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Review

# *Xenopus* oocytes as an expression system for plant transporters

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**Abstract**

The *Xenopus* oocyte provides a powerful system for the expression and characterisation of plant membrane proteins. Many different types of plant membrane proteins have been expressed and characterised using this system. As there are already several general reviews on the methodology for oocyte expression of channel proteins, we have summarised the particular advantages and disadvantages of using the system for the characterisation of plant cotransporter proteins. As an example of how the system can be used to identify transporters, we describe evidence for a low affinity nitrate transporter in oocytes injected with poly(A) RNA extracted from nitrate-induced barley roots. Furthermore, we describe evidence that the expression of some transporters in oocytes can modify the properties of endogenous membrane proteins. We conclude that although care must be taken in the interpretation of results and in choosing appropriate controls for experiments, oocyte expression is an excellent tool which will have an important role in characterising plant membrane proteins. 

*Keywords:* Transport; *Xenopus* oocyte; Plant; Carrier; Cotransport

## 1. Introduction

The application of molecular biology techniques to plant membrane transport has identified genes encoding different types of transporters. Often these genes were originally isolated by functional complementation of yeast transport mutants, but genome sequencing is also providing a complete catalogue of information. Sequence comparisons can identify if a newly isolated cDNA belongs to a known family of proteins (for examples, see other contributions to this special issue), but the function of these genes must be determined. The only way to characterise the function of a transport protein is to study the activity of the protein in a membrane.

Plant membrane transporters can be expressed in immature eggs (oocytes) from the South African clawed frog, *Xenopus laevis* [1]. Fig. 1 shows a diagrammatic summary of the steps involved in obtaining expression in an oocyte. The oocytes have accumulated stores of enzymes, organelles and proteins, all of which are required in the early developmental stages after fertilisation has occurred. Like a fully equipped factory waiting for an order from the customer, the message normally arrives at fertilisation to trigger production on the assembly line. This 'factory' can be exploited by supplying foreign information which can hijack the cell's protein manufacturing machinery and produce large quantities of the foreign protein. The most commonly used route for expression is to prepare mRNA from the cDNA and

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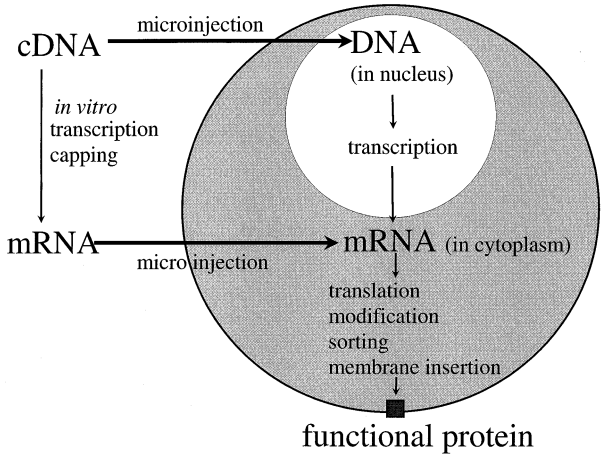


Fig. 1. Diagrammatic representation of the routes for expression of foreign membrane proteins in *Xenopus* oocytes.

then to inject this into the cytoplasm of the cell. Direct injections of DNA into the nucleus are technically more difficult because the nucleus must be located and can be damaged by the injection. The foreign plant protein can be synthesised, glycosylated, phosphorylated and targeted to the oocyte plasma membrane (Fig. 1). The final result is an oocyte expressing moderate amounts of a foreign transporter protein, and this cell can then be used to characterise a single gene product in isolation from other interacting proteins.

The activity of a foreign membrane protein expressed in oocytes can be studied by using conventional techniques such as uptake of a radiolabelled substrate or depletion from the external solution. Water channels have been assayed by measuring the rate of change in the volume of an oocyte after modifying the external osmolality of the bathing solution. If the transport is electrogenic, then electrophysiological techniques provide powerful tools for the characterisation of plant transport proteins. The large cell size of an oocyte favours using the two-electrode voltage clamp technique to assay the activity of the protein. The application of this technique to oocytes permits the transporter-mediated currents to be assayed as a function of membrane potential. This offers an extra dimension to analysis of the kinetic behaviour of the protein because for transporters which are electrophoretic, the membrane potential is a component of the driving force and is normally uncontrolled in transport assays. This characterisation of the carrier can include a complete kinetic analysis for each of the transported species.

In this review, we will not describe all the details of methodology steps shown in Fig. 1 because there are already several reviews describing this topic (see Table 1). The particular strengths of each of these previous background reviews are listed in Table 1. This review will describe information and methodology details, which are relevant to expressing plant membrane proteins in oocytes. These points include the choice of bathing solution, oocyte expression vectors and the appropriate controls. Finally, we describe some of the problems and advantages associated with using oocytes compared to other expression systems, and look ahead to possible future applications for the functional characterisation of plant membrane proteins.

