 (35S::Vsenbp1) or plasmid pCIB3089 (35S::bperu, negative control) was used. For each approach, five plates were bombarded. After bombardment, calli were cultivated for 20 hours on the high osmotic medium, subsequently stained in X-Gluc solution. Blue staining was very strong and no difference was detectable between the positive and negative samples during the course of the staining reaction.

3.2.8.2. Effect of VsENBP1 on transient expression of PsEnod12B in rice

If VsENBP1 is indeed a transcription activator of PsEnod12B, the bombardment of the VsEnbpl gene and the PsEnod12B together may result in a higher transient expression of PsEnod12B than the bombardment with PsEnod12B alone. Wild type rice suspension cultures were incubated for four days in medium without nitrogen (R2/N). Bombardment was performed following the rice transformation protocol. We used 0.5 µg DNA of 35S-E-EG (containing the 35S::Vsenbp1 and the PsEnod12B::uidA) or 35S-EG (negative control, containing only the PsEnod12B::uidA) per shot. After bombardment, calli were incubated for 24 hours on high osmotic medium, and subsequently stained for GUS activity. Neither with 35S-E-EG nor with 35S-EG any single blue spot was detected in the bombarded calli.

3.2.8.3. Effect of VsENBP1 on transient expression of MsEnod12A, MsEnod12B or PsEnod12b in rice protoplasts

In an initial experiment, rice protoplasts were transfected with 5 or 10 µg of pNan26 (containing MsEnod12A::uidA) or pNan10 (containing MsEnod12B::uidA) together with either 5 µg or 10 µg 35SE1 (35S::Vsenbp1) or pCIB3089 (negative control, containing the 35S promoter). No GUS activity
could be measured, neither with nor without the 35S::Vsenbp1. In a second experiment, rice protoplasts were transfected with 5 or 10 µg of 35S-E-EG or 35S-EG (Fig. 2.5.). No GUS activity could be measured, regardless of the presence of Vsenbp1.

In summary, in all the experiments with Vsenbp1, no effect of this gene was detectable in rice, neither on the expression of MsEnod12A, MsEnod12B, nor of PsEnod12B.
3.2. Auxin responsive promoter GH3

3.2.1. Bombardment of embryos and cell suspensions

In an initial experiment, 101 immature embryos were co-transformed with the plasmids pMP3631 (containing the *uidA* and *gfp* genes driven by the GH3 promoter) and pAcH1 (containing the selectable marker). Out of three regenerated plants, one contained the gene of interest in full length (line 41Z1, Fig. 3.18.d). Of the remaining two plants, one did not contain the complete construct and the third contained only a truncated copy.

In a second experiment suspensions were co-transformed with the plasmids pMP3631 and pAcH1. After bombarding 12 plates, in total 78 independent lines were regenerated. Eight of them showed extremely weak growth and died in the greenhouse. The genomic DNA of the remaining 70 plants was tested by Southern blot analysis, which proved 65 of them to be transgenic. Of the transgenic plants, 43 showed the expected band, suggesting to have incorporated the complete gene of interest. However, 24 of these 43 plants were sterile, therefore only 19 lines remained for analysis.

In a third experiment 257, immature embryos were co-transformed with the plasmids pMP3631 and pAcH1. The 36 resistant colonies were tested by GUS-staining and only those staining blue (indicating GH3 activity) were considered for further cultivation. Finally, four lines were regenerated from this experiment, showing all the expected size of the band as illustrated by Southern blot (Fig. 3.18.e).

In total 85 lines were regenerated. From those lines 48 showed the expected band by Southern blot and 24 of them were fertile (see Table 3.6.).

<table>
<thead>
<tr>
<th>Total of regenerated, independent lines</th>
<th>Tested by Southern blot to contain the complete gene of interest</th>
<th>Southern blot positive, fertile lines</th>
<th>Fertile, GUS positive lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>85</td>
<td>71</td>
<td>48</td>
<td>24</td>
</tr>
</tbody>
</table>

Table 3.6.: Summary of the regenerated lines from bombardments with pMP3631 and pAcH1.
Fig. 3.18.: a: Plasmid pMP3631, containing the auxin inducible promoter GH3, driving the uidA and the gfp genes. Indicated are the position of the probe, the two EcoR I sites to allow release of the gene of interest and the BamH I site to detect the copy number. b: Plasmid pAcH1, containing the selectable marker. c: Southern blot of 23 independent lines. DNA was digested with EcoR I to release the complete gene of interest. d: Southern blot of the transgenic line 41Z1. Shown are the T0 and the three T1 lines used for further work. e: Southern blot of the four lines generated after selection with hygromycin and on GH3 expression. Line GH3 1/1 and line GH3 1/2 are dependent, likewise lines GH3 23/1 and GH3 23/2.

- undigested DNA, E: EcoR I digestion to release the gene of interest, B: BamH I digestion to determine the copy number, pl: positive control, wt: negative control, M3: Marker M3, Boehringer.
3.2.2. Analysis of genomic DNA by Southern blot

To prove the integration of the bombarded sequences (pMP3631, Fig. 3.18.a), Southern blot analysis was performed. Genomic DNA was isolated from young leaves. In a first screening step, DNA was digested with EcoR I to release the complete gene of interest (Fig. 3.18.a). The DIG-labelled probe recognised a sequence located within the uidA gene (Fig. 3.18.a). Only lines showing the expected band of 3.7 kb were considered for further analysis. By restriction with BamH I, these plants were tested for the inserted number of copies of the construct (Fig. 3.18.a). Among the tested lines, almost all showed at least parts of the gene of interest (71 out of 85, corresponding to 84%; see Fig. 3.18.c). The few lines, which did not give a signal with the uidA probe, were probably not escapes, since they still may have contained the selection marker. However, we did not prove this. A high percentage of the transgenic lines contained only truncated copies (48 of 71, corresponding to 68%). Of the remaining lines containing the complete construct, 50% were sterile (24 out of 48; see as well Fig. 3.18.c). Only 24 lines remained for tests of the GH3 promoter activity (Table 3.6.). Finally, out of these 24 only 14 lines showed uidA expression (58%). Figures 3.18.d and 3.18.e show Southern blot analyses of the lines, which were used for the experiments. Line 41Z1 shows, besides of the complete gene of interest, at least five truncated copies (Fig. 3.18.d). Line GH3 1 contained more than ten copies, but most of them are truncated. Line GH3 6 contains four copies, three of them are complete and one is truncated. Line GH3 23 has seven copies, of which three to five are integrated complete. Two bands on the Southern blot are very faint, and make therefore a clear decision impossible. Line GH3 W2 also shows on the Southern blot a band pattern that is not fully interpretable. This line contains one complete and at least three, but most probably four, truncated copies.

3.2.3. Screening of the progeny of line 41Z1 by PCR

PCR allows testing of a high number of samples for the existence of the inserted gene in a short time. We established a screening of GH3-plants by PCR, using line 41Z1. DNA was extracted from leaf material and a PCR
performed with primers to sequences within the *uidA*-gene (Fig. 3.19.a). The result obtained for this line was a segregation of approximately 3:1 for the gene of interest. Since this line has several complete and truncated copies, this segregation ratio implies that probably all copies are integrated at the same integration site. Fig. 3.19.b shows a PCR-screening of the progeny of plant 41Z1/9. This plant proved to be heterozygous. From the progeny, 11 plants were shown to have the gene of interest and 3 proved to be segregants. All plants were tested for *uidA* expression to confirm the results obtained by PCR. The staining result verified the PCR result. In addition, it showed that the *GH3* promoter was active in all plants containing the gene of interest.

