

Markers of adventitious root formation

GJ De Klerk

Centre for Plant Tissue Culture Research, PO Box 85, 2160 AB Lisse, the Netherlands

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Summary — This article deals with markers for rootability of cuttings and markers for the successive phases in adventitious root formation. Since rootability depends on the ontogenetic, developmental and physiological state of the cuttings, markers for these states may be used as markers for rootability. For example, morphological characteristics that indicate the adult state, also indicate low rootability. For the phases in adventitious root formation, physiological parameters are reliable markers, in particular transient sensitivity to plant growth regulators applied as a pulse. The reliability of biochemical markers, in particular changes in peroxidase activity, is disputed. Molecular markers may be obtained from very sensitive screening techniques, from mutant-studies or from studies that do not deal with adventitious root formation, eg, research on auxin action, cell cycle activation or meristem organization. On the molecular level, research has started only recently. Molecular markers will require validation by physiological and histological studies.

auxin / peroxidase / regeneration / rootability / marker

Résumé — Marqueurs de la formation de racines adventives. Cet article traite des marqueurs d'enracinement des boutures et des phases successives de formation des racines adventives. Comme l'aptitude à former des racines dépend de l'état ontogénique, du développement et de la physiologie des boutures, des marqueurs de ces états peuvent être utilisés pour l'enracinement. Ainsi, des caractéristiques morphologiques indiquant le stade adulte traduisent une faible aptitude à la formation des racines. Pour la phase de formation des racines adventives, des paramètres physiologiques sont des marqueurs fiables, en particulier la sensibilité passagère à l'application des régulateurs de croissance apportés «en pulse». La fiabilité des marqueurs biochimiques, en particulier les changements dans l'activité peroxydase, sont contestés. Des marqueurs moléculaires peuvent être obtenus à partir de techniques très sensibles, d'étude de mutants ou des travaux ne concernant pas la phase de formation des racines adventives, par exemple les recherches sur l'action des auxines, l'activation du cycle cellulaire et l'organisation du méristème. La recherche de marqueurs moléculaires est récente ; ceux-ci doivent être validés par des études physiologiques et histologiques.

auxine / peroxydase / régénération / enracinement / marqueur

INTRODUCTION

A marker is a parameter that shows sufficient (preferably 100%) coincidence with a certain physiological, developmental or genetic state. Usually, markers are on the biochemical or mole-

cular level. Thus, proline accumulation is a marker of drought stress (Hanson and Hitz, 1982), storage protein synthesis of zygotic, somatic or microsporadic embryo maturation (Crouch, 1982) and glucuronidase activity of transformation. On other levels, useful parameters may also be

found. Root formation after application of auxin is a physiological marker for the ontogenetic (juvenile vs adult) state (Zimmerman et al, 1985). On the histological level, the presence of two opposite meristems connected by vascular tissue is a marker of a (somatic) embryo. Leaf shape is a morphological marker of the ontogenetic state (Zimmerman et al, 1985).

In adventitious root formation (ARF), markers may be used in various ways. Markers for rootability predict the success of a rooting treatment and have therefore a great practical importance. Since the various phases in ARF often have different hormonal requirements, markers are required to identify the beginning and end of the phases. Markers for the phases in ARF may also be used to establish in which phase recalcitrant cuttings become inhibited. Finally, markers may also provide an understanding of the underlying mechanisms in ARF.

The present article deals with histological, morphological, physiological, biochemical and molecular parameters studied in relation with rootability and the successive phases in ARF, and discusses their use as markers.

PITFALLS IN MARKER STUDIES

The degree of coincidence of a marker with a certain state determines its usefulness. To validate a marker, conditions must be created under which the state occurs to a different extent. In studies on markers of the phases in ARF, for example, conditions must be imposed that alter the rooting response (eg, besides auxin another inhibitory plant growth regulator may be added). The marker should show the appropriate change. For most markers discussed here, such analysis has not been carried out.

A second pitfall concerns markers for regeneration specifically. In the examples cited in the previous section, most or a large portion of the analysed tissue is in the state for which the marker is characteristic. However, in regeneration processes the situation is different because the number of cells that participate in regeneration processes is very small. For example, in 1 mm stem slices cut from apple microcuttings, ARF starts from a few cells in the primary rays between the vascular bundles three cell layers from the basal cutting surface (Jasik and De Klerk, 1997). At 72 h after the start of the rooting treatment, the time at which the meristemoids are being formed, the volume of a meristemoid is ca

0.001 mm³. The volume of a slice is ca 1 mm³. Thus, ten meristemoids (per slice five to ten roots are formed) account for 1% of the volume of a slice. Obviously, biochemical and molecular characteristics of the cells in the meristemoids are swamped by the other cells that account for 99% of the volume. Perhaps advanced techniques that may distinguish minor differences, eg, mRNA differential display, are successful. In embryogenesis from microspores in *Brassica*, as much as 30% of the cells may be involved in regeneration (Custers et al, 1994). In such a system, regeneration-related genes are more easily identified. For rooting, a similar system does not exist.