## 2. Oocyte injection

As shown in Fig. 1, there are two routes for introducing the foreign genetic information into the oocyte and both require injection of the cell. In an earlier review, we have discussed some ways of checking why expression may have failed, but it is very important to inject good quality RNA, which is not degraded and this can be easily checked on a gel [11]. The chief problem for cytoplasmic injections is the instability of the mRNA transcripts, this can be improved by adding a poly(A) tail. For a $K^+$ channel, increasing the polyadenylation of the mRNA

Table 1
Background reviews on the *Xenopus* oocyte expression system

| Year | Reference | Comments |
|---|---|---|
| 1984 | [2] | Background to the use of *Xenopus* oocytes |
| 1987 | [3] | Ion channels expressed in oocytes |
| 1990 | [4] | Membrane proteins in oocytes |
| 1994 | [5] | Practical guide to procedures |
| 1994 | [6] | Guide to receptor expression |
| 1992 | [7] | Endogenous electrophysiological characteristics |
| 1993 | [8] | Short background review on plant proteins |
| 1994 | [9] | Short review on plant $H^+$-coupled transporters |
| 1995 | [10] | Review of plant membrane proteins |
| 1995 | [11] | Practical details on expressing plant membrane proteins |
| 1995 | [12] | Plant proteins expressed in oocytes |
| 1998 | [13] | Ion channels in oocytes |

increased the current amplitude and led to higher levels of the protein (as assayed by Western blots) [14]. We have also found improvements in the expression of a plant sucrose carrier expressed in oocytes when a 75 poly(A) tail was added to the expression vector. An 8-fold increase in the sucrose transport activity was obtained in the same batch of oocytes. Most RNA molecules have a 5′ 7-methyl guanosine residue, the cap structure, which functions in the protein synthesis initiation process, protects the mRNA from degradation and is essential for oocyte expression [15]. The relative importance of both capping and the poly(A) tail of the mRNA for expression in oocytes changes during oocyte development, but the two processes work synergistically stimulating translation as the oocyte matures [16]. This is one reason for checking that the oocytes chosen for injection are at the correct developmental stage to optimise expression, other reasons will be described later.

To increase expression in oocytes, many papers report using a vector containing 3′ and 5′ untranslated regions (UTRs) of a *Xenopus* β-globin gene [15]. Injection of in vitro transcribed mRNA for the $K^+$ channel flanked by these UTRs resulted in very high expression and $K^+$ currents were detected only 10 h after injection of mRNA [17]. By 4 days after the mRNA injection, there was a 200-fold increase in the current mediated by this channel compared with the value for oocytes injected with mRNA without the flanking sequences [17]. In a recent review, the authors describe a *Xenopus* expression vector which includes both a poly(A) tail and the globin flanking sequences with a single *Bgl*II cloning site [13]. To avoid subcloning into an expression vector, there is a PCR-based method which begins with RNA which is reverse-transcribed to generate cDNAs [18]. This method requires sequence information without needing an actual cDNA clone to produce mRNA for oocyte injection, but although a poly(A) tail is included, there are no globin flanking sequences and as with any PCR-based approach, there is the possibility of introducing mutations.

A different approach is to directly inject the nucleus with double-stranded cDNA. The biosynthetic machinery of the oocyte nucleus then does the transcription, capping, polyadenylation and exporting to the cytoplasm of the processed mRNAs. However, the cDNA must be cloned into a suitable expression vector before nuclear injections can be performed. This involves inserting the cDNAs, in the correct orientation into a vector which contains a eukaryotic promoter, examples are viral promoters [19,20]. Plant regulatory elements have even been used to drive expression in oocytes. When microinjected into oocytes, the cauliflower mosaic virus 35S promoter and polyadenylation signal effectively promoted chloramphenicol acetyl transferase synthesis in a manner that was dependent on the promoter and probably also on the polyadenylation signals [21].

## 3. Plant membrane proteins expressed in oocytes

Membrane proteins from yeast [22], bacteria [23] and plants have been successfully expressed in oocytes showing that there can be no kingdom or codon usage limitation to oocyte expression. The first plant membrane proteins expressed in oocytes were a hexose carrier [24] and $K^+$ channels [25], but since then, many other different types have been characterised and these are listed in Table 2. The numbers of publications have gradually increased from three in 1992 and 1993, to eight in 1997, with success expressing plant examples of channels, carriers and aquaporins. The characterisation of aquaporins and $K^+$ channels in oocytes has been particularly successful and papers on these two types of membrane proteins account for more than 60% of the papers in Table 2.

The oocyte system has also been used to isolate cDNAs encoding mammalian membrane protein genes (e.g. [65]), but only recently was it applied to plants when a tobacco syntaxin-type cDNA was isolated by an abscisic acid-elicited electrical response [66]. This procedure involved extracting total poly(A) mRNA from a tissue and injecting this into oocytes. Potassium channel activity was identified in leaf RNA [25,62] and here we show an example in Fig. 2, where an oocyte was injected with total mRNA extracted from nitrate-induced barley roots. These oocytes showed a nitrate-elicited current which was not present in water-injected control oocytes. This current was pH-dependent and increased when the external nitrate concentration was increased from 1 to 50 mM, suggesting that there was a barley low