![Fig. 3.19.: PCR screening of line 41Z1/9 a: Plasmid used for transformation. Indicated are the used primers G3 and G5. b: Test of the progeny of line 41Z1/9 by PCR: the expected size of the amplified band is 530bp. 1-14: individual plants: 5, 7, 12 are not transgenic, all the others contain the gene of interest (confirmed by GUS-staining), wt1-wt4: negative control, containing DNA of 4 different wild type plants, H2O: negative control containing only water, pl: plasmid pMP3631, 1kb: 1kb ladder.](image)

### 3.2.4. *GH3* activity in embryos and calli

We studied activity of the auxin-inducible promoter *GH3* in rice. As marker for the *GH3* activity we used the *uidA* gene and visualised the expression by GUS histochemistry. One day after bombardment of embryos or suspensions, no expression could be detected by GUS-staining. Therefore, the promoter was either not active, or transient expression was too weak to give detectable *uidA* expression. After three weeks of selection with hygromycin, resistant calli were selected for plant regeneration. At this stage, we observed for the first time that the promoter is active in rice. Two different staining patterns could be observed (Fig. 3.20.). In almost all cases blue spots were visible, scattered all over the calli (Fig. 3.20.a). In only two cases the entire calli were stained (Fig. 3.20.b).
All cells should contain the gene of interest, since during regeneration of transgenic rice lines all cells of a callus originate from a single, transgenic cell. Therefore, they all should express the *uidA*-gene, even more since the regeneration mediums R2 and R2I contain 2,4-D at a concentration of 1 mg/l and 2 mg/l, respectively. In order to rule out an artefact causing this scattered expression pattern, due to non-clonal callus material, we used embryos of transgenic mature seeds from line 41Z1 to start cell cultures and cell suspensions. With this procedure we excluded that calli were chimeric. After staining these calli, we observed again the scattered staining pattern.

### 3.2.5. *Gfp* activity in calli and protoplasts

The construct we used to monitor *GH3* expression in rice contains besides the *uidA* gene also the gene encoding the green fluorescence protein (*gfp*). This marker has, compared with the GUS marker, the advantage that its expression can be monitored in living tissue.

Since GUS staining was shown in calli, we tested whether *gfp* expression is detectable as well. Therefore suspension-derived calli from the transgenic line 41Z1 were tested for *gfp* expression. The very strong auto-fluorescence did not allow detection of *gfp* expression. To eliminate background fluorescence of cell walls, protoplasts were produced from calli of line 41Z1. Again no GFP signal was detectable in these protoplasts. To check whether any activity of the *GH3* promoter would be detectable, protoplasts were stained for GUS activity. In some cells, blue staining precipitate was visible. The observation that only few of the protoplasts stained blue was consistent with the scattered staining pattern in calli. In order to exclude the possibility that the auxin concentration was too low for a significant induction of *gfp* expression, we incubated protoplasts in R2-
medium (with 13% sucrose) containing an additional amount of 2,4-D of $5 \times 10^{-8}$ M or $1 \times 10^{-6}$ M for one hour and 20 hours, respectively, but gfp expression was still not detectable.

### 3.2.6. GH3 activity in plants

#### 3.2.6.1. General staining pattern

Out of the 24 fertile, Southern positive lines, 10 showed no blue staining, neither without nor with 2,4-D treatment ($5 \times 10^{-8}$ M and $1 \times 10^{-6}$ M for 3 days). Therefore the 14 blue staining lines 41Z1, 62/28, 62 G30/1, 62/39, 62/44.3, 62/50.2, 62/53, 62G56, 73.2 2/2, 73.2/4, GH3/1, GH3/6, GH3/23 and GH3/W2 were used for further analysis.

In all 14 lines, 2,4-D treatment for 3 days ($5 \times 10^{-8}$ M and $1 \times 10^{-6}$ M) increased the activity of the GH3 promoter. Besides this common feature for all plants, two different expression patterns were observed in two groups of transgenic lines. Lines 41Z1, 62/39, 62/50.2, GH3/1, and GH3/23 showed distinct blue staining in untreated roots and a strong increase of uidA expression after auxin treatment. In the other lines, no uidA expression, or only in few cells, was visible in untreated roots. However, after auxin-treatment faint blue staining in a few cells was observed. Since in this group of transgenic lines the staining pattern was very irregular and not reproducible, we used lines of the other group, with stronger and reproducible expression, for our experiments. Lines 41Z1, 62/39, 62/50.2, GH3/1, and GH3/23 were used for expression without treatment. For further experiments, the lines 41Z1, GH3/1 and GH3/23 were considered, due to better seed set in the T0 and T1 generation.

#### 3.2.6.2. GH3 activity in stem and leaves

Young leaves were stained for 24 hours. UidA expression due to active GH3 promoter was found in the basal cell of the bi-cellular protuberances (Fig. 3.21.). No GH3 activity was detectable in the proximal cell of the bi-cellular protuberances and in the unicellular trichomes or in other parts of the stem and leaves.
Results

3.2.6.3. GH3 activity in roots

Roots of plants grown either on solid or liquid MS medium or on R2 medium were stained 24 hours for histochemical GUS activity. GUS staining was only detectable in the root cortex, in front or edgewise of lateral root primordia (LRP). Blue staining was observed in few cells (Fig. 3.22.a).

In most of the cases, four single blue cells (indicating activity of the GH3 promoter) were visible, grouped around the tip of the lateral root primordia (Fig. 3.22.a; arrows). The blue spots were located opposite the central cylinder. Hand and microtome sections confirmed that uidA expression was restricted to single cells (Fig. 3.22.c-g). Sometimes, these cells expressing uidA seemed to be destroyed by the growing lateral root primordium (Fig. 3.22.g).

Alternatively, a single blue cell or several cells forming a blue area (Fig. 3.22.b) were in front of the lateral root primordia. Very rarely blue staining cells were surrounding the tip of a lateral root primordium. As soon as the lateral root breaks through the main root, no blue staining is visible anymore. Localisation of the uidA mRNA by in situ hybridisation with a radioactive probe confirmed the results obtained by histochemical analysis of GUS activity (Fig. 3.22.h, i)
Fig. 3.22.: Activity of the GH3 promoter in rice roots. a: Low magnification view b: Microscopic view of an unsectioned root c: Handsection d-g: 4 μm microtome sections, Technovit embedded h: In situ hybridisation with the antisense probe i: In situ hybridisation, control with sense probe. Arrows indicate the location of GH3 activity.
3.2.6.4. Effect of an auxin transport inhibitor

The observed GUS activity in front of or edgewise of the lateral root primordia could be a result of an activation of the GH3 promoter either due to a higher sensitivity of its activating signal transduction chain to auxin, or due to a higher auxin concentration in these cells. Celenza et al. (1995) showed that LRP development depends on a local rise in the IAA level for continued cell viability, cell division, and the subsequent maintenance of the lateral root meristem. They suggest, that this additional IAA is either coming from the shoot meristem via an increased auxin transport, or from IAA synthesised de novo at this place. To test whether the activation of the GH3 promoter is due to increased auxin transport or due to de novo synthesised auxin, plants were grown on liquid MS-medium and the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) was added. Treatments were performed with a concentration of $1 \times 10^{-6}$ M for one or three days. This treatment induced slight changes in the root morphology. Growth of the root decreased and the induction of new LRP was inhibited. However, the expression pattern of GH3 did not change. As long as roots show lateral root primordia that did not break through the main root, the same expression pattern as in the treated control roots could be observed.

3.2.7. Effects of plant hormones

To investigate the effect of hormones on the expression pattern of the GH3 promoter, plants were grown on liquid R2-medium or MS-medium. The hormones 2,4-D, IAA, and kinetin were added in different concentrations (see below). Roots were cut off either after one or three days of treatment and stained for GUS activity for 24 hours.

3.2.7.1. Auxin treatment

Upon auxin treatment, four different regions can be distinguished on a root. These four regions are useful to describe the activity of GH3 after auxin treatment and also for other treatments. The following four regions were distinguished (Fig. 3.23.):
Results

RT-region: Root tip region; including the root cap, the meristematic region and the region of elongation. The RT-region shows a brownish colour after three days of auxin treatment.

ALR-region: Abnormal lateral root region; following the root tip region, comprising the root hair region and the region of lateral root primordia initiation. This region contains many abnormal lateral roots.

YLR-region: Young lateral root region; region with young lateral roots, which have been arrested in their development by the addition of auxin.

OLR-region: Old lateral root region; region of the old lateral roots.

Fig. 3.23.: Schematic overview of the four regions of an auxin treated rice root.