MARKERS FOR ROOTABILITY

In ARF, meristem formation is likely initiated from specific cells only (De Klerk et al, 1997a). As noted earlier, molecular and biochemical characteristics of these few cells will be swamped by the large number of surrounding cells. However, molecular and biochemical analyses may still be helpful: Since rootability differs greatly among cuttings with various developmental, physiological or genetic states, parameters characteristic for these states may be used as markers for rootability.

Adventitious roots easily regenerate from juvenile tissue. Thus, markers for rootability are to some extent the same as markers for the juvenile state. Markers for juvenility include morphological characteristics (Zimmerman et al, 1985), phenolic compounds (Girouard, 1969; Vieitez et al, 1987; Jay-Allemand et al, 1988), abscissic acid level (Noiton et al, 1992) and specific mRNA species (Murray et al, 1994; Woo et al, 1994).

Besides changes in the ontogenetic state, tissues may also show various other developmental changes such as chronological ageing (as opposed to ontogenetic ageing), dormancy development and rhythmic growth. These changes may cause variation in rootability during a growing season (Howard, 1996). In cuttings with different developmental or physiological states, it has been observed that rootability coincides with a high level of indoleacetic acid (IAA) (Weigel et al, 1984; Blakesley et al, 1991) and with specific phenolic compounds (Curir et al, 1990, 1993). Rhythmic growth (El-Morsy and Millet, 1996) and dormancy development (De Klerk and Gerrits, 1996) also occur in tissue culture and likely cause the large differences in

rootability among microcuttings from different subcultures (Denissen et al, 1992), among microcuttings of a single batch and among stem slices taken from the same cutting (De Klerk and Caillat, 1994). An example of the relation between rootability of individual microcuttings and a biochemical parameter (peroxidase activity) is shown in figure 1.

There have been several examinations on closely related genotypes that differ in rootability. Differences include the degree of sclerification of the primary phloem (Beakbane, 1961), endogenous IAA levels (Alvarez et al, 1989) and peroxidase activity (Gus'kov et al, 1989).

In conclusion, morphological, biochemical and molecular parameters characteristic for the ontogenetic state may be used as markers for rootability. However, their use is limited. For example, in *Hedera* the leaf shape is a reliable marker for the ontogenetic state but this does not mean that all cuttings with juvenile leaves develop adventitious roots after the proper stimulus has been given. For other developmental and physiological states that influence rootability, markers may be developed. Possibly, ARF depends on the occurrence of a specific cell type from which roots always develop when the proper stimulus is given. If such a cell type occurs, specific molecular probes may be identified.

MARKERS FOR THE PHASES IN ADVENTITIOUS ROOT FORMATION

Physiological markers

Christianson and Warnick (1983) concluded that regeneration consists of three successive phases: i) dedifferentiation during which cells become competent to respond to the organogenic or embryogenic stimulus, ii) induction during which the stimulus acts and the cells become committed to a developmental pathway and iii) differentiation during which the stimulus is no longer required and the previously determined cells form the organ/embryo. These authors established the beginning and end of the phases in shoot regeneration using differences in sensitivity of the three phases to added hormones. A similar study was conducted by our group for rooting. By giving pulses with benzylaminopurine (BAP, which may be considered as an auxin antagonist), indolebutyric acid (IBA), *p*-chlorophenoxyisobutyric acid (PCIB, an anti-auxin) and salicylic acid (SA, a monophenol that stimulates IAA-oxidase), the

timing of the three phases was determined in shoots and stem slices of the apple rootstock 'Jork 9' (De Klerk, 1995; De Klerk et al, 1995, 1997b). Results with BAP and SA pulses are shown in figure 2. Other authors have reported similar results (Mitsuhashi et al, 1969; Attfield and Evans, 1991; Burritt and Leung, 1996).