Table 2
Plant transporter proteins expressed in *Xenopus* oocytes

| Type of transporter | Plant and transporter | Year and reference |
|---|---|---|
| $H^+$/hexose cotransporter | *Arabidopsis thaliana*, STP1 | 1992 [24] |
| $K^+$ channels | *Zea mays* | 1992 [25] |
| | *A. thaliana*, KAT1 | 1992 [26] |
| Aquaporin | *A. thaliana*, γ-TIP | 1993 [27] |
| $H^+$/hexose cotransporter | *Chlorella kessleri*, HUP1 | 1993 [28] |
| $H^+/NO_3$ cotransporter | *A. thaliana*, CHL1 | 1993 [29] |
| Aquaporin | *A. thaliana*, PIP1-5 | 1994 [30,31] |
| $H^+$/hexose cotransporter | *A. thaliana*, STP1 | 1994 [32] |
| $H^+/K^+$ cotransporter | *Triticum aestivum*, HKT1 | 1994 [33] |
| $K^+$ channel | *A. thaliana*, KAT1 | 1994 [34] |
| Aquaporins | *Mesembryanthemum crystallium*, | 1995 [35] |
| | *A. thaliana*, α-TIP | 1995 [36] |
| $H^+/K^+$ cotransporter | *T. aestivum*, HKT1 | 1995 [37] |
| $K^+$ channels | *A. thaliana*, KAT1 | 1995 [38–41] |
| | *A. thaliana*, AKT2 | 1995 [14] |
| | *Solanum tuberosum*, KST1 | 1995 [42] |
| $H^+$/amino acid cotransporter | *A. thaliana*, AAP1 | 1996 [43] |
| $H^+$/sucrose cotransporter | *S. tuberosum*, StSUT1 | 1996 [44] |
| $Cl^-$ channels | *Nicotiana tabacum*, NtClC1 | 1996 [45] |
| | *A. thaliana*, CLC | 1996 [46] |
| $K^+$ channels | *A. thaliana*, KAT1 | 1996 [47,48] |
| $K^+$ channel | *A. thaliana*, AKT3 | 1996 [49] |
| Aquaporin | *Glycine max*, NOD 26 | 1997 [50] |
| | *A. thaliana* | 1997 [51] |
| $H^+$/silicon cotransporter | *Cylindrotheca fusiformis*[a] SIT1 | 1997 [52] |
| $H^+$/sucrose cotransporter | *A. thaliana*, AtSUC1 | 1997 [53] |
| $H^+$/amino acid cotransporter | *A. thaliana*, AAP5 | 1997 [54] |
| $K^+$ channels | *A. thaliana*, *S. tuberosum* | 1997 [55] |
| | *S. tuberosum*, KST1 | 1997 [56] |
| Aquaporin | *Spinacia oleracea*, PM28A | 1998 [57] |
| Aquaporin | *Raphanus sativus*, VM23 | 1998 [58] |
| $H^+$/nitrate cotransporter | *B. napus*, BnNRT1;2 | 1998 [59] |
| $H^+$/peptide | *Hordeum vulgare*, HvPTR1 | 1998 [60] |
| $K^+$ channel | *Oryza sativa*, KOB1 | 1998 [61] |
| | *S. tuberosum* | 1998 [62] |
| | *A. thaliana*, KAT1 | 1998 [63,64] |

[a]Marine diatom.

affinity $H^+$/nitrate cotransporter functioning in this oocyte. Size fractionation of this RNA by sucrose gradient centrifugation could then be used to identify the RNA fraction encoding the low affinity nitrate transporter. However, this is a laborious procedure and a much quicker route to isolate a cDNA for the transporter is to use probes based on sequence homology to known nitrate transporters, an approach already used to isolate a cDNA encoding a low affinity nitrate transporter homologue from *Brassica napus* [59]. Another method of cloning by hybrid arrest of translation in oocytes [67] has not yet been used for any plant examples, but is not in common use for mammals. This method is relevant when the mRNA is of low abundance or cannot easily be fractionated or if the protein of interest is comprised of multiple subunits.

## 4. Assaying plant cotransporter activity

For the electrophysiological characterisation of a membrane protein in oocytes, the two-electrode voltage clamp technique can be used. Two electrodes

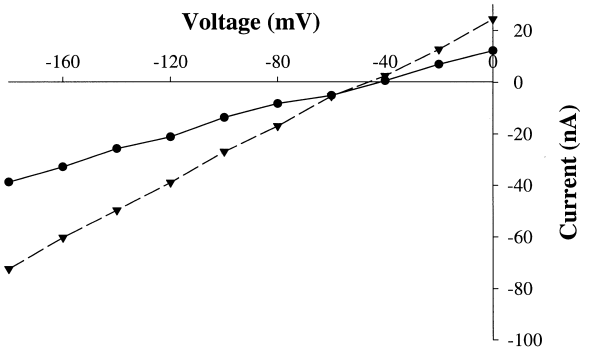


Fig. 2. Oocytes injected with total poly(A) RNA extracted from nitrate-induced barley roots showing the presence of a low affinity nitrate transporter activity. The *I–V* difference curves were obtained under conditions described previously [53]. Hydroponically grown barley roots were pretreated with 1 mM nitrate for 2 h before the RNA was extracted. Nitrate was applied in modified oocyte saline pH 7.2 at concentrations of 1 (●) and 50 mM (▼) [59]. Oocytes were injected 4 days before the *I–V* analysis.

inserted into an oocyte are used to measure voltage and current. One electrode reports the membrane potential while the other is used to pass current to maintain the voltage at a predetermined value. Current–voltage (*I–V*) relationships are obtained with a pulsed protocol generated by a computer linked to an A/D interface. The oocyte membrane potential is clamped at a particular voltage, the holding potential, from which the membrane is pulsed in a series of steps to a range of predetermined voltages. Usually, the membrane potential is returned to the holding voltage between each of these steps. Typically, the oocyte is voltage-clamped over the range +20 to −150 mV. To maintain the membrane voltage during each of these pulses, it is necessary to pass a recorded amount of current through the second electrode. The transported substrate is then added to the external bathing solution and the oocyte's membrane potential is again voltage-clamped through the range of values and the required current is measured. The oocyte is usually treated with the transported substrate for less than a minute to minimise the accumulation within the cell. This is done to avoid possible negative feedback on the activity of the transporter caused by accumulation of the substrate. Substrate-dependent currents are obtained by subtracting the currents measured before from those obtained after the addition of the transported substrate. The substrate-elicited currents are then plotted against each of the predetermined membrane voltages and an *I–V* profile is obtained. For a cotransporter expressed in an oocyte at any particular voltage, the steady-state substrate-dependent currents are measured as a function of external ligand concentration (either substrate or driver ion, usually $H^+$). These currents can be fitted to Michaelis–Menten kinetics and $K_m$ and $i_{max}$ values determined [68], and based on these measurements, a kinetic model for the cotransporter can be built [53]. This method enables many different possible substrates for a transporter to be tested quickly and cheaply without the need for radiolabelled substrates.