Plants were treated with 2,4-D at concentrations of $1 \times 10^{-6}$ M and $5 \times 10^{-8}$ M, and with IAA at a concentration of $1 \times 10^{-5}$ M, for one or three days. The activity of GH3 was increased much stronger by 2,4-D than by IAA, and 3 days treatment induced stronger uidA expression than only one day of treatment. In fact, IAA treatment for one day did not even produce any detectable effect. Treatment with 2,4-D ($1 \times 10^{-6}$ M and $5 \times 10^{-8}$ M) for one and three days and treatment with IAA ($1 \times 10^{-5}$ M) for three days had the same effect on GH3 activity. Effect of auxin on GH3 activity was observed mainly in the ALR-region and the YLR-region (Fig. 3.24.b,c). In the OLR-region only rarely and, if any, a weak reaction was visible (Fig. 3.24.d). This is in agreement with the morphological reaction to auxin treatment, which was also strongest in young root parts, and almost not visible in older root parts. In general, different roots of the same plant showed very different levels of uidA expression levels. However,
the expression pattern was the same in all roots, even though roots of weaker plants did almost not react to auxin.

The RT-region shows no staining of the root cap and in the meristematic region, followed by a band with strong staining (Fig. 3.24.a). Strongest GH3 expression was observed in the ALR-region (Fig. 3.24.b). GH3 expression was induced in all tissues with the exception of the epidermal layer and the ALRs.

Fig. 3.24.: Expression of the GH3 promoter in rice roots treated with auxin. a-d: treated with $1 \times 10^{-5}$ M 2,4-D for 1 day, e, f: treated with $1 \times 10^{-6}$ M 2,4-D for 3 days, arrows indicate the auxin-induced abnormal lateral roots.

The hand cut sections confirmed this expression pattern (Fig. 3.25.a, b). Strongest GH3 activity occurred in the cortical parenchyma. Activity was also visible in the solorenchyma in the region of the ALR and very rarely in the exodermis. The endodermis showed little uidA expression, whereas no expression was detectable in the central cylinder. Neither the epidermis nor the ALRs showed any activity of the GH3 promoter. In addition to hand cut sections, microtome sections were performed for several reasons. First, microtome
sections have a higher resolution than hand sections, due to lower thickness and the embedding of the material. Second, with hand sections it was almost impossible to get useful sections through lateral or abnormal lateral roots, most probably due to the different density of these structures compared to the neighbouring tissue. Embedding of material in plastic solved this problem. Microtome sections generally confirmed the observations made with hand sections (Fig. 3.25.d,e). GH3 activity was observed in the cortical parenchyma, the endodermis and very rarely in the exodermis. In the solorenchyma, uidA expression was located mainly opposite of induced ALRs. Abnormal lateral roots did not show any GH3 activity. In contrast to the observed uidA expression pattern in hand sections, the central cylinder showed clear blue staining in the plastic embedded, microtome sectioned samples. Within the central cylinder, the pericycle and the protoxylem elements were stained most strongly.

In the YLR-region the GH3 promoter reacted little to the auxin treatment (Fig. 3.24.c). The GH3 promoter was activated only little in the main root and in the lateral roots, there similar to the main root. Occasionally, ALRs were formed. In this case, in the tissue around these ALRs the GH3 promoter was activated.

In the OLR-region only the tips of the LRs were stained, and in addition, single solorenchyma cells of the main root (Fig. 3.24.d). Very rarely ALRs were formed on the lateral roots, then the GH3 promoter was activated in the cortical tissue around these ALRs.

Figures 3.24.e and f show the RT-region and the ALR-region of roots incubated for three days with 2,4-D. The roots showed much bigger ALRs and the GH3 promoter was more strongly activated than after one day of auxin treatment. However, the general staining pattern was similar to that of roots treated for one day. We found only one exception: behind the root tip and the very strong blue staining band a long region followed, which showed a brownish colour and did not stain. The entire remaining staining pattern (overview and cross sections) was similar to that observed after one day of 2,4-D treatment.

In summary, upon auxin application, the GH3 promoter was activated in the cortical parenchyma of transgenic rice plants. To a lower extent, the GH3
Results

Fig. 3.25.: Activity of the GH3 promoter in rice roots treated with $1 \times 10^{-6}$ M 2,4-D for 1 day. a-c: hand sections in the early (a, b) and late (c) ALR-region, d, e: 4 μm microtome sections in the late ALR-region. *: GH3 activity in the cortical parenchyma, +: GH3 activity in solorenychma cells, arrowheads mark the activity in the central cylinder.

promoter was also active in the solorenychma, the endodermis and the central cylinder.
3.2.7.2. Kinetin treatment

Kinetin treatment (1 x 10^-6 M) of the rice plants inhibited the formation of new lateral root primordia completely. Emergence of already induced LRPs was not affected. After three days of kinetin treatment, no lateral root primordia exist and no GH3 activity was detectable anymore. After one day of kinetin treatment, LRP that had not broken through the main root were still observed. GH3 activity was observed edgewise or in front of these LRPs, as observed in untreated roots.

3.2.7.3. GA3 treatment

Treatment with GA3 at a concentration of 1 x 10^-6 M for three days did not change the expression pattern compared to the expression pattern of untreated roots.

3.2.8. Activity under Nitrogen-starvation

Plants were grown on medium without nitrogen (R2/N). During the time of the experiment, seedlings grew almost normally, with only a weak bleaching of the leaves and a slightly reduced growth. In rice kernels, the aleurone layer is the site of protein storage. Due to a low germination frequency, a number of the seedlings had to be rescued, i.e. the embryos had to be pulled out of the seed coat. These seedlings showed clear effects of N-deficiency, since the nitrogen in the storage protein of the aleurone layer was no longer available. The phenotype was a loss of chlorophyll and finally growth was almost completely inhibited. In case plants showed normal growth despite the low nitrogen content in the medium, the basic uidA expression pattern and the expression upon hormone application were similar to the expression pattern on R2 medium. Plants that showed clear N-deficiency symptoms showed no uidA basic expression and no expression after treatment with kinetin and GA3 and TIBA. In contrast, they showed still auxin induced uidA expression, but to a much lower level than in plants grown on R2 medium.
3.2.9. Activity of the GH3 promoter during plant regeneration from bombarded suspensions or embryos

After bombardment of suspensions or embryos, no transient GH3 activity was detectable. However, after selection with hygromycin, and subculturing of resistant calli on callus increasing medium (R2I) or into liquid culture medium (R2), GH3 activity was detectable in these resistant calli. In most of the calli, GH3 activity was scattered, visible by a scattered distribution of blue precipitate (Fig. 3.20.a, 3.26.a). Even though the R2I and the R2 medium contain high concentrations of auxin (9 x 10^-6 M and 4.5 x 10^-6 M 2,4-D, respectively), not all cells expressed uidA at the same level. Hence we supposed that the cells expressing uidA must have characteristics distinct from cells not expressing uidA.

In primary transformants, as a result of chimeric calli, we can not exclude scattered distribution of GH3 expression. Therefore we induced clonal callus material from the scutellum of mature embryos of the transgenic line 41Z1. Histochemical demonstration of GUS activity in these calli resulted in the same scattered distribution of blue spots as observed already in T0 calli. Therefore we concluded that an endogenous factor was responsible for this scattered GH3 promoter activity in calli. In order to study the reason for this scattered distribution of GH3 activity, we made hand and microtome sections of callus material from the selection medium R2 and the callus increasing medium R2I (Fig. 3.26.).

In most cases, blue parts in calli proved to be cells contributing to a distinct structure. Some features are common in all sections: (i) uidA expressing cells were usually part of a distinct structure, clearly distinguishable from the surrounding tissue, (ii) blue cells (showing GH3 promoter activity) were in general large, compared to the surrounding, not staining cells, (iii) structures where GH3 was active were usually located at the periphery of the calli.

In most of the cases the blue staining structures looked strikingly similar to globular somatic embryos (Fig. 3.26.b, e, g, h; arrows). The structure in Fig. 3.26.h (arrow) consisted of four big and six small cells. This looked like a four-cell proembryo (asterisk) with a six-cell suspensor. The distribution of the
Fig. 3.26.: Activity of the GH3 promoter during somatic embryogenesis. All pictures are from calli cultured on R2I or R2 medium. a: low magnification view of a callus, b, c: Hand sections, d-h: microtome sections (4 μm). b: arrow: globular embryo, c, d: arrowhead: shoot apex of a mature somatic embryo. The sections show the same structure, as a hand section (c) and as a microtome section (d), e: arrow: globular embryo, uidA expressing, arrowhead: globular embryo, not expressing, f: arrow: globular embryo, asterisk: 4-cell proembryo, g: arrow: globular embryo, asterisk: distinct, uidA expressing structure, h: arrow: somatic globular embryo, asterisk: four-cell proembryo.
indigo precipitate is remarkable. Obviously, this four-cell proembryo just arose from a two-cell proembryo. The two mother cells showed strong GUS staining, whereas the two daughter cells showed weak staining. Their precipitate is most probably inherited from the mother cells.