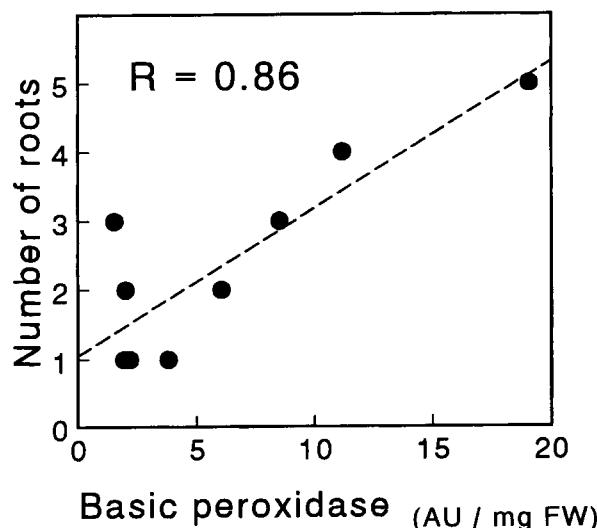


Fig 1. The number of roots regenerated from an apple microcutting plotted against the activity of a basic peroxidase component at the time of transfer to rooting medium. The basic peroxidase activity was determined in a 1 mm slice excised from the basal end of the microcutting (redrawn from De Klerk et al, 1990).

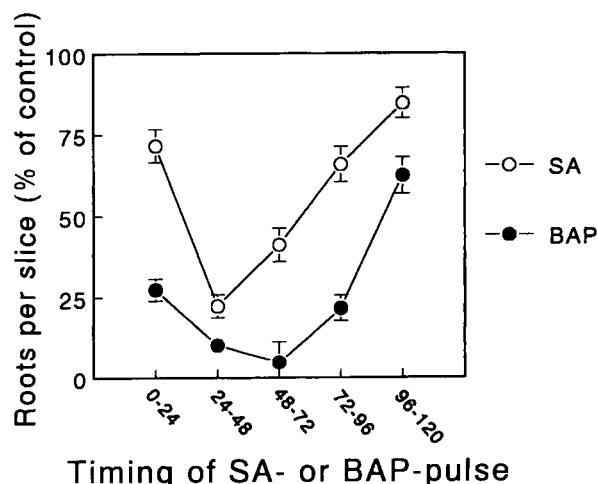


Fig 2. Effect of 24 h salicylic acid (SA) or benzylaminopurine (BAP) pulses given during the first 5 days of adventitious root formation in 1 mm thick apple-stem slices. Slices were cultured for 5 days on medium containing 100 μ M indoleacetic acid (IAA) (SA pulses) or 3 μ M indolebutyric acid (IBA) (BAP pulses). They were then transferred to hormone-free medium for 16 days. BAP (0.3 μ M) or SA (100 μ M) were added during the indicated periods (in hours). During the SA or BAP pulses, 100 μ M IAA or 3 μ M IBA were also present in the medium. The root number is expressed as a percent of the control. The experiments with BAP and SA pulses were carried out with two batches of 'Jork 9' microcuttings obtained in different subcultures.

The studies cited here show that a transiently increased sensitivity to plant growth regulators provides a reliable marker for the successive phases in regeneration. However, physiological markers have a limited use. Since they are only applicable when the cuttings are capable of rooting, physiological markers cannot be used to monitor in which phase recalcitrant cuttings become inhibited.

Histological markers

From numerous histological examinations on ARF, a general pattern can be deducted (Lovell and White, 1986). After auxin application, specific cells are activated. Then, after several cell divisions, a meristemoid is formed and subsequently a root primordium with a typical dome-shape. Histological studies indicate at what time recalcitrant cuttings become inhibited. Occurrence of cell divisions in stems without subsequent root formation is reported in several studies. For example, a tobacco mutant that is unable to form root meristemoids shows initially 'normal' cell divisions in response to auxin (Lund et al, 1996). A major problem in histological studies is that the physiological state cannot be derived from the observations until the typical root primordium has been formed. In particular, it is not known whether cells in a meristemoid are already committed to root formation or whether the commitment occurs before or after meristemoid formation. (The term 'commitment' is used in the physiological sense: after the cells have developed commitment to root formation, they no longer require the rhizogenic stimulus.) Recently, it was shown in 'Jork 9' microcuttings and stem slices that the commitment to root formation develops during meristemoid formation (see earlier; De Klerk et al, 1995; Jasik and De Klerk, 1997). Another disadvantage of histological studies is that they are very laborious. Since from a population a sufficiently large sample has to be taken, in each experiment large numbers of cuttings (> 50) have to be analysed.