One further refinement of the two-electrode voltage clamp method was the measurement of presteady-state currents and this analysis was shown in oocytes expressing a $Na^+$/glucose cotransporter [68]. These currents were measured in the absence of any external sugar and resulted from the translocation of the unloaded carrier in response to a positive pulse of the membrane potential. These currents required $Na^+$, but were abolished by sugar in the bathing solution. Various different 6-state kinetic models for the cotransporter could be eliminated based on the information provided by the presteady-state currents and the assumption that the unloaded carrier was negatively charged [69]. Later, the same type of analysis was applied to plant sugar cotransporters [32,44]. The information obtained from oocyte *I–V* analysis is ideal for the kinetic modelling of transporters.

The transport properties of membrane proteins expressed in oocytes appear to be similar to those obtained when the protein was expressed in other cell types (e.g. [24,70]), but it is difficult to make a direct comparison between various systems because the techniques used for characterisation may be different. For example, the determination of $K_m$ for a substrate in yeast is done by uptake of radiolabelled isotope and the membrane voltage was uncontrolled in these experiments so it may be difficult to make a direct comparison with results from oocytes without knowing the resting potential of the yeast cell. The $K_m$ values for both substrate and driver ion can depend on the membrane potential (e.g. [53]).

There are some differences between the oocyte and a typical plant cell which can provide some difficulties for the characterisation of a plant membrane

protein expressed in oocytes. The resting potential of an oocyte is usually only −30 to −40 mV, while the equivalent value for a plant cell is between −100 and −200 mV. Therefore, to study the activity of a plant membrane protein expressed in oocytes by using electrophysiological techniques, it is of interest to clamp the membrane voltage at values much more negative than the oocyte resting potential. One problem with such negative potentials is that the oocyte plasma membrane has a voltage-dependent chloride channel which is activated at potentials more negative than about −150 mV [71,72] and this channel can dominate the *I–V* difference curve and so hide all the properties of the foreign protein. However, the activity of this channel is variable between batches and perhaps developmental stage of the oocytes. The problem has been discussed previously and there are reported to be ways of decreasing this background current. For example, the use of glutamate salts rather than chloride in the bathing solution has been reported to decrease the current [10], a surprising result as this treatment would increase the driving force for chloride efflux. However, these endogenous currents have also been exploited, for example, by using the tail currents associated with the chloride channel activity, it was possible to measure intracellular anion concentrations in oocytes and to demonstrate the function of a plant nitrate transporter [59].

These differences between plant and animal cells are also emphasised by the composition of a typical bathing solution for plant cells when compared to that used for oocytes. The standard saline for maintenance of oocytes contains around 100 mM NaCl, e.g. Barth's saline [2], which is a higher salt concentration than usually encountered by most plant cells. The plant membrane proteins are thus subjected to a higher external salt concentration than that normally encountered in the apoplast. To avoid the problem of chloride transport by putative nitrate transporters, a mannitol-based bath saline has been described containing 0.15 mM $CaCl_2$, 230 mM mannitol and 10 mM HEPES pH buffer [10]. We have attempted to use this saline, but in control water-injected oocytes, we found inward currents elicited by a range of different external salt solutions. The size of these currents depended on the concentration of the salt solutions, so to avoid these background problems for all measurements, we used a more usual frog saline [59]. We have found that the oocytes can also be voltage-clamped by replacing sodium in the standard saline with choline and chloride by gluconate. For the characterisation of higher plant $K^+$ channels in oocytes, a standard frog saline has been used in which all the $Na^+$ was replaced with $K^+$ [10].

In plants, the uptake of nutrients usually occurs by cotransport coupled with the movement of protons across the membrane. One consequence of expressing a proton-coupled carrier in oocytes is that in order to optimise the substrate-elicited current, it is necessary to treat the oocyte with a bathing solution which is more acid than the normal frog saline. This acid treatment can cause problems for the oocyte because the in vivo environment for an oocyte is usually carefully regulated and the cell may be unable to maintain cytoplasmic ionic homeostasis. Changes in external pH can influence endogenous transport systems of oocytes. A pH-sensitive proton current has been measured in oocytes [73,74] and cytosolic pH is maintained by the proton-buffering capacity of the cytoplasm and the activity of an amiloride-sensitive $Na^+/H^+$ antiport in the plasma membrane [75,76]. Changes in external pH gave significant changes in the cytosolic pH which could have consequences for the activity of the $H^+$ cotransporter in oocytes. Changing the intracellular pH of oocytes has been shown to alter the intracellular calcium activity [77]. This may have consequences for the activity of both endogenous transport systems and foreign proteins expressed in the oocyte. For example, the activation of an endogenous oocyte membrane protein could be misinterpreted as a property of the foreign protein.