In some cases, the interpretation of the blue staining structures was not clear (Fig. 3.26.g; asterisk). However, even in this case, the uidA expressing cells seemed to be part of a distinct structure, distinguishable from the surrounding tissue. For the interpretation of such a structure, one had to take in account that an observed structure, depending on the relative orientation of the plane of the section, can be sectioned in any angle.

Nevertheless, most of the observed uidA expressing structures clearly resembled globular embryos, consisting of a proembryo and a suspensor (Fig. 3.26.e, g; arrows). However, not all morphological structures resembling a proembryo showed GH3 activity (Fig. 3.26.e; arrowhead).

In some sections, the blue staining structures exposed apparent similarity to the shoot apex of a somatic embryo (Fig. 3.26.c, d; arrowhead). In these sections, the structure of an almost mature embryo as described by Jones and Rost (1989) was clearly visible. The structure shown in Fig. 3.26.f can be considered as a GH3 expressing globular embryo (arrow), embedded in a globular structure (asterisk). On the other hand, the whole structure can also be considered to represent a somatic embryo with the blue-staining shoot apex (arrow).

In summary, most of the observed uidA expressing structures were globular somatic embryos. In some cases, the expressing structure seemed to be the apex of a mature somatic embryo.
4. Discussion

4.1. Comparison of lateral roots and auxin-induced structures

So-called nodule-like structures have been described for a number of monocotyledonous and dicotyledonous plant species. Nodule-like structures in monocots have been reported from rice (Ridge et al., 1993), wheat (Ridge et al., 1992a), and maize (Christansen Weniger and Vanderleyden, 1994b). Other names used for these structures are para-nodules (Gantar and Elhai, 1999; Elanchezhian and Panwar, 1997; Zeman et al., 1992; Tchan et al., 1991) or nodule-like tumours (El Khawas and Adachi, 1999; Christiansen Weniger, 1996).

The terms ‘nodule-like structures’, ‘para-nodules’, or ‘nodule-like tumours’ imply, due to the term ‘nodule’, a similarity in structure, and/or in function, to the known nodules of legumes, Sesbania, Parasponia, or actinorhizal plants. We studied anatomy and development of 2,4-D induced structures in rice roots in order to clarify whether these induced structures have any similarity to the known nodules. Cross-sections through auxin treated rice roots show clearly that the induced structures have their origin in the central cylinder, within the pericycle as well as in cell layers between two xylem vessels. This is a clear difference to the nodules known from legumes. In legumes, nodules originate either in the inner cortex (indeterminate nodules) or in the outer cortex (determinate nodules) (Newcomb et al., 1979). Sesbania rostrata develops determinate nodules on the roots and the stems. Root nodule primordia develop in the mid-cortex (Ndoye et al., 1994). Stem nodule primordia are formed in the mid-cortex of the dormant adventitious rootlets (Duhoux, 1984; Tsien et al., 1983).

Nodules of Parasponia and actinorhizal plants originate from the vascular tissue. In Parasponia, nodule primordia are formed in the pericycle of the root (Pawlowski and Bisseling, 1996). Parasponia nodules are indeterminate. In contrast to the legume nodules, they have a single central vascular bundle, surrounded by the central nodule tissue and therefore resemble deformed lateral roots. In actinorhizal plants, nodule primordia develop in the root pericycle (Pawlowski and Bisseling, 1996). This is the location of lateral root formation, and similar to lateral roots, actinorhizal nodules are indeterminate.
Therefore, the development of auxin-induced structures in rice shows some similarity to the nodules of *Parasponia* and actinorhizal plants. However, nodules of *Parasponia* and actinorhizal plants still resemble lateral roots and are only called nodules due to their function as a site of nitrogen fixation. Although several reports describe auxin-induced abnormal lateral root development in monocots (Ridge et al., 1992a; Francisco and Akao, 1993; Ridge et al., 1993; Christansen Weniger and Vanderleyden, 1994b; Christiansen Weniger, 1996), we are not aware of any proven *Rhizobium* association or nitrogen fixation within these structures. Therefore we conclude that the terms ‘nodule-like structures’, ‘para-nodules’, or ‘nodule-like tumours’ are misleading and suggest to use the term ‘abnormal lateral roots’ (ALRs). This term does not imply the association with nodules. The origin of these structures in the central cylinder speaks additionally in favour of the term abnormal lateral roots. The place of the induction, opposite to the protophloem vessels, is the same as the one of lateral root primordium induction in rice (Matsuo and Hoshikawa, 1993). We call these structures abnormal, since they do not show a clear internal organisation within ten days of $1 \times 10^{-6}$ M 2,4-D treatment and after 36 days of $4.5 \times 10^{-5}$ M 2,4-D treatment. In addition, the treatment with low IAA concentration ($5 \times 10^{-8}$ M) induced structures which resembled in their shape clearly lateral roots, however, these structures slowed down in their growth and became stunted. The reported internal organisation of the induced structures (Ridge et al., 1993) after treatment with $1 \times 10^{-5}$ M 2,4-D for several weeks was not detectable in our hands.

4.2. *MsEnod12A* and *MsEnod12B*

4.2.1. Activity of *MsEnod12A* and *MsEnod12B* in rice roots

Both promoters, *MsEnod12A* and *MsEnod12B* are active in rice. This confirms preliminary results reported by Terada et al. (1996). The patterns of expression of the *uidA* gene directed by the two promoters were similar. *MsEnod12A* is active in the root cap, the elongation zone, in root hairs, in the cortical parenchyma around lateral root primordia, the central cylinder, and weakly in the root tip. Lateral root primordia and young lateral roots do not express or only
Discussion

to a low extent, whereas lateral roots show the same expression pattern as the main root. The expression pattern of the uidA gene directed by the MsEnod12B promoter is largely the same as the one directed by the MsEnod12A promoter. Exceptions are the lateral root primordia, young lateral roots, and the root tip, where the MsEnod12B promoter directs a stronger activity.

In alfalfa, MsEnod12A activity is linked to meristematic activity (Bauer et al., 1997). Using a MsEnod12A::uidA construct, Bauer and co-workers showed transient activity of the promoter at the initiation site of lateral roots during the time cortical and endodermal cell layers surrounding the dividing pericycle cells were participating in lateral root primordium formation (Bauer, 1995). The promoter was active in all cells of the primordium. At a more advanced stage, MsEnod12A activity was found only in the root tip. The promoter was active in the region of the root meristem, the young differentiating root cells in front of the meristem as well as the quiescent centre and in few cells differentiating presumably into root cap cells. Sections showed that MsEnod12A was active in all cells of the still undifferentiated root apices as well as in the beginning differentiation zone of the root axis, but not in the elongation zone.

In Medicago sativa, MsEnod12B is expressed mainly in nodules (Bauer, 1995). Weak expression was found in roots, flowers, stems and leaves. Unfortunately, Bauer (1995) did not describe the expression pattern in roots in more detail.

Enod12 proteins are thought to have a function during cell wall synthesis (Scheres et al., 1990a; Bauer et al., 1994). In rice, the expression of MsEnod12A and MsEnod12B is observed mainly at places were new cell walls are formed.

It is obvious that cell wall synthesis takes place in emerging root hairs, likewise in lateral root primordia. The differentiation and elongation zones are also sites of cell wall synthesis. How can the observed activity of the two promoters in the cortex around lateral root primordia be explained? The consequences of lateral root emergence in the cortex are not clear yet (for an overview see Charlton, 1996). In Cucurbita maxima, cell divisions occur in the young endodermis and spread outward in adjacent layers of the cortex (Mallory, 1968). This process of dedifferentiation results in several layers of cortical cells becoming incorporated into the primordium. Only during the final stage of lateral root development the
primordium appears to break through the remaining cortical layers by physical pressure. In cereal roots, the lateral root epidermis and parent root cortex form a graftlike connection with considerable lignification (McGully, 1987). Therefore it is conceivable that in the endodermis and the root cortex around a developing lateral root primordium the cells synthesize cell wall material. This explains the activity of MsEnod12A and 12B in the vicinity of lateral root primordia. In the central cylinder of young roots, xylem and phloem cells still divide and elongate. In this part of a root, new xylem and phloem vessels are generated. This may explain the observed MsEnod12 activity in the central cylinder. Moreover, it explains the inactivity of the MsEnod12B promoter in the central cylinder of the old lateral root zone, were new vessels are no longer created.