In few histological studies, the cells that participate in ARF have been analysed on the biochemical or molecular levels. In 1 mm stem slices cut from 'Jork 9' microcuttings, Jasik and De Klerk (1997) found during the first 24 h of exposure to auxin an increase in starch grains in specific cells (fig 3). This is before the induction phase begins. Thus, the accumulation of starch grains may be a marker for the dedifferentiation

phase. After 24 h, these starch grains are degraded rapidly and the cells enter cell division. From some of these cells, root meristemoids develop. The increase and decrease of starch only occurs in the presence of IBA. In the apple stem slices, other correlations between starch and rooting have not yet been examined. In excised mung bean hypocotyls, many cells throughout the stem are activated as shown by the incorporation of labelled thymidine (also in the upper part where no or only few roots are formed). Activation occurs more rapidly in hypocotyls exposed to auxin (Tripepi et al, 1983). A third example is on *Hedera*. Chronologically young, mature petiole explants show a decrease of a mRNA for a proline-rich protein in specific cells from which the root meristemoids will develop. Such a decrease does not occur in chronologically old, mature petioles that are incapable of root formation (Sanchez et al, 1995).

Biochemical markers

Many studies deal with biochemical changes in cuttings during ARF. A characteristic course of the activity of basic peroxidase has been reported by several researchers: after excision of a cutting, peroxidase activity increases sharply, then decreases and subsequently gradually increases (for review see Gaspar et al, 1992). The decrease of peroxidase activity supposedly coincides with the initiation of cell division. Figure 4 shows the course of peroxidase activity during ARF in *Sequoiaadendron* shoots and figure 5 the results of an experiment in which the value of the peroxidase peak is associated with rooting of

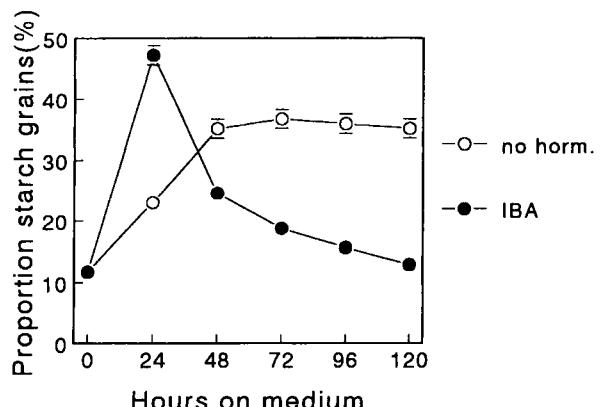


Fig 3. Proportion of starch grains in cells of the cambial region in the basal part of 1 mm thick apple-stem slices. The proportion of starch grains is given as a percent of plastid area. The slices were cultured with (●) or without (○) 3 µM indolebutyric acid (IBA) (redrawn from Jasik and De Klerk, 1997).

Cynara shoots rejuvenated by repeated subcultures. Some authors, however, did not observe the characteristic course but a continuous increase of peroxidase activity (Pythoud and Buchala, 1989; De Klerk et al, 1990). Several conditions have been created by which the rooting performance decreases. Sometimes the expected reduction of the peroxidase peak occurs (Moncousin and Ducreux, 1984), but in other cases not (Gus'kov et al, 1989; De Klerk et al, 1990). It may be that the characteristic changes in peroxidase activity is caused by auxin and is not related to rooting. The evolution of peroxidase with 2,4-dichlorophenoxyacetic acid (2,4-D), an auxin that induces only very few root meristems (De Klerk et al, 1997c), has not been examined.

Hormone levels, in particular IAA levels, constitute another possible biochemical marker for the phases in ARF. It has been reported that during ARF, IAA levels show the opposite course of peroxidase (fig 4; see also Moncousin et al, 1989). The inverse relationship between the endogenous IAA level and peroxidase activity may be explained by the fact that basic peroxidases likely oxidize IAA. It has not been determined whether the characteristic changes in endogenous IAA levels also occur independently of the rooting event. A complicating factor is that these studies are usually performed in IBA-treated cuttings. Applied IBA is metabolized into IAA (Van Der Krieken et al, 1993) and probably functions as a slow-release source of IAA.