When a proton cotransporter is expressed in oocytes, the activity of the transport mechanism imposes an additional acid load on the cell. There are now several mammalian and plant examples of $H^+$ cotransport proteins expressed in oocytes. The cytoplasmic pH of oocytes has been measured using $H^+$-selective microelectrodes, and these recordings have shown that imposing an increase in the external $H^+$ concentration can give an acidification of the cytoplasmic pH [9,78]. These changes in cytosolic pH only occurred when the external pH was decreased to pH 6 or less, but a new steady-state pH was established after 5–10 min [9,78]. We have used a $H^+$-selective microelectrode to measure the intracellular

pH when glucose was applied to an oocyte expressing a $H^+$/glucose cotransporter [9]. These measurements showed that the internal pH changed from 7.4 to 7.25 when the external pH was adjusted from 7.6 to 6, but the activity of the cotransporter itself, when glucose was applied to the oocyte, did not alter the internal pH of the oocyte. Cytosolic pH was restored to 7.4 in the oocyte when the bathing solution pH was returned to pH 7.6. These results showed that, under these conditions, the endogenous pH regulating system of the oocyte can cope with the acid load imposed by $H^+$/glucose cotransport activity, but the steady-state pH of the cytosol depends on the external pH. A contrasting result was obtained when a mammalian $H^+$/peptide cotransporter (PEPT1) was expressed in oocytes [78]. These authors found that the cotransport activity of PEPT1 could give significant changes in intracellular pH and they also found that changing the external pH from 7.4 to 5.5 also gave a significant change in oocyte cytosolic pH from 7.3 to 7.2. The apparent disparity between these two findings may be explained by the differences in expression levels of the two different $H^+$ cotransport activities, PEPT1 had $i_{max}$ ($V_{max}$) values which were twice those obtained for the $H^+$/glucose transporter. Recently, the activity of a rat $H^+$/monocarboxylate transporter was assayed in oocytes by measuring the associated changes in cytosolic pH using $H^+$-selective microelectrodes [79].

Changes in external pH are usually applied before the other transported substrate, and then oocytes should be allowed at least 5 min to establish a new steady-state before the application of another treatment. Curiously, when the first nitrate transporter was characterised by oocyte expression, the external pH was changed to 5.5 simultaneously with the application of 10 mM nitrate [29]. In our experience, the oocyte should always be given a minimum of 5 min to adjust to any changes in external pH before the application of any treatment because there can be transient changes in cytosolic pH and presumably endogenous transport systems during this time [9,53].

The preferred relatively high $Na^+$ concentration of oocyte saline can appear to be a disadvantage, but this can be turned into an advantage for determining the mechanism of transport. In order to measure which ions can drive transport in a cotransport system, the external solution was usually changed in such a way that the concentration of likely driver ions was changed, and the consequences for transport of the substrate were measured. In expression systems like yeast, one consequence of changing the external ionic environment may be to change the resting potential of the cell and hence the driving force for uptake. This problem is avoided in oocytes because the external pH can be changed and the membrane potential controlled. If transport of the substrate occurs when there is zero membrane potential and no proton gradient (alkaline external pH 8), then another ion must be cotransported with the substrate. The transport mechanism of an amino acid transporter (AAP1) and a sucrose transporter (StSUT1) expressed in oocytes was shown to be strictly dependent on $H^+$ [43,44], whereas some mammalian cotransporters can be driven by gradients of $Na^+$, $H^+$ and $Li^+$ [80,81]. However, there is evidence for plant $Na^+$-coupled transport and this was demonstrated in the oocyte expression system [37].

One way of controlling the ionic environment on both sides of the plasma membrane is to use the cut oocyte method [82,83]. This technique enables the bathing solution on both sides of the oocyte membrane to be controlled by perfusing away the cell contents but leaving the oocyte plasma membrane intact for transport measurements. This technique has not been widely used and is technically difficult to perform, probably because the resulting oocyte preparation requires very careful handling since the membrane is very fragile.

### 4.1. Choosing a suitable control

For structure/function studies comparing mutated forms of proteins expressed in oocytes, the wild-type protein provides a suitable control, but if the mutation results in loss of function, it is necessary to check that the protein is still present in the plasma membrane. For example, mutating a single arginine residue in a $Na^+$/glucose cotransporter blocked the normal trafficking of the protein to the oocyte plasma membrane [84]. The most widely used control for oocyte expression experiments is to use a cell from the same batch of eggs which has been injected with a similar volume of water. However, this is not an appropriate control for some experiments.

The types of endogenous transporter activity can vary with the batch of oocytes and the developmental stage of the oocyte. Therefore, it is important to identify that all the oocytes used for injections are at the same developmental stage. Thousands of oocytes at all stages of development are present in the ovaries of the adult female frog, these range from stage I which are the most immature to stage VI, the most mature [85]. Stage V oocytes used for oocyte expression studies have reached their maximum size with typical diameters of between 1 and 1.2 mm. At this stage, oocytes possess clearly defined hemispheres consisting of a dark animal pole towards which the nucleus is displaced (Fig. 1), and a pale yellow vegetal pole. They are surrounded by a vitelline envelope and a layer of follicle cells [86] which are usually removed before any electrophysiological measurements. Although most papers report using stage V or VI oocytes, the definition of these stages is difficult. The stages are defined by the oocytes reaching their maximum size with diameters of 1.2–1.4 mm, but stage VI is characterised by the appearance of an unpigmented equatorial band between the animal and vegetal hemispheres [85]. Choosing oocytes of the correct developmental stage may be important because the endogenous transport properties of the oocyte have been shown to change during development. For example, there are changes in the intracellular ion concentrations [87], membrane permeability [88] and the surface pattern of extracellular currents changes during oocyte maturation [89].