*Enod* genes were originally supposed to be activated only during nodulation (Van Kammen, 1984). However, some of these genes are also expressed in non-symbiotic organs of legumes (Scheres et al., 1990a; Kouchi and Hata; 1993; Bauer, 1995). In addition, several studies report the existence of homologues of the *Enod* genes in other plant species, such as *Enod40* in tobacco (Van De Sande et al., 1996), *Enod40* and *Enod93* in rice (Kouchi et al., 1999; Reddy et al., 1998a). In addition, Reddy et al. (1999) found a widespread occurrence of *Enod* homologues in *Oryza* species and related grasses. The detection of *Enod* homologues in a wide variety of plant species including dicots and monocots suggests: (i) a biological function which is not restricted to nodule organogenesis (Reddy et al., 1999), (ii) that these homologues are derived from ancestral *Enod* genes and that the subsequent evolution of the *Enod* genes with nodule-specific symbiotic functions resulted after the separation of dicots from monocots (Reddy et al., 1999). Therefore one may assume that the expression pattern of MsEnod12A and 12B in rice reflects the putative expression pattern of the endogenous *Enod12* gene of rice. A homologue of the *Enod12* in rice has indeed been reported (Reddy et al., 1999), but nothing is known about its expression pattern.

MsEnod12B is strongly active in the root tips of transgenic rice plants, whereas MsEnod12A is only weakly active there. This is somewhat surprising, since cell walls are synthesised in root tips. In *Medicago sativa*, the *Enod12A* promoter is indeed active in the root meristem. Since we have studied these promoters in a
heterologous system, it is possible that they are differently expressed in rice and in *Medicago*. Apparently, in rice the *MsEnod12A* and *12B* promoter expression is associated with cell division, differentiation and cell elongation, whereof the *MsEnod12B* promoter activity is higher during cell division than that of *MsEnod12A*.

In auxin induced abnormal lateral roots of transgenic rice, cell divisions are frequent. However, we did not detect any *MsEnod12A* or *12B* promoter activity. In contrast, in transgenic *Medicago sativa* containing *MsEnod12A:*uidA, auxin-induced lateral root primordia and lateral root bumps stained blue (Bauer, 1995). In dicotyledonous plants, auxin induces lateral root formation and in higher concentrations lateral root-derived outgrowths. In rice, 2,4-D in the concentrations applied induces abnormal lateral roots, growing in a non-organised way. It might be that in these structures signals are missing which are able to activate the *MsEnod12* promoter.

Surprisingly, *MsEnod12A* and *12B* are much more active in the heterologous system rice than in the homologous system alfalfa. Possibly, in the heterologous system rice, the two promoters get activated by a signal, which is in general active during cell wall synthesis. In contrast, in alfalfa the two promoters have a more specific function during nodulation related cell wall synthesis. This would explain the observed strong expression in rice.

The pattern of the *MsEnod12B* promoter activity in rice differs from the pattern found earlier by Terada *et al.* (pers. comm.). In that case, in the Japonica rice variety Kinuhikari transformed with *MsEnod12B:*uidA, GUS expression was mainly restricted to the central cylinder, with occasionally weak staining in the cortical parenchyma. The *MsEnod12B* promoter was not active in lateral root primordia and young lateral roots. The reasons for the differences between our results and those of Terada *et al.* are not clear. One explanation might be that different rice varieties have been used. Alternatively, positional effects of the integration site might also provide an explanation. However, the observations were made with several independent transgenic lines. This makes it unlikely that positional effects play an important role. A third possible explanation could be the different constructs used for transformation. Terada *et al.* co-bombarded the two plasmids pPB12B and pAcH1, whereas we used the plasmid pRIB9. In
this plasmid, the gene of interest and the selectable marker reside in the same construct. The selectable marker, driven by the strong *actin* rice promoter, is located upstream of and in the same direction as the gene of interest. We can not completely rule out an influence of this promoter on the gene of interest which is located downstream. Meanwhile constructs are available carrying the gene of interest upstream of the selectable marker.

Reddy *et al.* (1998b) report that *MtEnod12* is expressed in transgenic rice only under nitrogen limiting conditions. We were not able to confirm this differential expression as a function of the amount of nitrogen in the medium. Since we assume that in rice the *Enod12* has a function in cell wall synthesis, it is not surprising that this gene is expressed independent of the nitrogen supply of the plant.

### 4.2.2. Effect of Nod factor and chitin fragments

Bombardment of the Nod factor *NodRm-IV*(C16:2,Ac,S) to rice roots did not affect the basic expression at all, neither that controlled by the *MsEnod12A* promoter nor that controlled by *MsEnod12B* promoter. Incubation of dissected root sections in Nod factor solution did also not change the expression pattern. Our results contrast to those of Reddy *et al.* (1998b) and Terada *et al.* (pers. comm.). Earlier experiments conducted in our lab showed that Nod factor application to rice transformed with *MsEnod12B::uidA* led to a detectable response in the expression pattern of *MsEnod12B* (Terada *et al.*, pers. comm.). Plants were grown under nitrogen-limiting conditions and the Nod factor response was obtained only after a pre-treatment with auxin and when the Nod factor was delivered by microprojectiles. These results indicate that Nod factor may have an effect once delivered into the tissues or cells, but that cell walls and cell membranes could be part of a specific barrier for Nod factors (Terada *et al.*, pers. comm.). Furthermore, these results suggest a function of auxin in this interaction. Simply applying of Nod factor with or without auxin pre-treatment, or delivering the Nod factor by microprojectiles without auxin pre-treatment did not change the expression pattern. Unfortunately, plants regenerated in the experiment of Terada *et al.* turned out to be sterile and
consequently the lines were lost. Therefore it was not possible to explore the
differences between these lines and our lines.

One of the reasons for the observed differences might be the different plasmids
used for transformation (see above). A second possibility might be the different
rice variety used in the experiment. Terada et al. (pers. comm.) used the
Japonica rice variety Kinuhikari, whereas we used the Japonica rice variety
TP309. A third possibility could be that the used Nod factor was no longer
intact. However, we examined the used Nod factor by HPLC to exclude this
possibility. A fourth and most probable possibility might be an effect we
observed with the *MsEnod12B::uidA* construct: The activity of the promoter
changes during root development. In the younger root (which we usually
investigated), the activity was as described (Fig. 3.11.). Briefly, the central
cylinder shows clear *MsEnod12B* activity (Fig. 3.12.a). In older root parts the
activity of the promoter was in general much weaker. If *uidA* expression was
detectable at all, it was mainly in the cortical parenchyma and no expression
was detectable anymore in the central cylinder (Fig. 3.12.b). This change in the
expression pattern was detectable independent of the auxin pre-treatment and
Nod factor bombardment. Terada et al. (pers. comm.) reported a change in
*MsEnod12B* activity from the central cylinder to the cortical parenchyma after
Nod factor bombardment. It is conceivable that the observed change was a
function of root development rather than the result of Nod factor bombardment.

In our laboratory, differential display was performed to monitor changes in gene
expression upon bombardment of rice roots with the same Nod factor as used
in this work (S. Goormachtig, pers. comm.). The same conditions as used by
Terada et al. (pers. comm.) were chosen. In all experiments, no significant
changes in gene expression were detectable in response to Nod factor
treatment.

In conclusion, two independent experimental approaches of monitoring effects
of Nod factor treatment on rice roots were not able to show any effects.