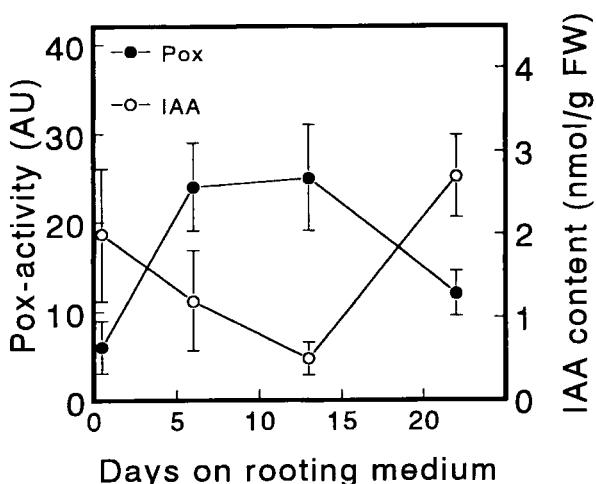


Fig 4. Peroxidase (Pox) activity and indoleacetic acid (IAA) level in *Sequoiadendron giganteum* shoots during adventitious root formation. The shoots were on medium with 1 mg.L⁻¹ naphthaleneacetic acid for 13 days, and subsequently transferred to medium without auxin and cultured for another 15 days. The complete shoot was extracted for the determinations (redrawn from Berthon et al, 1989).

Other compounds that show a characteristic course during ARF are ethylene (Moncousin et al, 1989; Hausman, 1993) and polyamines (Torrigiani et al, 1989; Hausman et al, 1994), but again in all cases it has not been properly examined whether the changes only occur in tissues in which ARF takes place.

Based on early, promising observations, many examinations have been carried out on biochemical markers for the phases in ARF, in particular on peroxidases. Occasionally, these markers have been used. For example, Berthon et al (1987) used the timing of the peroxidase peak to establish the time at which the shoots have to be transferred from an inductive to an expressive medium. Although the early findings have been confirmed, numerous exceptions occur. Particularly when conditions are created that do not allow ARF, the markers often do not show the appropriate changes. This puts into question the reliability of these markers.

Molecular markers

There are several approaches to study adventitious rooting on the molecular level. First, mRNA populations or newly synthesized proteins may be studied in cuttings that root and cuttings that are incapable of rooting, eg, by not supplying them with auxin. In this way, Dhindsa et al (1987) studied newly synthesized proteins and mRNAs in a cell-free protein synthesizing system. mRNA

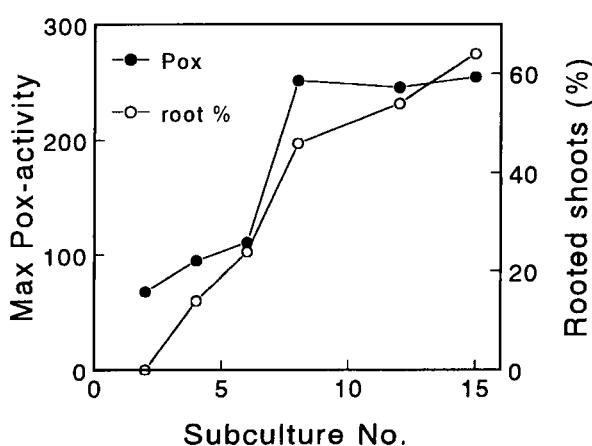


Fig 5. Maximal level of peroxidase (Pox) activity and rooting performance of *Cynara scolymus* shoots subcultured for increasing times. Cultures of *Cynara* were initiated from buds and subcultured at 8 week intervals. At the indicated subculture numbers, shoots were transferred to rooting medium, and both the rooting percentage and the maximal value of peroxidase activity during the rooting treatment were determined. For the peroxidase determination, the complete shoot was extracted (drawn from data of Moncousin and Ducreux, 1984).

populations have recently been examined with the differential display technique (Diaz-Sala et al, 1996). These studies suffer from the fact that only very few cells participate in the rooting process.

Second, probes that have been isolated in other studies, eg, probes related to auxin application, meristem organization or cell cycle (Hemerly et al, 1993) may be studied in cuttings during ARF.

Third, in a population of mutated plants (*Arabidopsis* seedlings) mutants incapable of ARF may be selected. Subsequently, the gene that has been mutated can be isolated. Such a study has recently been reported for lateral root primordia (Smith and Fedoroff, 1995). The identified gene was also a marker for adventitious root primordia.

Molecular studies have only been initiated recently. Their great advantage is their sensitivity. It is likely that the molecular studies will result in specific markers for the phases in ARF.

CONCLUSION

The various techniques used to root cuttings have been developed some 60 years ago (De Klerk and Ter Brugge, 1992). Ever since, there has been little or no progress in spite of the vast basic and applied research. Recently, however, progress has been made in the understanding of ARF. In particular, it has been shown that ARF is a process consisting of distinct phases, and physiological markers of these phases have been developed. In addition, molecular studies have been started. The development of molecular markers for the phases of rooting will allow us to establish when the block in recalcitrant cuttings occurs. These new developments may result in new rooting treatments.

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