In addition to changes occurring during development, there are also changes after removal of the oocytes from the frog. The magnitude of endogenous currents through chloride channels has been reported to decrease 2–3 days after isolation of oocytes [10] but in our experience, this is very variable and this chloride current can be present after 10 days. Controls for comparison should use oocytes at the same developmental stage, from the same frog and isolated at the same time. Careful choice of the oocytes used for both controls and the expression of foreign proteins can remove much of the background variation.

### 4.2. Interactions with endogenous transport activity

One of the key advantages of using the oocyte system is that there is little endogenous transport activity in the oocyte plasma membrane, i.e. there is no background transport activity to interfere with the characteristics of the foreign protein. This background activity is usually assayed by treating water-injected control oocytes with the transported substrate. For example, the endogenous glucose-elicited current was less than 1 nA in a water-injected control oocyte [24,68], while for sucrose, there was no background sucrose-elicited current [53]. The types of endogenous amino acid transport systems in oocytes have been reviewed [90], but their activity is minimal when compared with expressed transporters [43,54]. The endogenous electrophysiological characteristics of the oocyte plasma membrane have been reviewed [7]. However, what are appropriate controls for these experiments? The injection of a foreign mRNA in the cytoplasm of an oocyte may trigger the activation of other proteins or the expression of endogenous genes so water injection may not be the best control, but even the injection of antisense mRNA may not account for this possibility.

There are several examples of the expression of foreign proteins giving surprising interactions with the endogenous transport proteins. For example, the activity of endogenous channels was stimulated by the expression of foreign proteins [91,92]. These authors reported that high levels of heterologous expression of several different membrane proteins specifically induced a chloride current and a hyperpolarisation-activated, cation-selective current. However, a number of criteria can be used to distinguish endogenous currents from those due to heterologous expression of electrogenic proteins. These criteria were based on the different biophysical, pharmacological and regulatory properties of endogenous versus the foreign protein [92].

We have seen an example of the interaction of a foreign protein with endogenous membrane proteins during the expression of plant nitrate transporters in oocytes. The oocyte has an anion channel which was activated when the membrane potential was pushed to more negative values (hyperpolarised) [71,72] and this channel usually mediated the movement of chloride ions when it was open (see Section 4 above). The threshold voltage for the opening of this channel changed when the oocyte was expressing a low affinity nitrate transporter (Fig. 3). Oocytes injected with mRNA for CHL1 (AtNRT1;1) and BnNRT1;2 both

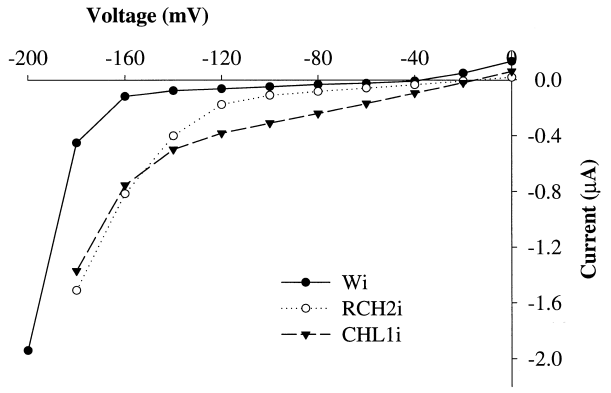


Fig. 3. Differences in the threshold voltage for activation of an endogenous chloride channel in oocytes injected with either water (●) or mRNA encoding two different low affinity nitrate transporters AtNRT1 (CHL1 in [29]) (▼) and BnNRT1;2 (○). Both the RNA-injected oocytes showed nitrate transport activity as measured by the presence of nitrate-elicited currents (not shown here). These *I–V* curves were all obtained in oocytes from the same frog and were bathed in modified nitrate-free oocyte saline pH 7.2 under voltage clamp conditions described previously [59]. Oocytes were injected 3 days before the *I–V* analysis. In contrast to Fig. 2, these are not *I–V* difference curves (note magnitude of current).

showed a significant change in the threshold value of the activation voltage. Fig. 3 shows how the threshold voltage needed to activate the endogenous chloride channel has changed from values more negative than −160 mV to −120 mV when comparing water-injected oocytes with mRNA-injected oocytes. The activation of the channel was shown by a large increase in the current on the *I–V* curve. We do not know the nature of this interaction between these low affinity transporters and the endogenous chloride channel and there could be two possible mechanisms how it was occurring. The first was that there was a direct interaction between the two different types of protein in the oocyte plasma membrane. Alternatively, the functional activity of the $H^+$/nitrate cotransporter may change the internal ion concentration, resulting in a change in the threshold voltage needed to activate the oocyte's chloride channel. However, this response from the oocytes did not require the presence of nitrate in the external solution as they had been stored in nitrate-free saline and chloride was not transported by BnNRT1;2 [59], so we conclude that this must be as a result of some direct protein–protein interaction between the endogenous chloride channel and the plant nitrate transporters. We did not observe such an effect in oocytes of the same batch injected with mRNA for plant $H^+$/sugar cotransporters. However, the expression of a mammalian $Na^+$/phosphate cotransporter was recently shown to induce an outwardly rectifying chloride conductance in oocytes [93].