Different conclusions can be drawn from this: (i) rice is not able to react to the
used Nod factor. This means either that rice is not at all able to react to any Nod
factor, or solely not to the specific Nod factor we used. Testing different, mainly
broad range, Nod factors, should provide an answer to this question. (ii) the
effects the Nod factor we used had on rice were too marginal to be detected by a changed expression pattern of \textit{MsEnod12} or by differential display. The Nod factor used, NodRm-IV(C16:2, Ac, S), is a specific Nod factor from \textit{Rhizobium meliloti}, which triggers nodulation in \textit{Medicago sativa}. This Nod factor was used because, in earlier experiments an effect, on \textit{Enod12B} activity in transgenic rice has been reported (Terada \textit{et al.}, 1996). However, it is not known whether rice possesses a receptor for such a specific Nod factor. The backbones of Nod factors consist of chitin fragments of varying length. Chitin fragments are known to induce defence responses or related cellular responses in many monocots and some dicots (Roby \textit{et al.}, 1987; Ren and West, 1992; Kaku \textit{et al.}, 1997). For rice, several reports of chitin fragment-induced cellular responses are known (see in Stacey and Shibuya, 1997). It was argued that Nod factors could have co-opted a plant defence response pathway, originally responding to chitinaceous or other elicitors (Long, 1996). Therefore chitin fragments may be more general elicitors than Nod factor. However, treatment of rice roots with a chitotetraose or chitopentaose did not change the expression pattern of \textit{uidA} controlled by the promoters of \textit{MsEnod12A} or \textit{MsEnod12B}.

Reddy \textit{et al.} (1998b) transformed three rice varieties (TP309, Chinsurah Boro, and IR58) with the \textit{MtEnod12::uidA} construct (Pichon \textit{et al.}, 1992). Under nitrogen-limiting conditions, normally no \textit{MtEnod12} expression was observed. However, incubation of whole roots or excised root segments in a mixture of NodNGR factors induced expression specifically in cortical parenchyma in the elongation zone behind the root tip and at the sites of lateral root emergence (Reddy \textit{et al.}, 1998b). In the presence of combined nitrogen, independent of the treatment with NodNGR factors, no \textit{MtEnod12} expression was found at all. These results implicate that rice roots perceive NodNGR factors, and that these rhizobial signal molecules are able to efficiently mediate the activation of \textit{MtEnod12::uidA} in nitrogen-starved rice roots. We have asked for seeds of the transgenic TP309 lines published in Reddy \textit{et al.} (1998b) in order to investigate the effects of the Nod factor and chitin fragments we used for our experiments on these lines. The seed material of the transgenic lines is presently being multiplied and the progeny screened for homozygous lines. Before any seed material of \textit{MtEnod12} expressing lines will be delivered, homozygous lines will
have to be identified and more seed material generated. The T1 progeny of the
published lines showed no MtEnod12 activity anymore, with or without
treatment with Nod factors (Swapan K. Datta, IRRI, Manila Philippines, pers.
comm.). The published experiments (Reddy et al., 1998b) were performed with
regenerated transgenic plants (T0 lines). This laboratory will continue to
investigate the gene expression or gene silencing in the progeny of these T0
lines (pers. comm.). Therefore the results published by Reddy et al. (1998b) still
need to be confirmed.

Additionally, one of the first reports of Nod factor action in non-legumes (Röhrig
et al., 1995) was recently seriously questioned (Schell et al., 1999). Following
the discovery of scientific fraud in the Department of Genetic Principles of Plant
Breeding at the Max Planck Institute for Plant Breeding in Köln, a wide-ranging
group of researchers was assembled to repeat some key experiments (Schell et
al., 1999). Among other findings, in their experiments Nod factors were unable
to promote auxin- or cytokinin-independent cell divisions in tobacco protoplasts,

In conclusion, most of presently known effects of Nod factors in non-leguminous
plants (Röhrig et al., 1995 1996; Reddy et al., 1998b; Terada et al., pers.
comm.) need proof or confirmation.

4.2.3. VsEnbp1

The DNA-binding protein ENBP1 from *Vicia sativa* (VsENBP1, Christiansen et
al., 1996) reportedly affects the expression of the early nodulin PsEnod12B in
transgenic *Vicia hirsuta* roots (Hansen et al., 1999). The authors propose that
proteins corresponding to ENBP1 will interact in a similar manner with *Enod12A*
in *Medicago sativa* and pea as well as with *Enod12* in *Medicago truncatula*. The
two *Enod12* promoters have homologous sequences as are found in the
ENBP1-binding site of the *PsEnod12B* promoter. However, these corresponding
proteins are not yet known. Therefore, we wanted to test whether ENBP1 of
*Vicia hirsuta* can influence *MsEnod12* expression in transgenic rice.

In a first experiment, cell suspensions of transgenic lines containing the
*MsEndod12A* or *MsEnod12B* promoter were bombarded with 35S::VsEnbp1. In
a second experiment, the constructs, used by Hansen et al. (1999) in their
experiments, were bombarded to rice wt-suspensions. In a third experiment, protoplasts were transfected with the same constructs, and in the last experiment, protoplasts were transfected with MsEnod12A::uidA or MsEnod12B::uidA, with or without 35S::VsEnbp1. In all these experiments, no effect of VsEnbp1 was detectable.

Cell suspensions of transgenic lines containing either MsEnod12A or 12B showed very strong activity of these promoters. Therefore, even if VsEnbp1 had a small effect, changes could most probably not have been detected. In all the other experiments, no expression of the promoters was visible at all.

After bombarding of MsEnod12A- or 12B::uidA to embryos or cell suspensions during rice transformation, no transient GUS staining was detectable. Apparently, these promoters (and therefore probably also the PsEnod12B promoter) are transiently not or only very weakly active. We conclude that if VsEnbp1 should have an activating effect on these promoters, it is too weak to be detected in transient. Stable transformation might be an alternative to test the effect of VsEnbp1 on MsEnod12A or 12B. However, this approach is quite dubious: (i) we do not know whether VSENBP1 has an effect on MsEnod12 at all, since the original experiment was performed with VSENBP1 and PsEnod12. Therefore, the effect must be shown in transient, before the effort for stable transformation is worthwhile, (ii) basic activity of the MsEnod12 promoter in rice is rather high. Therefore, a small activating effect of VSENBP1 might be hardly distinguishable from the basic expression of MsEnod12. However, a change of the expression pattern due to VSENBP1 should be detectable.

For stable transformation with VsEnbp1, it is advisable to transform rice with the two plasmids used by Hansen et al. (1999). The plasmid 35S-EG contains the PsEnod12::uidA construct. Plants transformed with this plasmid would be the negative control. In addition, these plants provide information on the activity of PsEnod12B in rice and how the promoter is expressed compared with MsEnod12. Plants transformed with plasmid 35S-E-EG, which contains besides PsEnod12::uidA also 35S::VsEnbp1, would give the information about a possible effect of VSENBP1 on PsEnod12B expression in rice.
4.3. Auxin responsive promoter GH3

4.3.1. GH3 activity in plants

We have shown that the auxin-inducible soybean promoter GH3 is expressed in transgenic rice and that the promoter is inducible by external auxin application. Until now, the GH3::uidA construct was proven to be active in the dicotyledonous plants tobacco and white clover (Hagen et al., 1991; Larkin et al., 1996). In our work we report for the first time that the GH3::uidA construct is active also in a monocotyledonous plant. Further, we showed that this construct responds to application of external auxin and offers a molecular tool to study effects of auxin in rice. The role of the GH3::uidA construct in white clover and tobacco as a useful marker to study hormone-mediated differentiation processes has already been mentioned (Larkin et al., 1996; Li et al., 1999).

In untreated rice roots GH3 expression was only detected edgewise and in front of lateral root primordia, before they break through the epidermis of the main root. Two lines of evidence suggest that indole-3-acetic acid (IAA) is a signal for lateral root initiation in planta. First, it has been known for 50 years that exogenous application of IAA induces the formation of lateral roots (Torrey, 1950; Blakely et al., 1982). Second, transgenic plants that overexpress the bacterial IAA biosynthetic genes have increased lateral root production (Klee et al., 1987; Kares et al., 1990). Sussex et al. (1995) suggested that the formation of a lateral root occurs in two distinct developmental steps. A primordium is formed, followed by a subset of cells within the primordium which begin to function as the apical meristem for the lateral root. In agreement with this, a model was proposed in which IAA is required for at least two steps in lateral root development (Celenza et al., 1995): First, to initiate cell divisions in the pericycle, and second, to promote cell divisions and maintain cell viability in the developing lateral root. In order to induce the pericycle to undergo initial cell divisions that create the primordium, IAA is presumed to be transported from other parts of the plant (Charlton, 1996). This auxin probably originates from the shoot apical meristem (Zhang and Hasenstein, 1999). Celenza et al. (1995) suggested that in the second step a lateral root primordium either must produce its own IAA or the primordium requires increased import of IAA. The lateral root
primordium depends on this local rise in the IAA level for continued cell viability, cell division, and the subsequent maintenance of the lateral root apical meristem (Celenza et al., 1995). From experiments with cultured root segments of *Haplopappus ravenii* it was reported that NAA was required throughout lateral root induction (Blakely et al., 1972).

Maclsaac et al. (1989) also reported that after exogenous application of NAA, auxin-induced differentiation of lateral roots is a two step process in primary roots of lettuce seedlings. High auxin levels were required to induce cell divisions in the pericycle. However, the subsequent cell divisions to form the lateral root primordia were auxin independent. In agreement with these findings, Pelosi et al. (1995) reported that in *Eucalyptus* seedlings, tissues had to be removed from the auxin containing medium for an efficient conversion of auxin-induced lateral root primordia to lateral roots.

In transgenic white clover roots, the GH3 promoter was active mainly in the xylem parenchyma, but also in other tissues of the central cylinder (Larkin et al., 1996). During lateral root formation, GH3 activity was first visible in pericycle cells in front of xylem elements before cell division (Rolfe et al., 1997). Later, the dividing pericycle cells also showed GH3 activity. After the initial primordium formation in the pericycle, the dividing cells no longer expressed of the GH3::uidA gene construct. Instead, cells in the outer cortex, in front of the growing lateral primordium, expressed GUS. A developing lateral root invariably grew towards and through this area of GUS expression. These observations partially overlap with our observations. In rice roots, we observed GH3 activity only in few cells edgewise or in front of lateral root primordia. In contrast to the GH3 activity in soybean, and to the GUS staining in transgenic clover, we never found any GH3 activity in the central cylinder in rice roots. This could be due to a lower amount of auxin in rice as compared to white clover and soybean, or due to a lower sensitivity of the signal transduction pathway which recognises auxin and activates the GH3 promoter. The blue staining in pericycle cells prior and during lateral root primordia induction was not observed in rice. Here again this could either be due to a lower auxin concentration in rice as compared to white clover, or more likely due to a lower sensitivity of the GH3 promoter in the heterologous system rice. During lateral root primordium growth, blue cells in
the outer cortex were present in rice, as already described in white clover (Larkin et al., 1996; Rolfe et al., 1997). In contrast to white clover, fewer cells were involved, but the lateral root was growing through this cell group as well. This indicates a higher sensitivity to auxin in this region, or, more likely, a higher auxin concentration in these cells. In the proposed two step model of lateral root growth by Celenza et al. (1995), the elevated auxin level is necessary in the lateral root tip, after the lateral root has penetrated the epidermis of the main root. Our results and the data of Larkin et al. (1996), suggest however, that already after lateral root primordium formation, a higher auxin concentration can be found in the cortex, indicating a function of auxin in the continuing lateral root developing process. These findings are in contrast to results of Maclsaac et al. (1989) and Pelosi et al. (1995), who reported that subsequent development of lateral root primordia was auxin independent. However, in both publications the conclusions were based on external application of auxin. Under these circumstances, the auxin concentration is increased in all cells that are accessible by diffusion. Such a high auxin concentration could cover an endogenous polarity of auxin distribution. Therefore, the data of these authors are not necessarily in contrast to our observations and those of Larkin et al. (1996), of higher endogenous auxin levels, restricted to few cells in the cortex.

A possible explanation for the function of this higher auxin concentration in the cortex has been proposed by Larkin et al. (1996). They suggested that in white clover a lateral flow of auxin from the central cylinder to the outer cortex is required for lateral root initiation and growth. However, rice treated for one day with the auxin transport inhibitor TIBA still showed blue cells in the outer cortex, indicating that the GH3 promoter is active. This speaks in favour of an auxin production in these very cells, rather than of a lateral flow of auxin. Therefore, one can speculate that the higher auxin concentration in the outer cortex is not important for the lateral root initiation, but may later function as a polarity signal for the growing lateral root primordia.

As mentioned above, Pelosi et al. (1995) reported that for an efficient conversion of lateral root primordia to lateral roots, tissue must be removed from medium containing high auxin concentration. Our results showed that rice grown on medium containing a high 2,4-D concentration forms abnormal lateral
roots which do not develop into normal lateral roots. On such a medium, there is no polarity signal for the lateral root primordium, since the auxin concentration is likely to be high in all cells. Missing a polarity signal, the auxin induced lateral root primordia continue dividing in all directions and form a callus like structure, instead of converting into lateral roots. This also supports the assumption that the high auxin concentration in these cells in the outer cortex act as a guide for lateral root growth. After penetrating the epidermis, the lateral root would be further dependent on a high auxin concentration as suggested by Celenza et al. (1995), who assumed that the auxin could be synthesised in the lateral root tip. During TIBA treatment of rice roots, no new lateral root primordia were initiated. This is in agreement with the hypothesis that shoot-derived auxin is the source for induction of lateral root primordia (Zhang and Hasenstein, 1999). Growth of already initiated primordia continued and they became normal lateral roots. After one day of TIBA treatment, the blue cells in the outer cortex were still visible. These cells may have guided the outgrowth of the remaining lateral root primordia. After three days of TIBA treatment, no lateral root primordia were visible anymore, nor were any blue cells in the outer cortex detectable. If no lateral root primordia are present, the polarity signal is obviously no longer necessary. Whether this increase in auxin concentration in the cortical cells is a direct consequence of an initiated lateral root primordium, and how this process is triggered is unknown.

We localised the GUS expression controlled by the GH3 promoter in a few cells edgewise or in front of lateral root primordia. Apparently, we were able to detect single cells with a higher auxin concentration, or higher auxin sensitivity, in comparison to their neighbouring cells. This was also reported for tobacco (Li et al., 1999). Therefore, the GH3::uidA::gfp construct might be a useful tool to monitor changes in single cells with respect to their auxin concentration or sensitivity.

In soybean, in situ hybridisation showed that the GH3 mRNA was most abundant in developing organs of flowers and pods (Gee et al., 1991). In transgenic tobacco, the GH3 promoter showed strongest activity in developing ovules and developing seeds, along with vascular tissues in the flower receptacle (Hagen et al., 1991). In transgenic white clover, the GH3 promoter
has been reported to be active in the stem, to a lower amount in leaves, but very strongly in reproductive organs (Larkin et al., 1996). Therefore, in all studied species the GH3 promoter was most active in the reproductive organs. In rice we did not yet study the expression pattern of the GH3 promoter in the reproductive organs.

The natural auxin IAA and the synthetic auxin 2,4-D both can activate the GH3 promoter. This is in agreement with the reported function of the GH3 gene as an auxin responsive gene (Hagen et al., 1984). The activity of the GH3 promoter in rice remained the same independent of the treatment for one or three days with $1 \times 10^{-6}$ M 2,4-D. However, the GH3 promoter reacted differently to the auxin treatment in different parts of the roots and in different tissues. The main activation occurred in the cortical parenchyma, and less activation was visible in the central cylinder. The epidermis never showed GUS staining, the exodermis showed single blue crystals, and in the solorencyma the GH3 promoter was mainly active in cells in front of the auxin induced abnormal lateral roots. Obviously, not all tissues and cells react to external auxin application to the same extent. Three causes could explain this phenomenon: (i) the necessary receptors or parts of the signal transduction pathway to activate the GH3 promoter are not present in all cells, therefore, the higher auxin concentration can not activate the GH3 promoter in all cells, (ii) tissue-specific differences in DNA methylation or mRNA turnover could influence the tissue specificity of GH3 activation by auxin (Gee et al., 1991), (iii) although exogenous auxin was applied, within some cells or tissues the auxin concentration may remain low, either due to an aggravated uptake or diffusion of auxin or due to a higher export of the auxin out of these cells.

We compared different regions of the root with respect to morphologically recognisable auxin responses and the induction of the GH3 promoter by auxin. Interestingly, in older root parts, where the root morphology did not change in response to auxin application, hardly any GH3 activity was detectable. In younger root parts, where external auxin strongly affects root morphology, the GH3 promoter activity was strongly increased. The accordance between the morphological reaction to auxin application and GH3 activation is striking. One possible explanation is that GH3 is involved in mediating the morphological
responses to auxin, either as part of the signal transduction pathway or as part of the morphological changes in the cells after auxin treatment. Another possibility is that the \textit{GH3} promoter is activated by a similar or the same pathway as the one leading to cell divisions and formation of the abnormal lateral roots. However, both explanations are not compatible with the observation that right in the induced abnormal lateral roots the \textit{GH3} expression is not induced.