### 4.3. Overexpression of proteins

Another example of changes in the properties of proteins expressed in oocytes may result from the increased abundance of the protein in the oocyte plasma membrane. For example, increasing the expression of a plant $K^+$ channel, KAT1, in oocytes by polyadenylation of the mRNA increased the current amplitude [14]. The increase in KAT1 expression in oocytes produced shifts in the threshold potential for activation to more positive membrane potentials and decreased half-activation times. These results suggest that in addition to tissue specificity, the level of expression can contribute to the functional diversity of plant $K^+$ channels [14]. A similar effect had previously been reported for a mammalian $K^+$ channel [94].

The concept of a protein having more than one function was recently reviewed and an example of how this process can occur is by the formation of oligomers of the protein which then have new functions [95]. Thus in oocytes, the increased concentration of the protein in the membrane can give new properties, but it remains to be seen if this is also occurring in plants. For the interpretation of data from any heterologous expression system, this possibility should be considered and another 'control' might be to deliberately attempt differing levels of expression by injecting smaller quantities of RNA and comparing the properties of the expressed protein.

## 5. Other heterologous expression systems

After the cDNA encoding the membrane protein has been isolated, there are several different alternative expression systems for the characterisation of the proteins functions. In addition to oocytes, these include yeast, insect cells using the baculovirus and various mammalian cells systems (reviewed in [9]).

Each has particular merits, for example yeast cells can be transformed with expression linked to a specific promoter so that production of the foreign protein can be controlled by supply of a particular substrate, while the baculovirus system can yield large quantities of protein. All of these host cells are small, making electrophysiological measurements more difficult to perform, nonetheless the properties of plant $K^+$ channels expressed in yeast have been determined [96]. A disadvantage of the oocyte system is that the expression is transient, the oocyte usually dies 2–3 weeks after removal from the frog. Although this lifetime can be extended by a few weeks if the oocytes are stored at 4°C, in our experience, their ability to express a foreign protein was decreased by this treatment. The main advantage of the system is that it is easy to study the activity of membrane transport proteins by electrophysiological measurements on oocytes. It is not a suitable system for preparing large quantities of proteins, nor is it the best method for cloning new membrane transport proteins.

The choice of an expression system may just depend on which system will produce functional protein. For example, the *Arabidopsis* $K^+$ channel, AKT1, was functionally expressed in yeast and the baculovirus/insect cell system, but not in *Xenopus* oocytes [97,70]. Recently, another AKT-type $K^+$ channel from potato was also expressed in insect cells and not oocytes [98] but a third gene, AKT3, was successfully expressed in oocytes [49]. Although the plant $K^+$ influx channels have been divided into the KAT and AKT subfamilies based on differences in structural domains [99], it appears that oocyte expression does not separate the two groups. A good illustration of the advantage of the oocyte expression over the insect cells for the characterisation of membrane proteins is provided by a comparison between two plant $K^+$ channels. Although the cDNAs for the first examples of these two types of $K^+$ influx channel were isolated at the same time [26,97], there are many more papers published on the characterisation of KAT1 compared with AKT1 (see Table 2). Choosing an animal cell can be advantageous for the expression of a plant protein because there are less likely to be similar endogenous transport systems present in the host cell. Perhaps there is no ideal expression system, not even if there was a plant equivalent of the *Xenopus* oocyte.

Other cells have been used to express plant membrane proteins, such as aquaporins in the slime mould, *Dictyostelium* [100], but expression in these cells does not offer any particular advantages. To find a plant alternative to the oocyte is difficult, but one interesting approach was described for the expression of a $K^+$ channel in plant cells which did not normally show any activity for this type of channel [101]. These authors found that mesophyll cells lacked the inward $K^+$ current found in guard cells and so they expressed and studied the *Arabidopsis* KAT1 gene in the mesophyll of tobacco leaves. To express and characterise the function of a plant membrane protein, it is necessary to find a model system that lacks the endogenous activity and which can be genetically transformed. This approach could be used for foreign membrane proteins in other cell types, for example the root hair may be used. The techniques for electrophysiological characterisation of root hairs have been described [102]. Human muscarinic receptors have been expressed in tobacco plants and cultured tobacco cells using *Agrobacterium*-mediated transformation. The membranes of the transgenic plants and calli were then shown to bind muscarinic ligands with appropriate affinities [103].

Another example of a plant expression systems is the giant internodal algal cells of *Chara*. Acetylcholine receptors were successfully expressed in these cells [104], but since then there have not been any more reports using this system suggesting that it may not be such a useful system. At first, *Chara* appears to be almost a plant equivalent of the oocyte, for example they are large cells which are amenable to electrophysiology. However, one of the prerequisites for a good expression system is that the activity of any endogenous membrane protein does not interfere with the foreign protein being expressed. As the *Chara* cell is a growing plant cell, endogenous transport activity may hide or interfere with the activity of a foreign protein. Furthermore, the endogenous transport regulation processes in *Chara* are likely to be more similar to those in other plant cells and so the activity of the foreign protein may be altered by post-translational regulation. The successful characterisation of function in a heterologous expression system depends on the fact that the expressed protein is 'foreign' in the host cell. The search for an alter-

native expression system is necessary only when expression of the plant membrane protein in oocytes cannot be obtained.

## 6. Future uses and conclusions

Many types of plant macronutrient transporters have been characterised by oocyte expression (see Table 2), although there are some exceptions, e.g. ammonium. Future use of the oocyte system is likely to include the characterisation of other types of plant carriers, such as phosphate and sulfate transporters and there is strong evidence that the activity of at least some classes of these anion carriers will be electrogenic. For example, mammalian phosphate transporters are electrogenic [105] favouring their characterisation in oocytes and there is good evidence that they may be related to plant phosphate transporters. Also, there is in plants evidence that the transport of phosphate and sulfate is electrogenic [106,107]. Other types of plant carriers which may be identified and certainly characterised by oocyte expression include those for micronutrients, the first example for a diatom has been reported [52]. Oocyte swelling experiments like those used to assay aquaporins have been used to demonstrate a water channel activity in some mammalian carrier proteins [108] and a similar activity may yet be found for plant carriers expressed in oocytes.

Structure/function studies of membrane proteins can be conveniently performed using the oocyte system. For example, single amino acid residues can be altered and the consequences for the properties of the transporter can be determined by comparison with the wild-type protein expressed in oocytes. Another approach for structure/function analysis is to combine yeast and oocyte expression systems if the transporter protein is functionally expressed in both systems (e.g. [37]). A particular selection medium is applied to yeast expressing randomly mutagenised forms of the transporter, for example very low concentrations of the driver ion for a cotransporter, and the selected mutants are then characterised in detail using oocytes. Furthermore, this approach could also be used to select for genes which are involved in regulating the activity of a particular transporter. Oocytes can also be conveniently used to determine the topography of a membrane protein, for example the Flag epitope was inserted at various locations throughout a $K^+$ channel to determine on which membrane face each of these positions was located [13]. The future use of oocytes is likely to include the expression of more than one protein to determine how a transporter is regulated. There are examples for mammalian membrane proteins which when coexpressed in oocytes can interact together to modify transport activity [109], this is also an exciting prospect for future plant transport research.

Many different types of non-plant membrane receptors have been cloned and functionally analysed using oocytes [6]. These receptors chiefly belong to the GTP binding protein family [110] and although plant homologues have been identified [111], there are no reports of expression in oocytes. The key to obtaining functional expression of plant homologues to this type of protein depends on the homology between the plant and *Xenopus* α-subunits. In order to obtain activation of endogenous oocyte channels giving the characteristic electrical response, it may be necessary to inject mRNA encoding both the plant receptor and the α-subunit. Plant homologues of glutamate receptors have also been reported [112] and this family of receptors was first cloned by functional expression in oocytes [113]. The large amount of success in expressing receptors in oocytes must make this class of plant membrane protein a favourite for future oocyte expression and characterisation.

The oocyte expression system can be used to study the regulation of plant transporters by protein kinases. Phosphorylation was shown to modify the transport activity of both mammalian channels [114] and cotransporters [115,116] expressed in oocytes. Delivery of foreign cotransporters to the oocyte plasma membrane was changed by phosphorylation of the proteins [115,117]. There are also plant examples of phosphorylation modifying the activity of water and ion channels [36,57,118,119]. The effect of phosphorylation on the properties of tonoplast and plasma membrane aquaporins was demonstrated on the proteins expressed in oocytes [36,57]. We have investigated the effect of protein kinase activity on plant sucrose carriers expressed in *Xenopus* oocytes. Changes in the kinetic properties of three different carriers could be measured after stimulation of the activity of endogenous protein kinases in oocytes,

indicating a role for phosphorylation in modifying the activity of these carriers (Zhou and Miller, unpublished results). For some of these plant proteins, the effects of membrane protein phosphorylation were on both targeting and substrate binding.

All the plant membrane proteins expressed in oocytes are assayed by their activity in the plasma membrane and so it is generally assumed that they are correctly targeted and so have the same membrane location in plants. However, this may be a dangerous assumption because a plant vacuolar membrane aquaporin was expressed in the oocyte plasma membrane [27], suggesting that this may be a default pathway for the expression of endomembrane proteins. More information is needed to identify the membrane location of plant transporters, some of the carriers already characterised in oocytes may yet have a vacuolar location in plants. The identification and characterisation of plant vacuolar membrane proteins is an area of research in which oocyte expression will surely be a useful tool. The endomembrane of the oocyte can be used for transport assays. For example, the oocyte outer nuclear membrane has been studied using patch clamp electrophysiology [120]. The possibility that foreign proteins could be studied in this way exists, but the endogenous activity could make characterisation of a foreign envelope protein difficult, e.g. the IP3 receptor [120].

Oocytes can be used as immunological vectors to produce antibodies to brain cell antigens [121]. In this method, oocytes translated a rat brain mRNA, then an oocyte membrane fraction, containing the foreign membrane protein, was used to immunise mice which had been made immunotolerant to antigens of native oocyte membrane. These immunised mice were then used to generate monoclonal antibodies which reacted specifically to a rat brain membrane protein. This approach has not yet been used for a plant membrane protein. Even when a protein was not actually produced by the oocyte's synthetic machinery, it can still be studied in the oocyte membrane. For example, the microinjection of *Xenopus* oocytes with P-glycoprotein-containing membranes from multidrug resistant cells resulted in the 'transplantation' of the protein into the plasma membrane of the oocytes. The presence of the protein in plasma membrane was then confirmed by Western blot analysis [122].

In conclusion, the information obtained from oocyte expression of a protein can be used in combination with the characterisation of the phenotype of a plant in which the gene encoding the protein is disrupted. The oocyte results provide information on how the physiology of the mutated plant may be changed. Although oocytes are unlikely to have an important role in the isolation of cDNAs for new transporter genes, their continued use in determining gene function is assured. The interpretation of data from any heterologous expression system must be regarded with some caution, but nonetheless the oocyte expression system is a powerful system with a certain future for the characterisation of plant membrane proteins.

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