The auxin induced abnormal lateral roots do not show any \textit{GH3} activity. This was surprising, since the formation of these structures is clearly auxin dependent. However, lateral root primordia in rice do not show any \textit{GH3} expression either. In white clover, the \textit{GH3} promoter is only active during the first cell divisions in the pericycle, thereafter it is no longer active during lateral root primordium outgrowth (Rolfe \textit{et al.}, 1997). Maybe in these cells of the lateral root primordia as well as in the cells of abnormal lateral roots, the receptor or parts of the signal transduction pathway that would activate the \textit{GH3} promoter are missing.

\textbf{4.3.2. \textit{GH3} activity during somatic embryogenesis}

During the regeneration of transgenic plants from bombarded suspensions or embryos, we observed a scattered GUS expression pattern in transgenic calli. To exclude the possibility of chimeric callus material, we established callus suspensions from mature embryos of transgenic lines containing the \textit{GH3::uidA::gfp} construct. Since these calli descend from an embryo, all cells of such a suspension should contain the same genomic information. However, the scattered GUS expression pattern was still observed. Hand and microtome sections showed that these blue spots might either be entire somatic proembryos or the shoot apex of mature somatic embryos.

According to the studies of Jones and Rost (1989), most of the structures showing blue staining in cross sections, are somatic embryos (Fig. 3.26.e-h). Still it is not clear, whether all somatic embryos stain. In Figure 3.26.e, the arrow designates a blue staining structure, most probably a somatic proembryo. In the same cross section, the arrowhead points to a structure which apparently is also a somatic embryo. According to Jones and Rost (1989), only part of the
initiated somatic embryos mature, the development of the others is arrested. Therefore, one can assume that the non-staining structures represent somatic embryos arrested in their development.

In some cases, the observed blue structures seem to be the shoot apex of an already mature somatic embryo (Fig. 3.26.c, d). Therefore, we conclude that in the beginning of the development the entire somatic embryo stains blue, whereas after differentiation of the diverse tissues only the auxin producing shoot apex stains blue.

Yatazawa et al. (1967) mentioned that in rice calli derived from roots, cells in outer regions were small in size and rich in cellular content, while cells in inner regions were large in size and poor in their content. During our work with calli derived from immature or mature embryos, we observed the opposite: in the inner callus, cells were small, whereas at the outer surface larger cells were visible. The small cells in the inner parts of calli never showed any GH3 activity. This is quite surprising, since the R2I medium contains $9 \times 10^{-6}$ M and the R2 medium $4.5 \times 10^{-6}$ M 2,4-D, respectively. Therefore, in all cells of such a callus the auxin concentration should be quite high, and apparently this auxin concentration is able to induce cell divisions. Why these small cells did not express $uidA$ is unclear. It was reported that possibly one or several classes of auxin receptors and auxin signal transduction pathways exist in plant cells, and that these receptors and pathways are not uniformly distributed among different cell types and tissues (Guilfoyle et al., 1998). We assume that in the small callus cells the receptor and signal transduction pathway leading to cell divisions is present, whereas the receptor and/or the signal transduction pathway, which would activate the GH3 promoter, are absent. This could explain the effect that these cells react to auxin but show no GH3 promoter activity.

The rather large cells of a proembryo stain blue. Why is the GH3 promoter active in cells that we consider being part of a proembryo? It could be due to the synthesis and, therefore, presence of an appropriate receptor and signal transduction chain, which enables the GH3 promoter to react to the high 2,4-D concentration in the medium. It might also be that these cells produce their own auxin, which leads to the activation of the promoter.
During the liquid culture of *Daucus*, non-embryogenic cells are sensitive to auxin, whereas cells of the so-called proembryogenic masses become insensitive to auxin (LoSchiavo *et al.*, 1991). By the time the embryo becomes polarized, auxin sensitivity is regained, and auxin has to be removed from the medium, otherwise the development of the embryos is arrested and they dedifferentiate. Whether this model of auxin sensitivity and auxin insensitivity can be transferred to rice is questionable, since during rice somatic embryogenesis the medium contains auxin during the entire regeneration process.

Furthermore, LoSchiavo *et al.* (1991) showed that when *Daucus* embryos reach the globular stage, auxin will initially be synthesized by a few cells, and others will respond to it and the generation of different tissues will ensue. Our results with the *GH3* promoter support these results. The *GH3* promoter is not active in callus cells, but is activated during the proembryogenic stages. We assume that during somatic embryogenesis the *GH3* promoter responds to endogenous auxin rather than to the externally applied auxin. Our observations show that the *GH3* promoter is already activated in very early stages of somatic embryogenesis, indicating that somatic embryos start to produce their own auxin early in development.

A difference between the *GH3* response to externally applied 2,4-D and endogenous IAA is, however, questioned through experiments reported by Michalczuk *et al.* (1992a, b): in carrot suspension cultures, 2,4-D in the medium appears to be effective through an increased IAA production. Therefore, one would expect the same *GH3* activity, whether being directly triggered by endogenous IAA or indirectly by external 2,4-D. Thus, we consider that during proembryo development a new set of proteins is expressed, with the result that the *GH3* promoter is activated by IAA.

Liu *et al.* (1993) suggest from their experiments with *Brassica* that polar transport of auxin in early globular embryos is essential for the establishment of bilateral symmetry. For *Arabidopsis* it was reported that establishment of bilateral symmetry, and perhaps polarity, in early embryos depends on the activity of the polar auxin transport system (Hobbie, 1998). In monocots this shift from radial to bilateral symmetry occurs during the so-called transition
phase (Fischer and Neuhaus, 1996). Fischer and co-workers concluded from their work with isolated zygotic embryos, that auxin is synthesised in the lower part of the embryo proper and transported polarly in two main directions, along the longitudinal axis towards the area, where the scutellum will differentiate, and laterally toward the area, where the shoot promeristem will differentiate (Fischer et al., 1997). Treatment of isolated zygotic embryos with auxin transport-inhibitors caused the development of abnormal embryos with multiple meristems and multiple organs (Fischer et al., 1997). The authors conclude that polar auxin transport plays an important role in the establishment of embryonic symmetry in Poaceae.

Reports about somatic embryo development in dicots (LoSchiavo et al., 1991; Liu et al., 1993), and experiments with zygotic embryos of wheat (Fischer and Neuhaus, 1996; Fischer et al., 1997) strongly suggest an important role of endogenous auxin during somatic embryogenesis in rice as well. The activity of the GH3 promoter in the young somatic embryos favours this assumption. With this promoter we could not define a certain region producing auxin, and whether an auxin polar transport takes place.

After the diverse tissues of an embryo have differentiated, the newly formed shoot apex is the site of auxin synthesis. This is also supported by the GH3 activity in mature rice embryos (Fig. 3.26.c), where the GH3 promoter is only active in the shoot apex, and the rest of the embryo does not stain anymore.
5. References


References


References


Francisco PB and Akao S (1993). The 2,4-D-induced wheat para-nodules are modified lateral roots with structure enhanced by rhizobial inoculation. Plant and Soil 157, 121-129.


Zeman AMM, Tchan YT, Elmerich C, and Kennedy IR (1992). Nitrogenase activity in wheat seedlings bearing para-nodules induced by 2,4-dichlorophenoxyacetic acid (2,4-D) and inoculated with Azospirillum. Research in Microbiology 143, 847-855.


Curriculum vitae

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Publications


Acknowledgements

I am very grateful to PD Dr. Christof Sautter that he accepted an ecologist, without molecular knowledge, in his group, and for all the support he gave me during the time in his group.

I thank the whole group of Christof Sautter for the nice working atmosphere. A special thank goes to Claire for her linguistic advice.

I would like to thank Prof. Dr. Ingo Potrykus for the opportunity to work in his laboratory.

Special thanks to everybody who helped me in some way during this time. Especially for the great time we had together and all the help I would like to thank Joanna, Sofie, Cécile, Stéphane, Renata, Marcel and Carola.

I am thankful to Prof. Dr. N. Amrhein for co-examining this dissertation.

Ich möchte meiner Mutter danken, die in schwierigen Situationen immer Zeit und ein offenes Ohr für mich hatte.